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The involvement of central L-type calcium channel subtypes $Ca_V1.2$ and $Ca_V1.3$ in alcohol dependence and comorbid mental disorders

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Für meine Familie

Abstract

Alcohol consumption is a broadly accepted part of many cultures around the world. While for some people it is not a problem to control their intake and use alcohol recreationally, others escalate their drinking until it becomes compulsive. Through cycles of excessive drinking and abstinence, alcohol dependence develops. This process is accompanied and supported by adaptations in the brain, including neurotransmitter and hormone systems as well as ion channels. Many of these systems are also altered in nicotine dependence, schizophrenia and depression, in part explaining the high comorbidity between these disorders and alcohol dependence.
For the development of new drug therapies, an endeavor necessitated by the lack of efficient medications, it is imperative to understand the underlying mechanisms of each of these disorders. One possible target are the L-type calcium channels (LTCCs), which are influenced by both alcohol and nicotine, and have also been implicated in the risk to develop schizophrenia and depression. However, the two central LTCC subtypes $Ca_V1.2$ (*Cacna1c*) and $Ca_V1.3$ (*Cacna1d*) may play different roles, which have not yet been defined.
This thesis aims to identify the individual involvement of $Ca_V1.2$ and $Ca_V1.3$ in alcohol dependence, and determine whether similar contributions of these subtypes can be found in comorbid disorders.

In Study I, *Cacna1c* mRNA levels are found to be dynamically regulated during intoxication, withdrawal, and protracted abstinence, with a strong increase in the amygdala and hippocampus after 21 days of abstinence. While *Cacna1d* mRNA remains unchanged at this time, $Ca_V1.2$ protein levels and currents are also increased. Furthermore, antagonism of central LTCCs prevents cue-induced reinstatement of alcohol seeking. Other genetic and functional models of alcohol dependence do not show a clear distinction between *Cacna1c* and *Cacna1d* mRNA expression (Study II). Transgenic mice with a $Ca_V1.2$ knockout (KO) in Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-positive neurons did not show a dependence-induced increase of alcohol intake, which their control littermates displayed clearly (Study III). Similarly to alcohol dependence, Study IV shows increased *Cacna1c* mRNA after chronic administration of nicotine and subsequent abstinence, while *Cacna1d* mRNA is increased one day after a single nicotine injection. An augmented nicotine sensitization after abstinence from chronic nicotine was prevented by nifedipine administration during abstinence. Additionally, $Ca_V1.2$ KO in CaMKII-positive neurons prevented nicotine self-administration behavior. Study V investigated *CACNA1C* and *CACNA1D* mRNA expression in postmortem samples of schizophrenia patients, alcoholics, and suicide completers. In schizophrenia, both subtypes were decreased in the prefrontal and temporal cortex. No changes caused by alcohol dependence or depression were found.

In conclusion, this thesis provides evidence for a crucial role of $Ca_V1.2$ in prolonged abstinence from chronic alcohol and nicotine, with effects on drug seeking and craving. It further underlines the importance of central LTCCs in some aspects of schizophrenia. Altogether, it highlights $Ca_V1.2$ as promising target for the development of new therapies for drug dependence and comorbid mental disorders.

Zusammenfassung

Alkoholkonsum ist ein weltweit akzeptierter Teil vieler Kulturen. Während manche Menschen ihren Konsum problemlos kontrollieren können und Alkohol ausschließlich zum Vergnügen in ihrer Freizeit trinken, eskaliert bei anderen das Trinken bis ins Zwanghafte. Durch mehrere Zyklen aus Trunkenheit und Verzicht entwickelt sich eine Abhängigkeit. Dies wird von Veränderungen von Neurotransmittern und Hormonsystemen sowie von Ionenkanälen im Gehirn begleitet. Viele dieser Veränderungen treten ebenso bei Nikotinabhängigkeit, Schizophrenie, und Depressionen auf, welche häufig gemeinsam mit Alkoholabhängigkeit auftreten.
Für die Entwicklung neuer Medikamente ist es notwendig die Mechanismen zu verstehen, die den Erkrankungen zugrunde liegen. Ein möglicher Angriffspunkt sind L-Typ Kalziumkanäle (LTCCs), die sowohl von Alkohol als auch von Nikotin beeinflusst werden, und die auch mit Schizophrenie oder Depressionen in Verbindung gebracht wurden. Die beiden zentralen LTCC Subtypen $Ca_V1.2$ (*Cacna1c*) und $Ca_V1.3$ (*Cacna1d*) spielen jedoch möglicherweise unterschiedliche Rollen, welche bisher nicht bestimmt wurden.
In dieser Arbeit soll die individuelle Beteiligung von $Ca_V1.2$ und $Ca_V1.3$ an Alkoholabhängigkeit und psychiatrischen Begleiterkrankungen identifiziert werden.

In Studie I wird eine dynamische Regulierung der *Cacna1c* mRNA-Level während Trunkenheit, Entzug, und langfristiger Abstinenz ermittelt, wobei die Expression nach 21 Tagen der Abstinenz in der Amygdala und dem Hippocampus deutlich erhöht ist. Während *Cacna1d* mRNA unverändert ist, sind das $Ca_V1.2$ Protein sowie der Ionenfluss durch $Ca_V1.2$ ebenfalls erhöht. Die Blockade von zentralen LTCCs verhindert auch den Reiz-induzierten Rückfall. Keine deutlichen Unterschiede zwischen *Cacna1c* und *Cacna1d* mRNA Expression konnten in anderen genetischen und funktionalen Mausmodellen für Alkoholabhängigkeit gefunden werden (Studie II). Transgene Mäuse mit $Ca_V1.2$ Knockout (KO) in Ca^{2+}/calmodulin-abhängige Proteinkinase II (CaMKII)-positiven Neuronen zeigen außerdem keine, durch Abhängigkeit verursachte, Eskalation des Alkoholkonsums, welche in Kontrollmäusen deutlich zu sehen ist (Studie III). In Studie II führt chronische Verabreichung und anschließende Abstinenz zu vermehrter *Cacna1c* mRNA, während *Cacna1d* mRNA einen Tag nach einmaliger Nikotinverabreichung ansteigt. Eine stärkere Sensibilisierung durch Nikotin aufgrund einer längeren Abstinenzperiode wird durch Nifedipin verhindert. Außerdem blockiert ein $Ca_V1.2$ KO in CaMKII-positiven Neuronen die Selbstverabreichung von Nikotin in Mäusen. Studie V untersucht die *CACNA1C* und *CACNA1D* mRNA Expression in postmortem Gewebe von Schizophrenie- und Alkohol-Patienten, sowie Suizidopfern. In Schizophrenie-Patienten sind beide Subtpyen im präfrontalen und temporalen Kortex verringert. Keine Veränderungen wurden bei Alkoholabhängigkeit und Depressionen festgestellt.

Diese Arbeit liefert Hinweise auf eine wichtige Rolle von $Ca_V1.2$ bei langfristiger Abstinenz von Alkohol und Nikotin, mit Einfluss auf das Drogen-Suchverhalten und -Verlangen. Die Wichtigkeit der zentralen LTCCs in einigen Aspekten der Schizophrenie wird außerdem unterstützt. Im Ganzen wird $Ca_V1.2$ als vielversprechender Angriffspunkt für die Entwicklung neuer Therapien für Drogenabhängigkeit und begleitende psychische Erkrankungen hervorgehoben.

Inhalt

Abbreviations

AA	-	alko, alcohol (rat model)
AC	-	adenylate cyclase
Acb	-	nucleus accumbens
AcbC	-	nucleus accumbens core
AcbS	-	nucleus accumbens shell
aCSF	-	artificial Cerebro-Spinal Fluid
ADE	-	alcohol deprivation effect
ANA	-	alko, non-alcohol (rat model)
AUD	-	alcohol use disorder
BAC	-	blood alcohol concentration
BDNF	-	brain-derived neurotrophic factor
BLA	-	basolateral amygdala
BNST	-	bed nucleus of the stria terminalis
BSA	-	bovine serum albumin
CA	-	Cornus Ammon
CaM	-	Ca^{2+}/calmodulin
CaMKII	-	Ca^{2+}/calmodulin-dependent protein kinase II
CaMKK	-	Ca^{2+}/calmodulin-dependent protein kinase kinase
cAMP	-	cyclic adenosine monophosphate
CeA	-	central amygdala
Cing	-	cingulate cortex
CPu	-	caudate putamen
CREB	-	cAMP response element-binding protein
CRH	-	corticotropine-releasing hormone
Crhr1	-	corticotropine-releasing hormone receptor 1
CS	-	conditioned stimulus
DA	-	dopamine
DG	-	dentate gyrus
DHP	-	dihydropyridine
DS	-	dorsal striatum
DSM	-	Diagnostic and Statistical Manual of Mental Disorders
ERK	-	extracellular signal-related protein kinase
FDA	-	Food and Drug Administration
GABA	-	γ-aminobutyric acid
GIRK	-	G-protein-activated inwardly rectifying K+ Channel
Glu	-	glutamate
HCl	-	hydrogen chloride
HPLC	-	high-performance liquid chromatography
HSC70	-	heat shock cognate protein 70
HSP90	-	heat shock protein 90
5-HT	-	serotonin

ICD	-	International Statistical Classification of Diseases and Related Health Problems
i.c.v.	-	intracerebroventricular
IL	-	infralimbic
i.v.	-	intravenous
KO	-	knockout
LTCC	-	L-type calcium channel
LTP	-	long-term potentiation
MAPK	-	mitogen-activated protein kinase
MATRICS	-	Measurement and Treatment Research to Improve Cognition in Schizophrenia
MDC	-	minimal detectable change
MDD	-	major depressive disorder
MeA	-	medial amygdala
msP	-	marchegian Sardinian preferring rats (rat model)
nAChR	-	nicotinic acetylcholine receptors
NC	-	nucleus caudatus
NIMH	-	National Institute of Mental Health
NMDA	-	N-methyl-D-aspartate
NP	-	non-preferring (rat model)
NPY	-	neuropeptide Y
OFC	-	orbitofrontal cortex
P	-	preferring (rat model)
PANSS	-	Positive and Negative Symptom Scale
PBS	-	phosphate buffered saline
PFC	-	prefrontal cortex
PKA	-	protein kinase A
PKC	-	protein kinase C
PLC	-	phospholipase C
PreL	-	prelimbic cortex
PVN	-	paraventricular nucleus
qRT-PCR	-	quantitative real-time polymerase chain reaction
RT	-	room temperature
SA	-	self-administration
SCC	-	standard saline citrate
SN	-	substantia nigra
SNP	-	single nucleotide polymorphism
TTX	-	tetrodotoxin
TUD	-	tobacco use disorder
VS	-	ventral striatum
VTA	-	ventral tegmental area
WHO	-	World Health Organization

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(Manuscript submitted to the Journal of Neuroscience; Study I)

A. C. Hansson*, **S. Uhrig***, E. Domi, R. Ciccocioppo, V. Grinevich, W.H. Sommer, R. Spanagel. *Oxytocin reduces cue-induced reinstatement in alcohol dependent rats*
(Manuscript in preparation)
*authors contributed equally

E. Domi, **S. Uhrig**, L. Soverchia, A. C. Hansson, R. Spanagel, M. Ubaldi. *Genetic deletion of neuronal PPARγ enhances the emotional response to stress and exacerbates anxiety: An effect reversed by rescue of amygdala PPARγ function.*
(Manuscript in preparation)

S. Uhrig et al. Cacna1c *and* Cacna1d *mRNA expression in animal models of alcoholism.*
(Manuscript in preparation; Study II)

S. Uhrig et al. *L-type calcium channel subtype $Ca_V1.2$ mediates dependence-induced increase in alcohol self-administration.*
(Manuscript in preparation; Study III)

S. Uhrig et al. *Decreased* Cacna1c *and* Cacna1d *mRNA expression in cortical brain regions of schizophrenia patients.* (Manuscript in preparation; Study V)

S. Uhrig et al. *Crhr1 overexpression in the central amygdala increases stress-induced reinstatement of alcohol-seeking.*
(Manuscript in preparation)

I. Introduction

1. Alcohol dependence

Although known as a psychoactive, dependence-inducing substance, alcohol has been and still is consumed in many cultures world-wide. As such, its dangers are often underestimated, when in fact 5.9 % of all deaths can be directly or indirectly attributed to harmful use of alcohol (World Health Organization, 2014). Over 200 diseases, such as mental and behavioral disorders, and injury conditions are caused by excessive alcohol consumption, adding up to 5.1 % of the global burden of disease and injury (World Health Organization, 2014). These numbers are hardly surprising when considering the high number of people who regularly indulge in heavy drinking: worldwide, this applies to 16 % of drinkers over 15 years of age (World Health Organization, 2014). In 2010, individuals over 15 years consumed an average of 13.5 grams of pure alcohol per day, summing up to 6.2 liters of pure alcohol per year (World Health Organization, 2014). In the EU the amount was even higher, with 10.2 liters of pure alcohol per person per year, which does not include unrecorded consumption of self-made alcoholic beverages (World Health Organization, 2013a).

1.1. Development of the disorder

Alcohol dependence constitutes the physical part of the disorder 'alcoholism', i.e. the appearance of withdrawal symptom when alcohol consumption is discontinued, whereas addiction describes the psychological and behavioral part (Le Moal and Koob, 2007).

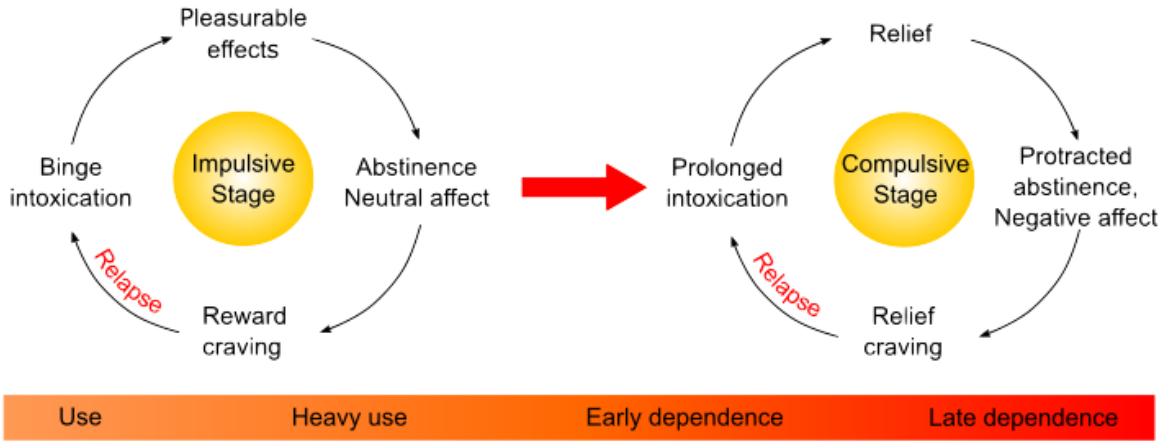

Figure 1. Development of alcohol dependence. Controlled use transits into compulsive alcohol consumption over time through multiple cycles of intoxication and abstinence. The motivation for alcohol consumption changes from pleasurable effects to the relief from a negative emotional state as neuroadaptations are taking place and dependence develops. Adapted from (Koob, 2009).

The combination of both leads to a person's inability to control their alcohol intake, and repeated cycles of binge drinking, abstinence, craving and relapse, or as Koob (Koob, 2009) described it, preoccupation/anticipation, binge/intoxication and withdrawal/negative affect. The compulsivity of alcohol intake increases and replaces the initial impulsivity with every repetition of the cycle (Koob, 2009), as neuroadaptations such as changes in neurotransmitter systems, hormones and ion channels are taking place (Spanagel, 2009; Vengeliene *et al*, 2008).

During the different stages, several neurotransmitter and hormone systems are altered. For the rewarding properties, or pleasurable effects, of alcohol, the mesolimbic dopamine system appears to play a key role (Hirth *et al*, 2016b; Koob and Volkow, 2010). Additionally, glutamatergic projections from the prefrontal cortex (PFC), hippocampus, and amygdala to the nucleus accubmens (Acb), and projections of inhibitory γ-aminobutyric acid (GABA) neurons from the Acb to the ventral tegmental area (VTA) help to mediate reward (Russo and Nestler, 2013) (Figure 2). GABAergic neurons in the central amygdala (CeA) (Hyytia and Koob, 1995) are involved, as well, while the dorsal striatum seems to exert its influence during the compulsive, rather than the impulsive stage (Everitt *et al*, 2008). At this later point, alcohol consumption is motivated by relief from negative affect instead of the initial pleasurable effects. This negative affect, caused by the abstinence from compulsive alcohol consumption, is mediated largely by the amygdala and the bed nucleus of the stria terminalis (BNST), regions which have previously been associated with fear conditioning (LeDoux, 2000). During the transition from impulsive to compulsive alcohol consumption, it appears that neurotransmitter system mediating pleasurable effects, such as dopamine, are decreased in function. At the same time, brain stress systems, such as the corticotropin-releasing hormone (CRH) system in the amygdala (Hansson *et al*, 2006a; Sommer *et al*, 2008), are activated. The increased anxiety-like behavior during withdrawal from alcohol is also mediated by the activation of the CRH system (Hansson *et al*, 2007; Hansson *et al*, 2006a; Sommer *et al*, 2008).

Craving, whether for reward or relief from negative affect, is often associated with the relapse to drug taking, even though it is difficult to even take clinical measures (Tiffany *et al*, 2000). None withstanding the fact that in several studies no correlation between the reported craving and relapse was found, this stage is the focus of treatment development (Koob *et al*, 2010). Therefore, the mechanisms underlying the anticipation, or craving, of alcohol consumption, have to be investigated thoroughly. Here, too, the amygdala appears to play a major role. The

basolateral amygdala (BLA) was shown to mediate cue-induced reinstatement (Everitt and Wolf, 2002; Weiss *et al*, 2001), and is also involved in the association of previously neutral cues to drug withdrawal (Schulteis *et al*, 2000). As for the reward circuitry, glutamatergic, GABAergic, and dopaminergic projections are also involved in the mediation of cue-induced reinstatement of drug seeking (Kalivas and O'Brien, 2008). During extended abstinence, which often fosters relief craving, the CRH and glutamate systems in the amygdala likely show an increased activity (De Witte *et al*, 2005; Valdez *et al*, 2002), further underlining the importance of this region for the development and maintenance of drug dependence.

Next to neurotransmitter and hormone systems, several ion channels undergo changes throughout the process of becoming drug dependent. L-type calcium channels (LTCCs) seem to be of particular interest for the effects of alcohol (Vengeliene *et al*, 2008; Wang *et al*, 1994). They are also expressed in brain regions crucial for the mediation of reward (Figure 2). Although the highest expression is found in the hippocampus, LTCCs are also expressed in the amygdala, and to a lesser extent in the PFC, Acb and VTA (Brimblecombe *et al*, 2015; Busquet *et al*, 2010; Cardozo and Bean, 1995; Hell *et al*, 1993; Lee *et al*, 2012; Liebmann *et al*, 2008).

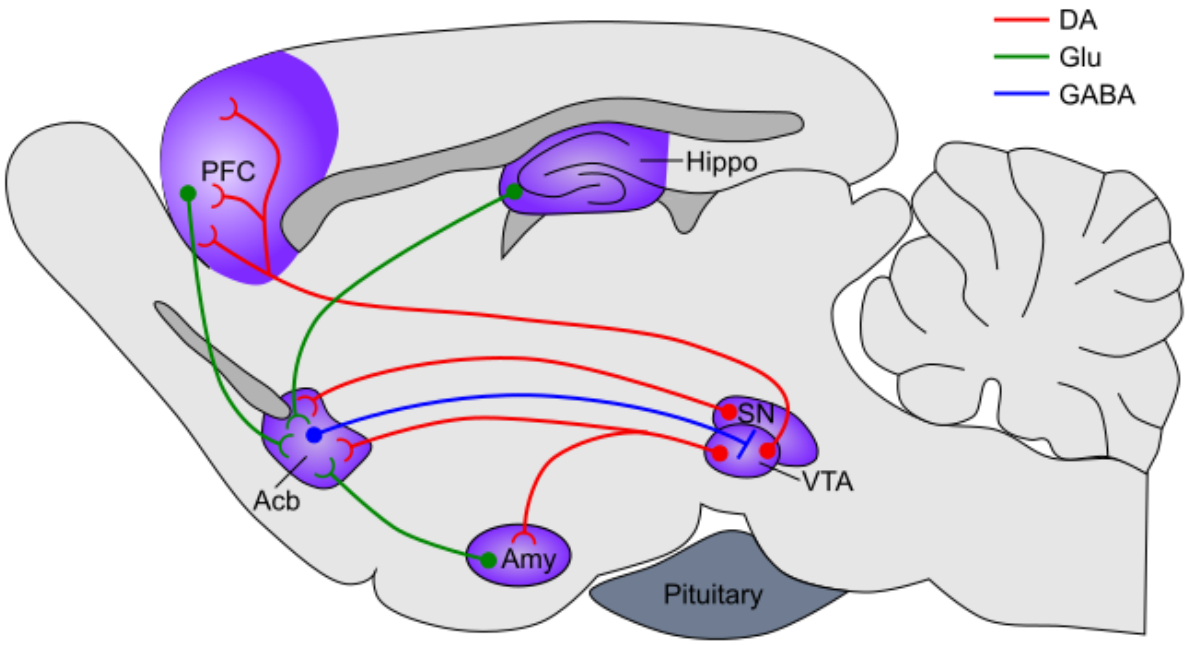

Figure 2. The brain reward circuitry including important neurotransmitter systems and LTCC expression. LTCCs are expressed in several brain regions relevant to the mediation of reward (indicated as purple areas). The neurotransmitters dopamine (DA, red), glutamate (Glu, green) and γ-aminobutyric acid (GABA, blue) play crucial roles in the communication between these brain regions. PFC, prefrontal cortex; Hippo, hippocampus; Acb, nucleus accumbens; Amy, amygdala; SN, substantia nigra; VTA, ventral tegmental area.

There are still many unanswered questions about the involvement of LTCCs and the individual subtypes, as well as other ion channels, neurotransmitters, and hormone systems, during the different stages of alcohol dependence. However, even if new treatments can be developed using information on these molecular changes, the first step in helping addicts recover is to diagnose them with the disorder.

1.2. Guidelines for diagnosis

As with mental disorders in general, the diagnosis of alcoholism is challenging. The symptoms vary greatly in their nature and severity among afflicted individuals and it is often difficult to determine when normal drinking behavior has developed into dependence and addiction. The Diagnostic and Statistical Manual of Mental Disorders (DSM) has been used as a guide for the diagnosis of mental disorders since 1952, evolving over time to accommodate new scientific and medical insights (NIH Publication No. 13-7999, July 2015). The latest edition, DSM-5 (American Psychiatric Association, 2014), was issued in 2013, and differs in several key points from the previous version DSM-IV. As such, the terminology of the disorder has been changed: what had been defined as two distinct disorders, alcohol abuse and alcohol dependence, in DSM-IV, has now been integrated to describe a single disorder, the alcohol use disorder (AUD). The division into three sub-classifications (mild, moderate, and severe) nevertheless ensures a subtle categorization.

The symptoms include

1) Alcohol is often taken in larger amounts or over a longer period than was intended.
2) Persistent desire or unsuccessful efforts to cut down or control alcohol use.
3) A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from it effects.
4) Craving, or a strong desire or urge to use alcohol.
5) Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home.
6) Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.
7) Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
8) Recurrent alcohol use in situations in which it is physically hazardous

9) Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
10) Tolerance, as defined by either of the following:
    a) A need for markedly increased amounts of alcohol to achieve intoxication or desired effect
    b) A markedly diminished effect with continued use of the same amount of alcohol
11) Withdrawal, as manifested by either of the following:
    a) The characteristic withdrawal syndrome for alcohol (refer to criteria A and B of the criteria set for alcohol withdrawal)
    b) Alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms

The World Health Organization (WHO) also introduced another diagnostic manual called "International Statistical Classification of Disease and Related Health Problems" (ICD-10) in 1992, which defines six criteria for drug dependence (World Health Organization, 1992). Although some differences exist between DSM-5 and ICD-10, both manuals are comparable and widely used. Following the positive diagnosis, efforts can be made to treat the disorder and prevent relapse.

1.3. Available pharmacological treatments

To date, four pharmacological treatments are available for the use in alcohol dependence. However, the efficacy of each treatment is somewhat limited, and improvements would be highly warranted.

The most widely used anti-relapse drug in the USA is acamprosate (Campral®) (Mark *et al*, 2009), which does not show an effect on craving (Umhau *et al*, 2011). Interestingly, acamprosate has been shown to act via calcium (Spanagel *et al*, 2014b).

Disulfiram (Antabus®) works through a completely different mechanism. It inhibits alcohol dehydrogenase, thereby eliciting very unpleasant effects of accumulated acetaldehyde in the body (Crowley, 2015). Unfortunately, it has shown little effect when it had to be taken without supervision, a scenario more closely related to reality than supervised administration, which has often been used in previous studies (Fuller and Gordis, 2004; Johnson, 2008).

A third treatment option is naltrexone (ReVia®, Vivitrol®), a mu opioid receptor antagonist. Although it has been shown to decrease relapse and to reduce heavy drinking in alcoholic

patients (Pettinati *et al*, 2006; Srisurapanont and Jarusuraisin, 2005), a meta-analysis found it only moderately effective on a subset of patients (Bouza *et al*, 2004; Heilig *et al*, 2010).
In 2013, nalmefene (Selincro®) was approved as medication for the reduction of alcohol consumption. Unlike naltrexone, it displays partial agonistic effects on one opioid receptor, next to its antagonistic activities on other opioid receptors (Bart *et al*, 2005). Animal studies suggest that nalmefene might help to decrease alcohol consumption more effectively in alcohol dependent than in non-dependent rats (Nealey *et al*, 2011; Walker and Koob, 2008), but human trials have yet to prove its efficacy (Stafford, 2014).

Given the insufficient efficacy of the available treatments, it is of great importance to investigate other possible intervention targets, such as the LTCCs, concerning their involvement in the development of alcohol dependence. A method of choice for preclinical analysis is the use of animal models for alcohol dependence.

1.4. Animal models for alcohol dependence

Modeling mental disorders in general using laboratory animals such as rats and mice is a necessary yet challenging task.
The eligibility of each model for the scientific investigation of a mental disorder is measured using three criteria, the face validity, predictive validity, and construct validity (Belzung and Lemoine, 2011; Willner, 1984). Several authors have given definitions of each validity criterion since the 1960's (Geyer and Markou, 1995; Koob *et al*, 1998; McKinney and Bunney, 1969; Willner, 1984), each emphasizing slightly different aspects. However, ultimately *face validity* can be seen as the similarity between the symptoms of the animal model and those of the human disorder. *Predictive validity* concerns the question whether human patients and animal models will react similarly to a treatment approach, pharmacological or otherwise. *Construct validity* may be the criterion with the most diverse and vague definitions. It describes the theoretical accuracy of the model concerning the underlying mechanisms.
Considering that psychological disorders manifest with multiple versatile symptoms, an animal model can always represent only certain aspects of the disorder. Accordingly, the model has to be carefully considered for each scientific question.

In alcoholism, two types of patients have been defined. In type I alcoholics, the onset of dependence happens during adulthood after prolonged excessive drinking. Here, genetic and environmental factors are involved, and the patients demonstrate anxious personality traits.

Loss of control and development of dependence are caused by the initial anxiety-relieving effects of alcohol. The severity and symptomology can also vary strongly. In contrast, type 2 alcoholism is almost exclusively determined by genetic factors, and sets in already during adolescence or early adulthood, with patients displaying antisocial personality traits (Cloninger *et al*, 1988). Here, we focus on type I alcoholics, as the animal models discussed and analyzed in this thesis display an increased sensitivity to stress, and in functional models alcohol exposure takes place during adulthood (Bjork *et al*, 2010; Ciccocioppo *et al*, 2006; Meinhardt and Sommer, 2015).

There are several animal models for the investigation of specific aspects of alcohol dependence. A genetic predisposition to increased alcohol consumption, for example, can be examined using selectively bred rat lines such as the marchegian Sardinian Preferring (msP), which show an intrinsic high alcohol consumption and preference, as well as increased innate anxiety and depressive-like behavior (Ciccocioppo *et al*, 2006). A similar approach, although with differences in the selection criteria, has been used as early as the 1960s to breed the AA (alko, alcohol) and ANA (alko, non-alcohol) rat lines (Eriksson, 1981; Sommer *et al*, 2006), and not long after that the P (preferring) and NP (non-preferring) rats (Lumeng *et al*, 1977; Murphy *et al*, 2002), amongst others. In humans, AUD is defined by environmental factors, but also by a genetic predisposition. This is reflected by the existence of single nucleotide polymorphisms (SNPs) known to indicate an increased risk of becoming alcohol dependent (Bach *et al*, 2015; Spanagel *et al*, 2010; Spanagel *et al*, 2013).

A different kind of animal model relies on the exposure to alcohol, with a broad variety of exposure time, means of exposure and environmental conditions. Depending on these factors, different aspects can be investigated (Spanagel, 2003). For example, relapse to drug-seeking after a period of abstinence can be examined using the reinstatement model, while the alcohol deprivation effect (ADE) model takes a look at compulsive alcohol intake ("binge drinking") and loss of control, as well as relapse behavior (Sinclair and Senter, 1967; Vengeliene *et al*, 2014; Vengeliene *et al*, 2009).

The model of choice for the study of dependence-induced neuroadaptations and medication development, however, is the postdependent state model (Meinhardt *et al*, 2015; Rimondini *et al*, 2002). Here, rodents are subjected to chronic intermittent cycles of alcohol exposure via inhalation of alcohol vapor, followed by a period of abstinence. After alcohol vapor exposure is discontinued, withdrawal symptoms comparable to the human condition can be observed. Furthermore, the postdependent animals show an increase in voluntary alcohol consumption

as well as heightened anxiety. Long-lasting neuroadaptations have been found after a three-week abstinence period, such as an increase in CRH receptor 1 (Crhr1) mRNA in the amygdala (Sommer *et al*, 2008).

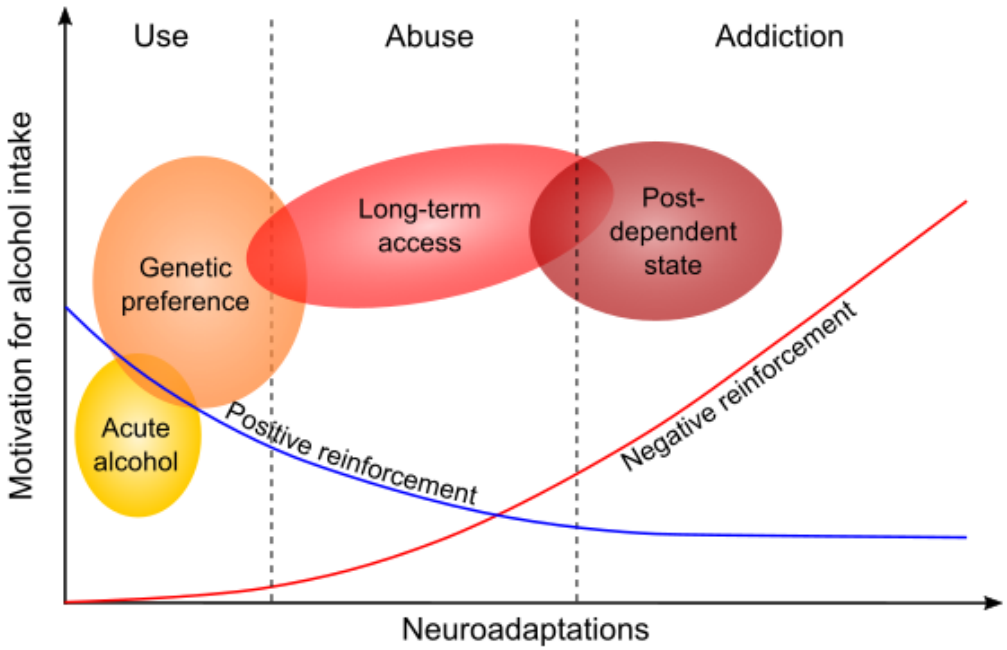

Figure 3. Animal models are used to investigate different aspects at different levels, from casual use to addiction. Initially, the pleasurable effects (positive reinforcement) of alcohol motivate consumption, but as alcohol intake becomes persistent and chronic, the focus shifts toward the amelioration of a negative emotional state (negative reinforcement. Several animal models are suitable to investigate the acute effects of alcohol or even the changes developing through prolonged alcohol consumption. To study addiction similar to the human condition chronic intermittent cycles of alcohol exposure are necessary, as rodents do not voluntarily consume intoxicating amounts of alcohol leading to withdrawal symptoms. Adapted from (Meinhardt *et al*, 2015).

Although dependence does not develop through voluntary alcohol consumption, rather than forced inhalation, the postdependent state model still shows *face validity* in terms of the similarities of symptoms to the human condition. Importantly, a high *predictive validity* can be attributed to the postdependent state model. One example is acamprosate, a modulator of the glutamatergic system which is used in human alcoholic patients to reduce the risk of relapse (Rosner *et al*, 2010). Acamprosate decreases the voluntary alcohol intake in postdependent rats (Rimondini *et al*, 2002). There also are several other pharmaceuticals (clinical or experimental) acting on a variety of neurotransmitter systems such as glutamate, GABA, dopamine and opioids, or on stress, which notably decrease alcohol intake in postdependent rats (for review, see (Meinhardt *et al*, 2015)).

The *construct validity* of the postdependent animal model is also well established, for example in studies combining human postmortem samples of alcoholics and controls with the

postdependent rat model to investigate brain glutamate levels (Hermann *et al*, 2012) and the dopamine system (Hirth *et al*, 2016b).

In summary, the postdependent animal model can be very effectively used to study neuroadaptations after chronic alcohol abuse, during the withdrawal period and abstinence, and has therefore been used for several studies in this thesis.

1.5. Comorbidity with other mental disorders

Comorbidity is the occurrence of at least one disorder or disease in addition to the primary disorder/disease. However, this definition is very broad and therefore unsuitable to effectively study and measure the phenomenon (Fortin *et al*, 2007). Nevertheless there are several studies investigating the comorbidity of mental disorders, in particular alcohol dependence, or AUD, with nicotine dependence, schizophrenia and depression.

The comorbidity of AUD with other mental disorders can partly be explained by the pronounced genetic influence in all of these disorders. The heritability is estimated at 50% or more in alcohol dependence (Heath *et al*, 1997; Prescott and Kendler, 1999; Sullivan *et al*, 2012), nicotine dependence (Li, 2006), schizophrenia (Fullard *et al*, 2016), and depression (Chaudhury *et al*, 2015). Genetic facts combined with environmental factors may act on the same neurotransmitter or hormone systems, or even the same ion channels, in drug dependence, schizophrenia, and depression. Through the investigation of the underlying cause of the disorders, the connections between them will be better understood.

1.5.1. Nicotine dependence

Nicotine is the main component in tobacco providing its reinforcing effects. As a legal drug, tobacco is consumed worldwide as part of many cultures, even though it is a well-known fact that it has often deadly consequences when used as intended by the manufacturer. According to the WHO, about six million lives are lost per year because of tobacco use. Not only would these often premature deaths be easily avoidable if people ceased smoking, a tenth of these deaths are the effects of second-hand smoke (World Health Organization, 2015).

Decreasing these deaths due to tobacco use is not easy, especially considering that nicotine has highly addictive properties. Once an individual becomes nicotine dependent, a relapse even after long periods of abstinence is very likely. In fact, only 3-6% of people who quit smoking manage to stay abstinent for six to twelve months. The majority relapses within the first eight days (Hughes *et al*, 2004).

The DSM-5 classification of Tobacco Use Disorder (TUD) (American Psychiatric Association, 2014) includes three main criteria: the consumption of larger quantities than intended over a longer period, the development of tolerance, and the appearance of withdrawal symptoms. Each criterion is divided into sub-features for a more comprehensive classification.

Neuroadaptations present in nicotine dependence can be studied in animal models of repeated nicotine exposure and are assessed according to tolerance development, sensitization, and withdrawal symptoms (Stolerman, 1999). In sensitization experiments, which were also performed in this thesis, the increase in locomotor activity as a result of repeated nicotine administration is measured. This effect can already be seen after few nicotine injections, depending on the dose, and appears shortly after the injection, lasting for over 90 min (Ksir *et al*, 1987).

Next to place conditioning and intracranial self-stimulation, nicotine self-administration (SA) is the paradigm of choice to study the rewarding effects of nicotine (O'Dell and Khroyan, 2009). Here, nicotine is used as a reinforcer of a behavioral task, most often the pressing of a lever. The voluntary administration of nicotine reflects a high degree of face validity. Furthermore, the SA model displays a strong predictive validity, as pharmacotherapies already in use for tobacco abuse have reportedly decreased nicotine SA in rodents (DeNoble and Mele, 2006; Lerman *et al*, 2007). There is also a strong construct validity, considering that smoking, just like nicotine SA, is influenced by drug-associated cues, stress, and a number of other factors (O'Dell *et al*, 2009).

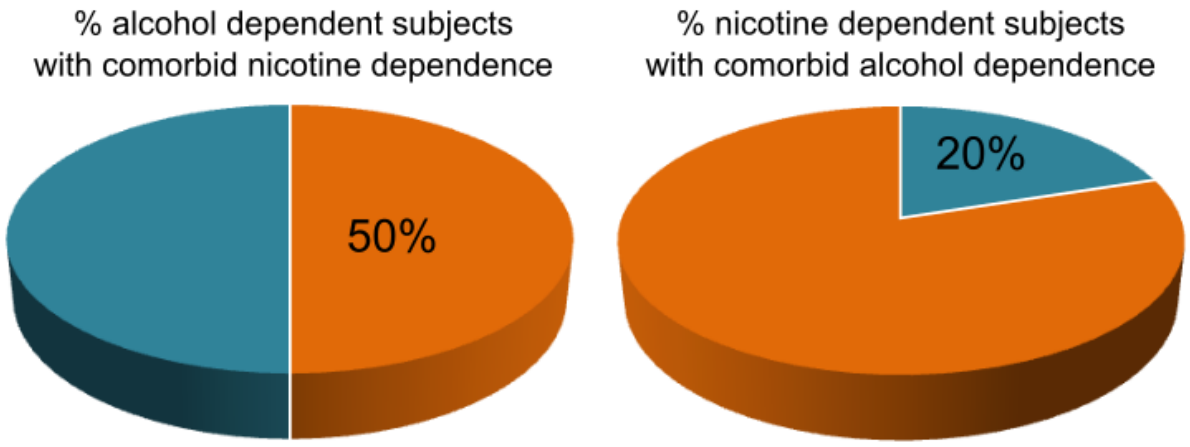

Figure 4. Alcohol and nicotine dependence show a high comorbidity. Alcohol dependence is shown in turquoise.

Studies on human subjects, rather than animal models, have shown a high comorbidity of nicotine dependence with alcohol dependence. In fact, alcohol dependent or abusing subjects are more likely to smoke than non-drinkers (Dawson, 2000). In 2004, a study showed that about 50% of alcohol dependent subjects were also dependent on nicotine, while almost every fifth smoker displayed signs of alcohol dependence (Grant *et al*, 2004). The severity of tobacco use and nicotine dependence rises in a dose-dependent manner with increasing alcohol consumption (Falk *et al*, 2006). It has been suggested that this relationship may in part be due to an interaction of the pharmacological effects of alcohol and nicotine, with cross-reinforcement and cross-tolerance as promotors of the development and maintenance of the use of and dependence to both substances (Oliver *et al*, 2013). Furthermore, brain regions controlling salience responses are activated by both alcohol and nicotine cues (Liu *et al*, 2014a), and the nicotinic acetylcholine system is modulated by repeated alcohol consumption, suggesting an influence of alcohol on nicotine-related neuroadaptations (Hillmer *et al*, 2014).

Apart from its comorbidity with and similarities to alcohol dependence, nicotine dependence also shows a high comorbidity with other mental disorders such as schizophrenia and depression (Tidey and Miller, 2015).

1.5.2. Schizophrenia

Schizophrenia is a chronic neuropsychiatric disorder with heterogeneous symptoms. In a study across ten countries, the WHO determined that about 1% of the word population are afflicted by schizophrenia (Jablensky *et al*, 1992). However, given the high diversity of manifestations, it is difficult to accurately estimate the incidence of schizophrenia.

There are three categories of symptoms of schizophrenia. Positive symptoms are generally an addition of traits which are not displayed by a healthy person. Amongst others, they entail alterations of perception, such as hallucinations or delusions. Negative symptoms describe the decrease in normal behavioral characteristics, for example the lack of motivation or pleasure, often leading to social isolation (Feifel *et al*, 2015). The third category are cognitive deficits, including visual and verbal learning and memory, attention/vigilance, working memory, reasoning and problem solving information processing speed, and social cognition. For each category, different or overlapping neurotransmitter and hormone systems are involved. For example, positive symptoms are due partly to a dysregulation of the dopamine system, while monoamine oxidases play a role in the appearance of negative symptoms, and the CRH

system is in part responsible for cognitive dysfunctions. Other neurotransmitters and hormones are involved in two or three symptom categories (Rich and Caldwell, 2015).

Although the three symptom categories are quite comprehensive, the manifestation is very diverse amongst schizophrenic patients. The definition of schizophrenia in the DSM-5 (American Psychiatric Association, 2014) accounts for that by differentiating between essential (delusions, hallucinations, and disorganized speech) and non-essential (grossly disorganized or catatonic behavior, and negative symptoms, i.e. diminished emotional expression or avolition) symptoms. The ICD-10 (World Health Organization, 1992) even distinguishes between different types of schizophrenia, such as paranoid, hebephrenic, or catatonic schizophrenia.

There are several antipsychotic drugs which can be used to treat the symptoms of schizophrenia, and new drugs are approved by the U. S. Food and Drug Administration (FDA) every year. At least 10 different drugs have been approved since the year 2000. However, existing antipsychotic drugs are mainly focused on reducing positive symptoms, whereas the treatment of the equally disabling negative symptoms and cognitive deficits is much less effective (Carpenter and Koenig, 2008; Feifel *et al*, 2015; Kirkpatrick *et al*, 2006).

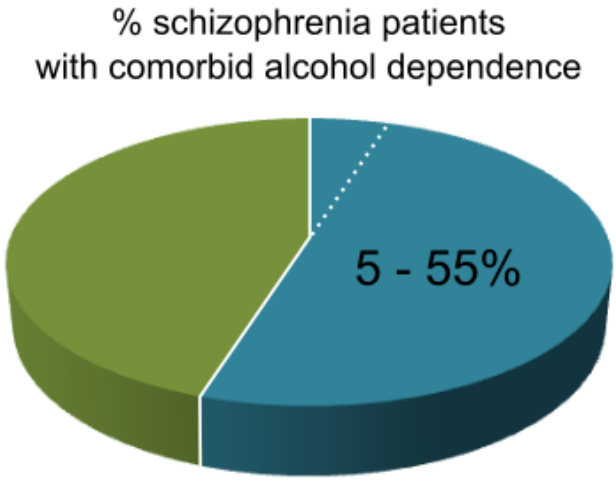

Figure 5. Accounts of schizophrenia patients with comorbid alcohol dependence varies across studies. Alcohol dependence in schizophrenia patients is shown in turquoise.

A problem in the treatment of schizophrenia is its high comorbidity with AUD. Adverse effects on adherence to the treatment, as well as an increase in negative disease outcomes have been attributed to the co-occurrence of schizophrenia and AUD (McLean *et al*, 2012; Murthy and Chand, 2012). In addition, comorbidity between AUD and schizophrenia has been linked to a significantly higher mortality (Hjorthoj *et al*, 2015). The exact percentage of

schizophrenia patients with AUD varies strongly throughout systematic reviews, ranging from 5% to 55% (Cantor-Graae *et al*, 2001; Koskinen *et al*, 2009; Mueser *et al*, 1990; Nesvag *et al*, 2015) due to the diversity of symptoms and differences in definitions. Regardless of these statistical values, the treatment of schizophrenia patients with comorbid AUD has to consider the impact of alcohol dependence on the treatment for schizophrenia itself. Fortunately, the administration of naltrexone and disulfiram in addition to antipsychotic medication has proven safe (Azorin *et al*, 2016).

1.5.3. Depression

Major depressive disorder (MDD) is a mood disorder with complex underlying causes and a ~40 % heritability (Kendler *et al*, 2006). With a lifetime prevalence of about 16.2% (Kessler *et al*, 2005) and a high suicide rate amongst affected subjects (Fairweather-Schmidt *et al*, 2009), MDD has devastating consequences on the life of patients and their families.

Although "depression" is a widely used term and easily self-diagnosed, there are actually very specific criteria laid out by the DSM-5 (American Psychiatric Association, 2014). Symptoms of MDD include a depressed mood for most of the day, diminished interest or pleasure, weight loss, fatigue or loss of energy, suicidal thoughts, and others. To classify for an MDD diagnosis, the symptoms have to be persistent for at least two weeks.

There are a variety of possible underlying causes of MDD. Next to a decrease in neurogenesis evidenced by reduced levels of the brain-derived neurotrophic factor (BDNF) (Brunoni *et al*, 2008), MDD patients often show signs of oxidative stress (Lopresti *et al*, 2014) and inflammation (Valkanova *et al*, 2013). In addition, there are anatomical and functional changes of the brain, such as a reduction in cerebellar volume (Soares and Mann, 1997) and decreased dorsolateral prefrontal cortex activation during tasks of emotional control (Hamilton *et al*, 2012).

Stress during early life or adulthood is often the cause of MDD (Darcet *et al*, 2016). Animal models of depression are therefore often anxiety or stress models, with stress occurring at various times and length (Blanchard *et al*, 2001; Chourbaji *et al*, 2005; David *et al*, 2009; Nishi *et al*, 2014). These studies have found severe cognitive impairments in stressed rodents, with a decrease in attention and executive function, as well as different types of memory dysfunctions (Darcet *et al*, 2016).

As with schizophrenia, there is a high comorbidity between AUD and MDD. About 27% of alcohol dependent patients are also affected by depression, while about 16% of MDD patients are affected by AUD (Regier *et al*, 1990). MDD also has an influence on the success of staying abstinent to alcohol: it was shown that male, severely depressed patients have a 20% higher likelihood of relapse (Beck *et al*, 1961).

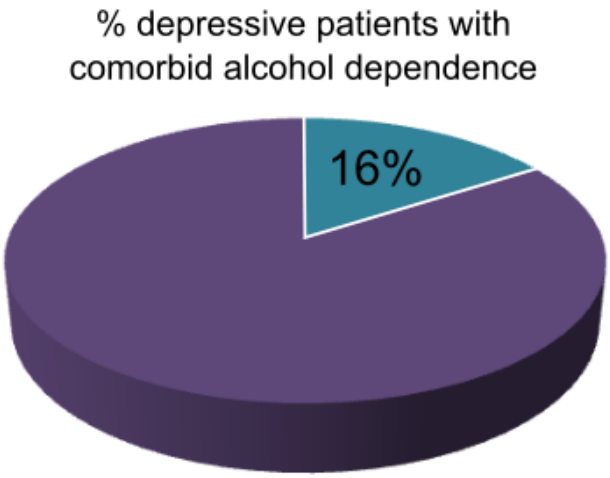

Figure 6. About 16% of depressive patients are also dependent on alcohol. Alcohol dependence is shown in turquoise.

There are several neurotransmitter and hormone systems which are dysregulated in both alcohol dependence and MDD. Considering the role of stress in the development of depression, alterations in the CRH system might at least in part explain the high comorbidity rates. In addition, LTCCs, which are both affected by alcohol and involved in the mediation of its effects, are regulated by the CRH system, which is to say by stress (Joels and Karst, 2012). They might therefore be a promising target for the development of a treatment targeting both comorbid disorders.

2. L-type calcium channels

The balance of intra- and extracellular calcium concentrations is crucial for many functions within and the survival of a cell, with intracellular calcium ranging between 10^{-7} to 10^{-5} M, and a higher extracellular concentration of 2 mM. Among a multitude of calcium channels regulating calcium concentrations and modulating cell functions through calcium signaling are high-voltage activated L-type calcium channels (LTCCs). Other types, namely N-, P-/Q-, and R-type, are also classified as high-voltage activated, while T-type calcium channels are considered to be low-voltage activated (Catterall *et al*, 2005; Moosmang *et al*, 2005b).

2.1. L-type calcium channels

Within the LTCC family, also named Ca_V1, four subtypes ($Ca_V1.1$ – $Ca_V1.4$) have been identified by their sensitivity to dihydropyridines (DHPs) (Moosmang *et al*, 2005b). In addition, they all show relatively slow activation kinetics, a large single-channel conductance and a long-lasting calcium-dependent inactivation (Lipscombe *et al*, 2004). The subtypes differ in their α_1 subunit (α_1S, α_1C, α_1D, and α_1F), a 2000 amino acid protein consisting of four repeated domains with six transmembrane segments (Catterall, 2000; Ertel *et al*, 2000; Tanabe *et al*, 1987).

Next to this pore-forming subunit (Catterall, 2000; Tanabe *et al*, 1987), LTCCs consist of $\alpha_2\delta$ and β subunits (Catterall, 2000; Curtis and Catterall, 1984; Striessnig *et al*, 1987) which enhance expression level of the LTCCs and regulate gating properties (Lacerda *et al*, 1991; Singer *et al*, 1991), and, depending on their location, γ subunits (Ahlijanian *et al*, 1990; Chang and Hosey, 1988; Curtis *et al*, 1984; Kuniyasu *et al*, 1992; Schneider and Hofmann, 1988) which seem especially involved in voltage-dependent inactivation (Singer *et al*, 1991).

LTCCs are widely expressed throughout the body. $Ca_V1.1$ is found in skeletal muscle cells (Burge and Hanna, 2012; Jorquera *et al*, 2013; Moosmang *et al*, 2005b; Striessnig *et al*, 2010), and $Ca_V1.4$ in the retina (Baumann *et al*, 2004; Doering *et al*, 2007; Moosmang *et al*, 2005b; Striessnig *et al*, 2010). $Ca_V1.2$ and $Ca_V1.3$ show a much more extensive expression pattern and are often expressed within the same tissues, such as ventricular cardiac muscle, smooth muscle, pancreatic cells and neurons (Bohn *et al*, 2000; Lipscombe *et al*, 2004; Moosmang *et al*, 2005b; Moosmang *et al*, 2003; Striessnig *et al*, 2010). On a subcellular level, in neurons LTCCs are localized mostly at the soma and proximal dendrites (Hell *et al*, 1993; Simon *et al*, 2003). Although $Ca_V1.2$ and $Ca_V1.3$ are found in the same compartments of the neurons, they still show a differential distribution within these general areas (Hell *et al*,

1993; Westenbroek *et al*, 1998), with $Ca_V1.2$ being organized in clusters and $Ca_V1.3$ showing an even distribution with decreasing density toward more distal parts of the dendrites (Hell *et al*, 1993).

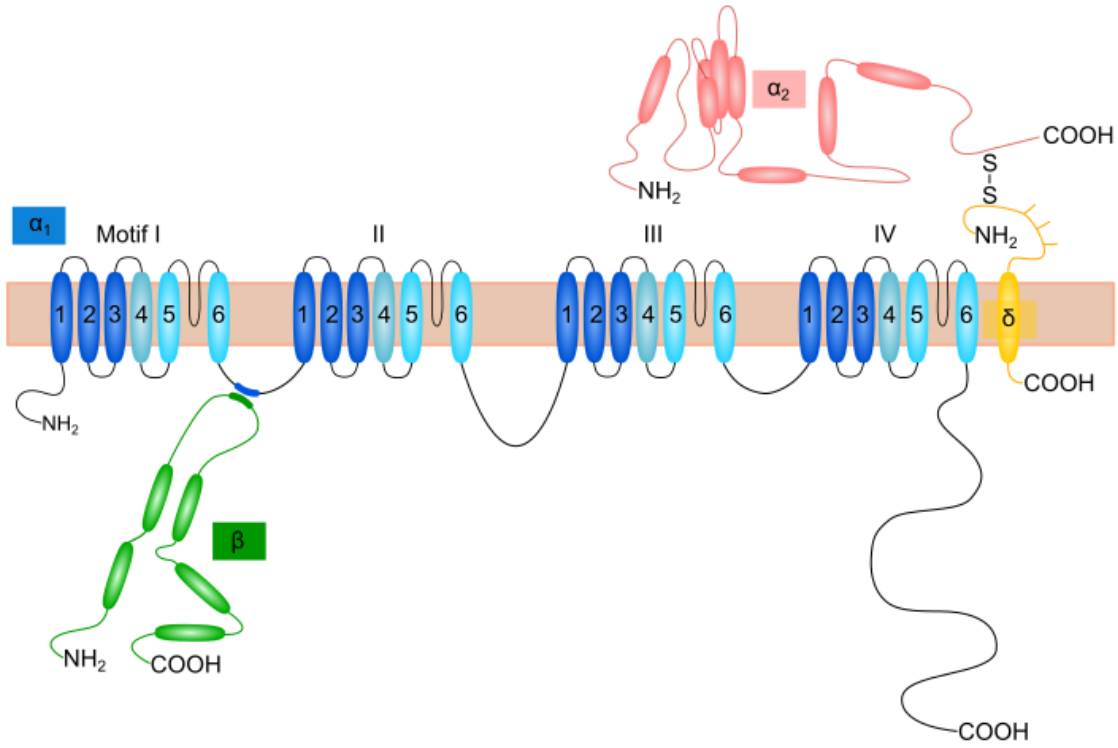

Figure 7. Structure of LTCC subunits found in all subtypes. The pore-forming α1 subunit consists of 4 homologous repeated motifs with 6 transmembrane segments each, containing interaction sites, voltage sensors, phosphorylation sites and other essential sites. The α2δ subunit is linked to the α1 domain through a transmembrane segment, while the β subunit interacts with the α1 subunit via a cytoplasmic linker. Adapted from (Bodi *et al*, 2005).

2.2. Subtypes $Ca_V1.2$ and $Ca_V1.3$ in neurons

As the only two LTCC subtypes expressed in neurons, $Ca_V1.2$ and $Ca_V1.3$ play important roles in processes such as gene expression (Bading *et al*, 1993; Deisseroth *et al*, 1998; Dolmetsch *et al*, 2001; Finkbeiner and Greenberg, 1998) and mRNA stability (Schorge *et al*, 1999), the release of neurotransmitters (Bean, 1989), synaptic efficacy (Christie *et al*, 1997) and the regulation of other ion channels (De Koninck and Cooper, 1995; Marrion and Tavalin, 1998). The signaling from LTCCs to the nucleus is mediated via different routes.

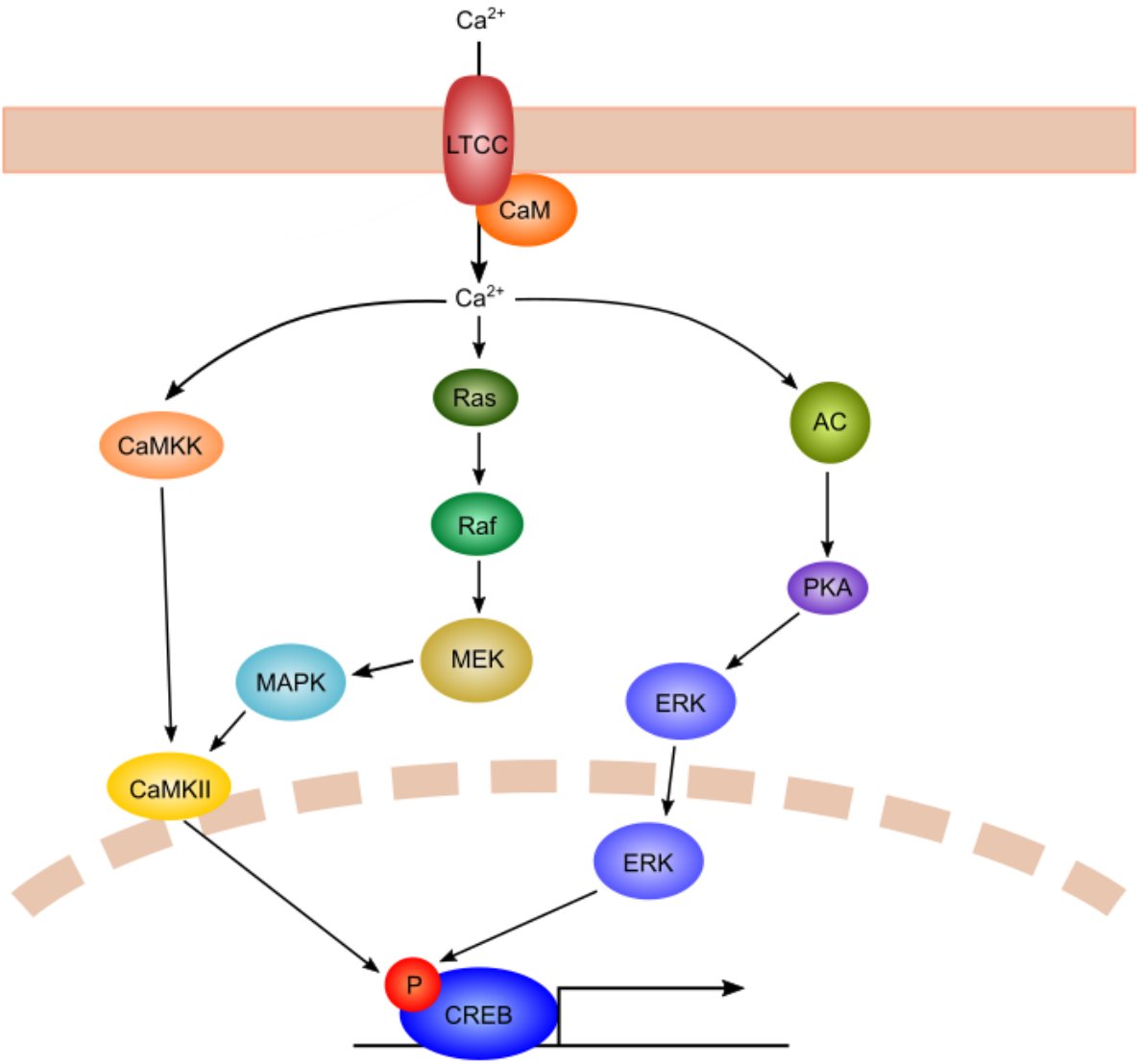

Figure 8. LTCC signaling pathways act alone or together in phosphorylating the transcription factor CREB. ERK is translocated to the nucleus, while the MAPK pathway and CaMKK both activate CaMKII, which then propagates the signal into the nucleus to change transcription of various genes.

The Ras/mitogen-activated protein kinase (MAPK) pathway and the extracellular signal-related protein kinase (ERK) pathway are activated through Ras and Raf (Ebert and Greenberg, 2013; West *et al*, 2002). Binding of Ca^{2+}-calmodulin (CaM) to the LTCCs is crucial for this signaling cascade (Dolmetsch *et al*, 2001). In the MAPK pathway, activation of CaM Kinase II (CaMKII) propagates the signal towards the nucleus (Jenkins *et al*, 2010; Lee *et al*, 2009; Wheeler *et al*, 2008), where phosphorylation of the cAMP response element-binding protein (CREB) regulates the transcription of various genes (Wheeler *et al*, 2008; Zhang *et al*, 2006). ERK is translocated to the nucleus with the help of the protein kinase A (PKA) and activates CREB (Impey *et al*, 1998). The PKA itself is activated by a calcium-dependent adenylate cyclase (AC) (Ebert *et al*, 2013). Another route is the activation of the CAMK kinase (CAMKK) by calcium influx through LTCCs. CaMKK in turn activates

CaMKII (Ebert *et al*, 2013). Transcription of many genes is influenced by a combination of these LTCC pathways, in addition to a multitude of other neurotransmitter and ion channel pathways. One example for transcription activation via CREB is the immediate early gene c-fos (Rubil *et al*, 2016).

Transcription of LTCCs themselves, in particular $Ca_V1.2$, is auto-regulated by a C-terminal cleavage product, which is formed at post-translational processing of the channel (Gao *et al*, 2001; Schroder *et al*, 2009). Additionally, CaMKII also regulates $Ca_V1.2$ expression (Ronkainen *et al*, 2011), and $Ca_V1.2$ activity is increased by the protein kinase A (PKA) (Fuller *et al*, 2014). The regulation of $Ca_V1.3$ has not been investigated in detail, although functional regulation of both LTCC subtypes is achieved through alternative splicing (Huang *et al*, 2013; Tang *et al*, 2004).

The individual contributions of $Ca_V1.2$ and $Ca_V1.3$ to their different functions are not well defined, which is mostly due to the fact that the differentiation between the subtypes is a challenging task. Although the two subtypes are not equally well inhibited by DHP antagonists (Koschak *et al*, 2001; Lipscombe *et al*, 2004; Xu and Lipscombe, 2001), there are no agonists or antagonists available which act only on one of the subtypes but not the other. Furthermore, the use of DHPs *in vivo* is complicated by the fact that cardiac and smooth muscle LTCCs are also affected in case of i.p. injection (Moosmang *et al*, 2005b). For an exclusive insight into the function of central LTCCs, i.c.v. injections are therefore necessary.

There are some differences between $Ca_V1.2$ and $Ca_V1.3$, however, which allow the distinction between these subtypes in certain experimental approaches. On a transcriptional level, *in situ* hybridization using subtype-specific probes can be used to map the mRNA expression of either $Ca_V1.2$ or $Ca_V1.3$ (Liebmann *et al*, 2008). Considering electrophysiological properties, although all LTCCs have been classified as high-voltage activated channels, $Ca_V1.3$ appears to differ from the other LTCC subtypes.

While $Ca_V1.2$ opens at a membrane potential of about -30 mV (Lipscombe *et al*, 2004), the $Ca_V1.3$ subtype starts to activate at a much lower voltage of -55 mV (Koschak *et al*, 2001; Lipscombe *et al*, 2004; Xu *et al*, 2001), thereby resembling rather the low-voltage activated T-type channels in this respect.

Especially in recent years, these properties and approaches have been used to identify specific roles of each subtype under normal conditions and in several disorders, mental or otherwise.

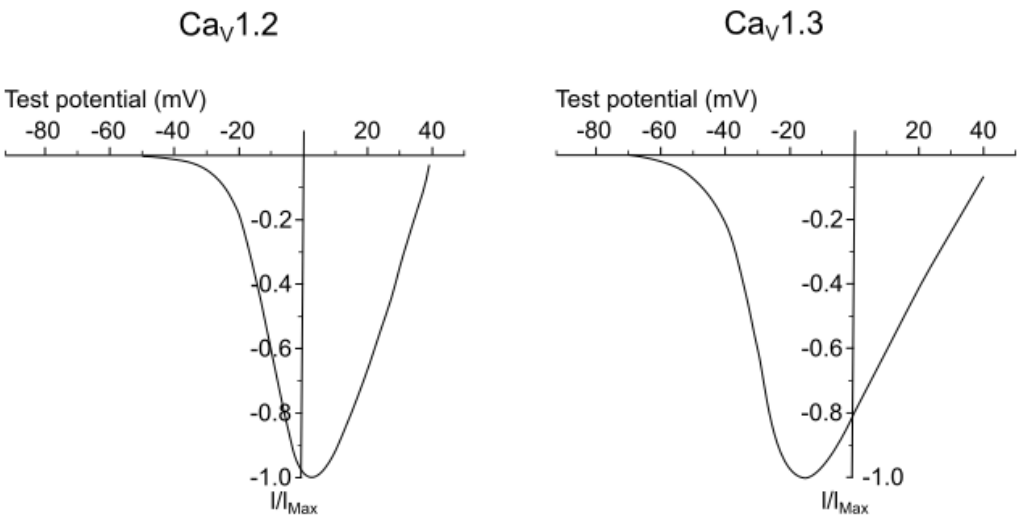

Figure 9. Activation of LTCC subtypes $Ca_V1.2$ and $Ca_V1.3$. Normalized peak current-voltage relationships differ between the subtypes, with an activation midpoint ($V_{1/2}$) of -5 mV for $Ca_V1.2$ and a much lower $V_{1/2}$ of -30 mV for $Ca_V1.3$. Adapted from (Lipscombe, 2002).

2.3. Pharmacology of LTCCs

LTCCs can be modulated by DHPs (nifedipine, amlodipine, isradipine, and others), phenylalkylamines, such as verapamil, or benzothiazepines, such as diltiazem (Moosmang *et al*, 2005b; Zamponi *et al*, 2015). The binding sites for all three classes are close to the pore-forming segments of the α1 subunit and overlap each other on many amino acid residues (Dilmac *et al*, 2003; Hockerman *et al*, 1997; Schuster *et al*, 1996). However, the mechanism by which they inhibit or activate the LTCC differs. DHPs can function as either activators or inhibitors (de Beun *et al*, 1996b) which leads to the conclusion that they do not block the pore but shift the channel toward the open or closed state (Moosmang *et al*, 2005b). Phenylalkylamines on the other hand occlude the pore from the cytoplasmic side (Triggle, 1991a, b), while benzothiazepines bind to the extracellular part of the pore (Hering *et al*, 1993; Seydl *et al*, 1993).

In our studies, we used the LTCC antagonists verapamil and nifedipine.

Verapamil

Verapamil is a phenylalkylamine which has been approved as an antiarrhythmic by the U. S. FDA in March 1982. In the WHO Model List of Essential Medicines from 2013, it is listed as antianginal and antiarrhythmic medicine (World Health Organization, 2013b). It has also been suggested as a treatment for cluster headaches (Leone *et al*, 2000).

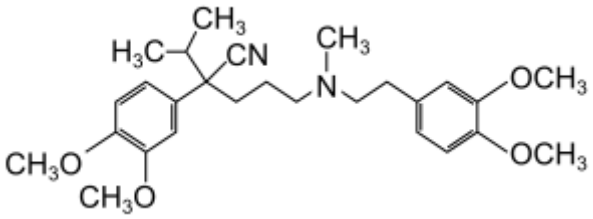

Figure 10. Chemical structure of verapamil.

Verapamil has been used in scientific studies as an LTCC antagonist (Nayler and Poole-Wilson, 1981), to investigate the role of LTCCs under healthy conditions and in mental disorders, e.g (Abe *et al*, 2009; Budzynska *et al*, 2012; Seoane *et al*, 2009). In many studies verapamil has been administered i.p., however this LTCC antagonist displays a low permeability for the blood-brain barrier (Bhat *et al*, 2012). For the investigation of central LTCCs, verapamil should therefore be administered either locally within specific brain regions, or i.c.v. for a more general brain-wide approach.

Nifedipine

The usage of nifedipine, a DHP, as antihypertensive medication has been approved by the U. S. FDA in December 1999. It is also listed in the WHO Model List of Essential Medicines from 2013, however here its use is described as antioxytocic (World Health Organization, 2013b).

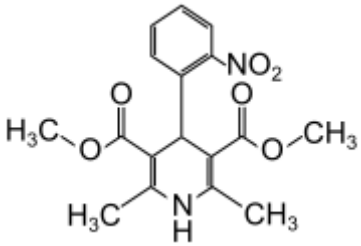

Figure 11. Chemical structure of nifedipine.

In scientific research, nifedipine has been used as a calcium channel antagonist as early as the 1980s (Nowicki *et al*, 1982) and is still used widely used today, e.g. (Bernardi *et al*, 2014; Daschil and Humpel, 2014; Daschil *et al*, 2015; Kouvaros *et al*, 2015). In addition, it is discussed as possible treatment for other disorders such as Parkinson's Disease and neuropsychiatric disorders (Striessnig *et al*, 2015). However, these hypotheses have not yet surpassed the stage of preclinical investigations.

2.4. Animal models to investigate $Ca_V1.2$ and $Ca_V1.3$

Unlike pharmacological manipulation the use of animal models allows for the explicit differentiation between $Ca_V1.2$ and $Ca_V1.3$. While conventional knockouts (KO) of $Ca_V1.2$ are not viable (Seisenberger *et al*, 2000), conventional KO of $Ca_V1.3$ experience deafness and sinoatrial node dysfunction (Platzer *et al*, 2000), both of which prohibit the useful interpretation of behavioral experiments. However, there are animal models which can be used to shed light on the different functions of both LTCC subtypes.

One such model is the $Ca_V1.2$DHP-/- mice, in which $Ca_V1.2$ has been rendered insensitive to the modulation by DHPs (Sinnegger-Brauns *et al*, 2004). Agonism or antagonism by DHPs therefore only affects $Ca_V1.3$, while $Ca_V1.2$ function remains intact. However, this requires the administration of a DHP agonist or antagonist, which adds more factors (for example dose and time of administration) to be considered.

Another way is to create conditional KOs. Here, the KO of either $Ca_V1.2$ or $Ca_V1.3$ can be switched on in a time- and cell type-specific manner. The Cre/loxP system is often used for the creation of conditional KOs. In this system, loxP ("locus of C-over of P1")-sites, 34-bp sequences, are introduced into a rodent so that they flank the gene of interest, or a functionally crucial part of the gene. When this rodent is mated with an animal expressing the Cre recombinase, the offspring will possess both the enzyme and the loxP sites. The Cre recombinase recognizes the loxP sites and excises the sequence in between, thus creating a functional KO (Jaisser, 2000).
The time- and cell type-specific manner of the KO depends on the expression and localization of the Cre recombinase. One system to achieve a time-specific KO is the Cre^{ERT2} system, based on tamoxifen-dependent cre recombinases (Branda and Dymecki, 2004). In this system, the Cre recombinase is coupled to the estrogen receptor, which is retained in the cytosol by the heat shock protein 90 (Hsp90). When tamoxifen, a specific antagonist of the estrogen receptor, is introduced, it displaces Hsp90 and thereby exposes a nuclear localization sequence on the estrogen receptor. The receptor, together with the Cre recombinase, is translocated to the nucleus, where the recombinase is free to excise the fragment flanked by loxP sites (Garcia and Mills, 2002).

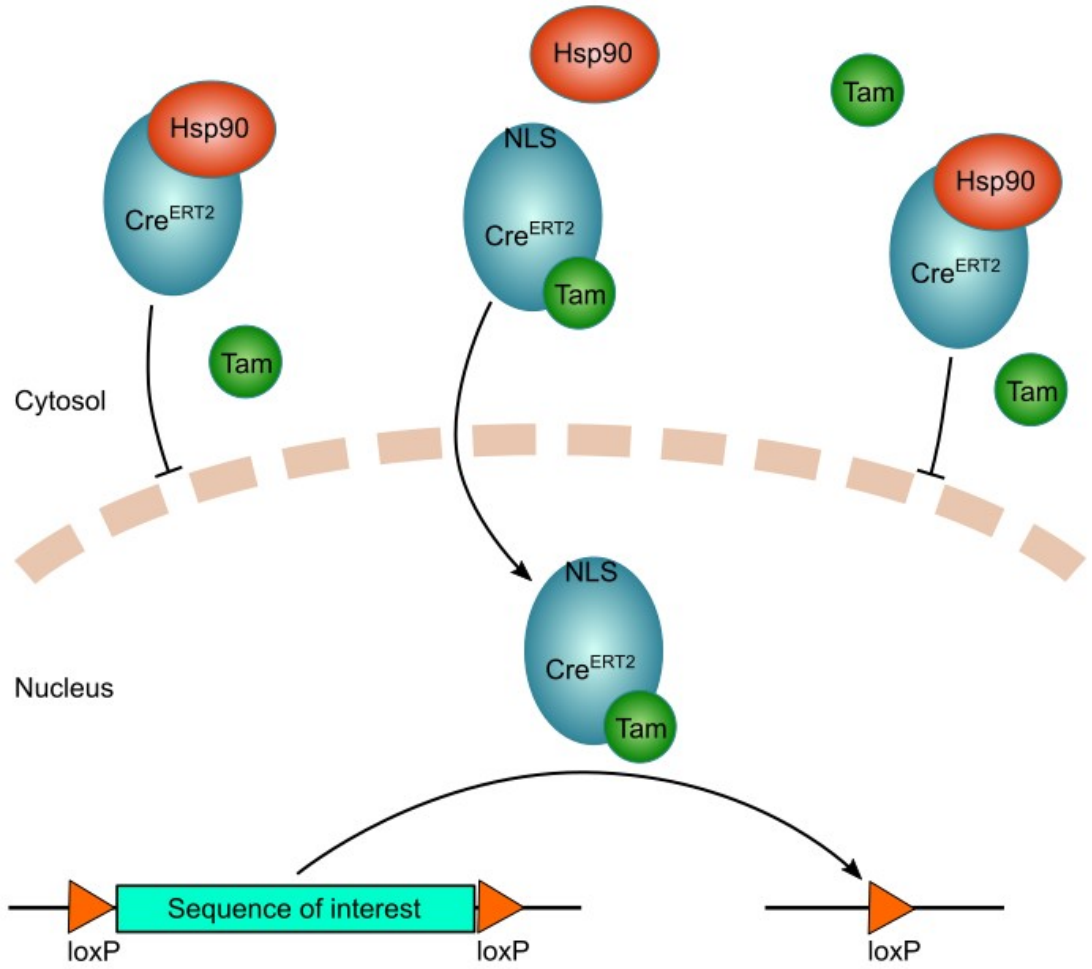

Figure 12. The CreERT2/loxP system enables a time- and cell type-specific conditional KO. Heat shock protein 90, Hsp90; nuclear localization sequence, NLS; tamoxifen, Tam.

For the cell type-specific KO, it is imperative to carefully choose the promotor under which the Cre recombinase is expressed. Two existing mouse lines are CaMKII-Cre^{ERT2} and Nestin-Cre mice. Both lines have been used for conditional KO of $Ca_V1.2$ and $Ca_V1.3$ in our lab. However, as nestin is expressed in neural progenitor cells and neural stem cells (Liang *et al*, 2012), the KO of the respective LTCC subtype early during development has led to motor impairment (Dr. Dusan Bartsch, personal communication). The CaMKII-Cre^{ERT2} line, on the other hand, allows for activation of the KO during adulthood, through the systemic injection of tamoxifen. Additionally, it is highly expressed throughout the brain, making up as much as 1% of total protein in the forebrain, and even 2% in the hippocampus (Erondu and Kennedy, 1985). Until recently, CaMKII was regarded as a marker for excitatory transmission (Benson *et al*, 1992; Jones *et al*, 1994). However, it has recently been shown that CaMKII may also be involved in inhibitory transmission of GABAergic interneurons to the basolateral amygdala (BLA) (Huang *et al*, 2014). Given its high expression and wide distribution within different

neurons, a conditional KO based on CaMKII expression is a promising tool for the analysis of individual $Ca_V1.2$ and $Ca_V1.3$ functions.

2.5. Single nucleotide polymorphisms as risk variants

SNPs, alterations of a single nucleotide, are found throughout the entire genome, often without any consequence. There are, however, SNPs which have been associated with the risk of developing diseases or disorders. One such SNP within the *CACNA1C* gene is rs1006737, located in the intron region 3 (Fiorentino *et al*, 2014), where a guanosine (G) has been replaced by adenosine (A). The ancestral homozygous GG does not confer any risk, while AA and AG carriers are at a higher risk to develop mental disorders (Nieratschker *et al*, 2015). The mechanism behind the SNP function is unknown, but an increase of *CACNA1C* expression has been found in the amygdala and hippocampus of risk allele carriers (Bigos *et al*, 2010).

2.6. LTCCs in mental disorders: state of knowledge

LTCCs have been implicated in several mental disorders, amongst them drug dependence, schizophrenia, depression (Backes *et al*, 2014), bipolar disorder (Uemura *et al*, 2015) and autism (Lu *et al*, 2012).

2.6.1. Alcohol dependence

The inhibitory effect of alcohol on central calcium channels has been discovered as early as 1980 (Harris and Hood, 1980). Three years later, voltage-gated calcium channels were proposed as one of the principal mediators of the effects of alcohol (Lynch and Littleton, 1983). Of the different types of voltage-gated calcium channels, only the expression of LTCCs is changed in alcohol dependent rodents (Dolin *et al*, 1987; Katsura *et al*, 2006; Katsura *et al*, 2005b) However, in these studies the rats and mice were only exposed to alcohol vapor for 7 and 8 days, respectively, and were intoxicated at the time of sacrifice. The increase of LTCC expression therefore is most likely due to the acute influences of alcohol, not dependence, which only sets in after several cycles of alcohol vapor intoxication and abstinence.

LTCCs also play an important part in the consumption of alcohol. It has been shown that systemic antagonism of LTCCs decreases the preference for alcohol in a free-choice paradigm (Engel *et al*, 1988). Interestingly, the preference and the consumption of alcohol is reduced in rats with a genetic disposition to alcohol drinking (AA rats, see 1.4) by systemic administration of the LTCC agonist BayK8644 (de Beun *et al*, 1996b) as well as several

LTCC antagonists, including verapamil and nifedipine (De Beun *et al*, 1996a). In alcohol-preferring (P) and "Sardinian ethanol-preferring" (sP) rats, alcohol consumption was decreased by verapamil and other LTCC antagonists (Fadda *et al*, 1992; Rezvani and Janowsky, 1990), with no effect on the non-preferring (NP) line (Rezvani *et al*, 1990).
Furthermore, alcohol withdrawal symptoms can be decreased in alcohol dependent rodents through the antagonism of LTCCs (Bone *et al*, 1989; Colombo *et al*, 1995; Little *et al*, 1986).
So far, investigations of alcohol consumption and preference have been conducted mostly using free-choice paradigms, either in wild-type rodents or in rats with a genetic predisposition to increased alcohol intake. There is, however, a lack of studies on the impact of LTCCs in alcohol dependent rats, especially considering the increased LTCC expression in alcohol dependent rodents. In our own lab, expression data from Affymetrix GeneChip arrays from the medial prefrontal cortex of 3 weeks abstinent alcohol dependent and non-dependent rats (Meinhardt *et al*, 2013) showed a gradual decrease of *Cacna1d* gene expression during the development of alcohol dependence (unpublished data), suggesting the LTCC subtypes as an interesting target for further research.

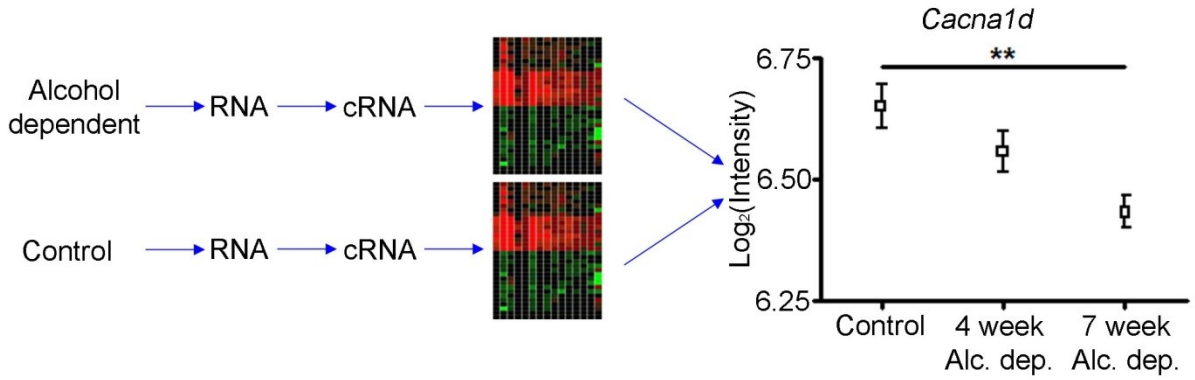

Figure 13. Transcriptome analysis from the medial prefrontal cortex of alcohol dependent and non-dependent rats suggests *Cacna1d* mRNA decrease over the course of dependence development. Rats were exposed to intermittent cycles of alcohol vapor for 4 weeks or 7 weeks and sacrificed 3 weeks after last vapor cycle.

Furthermore, attributable to the lack of subtype-specific LTCC modulators, little is known about the individual roles of $Ca_V1.2$ and $Ca_V1.3$ in alcohol dependence.

2.6.2. Nicotine-related behavior

Similar to alcohol dependence, LTCC expression is increased after chronic nicotine treatment (Hayashida *et al*, 2005; Katsura and Ohkuma, 2005a). Furthermore, nicotine-induced hyperlocomotion may be mediated by LTCCs, as it could be prevented by pretreatment with nimodipine (Hart *et al*, 1996). In an elevated plus maze paradigm, anxiety-related responses to an acute nicotine injection were decreased by LTCC antagonists (Biala and Budzynska,

2006). The development of tolerance after chronic nicotine administration was attenuated as well, with the antagonists being administered prior to every nicotine injection (Biala *et al*, 2006). LTCC antagonists were able to prevent nicotine sensitization as well as place preference, if injected prior to nicotine administration (Biala, 2003). There is also evidence for the involvement of LTCCs in the reinstatement of nicotine-induced place preference (Biala and Budzynska, 2008), and in nicotine withdrawal (Jackson and Damaj, 2009).
As in alcohol dependence, there is a substantial lack of studies investigating the potentially different functions of $Ca_V1.2$ and $Ca_V1.3$.

2.6.3. Schizophrenia

In schizophrenia, studies have mainly focused on $Ca_V1.2$, or rather *CACNA1C*, as several single nucleotide polymorphisms (SNPs) have been proposed as risk variants in human schizophrenics. There is one risk variant, rs1006737, which appears to be particularly relevant in schizophrenia (Jiang *et al*, 2015; Lancaster *et al*, 2015; Porcelli *et al*, 2015). An association with the improvement in the Positive and Negative Symptom Scale (PANSS), which is used to determine the severity of schizophrenic symptoms, has been determined for other, less commonly investigated SNPs (Porcelli *et al*, 2015).
Although the importance of *CACNA1C* SNPs suggests a role for this LTCC in schizophrenia, the extent to which the expression of these channels may be altered, or which role they might play in the development or expression of schizophrenic symptoms, is yet unknown.

2.6.4. Depression

LTCCs have been associated with MDD in both human and animal studies. As in schizophrenia, SNPs in *CACNA1C* are known as susceptibility markers for depression (Bhat *et al*, 2012). The polymorphism rs1006737, which is particularly relevant for schizophrenia as well, has been implicated in changes of functional connectivity of prefrontal brain regions and the cerebellum (Backes *et al*, 2014). The importance of $Ca_V1.2$ in depression is supported by a study in mice, where *Cacna1c* haploinsufficiency decreased exploratory behavior, response to amphetamine and antidepressant-like behavior (Dao *et al*, 2010). In addition, activation or blockage of LTCCs by DHPs has mood-modifying consequences (Sinnegger-Brauns *et al*, 2004). However, as noted earlier, MDD is a very diverse disorder with multiple underlying causes and a certain amount of heritability. There is much that has yet to be understood, including how external and internal challenges might interact with the *CACNA1C* polymorphisms, altering the risk to develop depression.

II. Aims of the thesis

Knowledge about neuroadaptations underlying alcohol dependence is crucial for the development and improvement of treatments and therapies, for example the lasting prevention of relapse. Studies on the LTCCs have suggested them as promising candidates. However, alcohol dependence, as all mental disorders, is complex and studies can only focus on certain aspects of the disorder, leaving many questions yet unanswered. Therefore, further research with respect to the involvement of the LTCCs, especially considering the individual subtypes, in alcohol dependence is needed to strengthen previous findings and investigate aspects which have so far not been sufficiently studied.

In addition, alcohol dependence shows high comorbidity with other mental disorders such as nicotine dependence, schizophrenia, and major depression, and LTCCs have been connected with each of these disorders, as well. Discerning the contributions of the LTCC subtypes $Ca_V1.2$ and $Ca_V1.3$ to the development and/or maintenance of these disorders may help to better understand the involvement of LTCCs in mental disorders in general.

The aims of this thesis are:

1. To determine differential roles of LTCC subtypes $Ca_V1.2$ and $Ca_V1.3$ in alcoholism

2. To define the involvement of $Ca_V1.2$ and $Ca_V1.3$ in nicotine-related behavior

3. To investigate LTCC subtype expression in schizophrenia and depression

The following studies were performed to achieve these aims:

Study I: Differential roles for L-type calcium channel subtypes in alcohol dependence (Aim 1)

Study II: Characterization of L-type calcium channel subtype expression in animal models of alcoholism (Aim 1)

Study III: L-type calcium channel subtype $Ca_V1.2$ mediates dependence-induced increase in alcohol self-administration (Aim 1)

Study IV: Functions of L-type calcium channel subtypes $Ca_V1.2$ and $Ca_V1.3$ in nicotine-related behavior (Aim 2)

Study V: Analysis of L-type calcium channel subtype expression in human postmortem samples of patients with mental disorders (Aim 3)

III. Materials and methods

1. Animal studies – alcohol dependence

1.1. Experimental animals

Wistar rats (Study I and II)

Male Wistar rats weighing 210 – 300 g at the beginning of the experiment were provided by Charles River (Germany). They were housed in standard cages at four rats per cage, if not indicated otherwise, under a 12 h light/dark cycle (lights off at 5 am or 2 am, depending on the experiment) with ad libitum access to food and water. Behavioral testing was performed during the dark phase. All experiments were conducted in accordance with the ethical guidelines for the care and use of laboratory animals, and were approved by the local animal care committee (Regierungspraesidium Karlsruhe, Germany, Aktenzeichen 35-9185.81/G-163/13).

Marchigian Sardinian alcohol-preferring (msP) rats (Study II)

msP rats were selectively bred from Wistar rats for high voluntary alcohol consumption (Bjork *et al*, 2010; Ciccocioppo *et al*, 2006) and brain samples were kindly provided by Dr. Roberto Ciccocioppo, University of Camerino, Italy.

AA (alko, alcohol) and ANA (alko, non-alcohol) rats (Study II)

Brain samples of AA and ANA rats were kindly provided by Dr. P. Hyytiä, University of Helsinki, Finland. They were selectively bred for high and low voluntary alcohol consumption, using Wistar rats as initial rat strain (Bjork *et al*, 2010; Eriksson, 1968).

$Ca_V1.2$flox x CaMKII-Cre^{ERT2} mice (Study III and IV)

These mice were breed at the breeding area of the Central Institute, by cross-breeding two lines: the $Ca_V1.2$flox (kindly provided by Prof. Dusan Bartsch, Mannheim) and the CaMKII-Cre^{ERT2} line (kindly provided by Prof. Günther Schütz and colleagues of the DKFZ, Heidelberg), both with C57Bl/6N background.

$Ca_V1.2$flox: In this mouse line, the exons 14 and 15 of the *Cacna1c* gene (coding for the transmembrane segments IIS5 and IIS6, as well as the pore loop in domain II) were flanked by two loxP sites. These 34 bp sequences were oriented in a way that in the presence of the Cre recombinase, exons 14 and 15 would be removed (Figure 28). In addition, there would be

a premature stop codon in exon 16, leading to a loss of function of $Ca_V1.2$ (Moosmang *et al*, 2005a; Seisenberger *et al*, 2000).

CaMKII-Cre^{ERT2}: Here, the gene for the Cre recombinase is expressed under the promotor for the CaMKII, which is mainly expressed in glutamatergic forebrain neurons. Import of the Cre recombinase into the nucleus can be activated by

Breeding of homozygous $Ca_V1.2$flox with heterozygous CaMKII-Cre^{ERT2} mice yielded Cre recombinase-expressing mutants and non-expressing controls.

10-12 week old male mice (mutants and controls) were group-housed in a reversed dark-light cycle (lights on at 7 pm). Food and water were available *ad libitum*. All experiments were conducted during the dark phase and were approved by the Regierungspräsidium Karlsruhe (Aktenzeichen 35-9185.81/G-301/14). The knockout (KO) was induced through one week of tamoxifen injections. All mice were treated with tamoxifen, to avoid any interference of the treatment (Vogt *et al*, 2008). 1 mg/100 µl tamoxifen in neutral oil was i.p. injected every 12 h for 5 days. Animals were then allowed 6 weeks of recovery.

1.2. Genotyping of $Ca_V1.2$flox x CaMKII-Cre^{ERT2} mice

Knockout (KO) of $Ca_V1.2$ in CaMKII-positive neurons of mutant mice was verified by PCR using cDNA and genomic DNA.
Taq DNA-Polymerase (5 U/µl, Sigma-Aldrich) was used as thermostable DNA-Polymerase, and 10 ng cDNA or 10-20 ng Plasmid DNA was added to each mix. The mix also included Primer A (10 pmol/µl), Primer B (10 pmol/µl), and 10x PCR-Buffer. Water was added for a reaction volume of 30 µl. After an initial denaturation at 94°C for 3 min, there were 28 cycles of denaturation (94°C, 30 s), primer annealing (60°C, 1 min), and polymerization (72°C, 1 min), followed by a final extension of the elongation (72°C, 10 min).

Two different primer pairs were used: The VL8/VL10 primer pair amplifies a sequence between exon 13 and 16, yielding either a larger fragment with the entire sequence, or in case of a deletion of exons 14 and 15 a 281 bp fragment (VL8: AGGGGTGTTCAGAGCAA; VL10: CCCCAGCCAATAGAATGCCAA). The primers VS11 (5′-CTG GAA TTC CTT GAG CAA CCT TGT-3′) and VS16 (5′-AAT TTC CAC AGA TGA AGA GG- ATG-3′) are located within exons 14 and 15, respectively. Genomic PCR yields a fragment of 1096 bp for primer pair VS11/VS16, which includes only DNA from exons 14 and 15. A complete KO

would not show any PCR product for these primers, while in a partial KO less PCR product than in WT mice is found.

1.3. Locomotor activity in mice: Open Field

Mice were acclimatized to the room for at least 30 min before the start of the test session. A 50x50 cm^2 white Open Field surrounded by dark walls was illuminated from above by 25 lux. Mice were placed individually into the center of the arena, and the activity was monitored for 10 min by a Video camera (Sony CCD IRIS). The image processing system EthoVision X8 (Noldus Information Technology, Wageninen, Netherlands) was used to analyze the total distance moved, velocity, and time spent in the center (10 cm distant from the walls).

1.4. Induction of alcohol dependence

1.4.1. Rats

Rats were exposed to daily intermittent cycles of alcohol vapor intoxication and withdrawal, and established paradigm to induce alcohol dependence on a molecular and behavioral level (Hansson *et al*, 2008; Rimondini *et al*, 2002; Sommer *et al*, 2008). Rats were exposed to alcohol vapor or normal air using a rodent alcohol inhalation system as described previously (Rimondini *et al*, 2002). Briefly, alcohol was delivered into glass/steel chambers (1x1x1m) through electrically heated stainless steel coils (60°C) by high-performance liquid chromatography (HPLC) pumps with an air flow of 18L/min. The conditions for each chamber can be individually adjusted with separate pressure gauges. After 1 week of habituation rats were exposed to five cycles of 14hr alcohol vapor per week (0:00 a.m. – 2:00 p.m.), separated by 10hr periods of withdrawal and an additional 58hr withdrawal at the end of each weekly cycle. Blood alcohol concentrations (BACs) were determined twice per week by analyzing blood (~20µl) sampled from the lateral tail vein with an AM1 Analox system (Analox Instruments Ltd, London, UK), attaining BACs of 266.2 ± 12.6 mg/dl. After seven weeks of alcohol exposure rats were subjected to three weeks of abstinence.

1.4.2. Mice

The alcohol vapor exposure in mice differed from the procedure in rats in several aspects. Mice were housed in their home cages during withdrawal periods and were transferred into vapor chambers for 16 h/day. Vapor chambers were divided into compartments by wire mesh fence, each housing 2-3 mice, to limit mutual injury while still maintaining an atmosphere of

group housing. Alcohol vapor delivery into the chambers was achieved by vaporization of alcohol. A pressure gauge supplied an air flow of ~6 l/min, leading to alcohol concentrations of 15-20 mg/l air within the chamber. Prior to each vapor exposure cycle, mice were injected with either 1 mmol/kg pyrazole (air exposed controls) or 1 mmol/kg pyrazole + 1.6 g/kg ethanol (alcohol vapor exposed mice).

The animals were vapor exposed for 5 days/week, followed by an extended withdrawal of two days. During the first two weeks, the alcohol concentration in the chamber was gradually increased to achieve the appropriate BACs of 150 – 300 mg/dl. When the BACs were at the desired level, the mice were vapor exposed for 4 more weeks to induce dependence. After the last exposure, withdrawal severity was measured at different time points: immediately after exposure, 4 h, 8 h, and 12 h after exposure (Mutschler *et al*, 2010). Each mouse was monitored for up to 5 min to assess tremor, tail rigidity, piloerection, vocalization, wet dog shake, and teeth chattering. Severity of each withdrawal sign was ranked as 0 (non-existant), 1 (mild), and 2 (severe), added up and expressed as the % of total withdrawal.

1.5. Alcohol self-administration with repeated deprivation phases

Wistar rats (Charles River, Germany) were allowed to habituate to the animal room for two weeks. They were then given ad libitum access to alcohol solutions (5%, 10%, and 20% v/v) and water. The position of the bottles was changed weekly to avoid location preference. After 8 weeks of alcohol access, rats were deprived of alcohol for two weeks, during which they had free access to water. This was followed by four cycles of 5-week alcohol access and 2-week deprivation. At the beginning of each cycle (access to alcohol), rats showed binge-drinking behavior, which increased with each cycle (Alcohol Deprivation Effect, ADE) (Vengeliene *et al*, 2014). This procedure was conducted by Dr. Valentina Vengeliene. All experiments were approved by the Regierungspräsidium Karlsruhe (Germany, Aktenzeichen 35-9185.81/G-209/11).

1.6. Chronic treatment with haloperidol or clozapine

Male Sprague Dawley rats (Taconic, Denmark), were fed haloperidol (Haloneurol®, Hexal, Germany; 1 mg/kg/day) or clozapine (Leponex®, Novartis, Germany; 20 mg/kg/day) from postnatal day 85 for 12 weeks. A control group did not receive any antipsychotic treatment. Animals were anaesthetized by pentobarbital (Narcoren®, Merial, Germany) on postnatal day 169, twelve hours after food removal. Brains were removed and frozen immediately by liquid nitrogen-cooled 2-methylbutane. They were stored at -80°C before cryosectioning. The

animal experiments were approved by the local animal care committee (Landesamt für Natur-, Umwelt- und Verbraucherschutz Nordrhein-Westfalen, Germany AZ 9.93.2.10.34.07.227).

1.7. *In situ* hybridization

1.7.1. Preparation of brain sections

After seven weeks of alcohol vapor exposure and varying times of abstinence (0, 1, 3, 7, 21 days, with BACs of 273 ± 52 mg/dl on day 0), rats were decapitated, their brains removed and snap-frozen in -40°C isopentane. They were then stored at -80°C until 12 µm coronal cryosections were taken at Bregma levels (i) +3.2 mm, (ii) +1.2 mm, (iii) -0.26 mm, (iv) -1.8 mm, (v) -2.3 mm, and (vi) -5.2 mm according to The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 1998). Mouse brains were equally extracted and cryosections taken at Bregma level -1.34 mm according to The Mouse Brain in Stereotaxic Coordinates (Paxinos and Franklin, 2001).
The sections were then mounted on SuperFrost slides and stored at -80°C.

1.7.2. RNA probe generation

Riboprobes ($Ca_v1.2$ (*cacna1c*: position 51 bp to 328 bp on rat cDNA, gene reference sequence: NM_012517, 90% homology to mouse cDNA sequence) and $Ca_v1.3$ (*cacna1d*: position 51 bp to 576 bp on rat cDNA, gene reference sequence: NM_017298.1, 96 % homology to mouse cDNA sequence)) were designed using the PubMed database (http://www.ncbi.nlm.nih.gov/Entrez) gene reference sequences. Riboprobes were kindly provided by Prof. M. Knipper. To synthesize antisense and sense probes, 200 ng DNA were incubated in transcription buffer (Ambion® Applied Biosystems, Darmstadt, Germany), 12.5 nmol ATP, CTP, GTP, 50pmol UTP and 125pmol [α-^{35}S]UTP (1250 Ci/mmol, Perkin Elmer, Rodgau, Germany), 1 U RNase inhibitor and 1 U RNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) at 37°C for 90 min. RNase-free DNase I was used to subsequently digest the DNA templates via incubation for 20 min at 37°C, and the transcripts purified in microspin columns (illustraTM MicrospinTM S-200 HR Colums, GE Healthcare, Munich, Germany). As quantification, the counts per minute (cpm) were measured with a Liquid Scintillation Analyzer (1600 TR).

1.7.3. Hybridization

Brain sections were fixed in 4% formaldehyde in PBS pH 7.0 for 15 min after reaching room temperature (RT). They were then washed in PBS pH 7.4 for 10 min and twice in sterilized

water for 5 min. For deproteination, the tissue was treated with 0.1 M HCl for 10 min, and washed again twice in PBS pH 7.4 for 5 min. Triethanolamine pH 8.0 with 0.25% acetic anhydride was used to acetylate positively-charged amino groups, by incubating for 20 min, followed by two additional washing steps in PBS pH 7.4 for 5 min. Subsequently, the sections were dehydrated in graded alcohol and air dried, before pre-hybridization with pre-hybridization buffer (50 % deionized formamide, 50 mM Tris-HCl pH7.6, 25 mM EDTA pH8.0, 20 mM NaCl, 0.25 mg/ml yeast tRNA, 2.5 x Denhardt's solution (Invitrogen, Darmstadt, Germany)), in a humidified chamber at 37°C for 2-4 hours. The pre-hybridization buffer was removed and 100 µl hybridization buffer (50% deionized formamide, 20 mM Tris-HCl pH 7.6, 10x Denhardt's solution, 5 mg/ml yeast tRNA, 1 mg/ml polyadenylic acid, 10 mM EDTA pH 8.0, 150 mM DTT, 330 mM NaCl, 10% dextransulphate) containing 1 x 10^6 cpm of either the labeled antisense RNA or sense RNA) was applied to each slide. For incubation at 55°C in a humidified chamber overnight, the sections were covered with siliconized coverslips, which were removed by washing with 1x standard saline citrate (SCC) at 42°C for 40 min on the following day. After two additional washing steps (1x SSC, 42°C, 30 min), sections were incubated in 0.5x SSC/50 % formamide for 1 h at 42°C, then washed twice again in SSC for 30 min. Unbound RNA was removed via treatment with 1 µg/ml RNase A in RNase buffer (0.5 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 7.5) for 1 h at 37°C, followed again by two washing steps in SSC (55°C, 30 min) and a brief rinsing in SSC at RT, before dehydration in graded alcohol. The sections were then air-dried, and BAS-SR 2025 imaging plates were exposed to the sections for 7 d and scanned by the phosphorimager (Fuji phosphorimager Typhoon FLA 700, GE Healthcare Life Sciences, Pittsburgh, USA). Densitometric analysis of the brain regions was performed with MCID Image Analysis Software (Imaging Research Inc., UK). Depending on the study, following regions were analyzed: prefrontal cortex (PFC)[cingulate cortex (Cing), prelimbic cortex (PreL), infralimbic region (IL), and orbitofrontal cortex (OFC)], motor cortex M1, nucleus accumbens [core (AcbC) and shell (AcbS)], caudate putamen (CPu), extended amygdala [bed nucleus of the stria terminalis (BNST), central amygdala (CeA), medial amygdala (MeA), and basolateral amygdala (BLA)], and the hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1, CA3, and CA4] (see Figure 14). Mean density values were determined as minimal detectable change (MDC) units per mm^2, and transformed into nCi/g by comparison with standard curves generated with [^{14}C]-Microscales.

1.8. Western blot analysis

Punched samples from the CA1 region of 3-weeks abstinent alcohol dependent and non-dependent rats were lysed in RIBA buffer containing protease inhibitors (Roche, Mannheim, Germany), followed by heating to 95°C for 10 min. 6% SDS-PAGE was used to separate protein samples, which were then transferred to 0.45 µm polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). A $Ca_V1.2$-specific (Moosmang *et al*, 2005a) and a heat shock cognate protein 70 (HSC70)-specific antibody (Santa Cruz, Heidelberg, Germany) were used for probing over night at 4°C. Blots were then washed and incubated with the respective secondary horseradish peroxidase-conjugated antibodies for 1 h at RT. Visualization of immune-reactive bands was done in a ChemiDoc station and the results analyzed with Image Lab (Bio-Rad, Munich, Germany). The $Ca_V1.2$ protein data was normalized to HSC70.

1.9. Electrophysiology

1.9.1. Preparation of acutely dissociated neurons

After seven weeks of intermittent alcohol vapor/air exposure and three weeks of abstinence, rats were anaesthetized with halothane gas, decapitated, and the brains quickly removed. 250 µm transverse hippocampal slices were dissected and kept in ice-cold cutting solution (2.5 mM KCl, 1.25 mM NaH_2PO_4, 10 mM $MgSO_4$, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 234 mM Sucrose, and 11 mM Glucose (saturated with 95% O_2 and 5% CO_2, pH= 7.10)). Slices were then transferred to oxygenated artificial Cerebro-Spinal Fluid (aCSF) (119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 11 mM Glucose) at RT, followed by aCSF solution with 0.2 mg/ml proteinase K (Sigma-Aldrich, Milan, Italy) at 37°C for 5 min. After wash-out of proteinase K, 1 mg/ml trypsin (Sigma-Aldrich, Milan, Italy) was added for 30 min. Trypsin was washed out and the slices transferred into oxygenated aCSF solution at RT.

Viability was given for all slices throughout the experiments. Isolation of neurons was achieved by mechanical dissociation with a fire-polished Pasteur pipette in a Tyrode's standard solution (130 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose; pH 7.4) with 0.5 mg/ml bovine serum albumin (BSA).

1.9.2. Electrophysiological recordings

Prior to electrophysiological recordings, dissociated neurons of the CA1/CA3 regions of the hippocampus were given time to adhere to poly-L-lysine coated petri dishes for 10 min.

Intracellular solution contained 20 mM Cs-$MeSO_3$, 90 mM CsCl, 2 mM $MgCl_2$, 5 mM EGTA, 4 mM ATP, 15 mM phosphocreatine, and 10 mM HEPES, pH 7,4 (with CsOH, Sigma-Aldrich, Milan, Italy). Extracellular solution consisted of 135 mM TEA, 2 mM CaCl, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4 (with TEA-OH; Sigma-Aldrich, Milan, Italy). 300 nM of tetrodotoxin (TTX) were present during calcium current measurements. To isolate the L-type component, saturating concentrations (3 µM) of nifedipine were added. Voltage-clamp recordings were performed at RT, using a multiclamp 700-B amplifier and pClamp 10.0 software (Molecular Devices, Sunnyvale, CA, USA) (Marcantoni *et al*, 2010b; Vandael *et al*, 2012). Traces were sampled at 10 KHz using a digidata 1440 A acquisition interface (Molecular Devices, Sunnyvale, CA, USA) and filtered using a low-pass Bessel filter set at 1-2 KHz. Borosilicate glass pipettes (Kimble Chase life science, Vineland, NJ, USA) with a resistance of 5-7 MΩ were dipped in an eppendorf tube containing intracellular solution, before being back filled with the same solution. Experiments with a series resistance above 20 mΩ were excluded and Rs was compensated by 80% and monitored throughout the experiment. Fast capacitive transients during step-wise depolarisations (in voltage-clamp mode) were minimized online by the use of the patch clamp analogue compensation. Uncompensated capacitive currents were further reduced by subtracting the averaged currents in response to P/4 hyperpolarising pulses.

1.10. Operant alcohol self-administration experiments in rats (Study I)

1.10.1. Drugs

Alcohol (10% v/v ethanol) was prepared using 96%-97% ethyl alcohol (Sigma-Aldrich®) and tap water.

Verapamil (R&D Systems, Wiesbaden-Nordenstadt, Germany) was dissolved in aCSF for intracerebroventricular (i.c.v) injection (120 µg/5 µl).

1.10.2. Training sessions

All training and test sessions took place during the dark phase of the light cycle. Rats were trained to self-administer 10% (v/v) alcohol in operant chambers (MED Associates), placed in ventilated sound-attenuating cubicles. The chambers were equipped with two retractable response levers, located on opposing sides of the chamber. Upon lever activation of the left lever, 30 µl of fluid was delivered into a liquid receptacle next to the lever via a syringe pump. A light stimulus (house light) was located above the right response lever of the self-administration chamber and was activated at fluid delivery (3 s blink light,

conditioned stimulus, CS), simultaneously indicating a "time-out" period during which additional lever pressing did not result in fluid delivery. Activation of the right lever did not result in either alcohol delivery or CS. An IBM-compatible computer controlled the delivery of fluids, presentation of stimuli, and data recording.

During the first 3 d of training, rats were water-deprived in their home cages for 16 h/d, after that food and water were available *ad libitum*. Each session was 30 min long, and during that time an orange odor (drops of orange extract on the bedding) was present as an additional environmental cue for the availability of alcohol.

Rats were trained until a steady baseline was reached, at which point they were divided into two groups, one of which was then exposed to alcohol vapor for 7 weeks (alcohol dependent), while the control group breathed normal air.

1.10.3. Stereotaxic placement of intracerebroventricular (i.c.v.) guide cannulas

Guide cannula placement took place during the two weeks of abstinence following alcohol vapor exposure. The animals were anaesthetized with isoflurane and the head fixed in a Kopf stereotaxic instrument. The skull was carefully exposed and a unilateral guide cannula (Bilaney Consultants GmbH, Düsseldorf, Germany) inserted into the lateral ventricle (AP = -0.8; ML = +/-1.4; DV = -2.0 relative to Bregma; the ML side was chosen randomly). It was fixed with dental cement and the rats were single-housed to minimize injuries. Before re-training of the operant self-administration procedure, rats were allowed a recovery time of at least five days.

1.10.4. I.c.v. injections

For i.c.v. injections, stainless-steel injectors fitting the 2.5 mm guide cannula with a 1.6 mm projection were attached to a 25 µl Hamilton syringe via 40 cm tubing (connector cannula, Bilaney Consultants GmbH, PlasticsOne®). A volume of 5 µl was administered at a flow rate of 2.5 µl/min using an infusion pump (PHD 2000 Infusion, Harvard Apparatus). To avoid backflow, the injector was left in place for 30 s after the injection.

1.10.5. Alcohol self-administration test

Rats were re-trained to self-administer alcohol after recover from surgery until they again reached a stable baseline. In the subsequent self-administration test, all conditions were exactly as during training sessions, with the exception that immediately before the start of the session they received an i.c.v. injection. Alcohol-dependent and non-dependent were each divided into groups: aCSF (5 µl), and Verapamil (120 µg/5 µl).

1.10.6. Extinction of alcohol self-administration behavior

Following the alcohol self-administration test, there were extinction sessions. During these sessions, lever pressing did not result in either alcohol delivery or CS, and no orange odor was present. Daily extinction sessions were performed until the rats had reached the extinction criterion, which was an active lever response of <10% of the baseline for three consecutive days.

1.10.7. Cue-induced reinstatement

As for the alcohol self-administration test, i.c.v. injections took place immediately before the start of the cue reinstatement session. The orange odor was again presented and upon active lever pressing, the CS was activated, however no alcohol was delivered.

1.11. Operant alcohol self-administration experiments in mice (Study II)

Mice were trained to self-administer alcohol by lever pressing in operant chambers (TSE Systems). On two opposing sides of each chamber were ultrasensitive levers. The left lever was defined as the active lever, where pressing resulted in the delivery of 10 µl of a solution (saccharine, alcohol, or a combination, see below) in a receptacle next to the lever, as well as the activation of a house light (only in sessions where 10% alcohol was delivered). Pressing of the right, inactive lever did not have any programmed consequences.

1.11.1. Saccharine fading procedure

During the first sessions, pressing of the left lever resulted in the delivery of 0.2% saccharine. After 12 sessions a stable baseline was achieved. The following sessions, 5% alcohol was added to the saccharine, again until the lever pressing activity was stable, then followed by only 5% alcohol, saccharine + 8% alcohol, 8% alcohol, saccharine + 10% alcohol, and finally 10% alcohol. As soon as the mice received exclusively 10% alcohol, the house light was added as a conditioned cue.

1.11.2. Self-administration

After self-administration training, the mice were alcohol vapor/air exposed. Following this procedure, 5 self-administration sessions were conducted, during which pressing of the left lever resulted in the delivery of 10% alcohol and activation of the house light. For the evaluation of self-administration behavior, the mean of all five sessions was calculated.

1.12. Statistical analysis

The Statistica software (StatSoft, Hamburg, Germany) was used for statistical evaluation. A region-wise one-way ANOVA (followed by Bonferroni's correction) was applied to analyze *in situ* hybridization of 3-week abstinent rats, for time course experiment, a two-way ANOVA for time and treatment, followed by Fisher's LSD post-hoc test was used. Western blot analysis was done by t test. For evaluation of alcohol self-administration behavior, a repeated measures two-way ANOVA (genotype*treatment, considering active and inactive lever) was used, followed by Newman Keuls post-hoc test. Cue-induced reinstatement of alcohol-seeking was evaluated by repeated measures two-way ANOVA including extinction values, followed by Newman-Keuls post-hoc test.

Statistical analysis of locomotor behavior in mice was performed using the statistic program SPSS 23 for Windows. One-way ANOVA was used to calculate changes in total distance moved (m), velocity (cm/s), and center time (%).

2. Animal studies – nicotine dependence

2.1. Experimental animals

C57Bl/6N mice

Male C57Bl/6N mice (Charles River, aged 10-12 weeks at the start of experiment) were single-housed at a temperature of 21°C and with a 12 h light-dark cycle (lights on at 7 am). The experiments were conducted during the light phase, and food and water were available *ad libitum*. All experiments were conducted in adherence with the European Communities Council Directive (8676097EEC) on the care and use of laboratory animals and were approved by the Regierungspräsidium Karlsruhe (Germany)(Aktenzeichen 35-9185.81/G-244/12 and Aktenzeichen 35-9185.81/G-301/14).

$Ca_V1.2$flox x CaMKII-Cre^{ERT2} mice

The same mouse line as in "2. Animal studies – alcohol dependence" was used. The decrease in functional $Ca_V1.2$ was equally induced through tamoxifen injections (see Study III; Figure 28.)

2.2. Drugs

Nicotine hydrogen tartrate salt (Sigma-Aldrich) was dissolved in physiological saline (0.9% NaCl) and the final solution adjusted to an approximate pH 7 using NaOH. It was either i.p. administered at a dose of 0.175 mg/kg or infused intravenously (i.v.) at 0.01 mg/kg/35 µl.
The non-selective L-type calcium channel antagonist Nifedipine (Sigma-Aldrich) was sonicated in 100% Cremophor EL (Sigma-Aldrich) at 16 mg/ml. For a final concentration of 10 mg/kg Nifedipine, PBS was added (5% Cremophor EL/95% PBS).

2.3. *In situ* hybridization

For *in situ* hybridization, mice received 14 daily nicotine (0.175 mg/kg) or vehicle (0.9% saline) i.p. injections and were sacrificed either 24 h or 7 d after the last injection. Another group of mice was injected with vehicle for 13 d, followed by a single injection of nicotine on day 14. They were sacrificed 24 h after the nicotine injection.
In situ hybridization was carried out as previously described (see 2.4).

2.4. Locomotor activity measurements

Seven TruScan activity monitors (Coulbourn Instruments) were used for the measurement of locomotor activity. The monitoring unit consisted of infrared photocell emitter/detector pairs which were evenly spread along the clear acrylic plastic cage (22x22x40 cm). Ambulatory beam interruptions were measured by a connected computer.

2.4.1. Nifedipine dose response

A dose response using vehicle (10 ml/kg) or doses of 10 or 25 mg/kg nifedipine was applied to determine the best dosage for the locomotor sensitization experiment. Mice were i.p. injected 30 min before a 60 min locomotor activity measurement. 24 h after the injection there was another locomotor activity session. Mice were again injected with vehicle or nifedipine 6 d after the second test and their locomotor activity was measured 12 h after the injection.

2.4.2. Locomotor sensitization

After three daily habituation sessions (60 min), during which mice were injected with saline (10 ml/kg) and placed in the activity monitors, chronic nicotine administration was performed as for *in situ* hybridization experiments. On day 1 and 14, locomotor activity was measured. During the subsequent abstinence period, control and nicotine-treated mice were each divided into two groups, which were then injected approximately every 12 h with either vehicle (10

ml/kg, i.p.) or nifedipine (10 mg/kg, i.p.), resulting in four treatment groups (sal/veh, sal/nif, nic/veh, nic/nif). Based on the results of the dose response experiment, the final injection 12 h before testing on day 21 was omitted. On day 21, locomotor activity was assessed again for 60 min after an i.p. injection of either nicotine or saline (according to their groups from previous nicotine injections). Distance traveled (cm) was recorded in each locomotor activity test session.

2.5. Nicotine self-administration

12 operant chambers (24.1 x 20.3 x 18.4 cm; Med Associates, USA) in light- and sound-attenuating cubicles were used for nicotine self-administration experiments. Each chamber contained a left and a right lever, a food dispenser, and a drug delivery system connected via infusion pump (PHM-100, Med Associates, USA). Operant chambers were controlled using Med-OC IV (Med Associates, USA) software.

For initial lever training, mice received 14 mg sweetened food pellets (TestDiet, USA) under a fixed ratio 1 (FR1) schedule as previously described (Bernardi and Spanagel, 2013). Mice were trained for 60 min per session. The active lever alternated between left and right daily, and changed after 1 cycle defined as the receipt of 10 food reinforcers. Lever training was considered complete when the mice achieved 2 cycles per lever on at least 2 separate days. After successful lever training, an indwelling intravenous catheter was implanted into the jugular vein. 0.15 ml heparinized saline (100 i.u./ml) containing Baytril (0.7 mg/ml) were administered daily throughout the experiment to maintain catheter patency. Mice were allowed 3 d of recovery from the surgery, followed by 8 consecutive days of nicotine self-administration sessions, 2 h each. Pressing of the active lever resulted in the delivery of nicotine under an FR2 schedule (two presses results in one reinforcer). A 20 s blinking light stimulus was presented during each nicotine delivery as a CS, and indicated a timeout period, during which additional lever presses did not lead to the additional delivery of nicotine. Inactive lever presses were recorded but had no programmed consequences. All behavioral testing was performed during the light phase.

2.6. Statistical analysis

The Statistical software (StatSoft) was used for statistical analysis. For *in situ* hybridization, expression was evaluated by region-wise one-way ANOVA. A Bonferroni correction (p value multiplied by the number of analyzed brain regions) was applied.

Nifedipine dose-response data were evaluated by one-way ANOVA, and two-way ANOVA was used to assess locomotor sensitization data (day [repeated measures] x treatment [saline vs. nicotine or vehicle vs. nifedipine]), followed by post-hoc analysis if applicable. Significance was set at $p < 0.05$. For nicotine self-administration, a three-way ANOVA (lever [active vs. inactive] x day [repeated measures] x genotype) was performed, followed by post-hoc t-test, and the number of nicotine reinforcers was evaluated by two-way ANOVA (day [repeated measures] x genotype).

3. Human studies

3.1. Postmortem tissue samples

3.1.1. Alcoholic patients

Human brain tissue samples were obtained from the New South Wales Tissue Resource Centre at the University of Sydney, Australia (http://sydney.edu.au/medicine/pathology/trc/index.php). Macrodissected tissue pieces of the nucleus caudatus (NC) and ventral striatum (VS) used for this study were obtained from 25 male subjects of European descent consisting of 43 chronic and heavy alcoholic and 43 control subjects (Table 1). The Diagnostic Instrument for Brain Studies – Revised (DIBS-R), which is consistent with the criteria of the Diagnostic and Statistical Manual for Mental Disorders, 4th edition (DSM-IV) (American Psychiatric Association, 1994) was used for postmortem confirmation of subject affiliation to the alcoholic or control group. All alcoholics had consumed 50g to >80g of alcohol per day, but had no measurable BAC at the time of death, while the daily consumption of the control subjects had an average of <20g. Demographic details are listed in Suppl. Table 1.

3.1.2. Schizophrenic patients

Postmortem brain tissue from inpatients with DSM-IV residual schizophrenia and matched control subjects were provided by the Department of Neuropathology, Mental Hospital Wiesloch, Germany. Experienced psychiatrists at the Mental Hospital Wiesloch obtained a complete clinical history and diagnosis for all patients, including the medication within the last ten years of the patients' lives. Information on patients and control subjects are given in Suppl. Table 2. Permission for autopsy was given by the donor or a family member, and all assessments and postmortem evaluations were approved by the Ethics Committee of the Faculty of Medicine, University of Heidelberg, Germany.

The Brodmann area 10 and 21, as well as the nucleus caudatus (NC) and the vermis of the cerebellum, according to a brain atlas (Nieuwenhuys *et al*, 2007), were used for quantitative real-time PCR. Demographic data is listed in Suppl. Table 2.

3.1.3. Depressive patients

Postmortem brain tissue of 28 suicide completers (19 males and 9 females) with established major depression as defined by DSM-IV criteria (American Psychiatric Association, 1994), and 79 controls (48 males and 31 females) with no history of mental illness were obtained from the Human Brain Tissue Bank, Budapest. The autopsy was performed at the Department of Forensic Medicine of the Semmelweis University Medical School (Budapest, Hungary). The procedures were approved by the ethics committee of the Semmelweis University.
Control subjects had no history of depression, alcohol or drug abuse. The anterior cingulate cortex (ACC) and the Brodmann Area 9 (BA9) were dissected and used for qRT-PCR experiments. Details on patient history and characterization of the tissue are found in Suppl. Table 3.

3.2. Genotyping for the *CACNA1C* single nucleotide polymorphism rs1006737

Genomic DNA was isolated from tissue samples using the QIAam DNA micro kit (Qiagen, USA) and the *CACNA1C* SNP rs1006737 was detected by TaqMan® SNP Genotyping Assay (C_2584015_10; Applied Biosystems, Carlsbad, USA) on an ABI 7900 HT RT-PCR system with SDS 2.2.2 software (10 µl reaction volume containing 10 ng genomic DNA, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min).

3.3. Quantitative real-time PCR (qRT-PCR)

RNA extraction and analysis was previously described (Meinhardt *et al*, 2013; Sommer *et al*, 2010). Briefly, total RNA was extracted using trizol reagent (Life technologies) and cleaned up via RNeasy (Quiagen) columns according to the manufacturer's instructions. RNA integrity (RIN) values were determined for all samples, and absorption ratios (A260/280) were between 1.9 and 2.1 RNA analysis was done on triplicates of each sample in a total reaction volume of 20 µl using Power SYBR®Green PCR Master Mix (ABI) on an ABI 7900 HT RT-PCR System (40 cycles of 95°C for 15 sec and 60°C for 1 min), with a melting profile being recorded after each PCR. Melting curves showed single fluorescence change peaks at the appropriate melting temperatures for all primers, which were designed to gain amplicons of 95 – 110 bp with a melting temperature >75 °C. The National Center for Biotechnology

Information (NCBI) reference sequence database was used to design primers toward the 3' end of the coding sequence, factoring in exon-exon junctions. Primers are listed in Table 1. ABI's SDS 2.2.2 software was used for analysis of SYBR Green fluorescence intensity and Ct-values (theoretical cycle number when a defined threshold was passed), applying *GAPDH* as an internal normalizer to calculate the ΔCT. All statistical analyses were performed on ΔCT values.

Table 1. Primers for qRT-PCR on human postmortem samples.

mRNA	Accession No	Forward primer	Reverse primer
CACNA1C	NM_199460	5'-GCAGGAGTACAAGAACTGTGAGC-3'	5'-CGAAGTAGGTGGAGTTGACCAC-3'
CACNA1D	NM_000720.3	5'-CTTCGACAACGTCCTCTCTGCT-3'	5'-GCCGATGTTCTCTCCATTCGAG-3'
GAPDH	NM_002046.4	5'- ATGAGAAGTATGACAACAGCCT-3'	5'- AGTCCTTCCACGATACCAAAGT-3'

3.4. Statistical analysis

The Statistica software (StatSoft, Hamburg, Germany) was used for evaluation of the data. Significance was determined by region-wise one-way ANOVA. An analysis of covariants was performed for alcoholic patients and suicide completers.

IV. Results

1. Study I: Differential roles for L-type calcium channel subtypes in alcohol dependence

LTCC-mediated currents, as well as LTCC expression, are altered by alcohol. This type of voltage-gated calcium channel has also been implicated in several aspects of alcohol-related behavior. However, there are few studies investigating the changes in LTCCs in alcohol dependence, and their influence on the development and maintenance of this mental disorder. The importance of the subtypes $Ca_V1.2$ and $Ca_V1.3$ has, so far, not been discovered either.

In this study, the changes in subtype-specific channel expression and calcium currents in alcohol dependence were analyzed by *in situ* hybridization, Western Blot analysis and electrophysiological current measurements. The effects of i.c.v. verapamil administration on alcohol self-administration and cue-induced reinstatement were measured in operant chambers.

1.1. Increased *Cacna1c* mRNA in the hippocampus and amygdala of alcohol dependent rats

Cacna1c and *Cacna1d* mRNA levels were measured in several brain regions of alcohol dependent and control rats after 3 weeks of abstinence using subtype-specific rat riboprobes, to examine long-term neuroadaptations.

There was a significant increase of *Cacna1c* mRNA in the hippocampus (CA1: 76% increase; CA3: 24% increase; CA4: 33% increase) and in the amygdala (CeA: 40% increase; BLA: 70% increase) of alcohol dependent rats. No differences between alcohol dependent and non-dependent rats were observed in the PFC (Cing, PreL, IL, OFC), the motor cortex M1, the striatum (AcbC, AcbS, CPu), the DG, BNST, MeA, or the PVN.

No difference in *Cacna1d* expression was observed in any regions. Absolute values and statistics are summarized in Suppl. Table 4.

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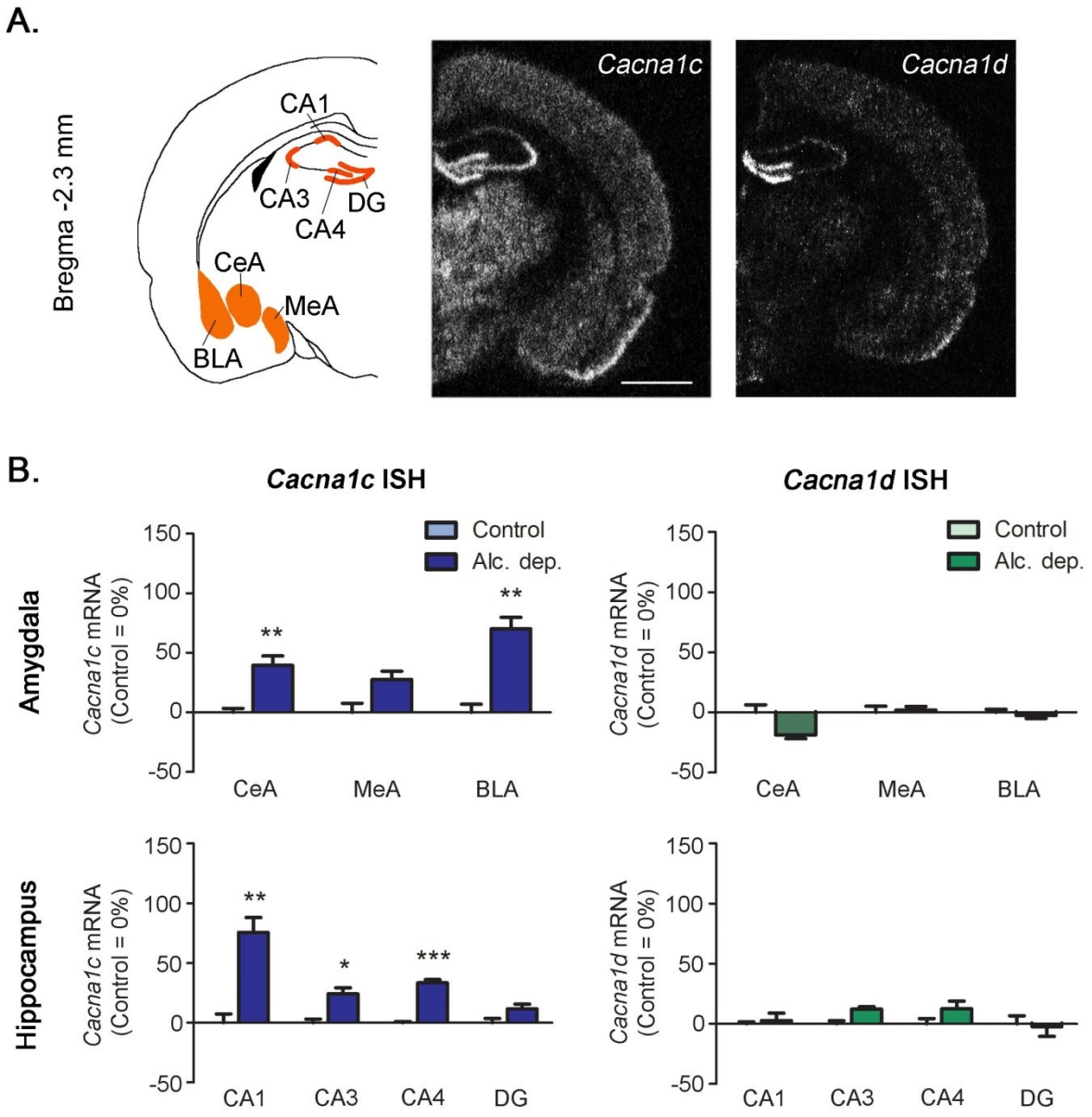

Figure 14. *Cacna1c* mRNA is increased in the hippocampus and amygdala of 3 weeks alcohol abstinent rats. A. Schematic representation of measured areas and mRNA expression pattern of *Cacna1c* and *Cacna1d* at Bregma 2.3 mm. CeA, central amygdala; MeA, medial amygdala; BLA, basolateral amygdala; and hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1, CA3, CA4]. Scale bar: 2.5 mm B. Changes of *Cacna1c* and *Cacna1d* mRNA expression in alcohol dependent rats measured by *in situ* hybridization using subtype-specific riboprobes. Bar graphs show *Cacna1c* and *Cacna1d in situ* hybridization values relative to control rats (Control = 0% regulation) and are expressed as mean ± SEM. Statistical analysis was performed by region-wise one-way ANOVA followed by Bonferroni's correction, n=6-7/group, p values: *p<0.05, **p<0.01, ***p<0.001. Absolute values of all measured regions are listed in Suppl. Table 4.

1.2. $Ca_V1.2$ protein increase in the CA1 matches observations on mRNA level

An increase in mRNA does not necessarily translate to protein level. Therefore we performed a Western Blot analysis on samples from the CA1 of alcohol dependent and non-dependent rats. The rats were alcohol vapor exposed by myself and brain samples were sent to Dr. Nina Dedic and Dr. Jan Deussing, who performed the Western Blot analysis.

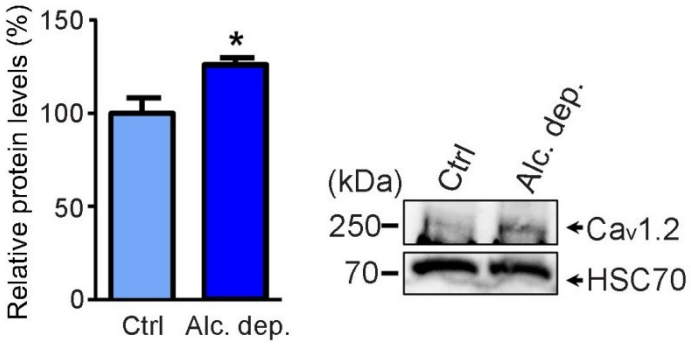

Figure 15. $Ca_V1.2$ protein level matches increase in mRNA in the CA1 in 3 weeks alcohol abstinent rats. Bar graphs of western blot analysis are expressed as mean ± SEM. Statistical analysis was performed by student's t test, n=8/group, p values: *p<0.05. Representative western blot is shown on the right. The data was produced by Dr. Nina Dedic and Dr. Jan Deussing.

$Ca_V1.2$ protein was increased by 26% in the CA1 of alcohol dependent rats compared to non-dependent controls ($p<0.05$), thereby confirming our mRNA data on the protein level.

1.3. *Cacna1c* expression levels show dynamic regulation from acute intoxication to prolonged abstinence

To better understand the changes in *Cacna1c* transcription, the mRNA was examined over a period of time, starting with acute intoxication, over withdrawal, to prolonged abstinence (0, 1, 3, 7, or 21 d of abstinence). The regions with most pronounced effects in 3 week abstinent rats (CA1, CeA, and BLA) were chosen for this investigation.

Two-way ANOVA revealed significant main effects of treatment (CA1: $F[1,75]=49.48$, $p<0.001$; CeA: $F[1,73]=137.4$, $p<0.001$; BLA: $F[1,77]=46.94$, $p<0.001$) and time (CA1: $F[5,75]=13.46$, $p<0.001$; CeA: $F[5,73]=43.3$, $p<0.001$; BLA: $F[5,77]=19.29$, $p<0.001$), as well as a significant interaction of treatment*time (CA1: $F[5,75]=13.46$, $p<0.001$; CeA: $F[5,73]=43.3$, $p<0.001$; BLA: $F[5,77]=19.29$, $p<0.001$).

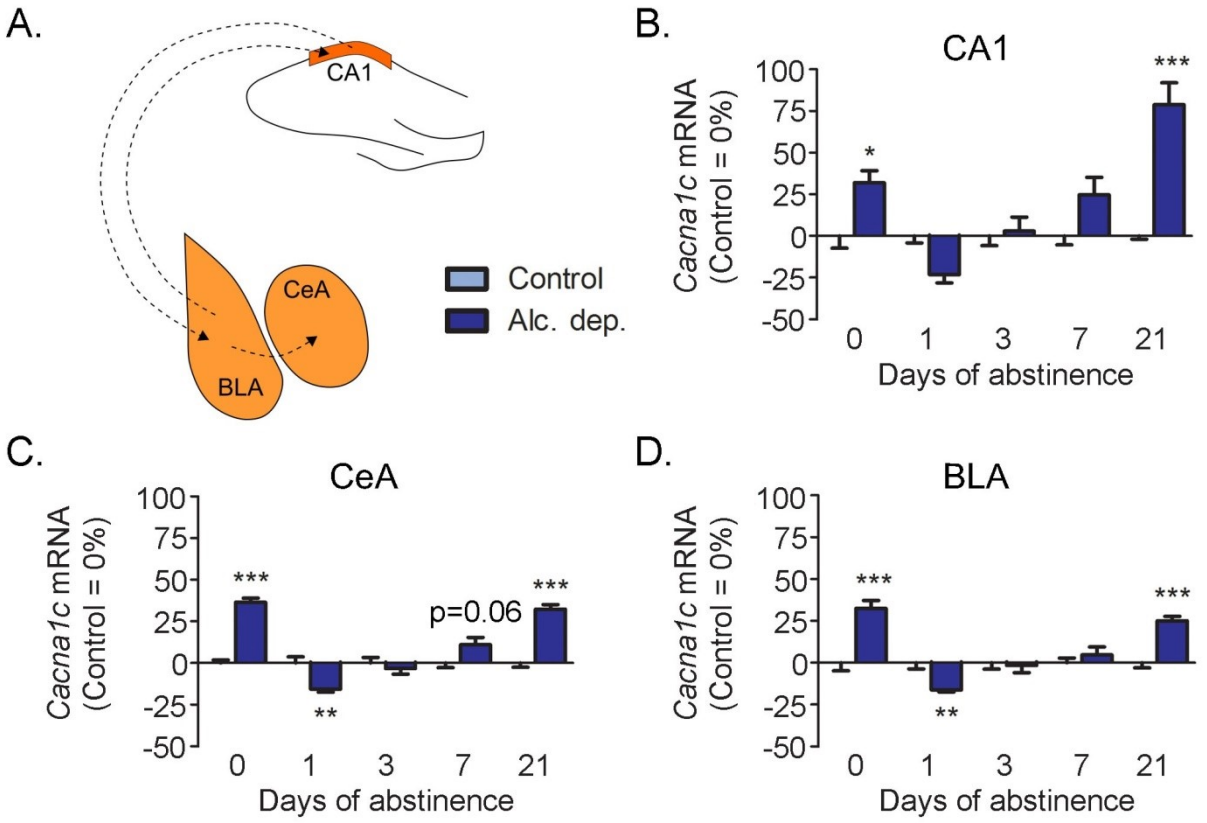

Figure 16. Dynamic changes in *Cacna1c* mRNA expression during abstinence. A. Schematic showing established anatomical connections between CA1, BLA and CeA (Kelley, 2004; LeDoux, 2003; Mandyam, 2013). B. – D. *Cacna1c* mRNA levels during acute intoxication, withdrawal, and prolonged abstinence in the CA1 (B.), BLA (C.), and CeA (D) of previously 7 weeks alcohol vapor exposed rats. Bar graphs are normalized to control levels (changes in regulation: control = 0%). Statistical analysis was performed by region-wise two-way ANOVA (time, treatment) followed by Fisher's LDS post-hoc test and Bonferroni's correction, n=6-8/group, p values: *p<0.05, **p<0.01, ***p<0.001.

Fisher's LSD post-hoc test followed by Bonferroni's correction showed an increase of *Cacna1c* mRNA in the CA1 of alcohol dependent rats during acute intoxication (0 d, $p<0.05$). This was followed by a non-significant decrease after 1 d, before *Cacna1c* transcription levels normalized on day 3 (=n.s.). On day 7 there was again an increase in mRNA which did not reach significance, and day 21 matched the previous experiment with a strongly significant increase in *Cacna1c* transcripts ($p<0.001$).

Similar observations could be made in the amygdala, where *Cacna1c* transcripts were increased in both the CeA ($p<0.001$) and the BLA ($p<0.001$) during acute intoxication. A decrease on day 1 (CeA: $p<0.01$; BLA: $p<0.01$) was followed by a return to control values on day 3 (CeA: p=n.s.; BLA: p=n.s.). On day 7, *Cacna1c* mRNA levels were increased slightly compared to controls in the CeA (trend towards significance, $p=0.06$), while no difference was observed in the BLA (p=n.s.). However, on day 21 both regions showed a strong increase

(CeA: $p<0.001$; BLA: $p<0.001$), which is again in line with the findings in the previous experiment. Absolute values are listed in Suppl. Table 5.

1.4. Alcohol-dependent rats display increased $Ca_V1.2$ currents in the hippocampus

Quantitative measurements of Ca_V1 channel currents were performed in acutely dissociated CA1 neurons to functionally validate our *in situ* findings. The rats were exposed to alcohol vapor by myself and sent to Dr. David Vandael, Dr. Andrea Marcantoni, and Dr. Emilio Carbone, who performed the current measurements.

Although no significant difference between total whole-cell calcium currents in neurons of alcohol dependent and non-dependent rats could be found, there was a trend toward increased calcium currents in the dependent animals. There was also no difference in the normalized conductance ($G_{norm}(V)$) of calcium currents. Half maximal activation was -15.2 mV, which coincides with typical high-voltage activated calcium channels (Mahapatra *et al*, 2011; Marcantoni *et al*, 2010a).

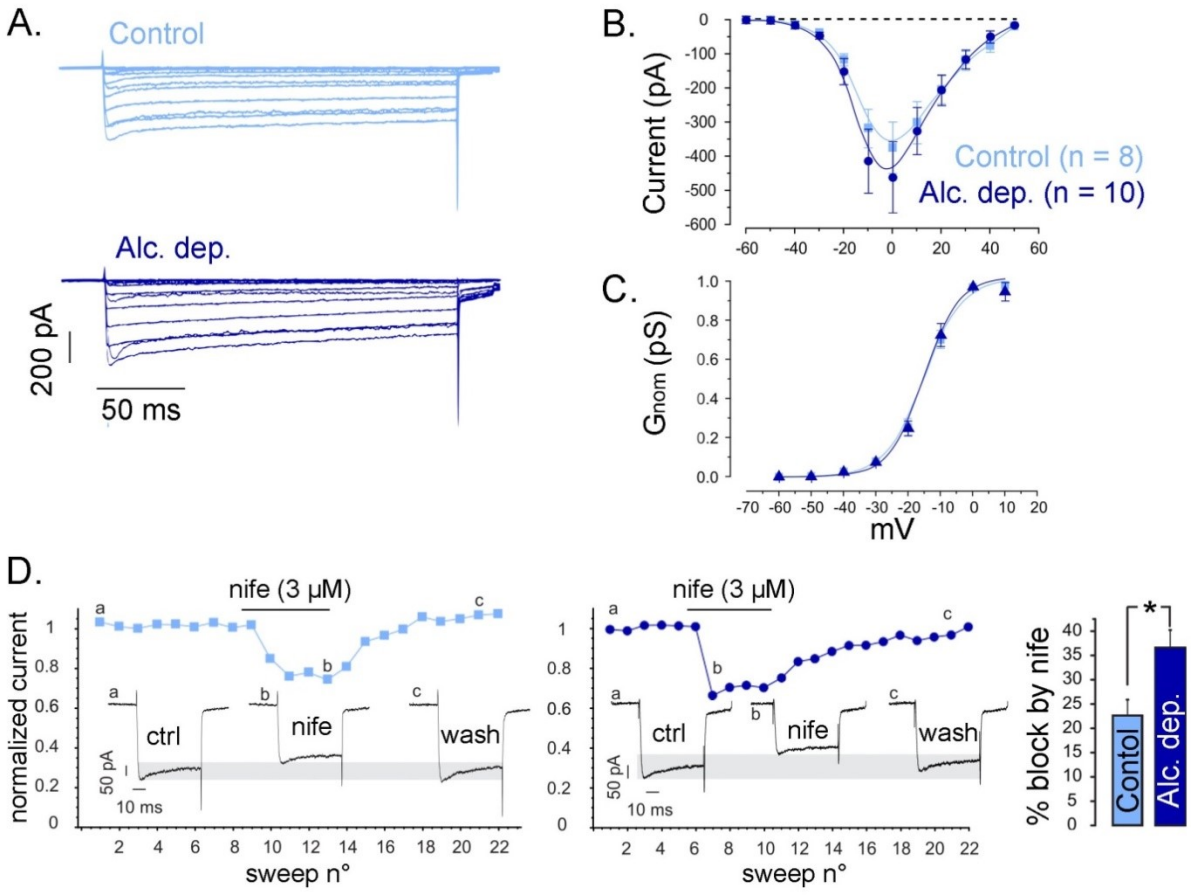

Figure 17. Functional LTCCs are increased in 3-weeks abstinent alcohol dependent rats. A. Representative traces of whole cell calcium currents from alcohol dependent (dark blue) and control (light blue) rats. B. Current-voltage relationship of calcium currents measured in high extracellular (135 mM) Tetraethylammonium and 300 nM TTX. C. Normalized conductance ($G = I/V\text{-}E_{Ca}$) of whole cell calcium currents. Reversal potential of

calcium currents is 50 mV, data were fit by Boltzman equations. D. Representative traces of the block by the selective LTCC antagonist nifedipine (3 µM), summary as bar graph. p value: *p<0.05. The data was produced by Dr. David Vandael, Dr. Andrea Marcantoni, and Dr. Emilio Carbone.

Nifedipine, added to block the L-type component was significantly more effective in alcohol dependent than in control rats (p=0.05) at 0 mV, revealing a contribution of LTCCs of 22.4 ± 3.1 % in controls and 36.6 ± 3.6 % in alcohol dependent rats (p<0.05, *t* test). Given the relatively high half maximal activation value, it can be assumed that almost all LTCC current is mediated by $Ca_V1.2$, with little or no contribution of $Ca_V1.3$.

1.5. Alcohol self-administration is not susceptible to LTCC antagonism

As a means to determine the influence of central LTCCs on voluntary alcohol consumption, a self-administration paradigm was employed. Animals were trained to press a lever to receive a drop of alcohol, were then alcohol vapor or air exposed, and re-trained, before alcohol self-administration under the influence of i.c.v. verapamil was tested.

Although two-way ANOVA revealed a significant effect of alcohol vapor exposure (alcohol vs. air exposure; F[1,23]=5.35, p<0.05), there was no significant main effect for treatment (verapamil vs. vehicle) and or the interaction of alcohol vapor exposure*treatment. Newman Keuls post-hoc test showed no difference between groups.

1.6. Cue-induced reinstatement of alcohol-seeking is blocked by verapamil in alcohol dependent rats

Cue-induced reinstatement is used to investigate relapse-like behavior in drug dependence. Here, the association between lever pressing and the receipt of alcohol was extinguished after the self-administration test until the lever pressing behavior was decreased to <10 % of the baseline. During the reinstatement session, the conditioned as well as environmental cue were presented, which should lead to an increase in lever pressing behavior without the reinforcement of alcohol.

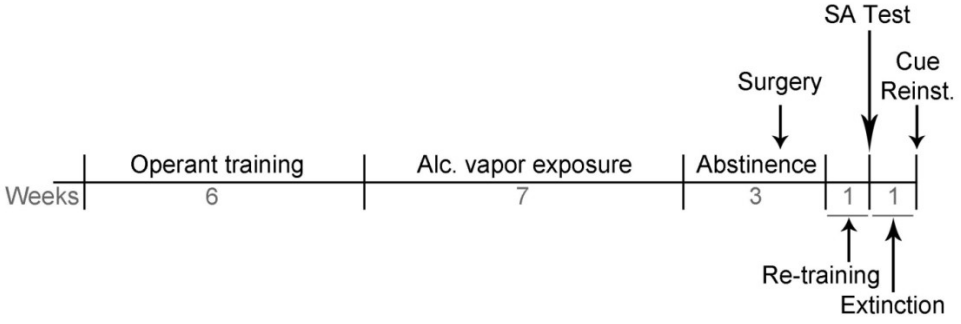

Figure 18. Timeline of cue-induced reinstatement experiment.

Repeated measures two-way ANOVA showed significant main effects for lever (active vs. inactive; $F[1,23]=186.93$, $p<0.01$), lever*group ($F[3,23]=6.21$, $p<0.01$), session (extinction vs. reinstatement; $F[1,23]=105.08$, $p<0.001$), session*group ($F[3,23]=4.52$, $p<0.05$), the interaction between lever*session ($F[1,23]=92.71$, $p<0.001$) and lever*session*group ($F[3,23]=7.09$, $p<0.01$).

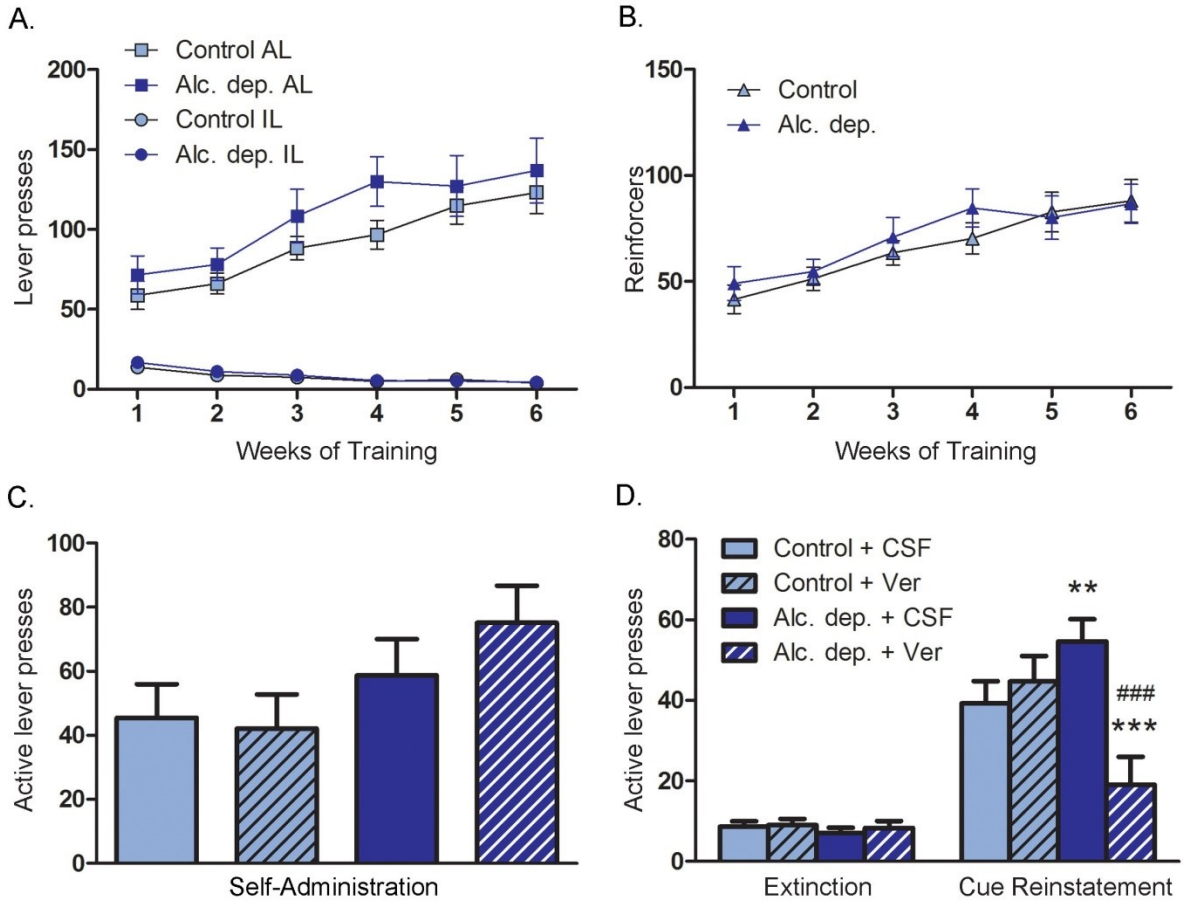

Figure 19. Central verapamil administration prevents cue-induced reinstatement of alcohol-seeking in alcohol abstinent rats. A. Graph shows lever presses during 6 weeks of self-administration training with active lever (AL) presses steadily increasing and inactive lever (IL) at an expected low level. B. Reinforcers received during sessions vary from AL presses because of the timeout period, but also steadily increase. C. Alcohol self-administration was not significantly altered by verapamil administration. D. Active lever presses during extinction and cue reinstatement. Verapamil prevents cue-induced reinstatement in alcohol dependent rats. n=5-8/group. Statistical analysis was performed by repeated measures ANOVA, followed by Newman-Keuls post-hoc test when applicable. P values: **p<0.01, ***p<0.001 alc.-dep. vs. control; ###p<0.001 verapamil vs. CSF.

Newman Keuls post-hoc test revealed that in alcohol-dependent rats, verapamil was effective in preventing cue-induced reinstatement (p=n.s.). All other groups showed cue-induced reinstatement (extinction vs. reinstatement: Non-dependent + CSF: $p<0.001$; Non-dependent + Verapamil: $p<0.001$; Alcohol-dependent + CSF: $p<0.001$). Alcohol dependent rats also showed significantly less active lever pressing than control rats when treated with verapamil

($p<0.001$). Furthermore, there was a significant difference between verapamil-treated and CSF-treated alcohol dependent rats ($p<0.001$), and between alcohol dependent and non-dependent CSF-treated rats ($p<0.01$).

1.7. Summary of Study I

In this study, we found increased *Cacna1c* mRNA in the amygdala and hippocampus, and $Ca_V1.2$ protein and current in the hippocampus, of 3 weeks abstinent rats. Furthermore, we observed a dynamic regulation of *Cacna1c* mRNA from alcohol intoxication, over withdrawal, to prolonged abstinence. Although verapamil had no effect on alcohol self-administration, central verapamil application completely blocked cue-induced reinstatement of alcohol-seeking. The data suggests that central LTCCs, most likely $Ca_V1.2$, play a role in relapse-related behavior.

2. Study II: Characterization of L-type calcium channel subtype expression in animal models of alcoholism

As *Cacna1c* and *Cacna1d* showed distinct expression changes in 3 weeks abstinent rats (Study I), this study was aimed at characterizing the expression of these LTCC subtypes in several animal models of alcohol dependence. The models shed light on different aspects of alcohol dependence, therefore determining the LTCC subtype expression will help to define the specific role of $Ca_V1.2$ and $Ca_V1.3$. In addition, it will give an overview of which models are suitable to study LTCCs in alcoholism in the future.

In my Master Thesis (Uhrig, 2012), I already measured *Cacna1c* and *Cacna1d* mRNA expression in Marchigian Sardinian alcohol-preferring (msP) rats (which were kindly provided by Dr. Roberto Ciccocioppo, University of Camerino, Italy) (Ciccocioppo *et al*, 2006; Hansson *et al*, 2006a). In the amygdala and hippocampus, we found an increase of *Cacna1c* and *Cacna1d* mRNA.
The expression pattern differs from the expression found in 3 weeks alcohol abstinent rats (Study I), especially since there are no changes of *Cacna1d* mRNA expression in the 3 weeks alcohol abstinent rats. These findings encourage the characterization of further animal models for a comprehensive interpretation of the role of both subtypes in alcohol dependence.

2.1. Differential *Cacna1c* and *Cacna1d* mRNA in the amygdala and hippocampus of alcohol preferring AA and non-preferring ANA rats

Alcohol preferring AA and non-preferring ANA rats were selectively bred for high or low voluntary alcohol consumption, respectively. These rat lines were originally derived from a foundation stock that included Wistar and Sprague-Dawley strains, and later crossed with F1 hybrids from Lewis and Brown Norwegian rats (Sommer *et al*, 2006).

AA and ANA rats were compared to each other and to Wistar rats, which were used as non-selected controls. Although AA and ANA rats were derived from many different strains, Wistar rats have been used as controls in other studies in this thesis, as well as other studies on AA/ANA rats (Caberlotto *et al*, 2001; Sommer *et al*, 2001). One-way ANOVA followed by Fisher's PLSD test and Bonferroni's correction found an increase of *Cacna1c* mRNA in the BLA for both AA and ANA lines (18% and 16% increase, respectively), while *Cacna1d* mRNA is decreased in the MeA of ANA rats (22% decrease). In the hippocampus, *Cacna1c* mRNA is increased in AA rats (CA1: 27%, CA4: 14%, DG: 22%). In the ANA rats, *Cacna1c* mRNA is only decreased in the CA4 (10% decrease), with no changes in the other

hippocampal regions. A similar pattern is found for *Cacna1d* mRNA, as it is increased in the CA4 (10% increase) and DG (27% increase) of AA rats, and decreased in the CA1 (13% decrease) and CA4 (16% decrease) of ANA rats. Interestingly, the changes in AA and ANA rats for each subtype sometimes coincide (e.g. *Cacna1c* in the BLA) and sometimes are opposed (e.g. *Cacna1c* and *Cacna1d* in the CA4). In the interpretation of the results, the specific characteristics of the lines and the functions of each region therefore have to be especially considered. Absolute values and statistics are listed in Suppl. Table 6.

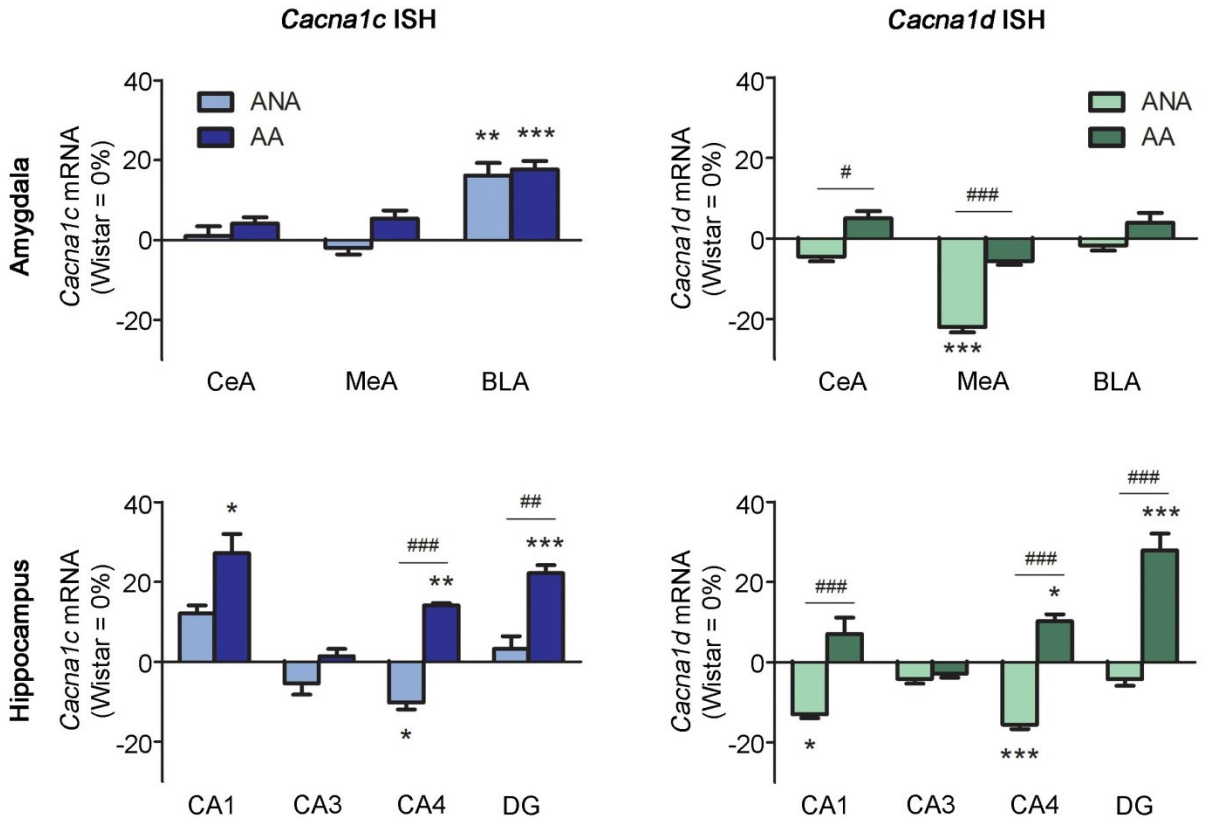

Figure 20. Expression changes of *Cacna1c* and *Cacna1d* mRNA in AA and ANA rats compared to Wistar rats. In situ hybridization shows distinct expression pattern for each subtype in the amygdala (central amygdala, CeA; medial amygdala, MeA; basolateral amygdala, BLA) and hippocampus (Cornus Ammon (CA) regions 1, 2, and 4; dentate gyrus, DG). Bar graphs represent changes in expression with Wistar rats defined as 0%. Data are expressed as mean ± SEM. Statistical analysis was performed by region-wise one-way ANOVA followed by Fisher's PLDS test and Bonferroni's correction, n=5-6/group, p-values: *p<0.05; **p<0.01; ***p<0.001 vs. Wistar; #p<0.05; ##p<0.01; ###p<0.001 AA vs. ANA. Absolute values and statistics are listed in Suppl. Table 6.

2.2. Induction of the alcohol deprivation effect (ADE) does not influence central LTCC expression in the amygdala and hippocampus

Next to genetic models of alcoholism, functional models are of great importance for the investigation of neuroadaptations. The ADE model is based on prolonged periods of free

access to alcoholic solutions with intermittent periods of abstinence. As soon as alcohol is available again after abstinence, rats increase their alcohol intake in a manner similar to the binge drinking in humans. This model therefore is aimed at the loss of control over drinking habits.

However, LTCCs in the amygdala and hippocampus do not appear to have an important role in this process, as no changes in mRNA expression was found for either *Cacna1c* or *Cacna1d*. On the other hand, the results have to be considered as preliminary, because of the small sample size (N=3-5/group). Data and statistics are listed in Suppl. Table 7.

2.3. 4 weeks of alcohol vapor inhalation is sufficient to increase *Cacna1c* mRNA levels

It was previously shown that 7 weeks of intermittent alcohol vapor exposure followed by 3 weeks of abstinence increases alcohol self-administration and preference in rats, while after only 4 weeks of alcohol exposure and 3 weeks of abstinence, no such increased alcohol-seeking was found (Rimondini *et al*, 2003). In Study I, we showed increased *Cacna1c* mRNA levels after 7 weeks of alcohol vapor exposure and 3 weeks of abstinence.

Here, we investigated the changes in *Cacna1c* mRNA expression after 4 weeks of vapor exposure followed by 3 weeks of abstinence, in order to determine whether this LTCC subtype is already altered by a shorter period of alcohol exposure. As all animals arrived at the institute simultaneously and all groups were sacrificed on the same day, controls for 4 and 7 weeks were the same.

We found a strong increase in *Cacna1c* mRNA in the amygdala (CeA: 66%, MeA: 62%, BLA: 47% increase) and hippocampus (CA1: 86%, CA3: 45%, DG: 29% increase) after 4 weeks of alcohol vapor exposure and 3 weeks of abstinence. This is an even stronger increase than after 7 weeks exposure and 3 weeks of abstinence, except in the CA1, where there is an equally strong increase for both time periods. Raw data is listed in Suppl. Table 10.

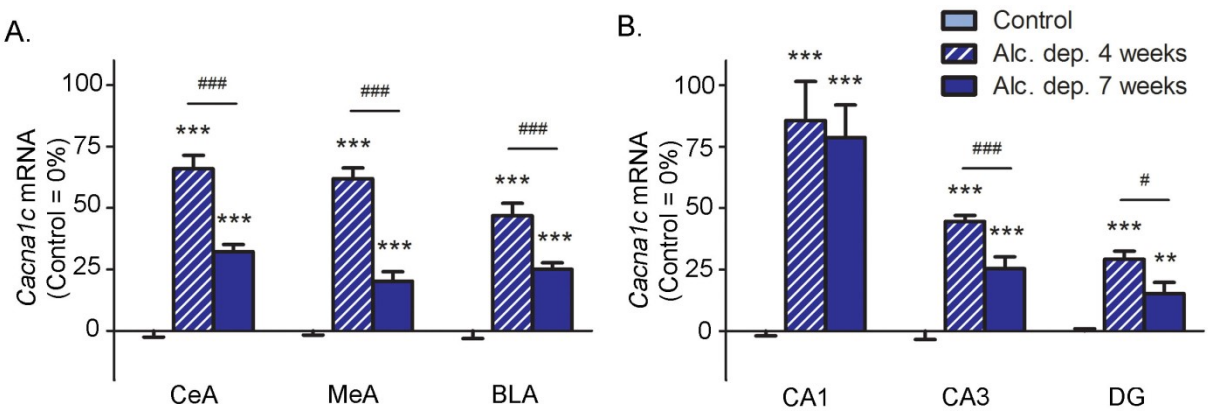

Figure 21. *Cacna1c* mRNA expression is increased in 3 weeks abstinent rats, both after 4 weeks and 7 weeks of alcohol vapor exposure. *Cacna1c* mRNA levels in A. the amygdala, and B. the hippocampus. Rats were exposed to alcohol vapor for 4 weeks or 7 weeks, each followed by 3 weeks of abstinence. All animals were sacrificed on the same day, therefore controls were the same for 4 weeks and 7 weeks exposure. Bar graphs are normalized to control levels (changes in regulation: control 4 weeks = 0%). Statistical analysis was performed by region-wise two-way ANOVA (time, treatment) followed by Fisher'sP LDS post-hoc test and Bonferroni's correction, n=6-8/group, p values: ***p<0.001 vs controls; #p<0.05, ###p<0.001 4 vs. 7 weeks.

In a previous study it was shown that the CRH receptor 1 (Crhr1) is increased in the amygdala of alcohol dependent rats after 7 weeks of alcohol vapor exposed 3 weeks of abstinence. The CRH system is crucial for anxiety-like behavior, which is also increased at this point during alcohol dependence (Hansson *et al*, 2006a; Meinhardt *et al*, 2015; Sommer *et al*, 2008). It would be interesting whether the CRH system is already activated after 4 weeks of alcohol vapor exposure and 3 weeks of abstinence, or the activation takes place after the temporal threshold described in (Rimondini *et al*, 2003). Therefore we investigated *Crhr1* and *Crh* mRNA expression in another group of rats which had been exposed for 4 or 7 weeks to alcohol vapor, each followed by 3 weeks of abstinence. Here, animals were not exposed simultaneously for practical reasons, which necessitated separate control groups for 4 and 7 weeks.

After 4 weeks of alcohol vapor exposure and 3 weeks of abstinence, no significant difference of *Crhr1* or *Crh* mRNA expression was found between alcohol dependent rats and controls (*Crh* mRNA: Control 4 weeks: 14.85 ± 3.5 nCi/g, Alc. dep. 4 weeks: 11.57 ± 1.2 nCi/g). Animals which had been exposed for 7 weeks, on the other hand, showed strongly increased *Crhr1* mRNA expression in the CeA (52% increase compared to "Control 7 weeks") and BLA (69% increase compared to "Control 7 weeks"). At this time, *Crh* mRNA was significantly increased in the CeA (50% increase compared to "Control 7 weeks"; Control 7 weeks: 12.67 ± 1.7 nCi/g, Alc. dep. 7 weeks: 20.16 ± 2.2 nCi/g, p<0.05), as well. For *Crhr1*, there was also a highly significant increase in 7 weeks-exposed rats compared to 4 weeks-

exposed rats in the CeA, MeA, and BLA, although these increases have to be considered in the context that "Control 4 weeks" also differs from "Control 7 weeks". Absolute values for *Crhr1* mRNA are listed in Suppl. Table 11.

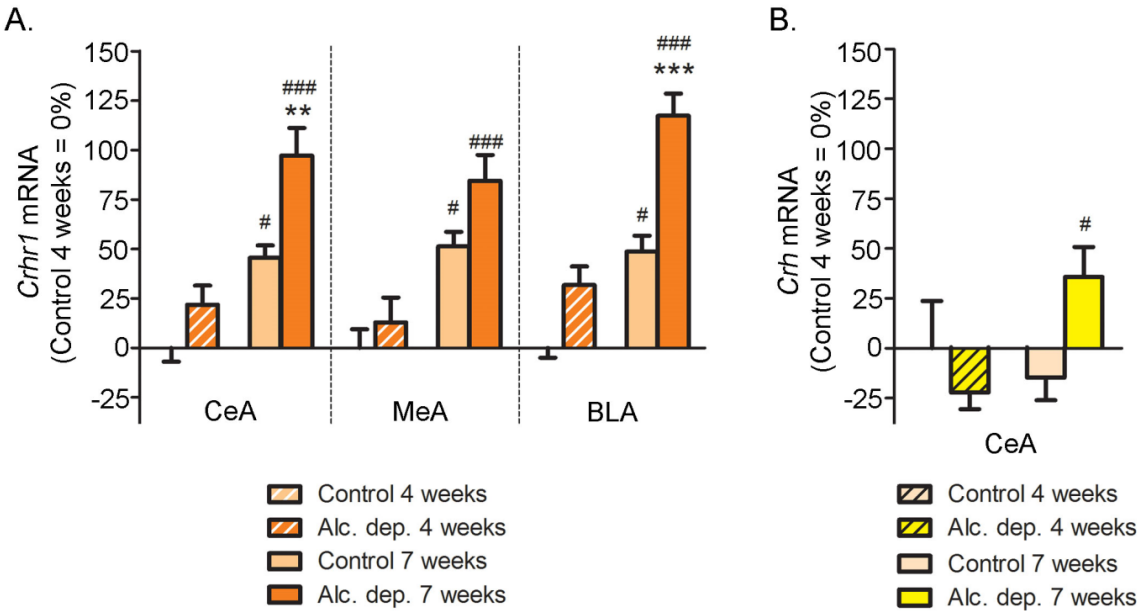

Figure 22. *Crhr1* and *Crh* mRNA levels are increased in the amygdala of rats exposed to alcohol vapor for 7 weeks, but not 4 weeks. Animals were exposed to alcohol vapor or normal air for 4 weeks or 7 weeks, each followed by 3 weeks of abstinence. Bar graphs are normalized to Control 4 weeks (changes in regulation: Control 4 weeks = 0%). Statistical analysis was performed by region-wise two-way ANOVA (time, treatment) followed by Fisher's PLSD post-hoc test and Bonferroni's correction, n=3-7/group, p values: **p<0.01, ***p<0.001 vs. respective controls; #p<0.05, ###p<0.001 4 vs. 7 weeks. Central amygdala, CeA; medial amygdala, MeA; basolateral amygdala, BLA; Cornus ammon (CA) regions of the hippocampus 1, and 3;dentate gyrus, DG.

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2.4. Voluntary alcohol consumption decreases *Cacna1c* mRNA expression and differentially regulates *Cacna1d* mRNA levels

Alcohol dependence induced through intermittent cycles of alcohol vapor intoxication and abstinence has been shown valuable in the search for potential anti-relapse medication (Meinhardt *et al*, 2015). Although alcohol consumption is not voluntary, the model offers good face, predictive, and construct validity (see 1.4). However, this raises the question how voluntary alcohol consumption influences *Cacna1c* and *Cacna1d* mRNA expression.

To address this, we performed *in situ* hybridization on alcohol dependent and non-dependent rats which had been allowed free access to an alcohol solution for 3 weeks. Brain tissue was kindly provided by Dr. Roberto Rimondini (Bologna University, Italy).

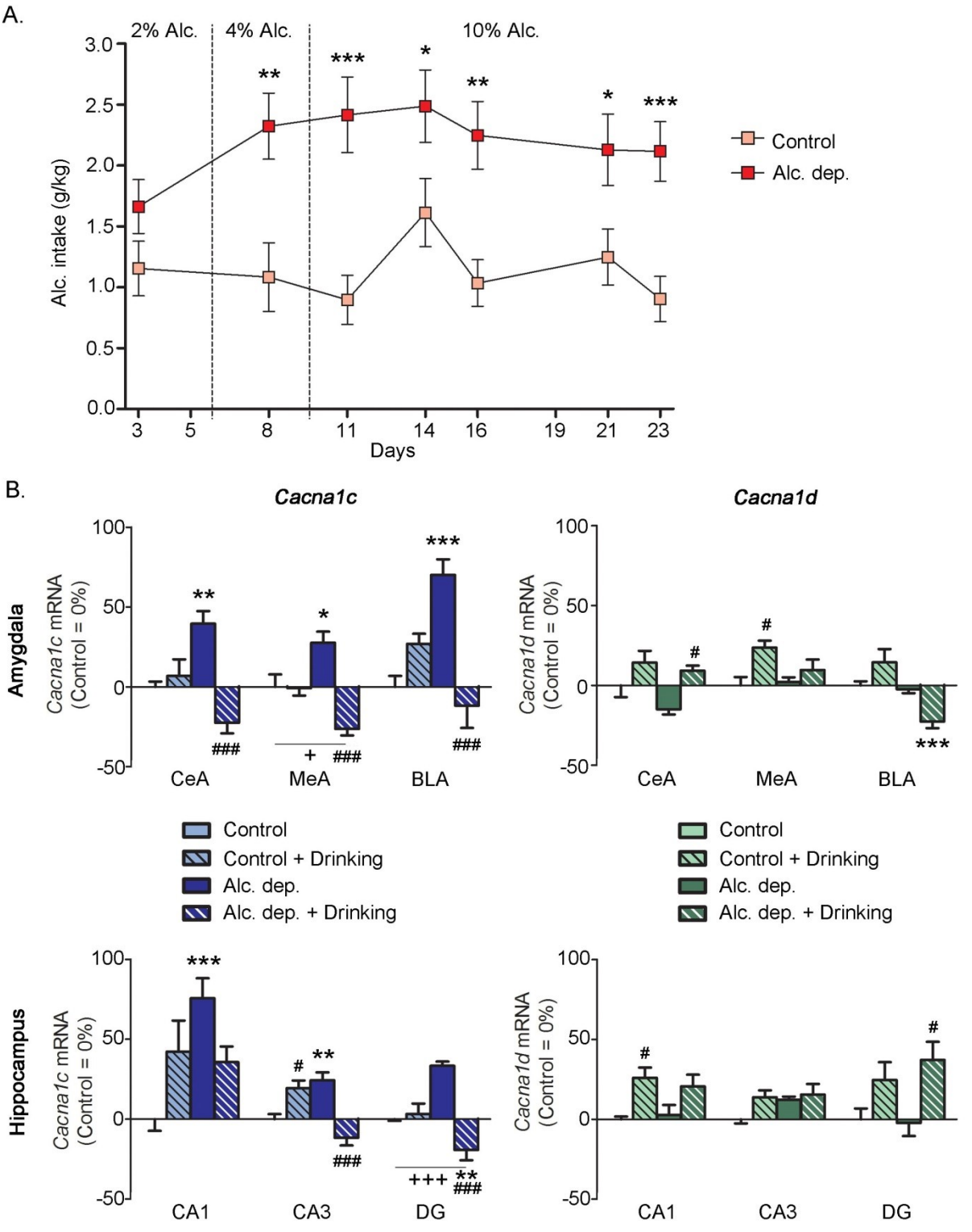

Figure 23. Free access to alcohol affects *Cacna1c* and *Cacna1d* mRNA expression differentially. Groups of alcohol dependent or control rats (n=5-7) were given *ad libitum* access to a 10% alcohol solution. Water was present in the other bottle. A. Alcohol intake over 23 days of free access. During the first days, alcohol was presented at a concentration of 2% and 4%, After the measurement on day 8, a 10% alcohol solution was presented. B. *Cacna1c* and *Cacna1d* mRNA measurements in the amygdala and hippocampus. p values: *p<0.05; **p<0.01; ***p<0.001 vs. respective control group; #p<0.05; ##p<0.01; ###p<0.001 respective Alc. dep. vs. Control groups; +p<0.05; +++p<0.001 Alc. dep. + Drinking v.s. Control. Central amygdala, CeA; medial amygdala, MeA; basolateral amygdala, BLA; cornus ammon (CA) regions 1 and 2; dentate gyrus (DG).

As shown previously in Study I, alcohol dependent rats showed strongly increased *Cacna1c* mRNA expression in the amygdala and hippocampus. However, in alcohol dependent rats which additionally consumed alcohol voluntarily *Cacna1c* mRNA was significantly decreased compared to alcohol dependent rats without voluntary alcohol consumption. The decrease compared to controls without voluntary drinking experience reached significance only in the MeA (26% decrease) and the DG (30% decrease). In the amygdala, *Cacna1c* mRNA was also decreased in drinking alcohol dependent rats compared to drinking controls, but it did not reach significance (CeA: p=0.057; MeA: p=0.06; BLA: p=0.052; not shown in Figure 23).

Cacna1d is only altered in animals with voluntary alcohol consumption. The changes are not as consistent as for *Cacna1c,* differing across regions. In the CeA and DG, *Cacna1d* mRNA is increased in alcohol dependent rats with voluntary alcohol consumption compared to alcohol dependent rats. In the MeA and CA1, there is an increase in drinking controls compared to non-drinking controls, and in the BLA, *Cacna1d* mRNA is strongly decreased in alcohol dependent drinking rats compared to control rats with alcohol consumption. Considering that all p values were corrected for multiple analyses, the results are nevertheless robust, and indicate a differentiated role for $Ca_V1.3$ in the effects of alcohol consumption which warrants further investigation. Absolute values and statistics are listed in Suppl. Table 8 and Suppl. Table 9.

2.5. Stress increases *Cacna1d* mRNA expression in the hippocampus

In alcohol dependence, stress is of great importance. Animal models of alcoholism such as the the functional model of alcohol vapor intoxication and the msP rats, a genetic model considered to be the phenocopy of the alcohol vapor model, are characterized by an increased sensitivity to stress (Bjork *et al*, 2010; Ciccocioppo *et al*, 2006; Meinhardt *et al*, 2015). Here, we investigated to which extent *Cacna1c* and *Cacna1d* are regulated by exposure to restraint stress.

4 h after a 1 h restraint period, the rats showed a significant decrease of *Cacna1c* mRNA in the MeA ($F[1,10]=15.08$, $p=0.021$), with no further changes in the amygdala or hippocampus. *Cacna1d*, on the other hand, was increased in the hippocampus (CA1: $F[1,10]=18.48$. $p=0.011$; DG: $F[1,10]=17.8$, $p=0.006$). In the CeA, BLA, CA3, and CA4, *Cacna1d* mRNA increase did not reach significance. Mirroring Cacna1c expression, Cacna1d mRNA is

decreased in the MeA, although not significantly. For mean values and statistical evaluation see Suppl. Table 12.

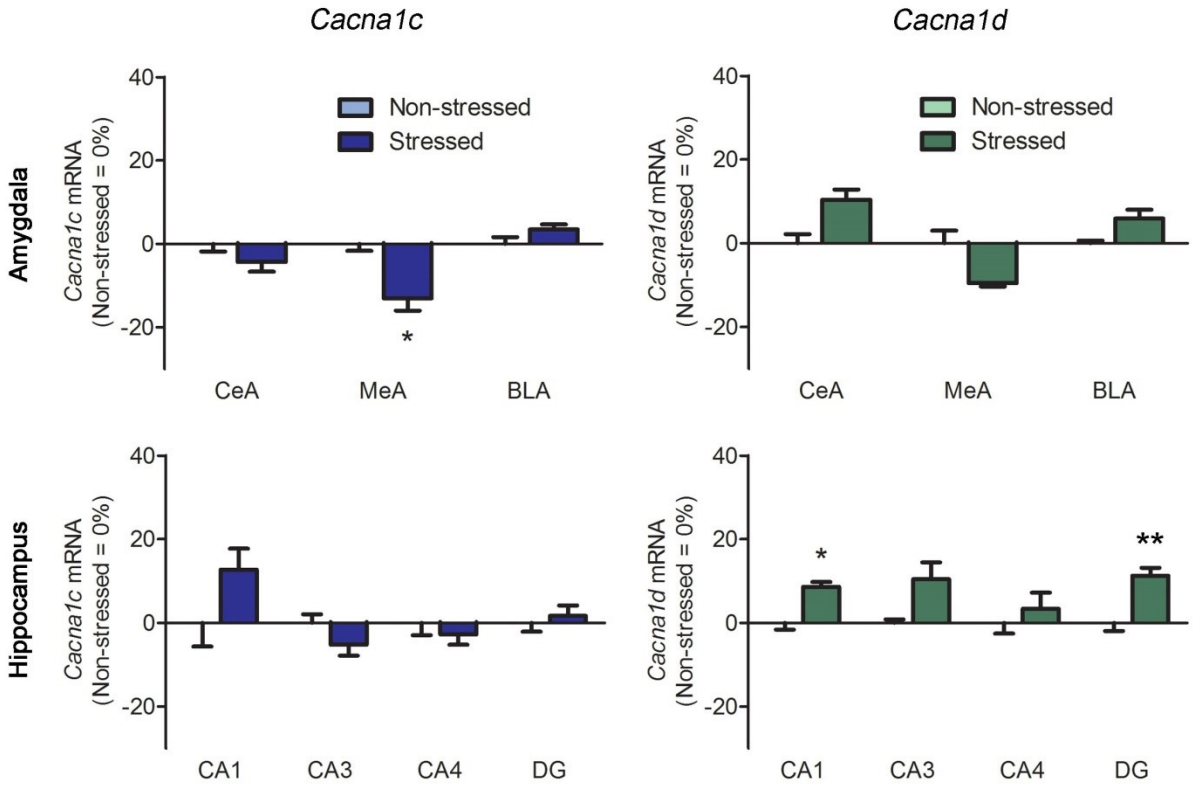

Figure 24. *Cacna1d* mRNA is significantly increased in the hippocampus of Wistar rats 4 hours after restraint stress. Bar graphs show mean ± SEM, values are normalized to non-stressed controls (Non-stressed = 0% change). *p<0.05 after Bonferroni's correction, n=4-6/group. Central amygdala, CeA; medial amygdala, MeA; basolateral amygdala, BLA; Cornus ammon (CA) regions of the hippocampus 1, 3, and 4;dentate gyrus, DG.

2.6. Summary of Study II

Here, we analyzed *Cacna1c* and *Cacna1d* mRNA expression in genetic and functional animal models of alcohol dependence. In animal models of alcoholism, both LTCC subtypes are generally increased in the amygdala and hippocampus. In non-preferring rats, *Cacna1c* and *Cacna1d* mRNA are mostly decreased. The functional model for binge drinking and craving does not show any changes in LTCC expression. However, alcohol vapor intoxication strongly increases *Cacna1c*, but not *Cacna1d*, mRNA expression, whereby a shorter alcohol exposure of 4 weeks leads to a stronger increase than the often used 7 weeks of exposure. After 7 weeks of exposure the CRH system, which is strongly involved in stress and anxiety-related behavior, is also activated. Voluntary alcohol consumption on its own increases *Cacna1d* mRNA expression, while *Cacna1c* mRNA is strongly decreased in alcohol dependent rats which also had free access to alcohol. Restraint stress also increases *Cacna1d*

expression. Together with the findings from Study I, Study II shows that the alcohol vapor exposure appears to be the best animal model to study the role of central LTCC subtypes in alcohol dependence.

Table 2. Summary of *Cacna1c* and *Cacna1d* mRNA changes in animal models of alcoholism. ↑/↓p<0.05; ↑↑/↓↓p<0.01; ↑↑↑/↓↓↓p<0.001 increase/decrease compared to Wistar rats after Bonferroni's correction; (↑)/(↓)p<0.08 (trend); ↔ no changes; ↔ Study I; N/A: not measured. *(Uhrig, 2012)

Animal model		mRNA	Cing	PreL	IL	CPu	AcbC	AcbS	CeA	MeA	BLA	CA1	CA3	DG
msP*		*1c*	↑↑↑	↑↑↑	↔	↑↑↑	N/A	N/A	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↔	↑
		1d	↑↑↑	↔	↑	↑↑↑	N/A	N/A	↑↑↑	↑↑↑	↑↑↑	↔	↑	↔
AA		*1c*	↔	↔	↔	↔	↔	↔	↔	↔	↑↑↑	↑	↔	↑↑↑
		1d	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↑↑↑
ANA		*1c*	↔	↔	↔	↔	↔	↔	↔	↔	↑↑	↔	↔	↔
		1d	↔	↔	↔	↓↓	↔	↔	↔	↓↓↓	↔	↓	↔	↔
ADE		*1c*	N/A	N/A	N/A	N/A	N/A	N/A	↔	↔	↔	↔	↔	↔
		1d	N/A	N/A	N/A	N/A	N/A	N/A	↔	↔	↔	↔	↔	↔
Alc. dep.	4 wk	*1c*	N/A	N/A	N/A	N/A	N/A	N/A	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
	7 wk	*1c*	↔	↔	↔	↔	↔	↔	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑
Drinking Control		*1c*	↓↓↓	↓↓↓	↓↓	↔	↓	↓	↔	↔	↔	↔	↑	↔
		1d	↑	↔	↔	↔	↔	↔	↔	↑	↔	↑	↔	↔
Drinking Alc. Dep.		*1c*	↓↓↓	↓↓↓	(↓)	↔	↔	↓	↓↓↓	↓↓↓	↓↓↓	↔	↓↓↓	↓↓↓
		1d	↔	↑	↑↑↑	(↑)	↑↑↑	↑↑	↑	↔	↓↓↓	↔	↔	↑
Stress		*1c*	↔	↔	↔	↔	↓↓↓	↓	↔	↓	↔	↔	↔	↔
		1d	↔	↓↓	↔	↑↑	↔	↔	↔	↔	↔	↑	↔	↑↑

3. Study III: L-type calcium channel subtype $Ca_V1.2$ mediates dependence-induced increase in alcohol self-administration

In Study I, we indirectly examined the involvement of $Ca_V1.2$ in alcohol dependence. Although the i.c.v. injection of verapamil could not distinguish between $Ca_V1.2$ and $Ca_V1.3$, the *Cacna1c* mRNA expression data and electrophysiological readings strongly suggest a role for $Ca_V1.2$, rather than $Ca_V1.3$, after prolonged abstinence.

In this study, we aimed to further investigate this hypothesis using transgenic mice with a KO of $Ca_V1.2$ in CaMKII-positive neurons, thus clearly distinguishing between the two central LTCC subtypes.

3.1. Locomotor activity does not differ between mutants and wildtypes

Before taking a closer look at the behavior in alcohol self-administration, the Open Field test was used to determine any changes in locomotor activity, which could also influence lever pressing in operant chambers. These experiments were performed jointly with Dr. Miriam Vogt.

Open Field measurements of distance travelled and velocity did not show a difference between $Ca_V1.2$ mutant mice and their littermate controls. Time spent in the center was equally comparable between the two groups.

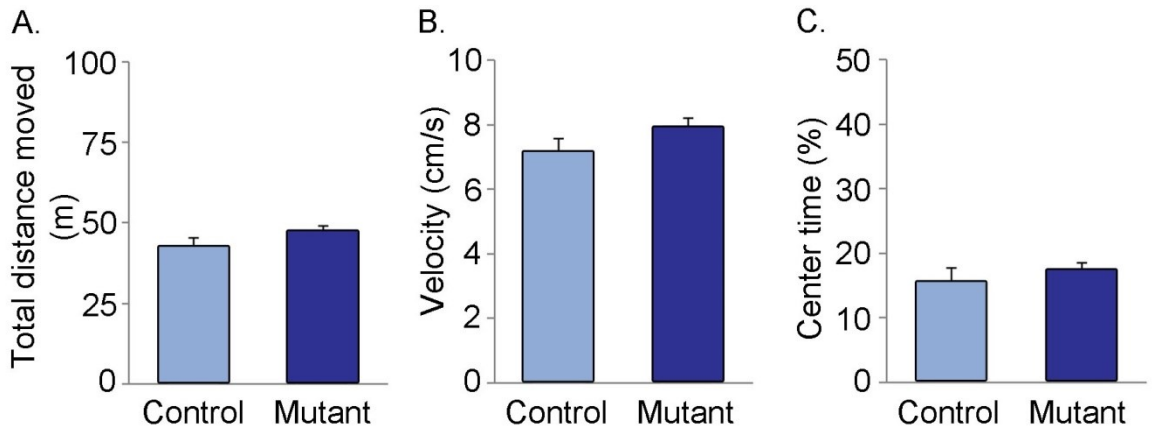

Figure 25. No change in locomotor activity in mice with a decrease in $Ca_V1.2$ was found. Total distance moved (A.), velocity (B.) and time spent in the center (C.) were equal to littermate controls. Bars show mean ± SEM, One-way ANOVA did not reveal any differences between groups, n= 20-22/group.

3.2. Lack of $Ca_V1.2$ blocks alcohol dependence-induced increase in self-administration

Mice were trained to press a lever for a drop of alcohol. Subsequently, they were either alcohol vapor or air exposed before their self-administration behavior was measured again. This experiment was designed to determine the motivation to perform a task to receive alcohol, and was performed jointly with Merle Kochan, and Dr. Ainhoa Bilbao.

During training sessions, including the saccharine fading procedure, there was no significant difference between the genotypes and the (putative) treatment groups. Successful induction of alcohol dependence was verified by measuring Withdrawal Scores after the last alcohol vapor exposure. Blood alcohol concentrations (BACs) were also determined, and alcohol exposed showed intoxicating BACs directly after the end of vapor exposure.

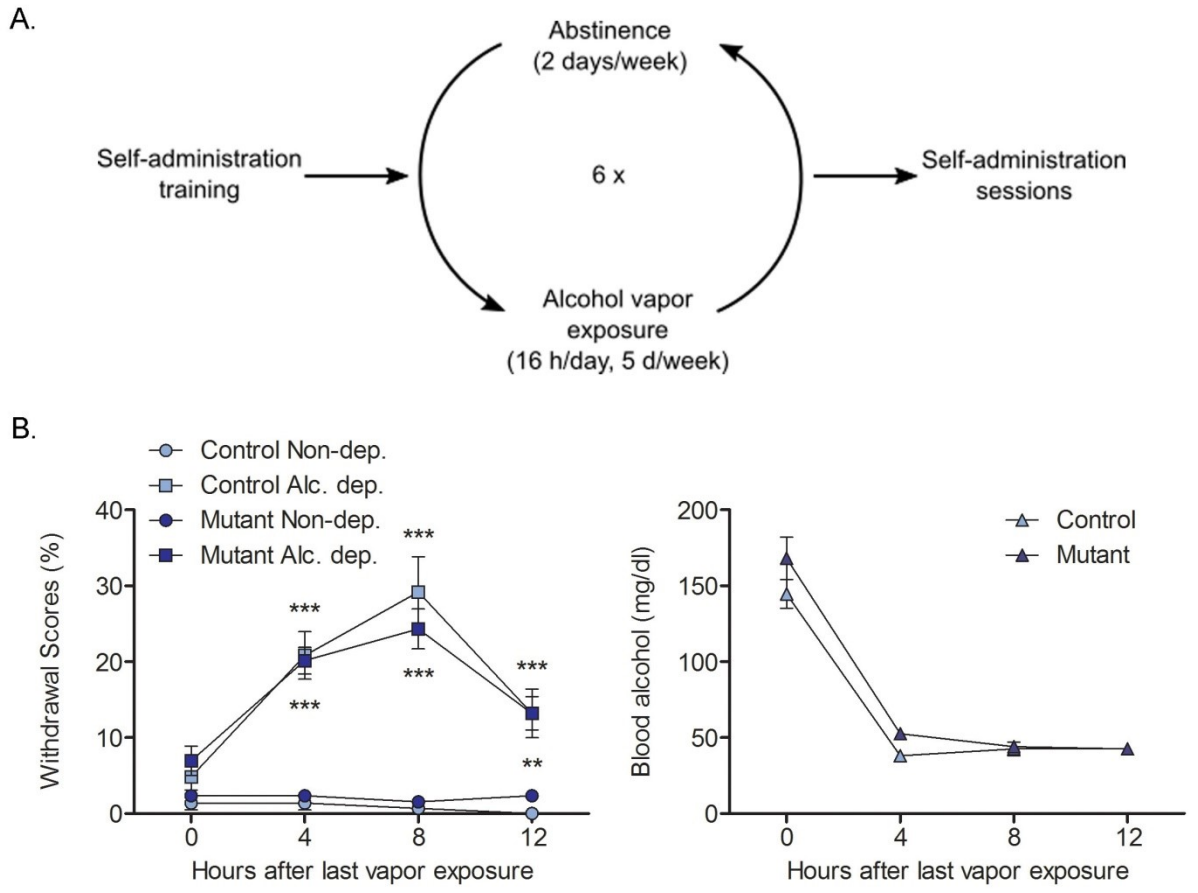

Figure 26. Alcohol dependent mice show strong withdrawal signs and high BACs after the last alcohol vapor exposure. A. After alcohol self-administration training alcohol dependence was induced through repeated cycles of vapor inhalation and abstinence. After 6 cycles, alcohol self-administration was measured. B. Left graph: Withdrawal signs are strongly increased after 4, 8 and 12 h of abstinence. ***p<0.001, **p<0.01 compared to respective non-dependent group. Right graph: Blood alcohol concentrations (BACs) of ~150 mg/dl (0 h) are comparable to alcohol intoxication in humans. BACs rapidly decrease during abstinence.

Alcohol self-administration after alcohol vapor/air exposure showed no main effect for genotype, but a trend toward a significant genotype*treatment interaction ($F[1,17]=4.38$, $p=0.05$) and a significant main effect for treatment ($F[1,17]=8.02$, $p<0.05$).

Newman Keuls post-hoc test revealed a strongly increased lever pressing of alcohol dependent control mice compared to air exposed controls ($p<0.001$), while alcohol dependent mutant mice did not differ from air exposed mutants. There was also no significant difference between air exposed controls and mutants, while alcohol dependent controls had a significant increase in lever pressing compared to alcohol dependent $Ca_V1.2$ mutant mice ($p<0.05$).

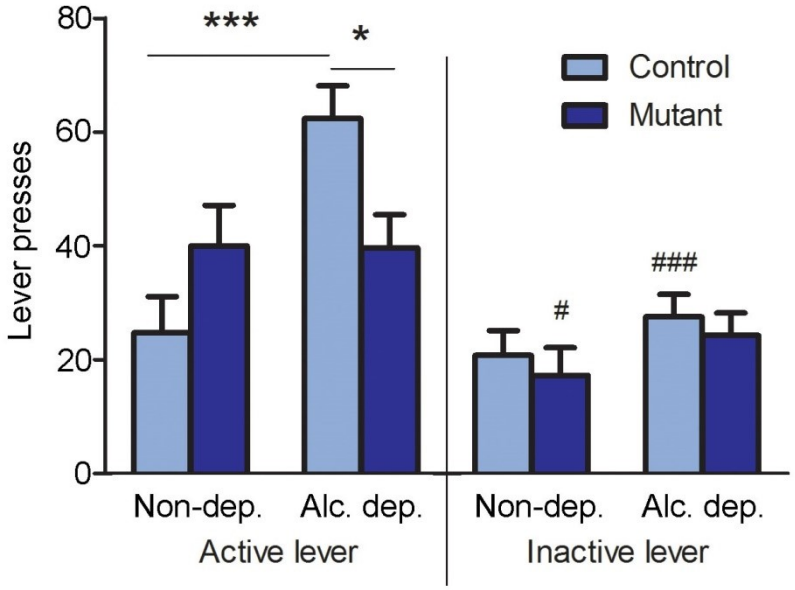

Figure 27. Alcohol dependence does not induce an increase in alcohol self-administration in $Ca_V1.2$ mutant mice. Control mice show a significant increase in alcohol self-administration after chronic intermittent alcohol vapor exposure, which is not observed in mutants. Bars indicate mean ± SEM of lever presses. Active (left side) and inactive (right side) lever presses were considered for repeated measures two-way ANOVA (lever*genotype*treatment). *$p<0.05$, ***$p<0.001$ comparison of active lever presses as indicated; #$p<0.05$; ###$p<0.001$ inactive lever vs. respective active lever; n=4-6/group. The data was produced jointly with Merle Kochan and Dr. Ainhoa Bilbao.

3.3. Demonstration of the $Ca_V1.2$ knockout

We performed PCRs on genomic DNA and cDNA (RNA) as well as *in situ* hybridization for *Cacna1c* and *Cacna1d* to verify the KO of $Ca_V1.2$ in CaMKII-positive neurons. The hippocampus was chosen as region of interest for both PCR and *in situ* hybridization because of its strong LTCC expression. CaMKII plays an important role in the hippocampus, such as the mediation of long-term potentiation (LTP) (Stanton and Gage, 1996) and hippocampus-dependent memory and learning functions (Tan and Liang, 1996). Hence, it is expressed in

many, but not all, hippocampal neurons, which allows for a partial $Ca_V1.2$ KO in the hippocampus.

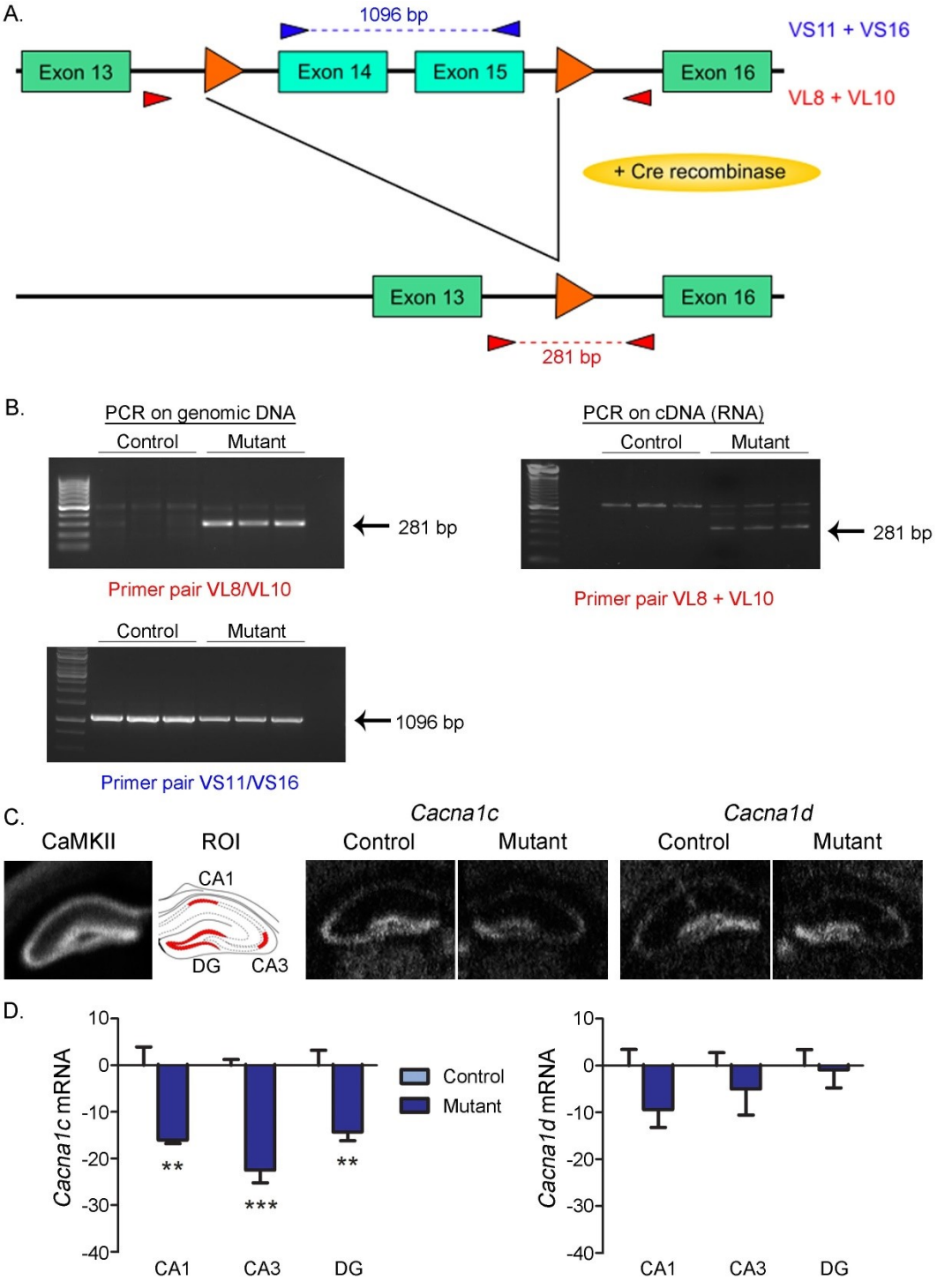

Figure 28. Demonstration of $Ca_V1.2$ KO in CaMKII-positive neurons. A. Schematic representation of exons 13 to 16 of *Cacna1c*. loxP sites are indicated by orange arrows. Cre recombinase cuts out DNA between loxP sites. Primers for validation of *Cacna1c* mRNA decrease are indicated in blue and red. B. PCR on genomic hippocampal DNA of *Cacna1c* (exons 14 and 15) shows an empirical decrease in mutant mice compared to controls (VS11/VS16) and a smaller fragment of 281 bp (VL8/VL10). Genomic PCR data was produced by Dr. Kai Schönig (CIMH, Mannheim). The smaller fragment was also found by PCR on cDNA of the same samples; n=3/group. C. Representative in situ hybridization of CaMKII (left), and regions of interest (ROI), and

comparisons of control and mutant mice by in situ hybridization for *Cacna1c* (middle) and *Cacna1d* (right). Subtype-specific riboprobes were kindly provided by Prof. M. Knipper (University of Tübingen). D. Quantitative evaluation of in situ hybridization of *Cacna1c* (left) and *Cacna1d* (right). Bars represent data as mean ± SEM, values are normalized to controls (controls = 0% change). **p<0.01; ***p<0.0001, n=5-6/group.

For PCR, two different sets of primers were used (Figure 28). Primer pair VL8/VL10 spans a fragment between exons 13 and 16, and excision of exons 14 and 15 would yield a shorter, 281 bp fragment. Primer pair VS11/VS16 is located within exons 14 and 15, so that in a complete KO no fragment should be found.

PCR on both genomic DNA and cDNA (RNA) showed the 281 bp fragment in mutant mice (Cre^+) using primers VL8/VL10, in addition to the larger fragment of control mice (Cre^-). The appearance of the larger fragment is due to *Cacna1c* DNA and mRNA expression in neurons without CaMKII. Additionally, PCR using primers VS11/VS16 on genomic DNA yielded less PCR product in mutant than in control mice. Although this is an empirical estimation rather than a quantitative measurement, it provides evidence for decreased *Cacna1c* expression in mutants.

In situ hybridization revealed a decrease in *Cacna1c* mRNA, but not *Cacna1d* mRNA, in the hippocampus of mutant mice. In the CA1, *Cacna1c* mRNA was decreased by 16% ($F[1,9]=13.18$, $p<0.01$; mutants: 18.4±0.2 nCi/g, controls: 21.9±0.9 nCi/g), in the CA3 by 22% ($F[1,9]=64.58$, $p<0.001$; mutants: 46.4±1.6 nCi/g, controls: 59.9±0.7 nCi/g), and in the DG by 14% ($F[1,9]=13.02$, $p<0.01$; mutants: 48.6±1.1 nCi/g, controls: 56.7±1.8 nCi/g).

3.4. Summary of Study III

$Ca_V1.2$flox x $CaMKIICre^{ERT2}$ mice showed a decrease in *Cacna1c* mRNA in the hippocampus, with no changes in locomotor activity. While alcohol dependent control mice increased their in alcohol self-administration compared to non-dependent controls, the decrease in $Ca_V1.2$ completely abolished this effect, as no difference between alcohol dependent and non-dependent mutant mice was observed.

Our results give further insights into the role of $Ca_V1.2$ in alcohol dependence, indicating an influence of this particular LTCC subtype on alcohol self-administration.

4. Study IV: Functions of L-type calcium channel subtypes $Ca_V1.2$ and $Ca_V1.3$ in nicotine-related behavior

L-type calcium channels have been implicated in the effects of nicotine exposure, development of tolerance, nicotine-induced reinstatement of conditioned place preference, and withdrawal symptoms. However, just as in alcohol dependence, the function of each subtype has yet to be determined.

In this study, *in situ* hybridization experiments after acute and chronic nicotine exposure, as well as during abstinence after chronic nicotine, were used to determine changes in *Cacna1c* and *Cacna1d* mRNA levels. On a behavioral level, locomotor activity was measured in response to nicotine sensitization. Furthermore, a preliminary nicotine self-administration study was performed.

4.1. *Cacna1c* and *Cacna1d* mRNA levels change during acute and chronic nicotine exposure and abstinence

mRNA levels of *Cacna1c* and *Cacna1d* were determined by *in situ* hybridization with subtype-specific riboprobes in mice after a single or chronic nicotine exposure and 24 h or 7 d of abstinence.

We found a downregulation of *Cacna1c* transcripts in the IL and CA1 (14% and 26%, respectively) after a single nicotine injection and 24 h of abstinence. In all other measured regions there was no difference between nicotine- and saline-injected animals. *Cacna1d* on the other hand was strongly upregulated in the PFC (Cing and OFC: 46% - 50%), CPu (27%), and AcbS (45%). No differences of *Cacna1d* mRNA levels were observed in the PreL, IL, AcbC, and hippocampus.

After chronic (14 d) nicotine exposure and 24 h of abstinence, *Cacna1c* mRNA levels were downregulated in the Cing (14%), PreL (17%), CPu (9%), VTA (16%), and CA3 (17%). There were no changes of *Cacna1d* transcription in any region except for the DG, where the expression was upregulated by 12%.

During abstinence (7 d after chronic nicotine exposure), we found a strong upregulation of *Cacna1c* transcripts in almost all regions (11% - 30%), excluding the Cing, AcbC, and VTA. In the CPu there was also an increase of 13% of *Cacna1d* mRNA, but in all other regions *Cacna1d* transcription was unaltered. Absolute values and statistical evaluation are listed in Suppl. Table 13 and Suppl. Table 14, respectively.

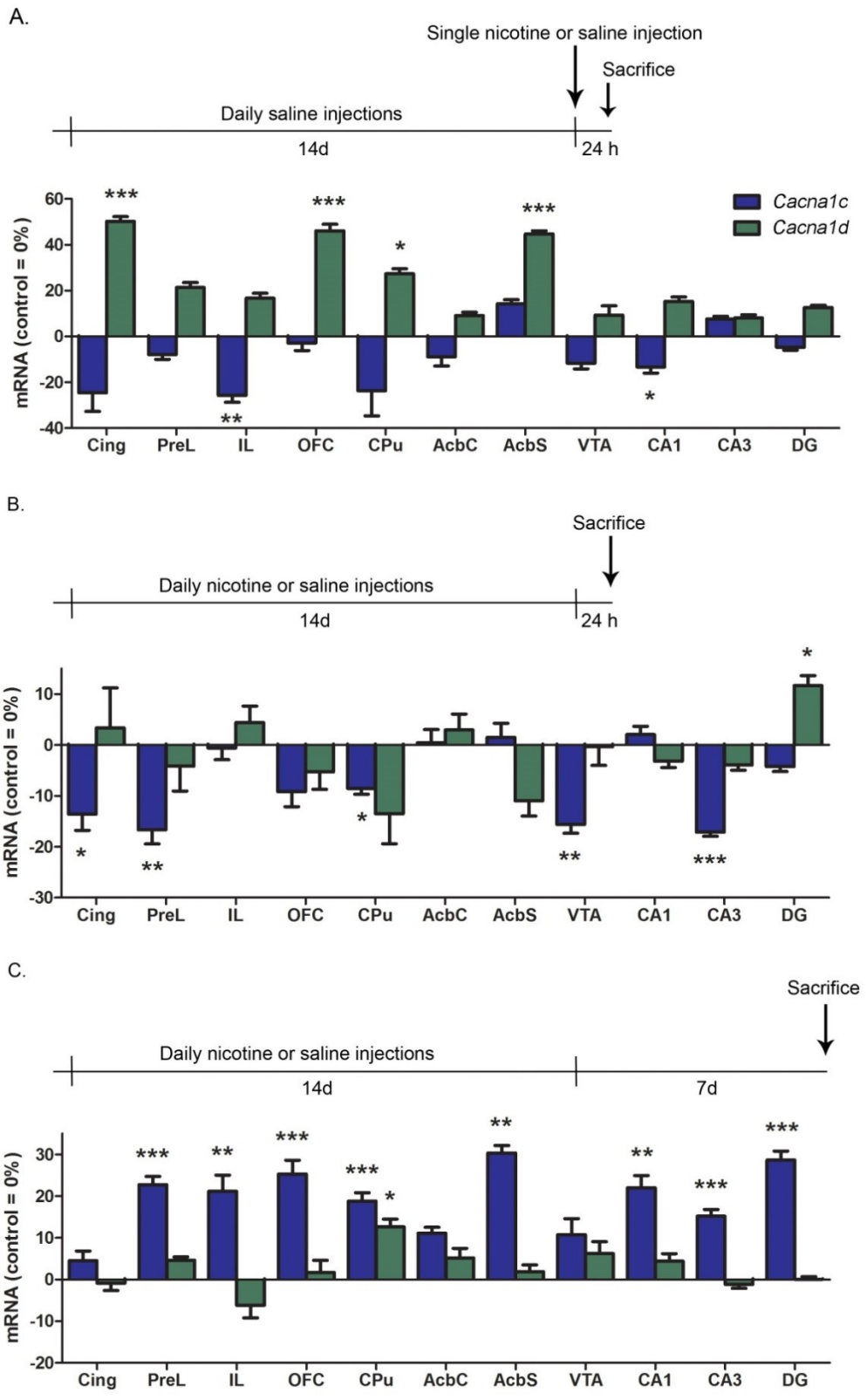

Figure 29. *Cacna1c* and *Cacna1d* mRNA expression is differentially regulated by nicotine exposure and abstinence. Experimental outline is shown above the respective graphs, with nicotine injected at 0.175 mg/kg, i.p., and saline at 0.9%, i.p. Bar graphs show *in situ* hybridization data of *Cacna1c* (blue) and *Cacna1d* (green) normalized to respective saline control group (mean ± SEM). Region-wise one-was ANOVA was used for statistical evaluation, followed by Bonferroni's correction, n=4-8/group, corrected p-values: *p<0.05, **p<0.01, ***p<0.001. For abbreviations see Materials and Methods. nCi/g values are summarized in Suppl. Table 13.

4.2. Nifedipine attenuates locomotor activity after 12, but not 24 h

Two doses of the non-selective LTCC antagonist nifedipine were tested concerning their effect on locomotor activity to determine the best dose for sensitization experiments. These experiments were performed jointly with Dr. Rick E. Bernardi.

30 min and 12 h after injection of 10 and 25 mg/kg nifedipine, there was a significant locomotor depression for both doses, while neither dose had an effect 24 h after injection. Significant effects were revealed by one-way ANOVA for 30 min ($F[2,19]=7.8$, $p<0.005$) and 12 h ($F[2,19]=4.1$, $p<0.05$), while there was no effect at 24 h ($F[2,19]=1.8$, p=n.s.). Fisher's PLSD test detected a decrease in locomotor activity at 30 min for both 10 mg/kg ($p<0.01$) and 25 mg/kg ($p<0.005$), as well as at the 12 h interval (both $p<0.05$) in comparison to vehicle injections.

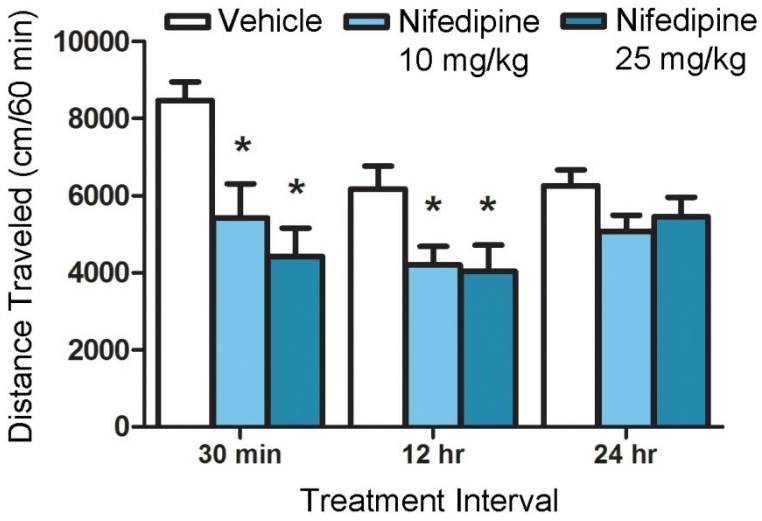

Figure 30. Nifedipine decreases locomotor activity 30 min and 12 h, but not 24 h, after i.p. injection compared to vehicle. The effects were observed for both 10 mg/kg and 25 mg/kg. Data are expressed as means ± SEM. *p<0.05 vs. vehicle. The data was produced jointly with Dr. Rick E. Bernardi.

The usage of 10 mg/kg nifedipine in the following nicotine sensitization experiment was determined by these findings, in addition to the consideration of the half-lives of nifedipine doses (Waltereit *et al*, 2008).

4.3. Nicotine-treated mice show increased locomotion

Locomotor responses were measured on day 1 and day 14 of chronic nicotine or saline treatment. These experiments were performed jointly with Dr. Rick E. Bernardi.

A two-way ANOVA (drug treatment x day) showed significant main effects of drug treatment ($F[1,31]=5.6$, $p<0.05$), day ($F[1,31]=50.4$, $p<0.001$), and drug treatment x day interaction ($F[1,31]=10.1$, $p<0.005$). Both nicotine-treated and saline-treated mice displayed increased locomotor activity on day 14 compared to day 1 in a paired *t* test ($t(16)=8.7$, $p<0,001$; and ($t(15)=2.4$. $p<0.05$, respectively). However, on day 1 there was no significant difference between nicotine- and saline-treated mice ($_t(31)=0.7$, p=n.s.), while on day 14 nicotine-treated mice displayed a significantly higher locomotor response compared to saline-treated controls ($t(31)=3.6$, $p<0.005$).

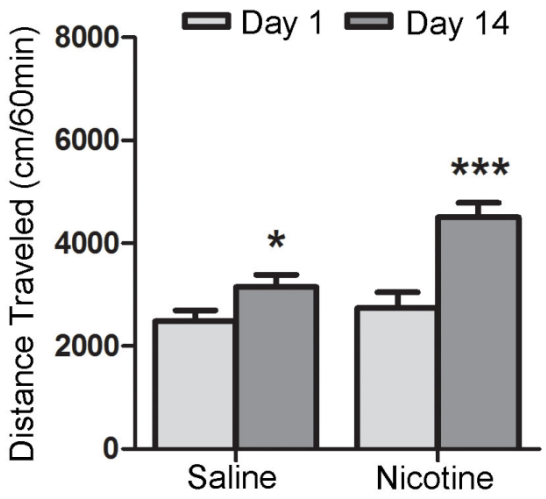

Figure 31. Locomotor activity increase in saline- and nicotine-treated mice after 14 d of repeated i.p. injection. After locomotor activity measurement on day 1, animals were injected with either saline or nicotine (0.175 mg/kg, i.p.) daily. The increase in locomotion was stronger in nicotine-treated than in saline-treated mice. *p<0.05, ***p<0.001 vs. day 1. The data was produced jointly with Dr. Rick E. Bernardi.

4.4. Nifedipine prevents increased nicotine sensitization after abstinence

14 d of chronic nicotine or saline treatment were followed by a 6 d abstinence period, during which nifedipine was administered every 12 h. Nicotine sensitization was then again measured on day 21. These experiments were performed jointly with Dr. Rick E. Bernardi.

The groups (vehicle and nifedipine, each in nicotine- and saline-treated mice) were chosen so that vehicle- and nifedipine-treated mice did not differ in their locomotion on day 14 (Fs<1).
In saline-treated mice, nifedipine did not show any effect on locomotion. Two-way ANOVA (treatment [vehicle vs. nifedipine] x day [day 14 vs. day 21]) revealed neither significant main effects nor a significant interaction (Fs<1), indicating that nifedipine did not result in any unspecific changes in locomotion.
Nicotine-treated mice which had received vehicle injections during the abstinence period displayed a sensitized response to the nicotine challenge on day 21, which was blocked in the

nifedipine-treated group. A two-way ANOVA (treatment [vehicle vs. nifedipine] x day [day 14 vs. day 21]) revealed a significant treatment x day interaction ($F[1,15]=5.2$, $p<0.05$). There was also a main effect of day ($F[1,15]=27.7$, $p<0.001$), but no main effect of nifedipine vs. vehicle treatment ($F[1,15]=1.1$, p=n.s.). Further analysis by paired *t* test confirmed that in nicotine-treated vehicle mice, there was a significant sensitization effect from day 14 to day 21 ($t(7)=5.0$, $p<0.005$). Treatment with nifedipine during abstinence prevented this increase ($t(8)=2.3$, p=n.s.).

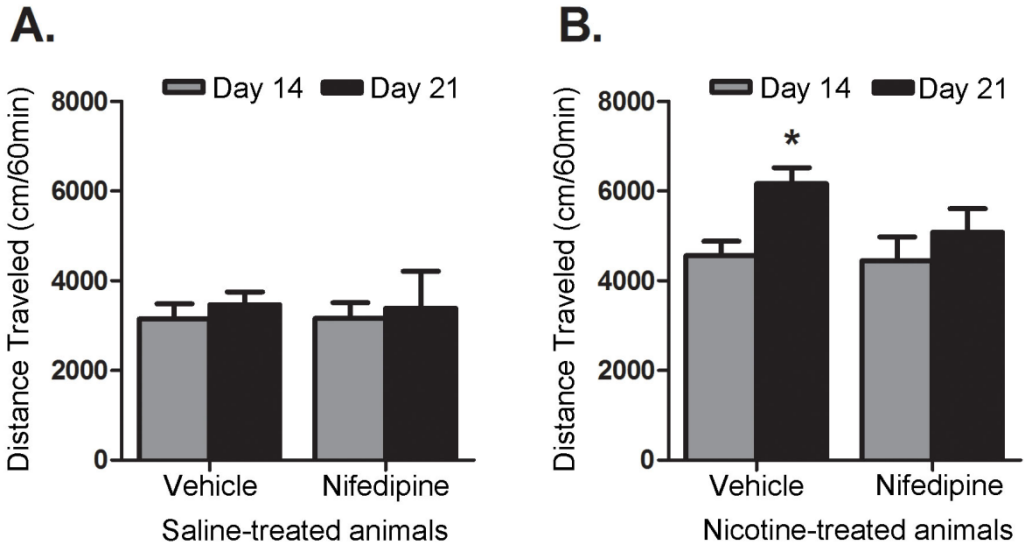

Figure 32. Increased nicotine-sensitization after 7 d abstinence is prevented by nifedipine. Saline-treated (A.) and nicotine-treated (B.) mice were each divided into two groups for either vehicle or nifedipine (10 mg/kg, i.p.) injections on day 15 – 20. Locomotor activity measurement on day 21 revealed nicotine-sensitization in vehicle-treated mice. This sensitization effect was not observed in nifedipine-treated mice. Nifedipine did not show an effect in mice which had received saline on day 1 - 14. *p<0.05 vs. day 14. The data was produced jointly with Dr. Rick E. Bernardi.

4.5. Decreased $Ca_V1.2$ attenuates nicotine self-administration

Nicotine self-administration (SA) behavior was assessed in a preliminary study using $Ca_V1.2$flox x CaMKIICre^{ERT2} mice with a decrease of $Ca_V1.2$ in CaMKII-positive forebrain neurons. Mice were first trained with food pallets, and an i.v. catheter was implanted into the jugular vein. Self-administration testing was performed on 8 consecutive days for 2 h each, on a FR2 ratio. This experiment was performed by Dr. Rick E. Bernardi and the data were included to support the interpretation of our data.

Mutants and control mice differed in the acquisition of nicotine SA, as revealed by three-way ANOVA (lever [active vs. inactive] x day [repeated measures] x genotype). There were significant main effects of lever ($F[1,16]=6.58$, $p<0.05$) and day ($F[2.2,34.5]=7.63$, $p<0.005$). The interaction of lever x genotype almost reached significance ($F[1,16]=4.32$, $p=0.054$), but

no other effects were found (lever x day: F<1; genotype: F[1,16]=3.61, p=0.076; day x genotype: F[2.2,34.5]=1.56, p=0.22). Post-hoc t-tests showed a decrease in active lever presses in mutants compared to controls (t(16)=2.42, p<0.05), while there was no difference in the responses to the inactive lever (t(16)=0.86, p=0,4).

The number of nicotine reinforcers acquired during the SA sessions was also decreased in mutant mice compared to controls, with a significant day x genotype interaction (F[3.2,50.4]=3.56, p<0.05) and significant main effects of day (F[3.2,50.4=7.7, p<0.0001] and genotype (F[1,16]=5.1, p<0.05).

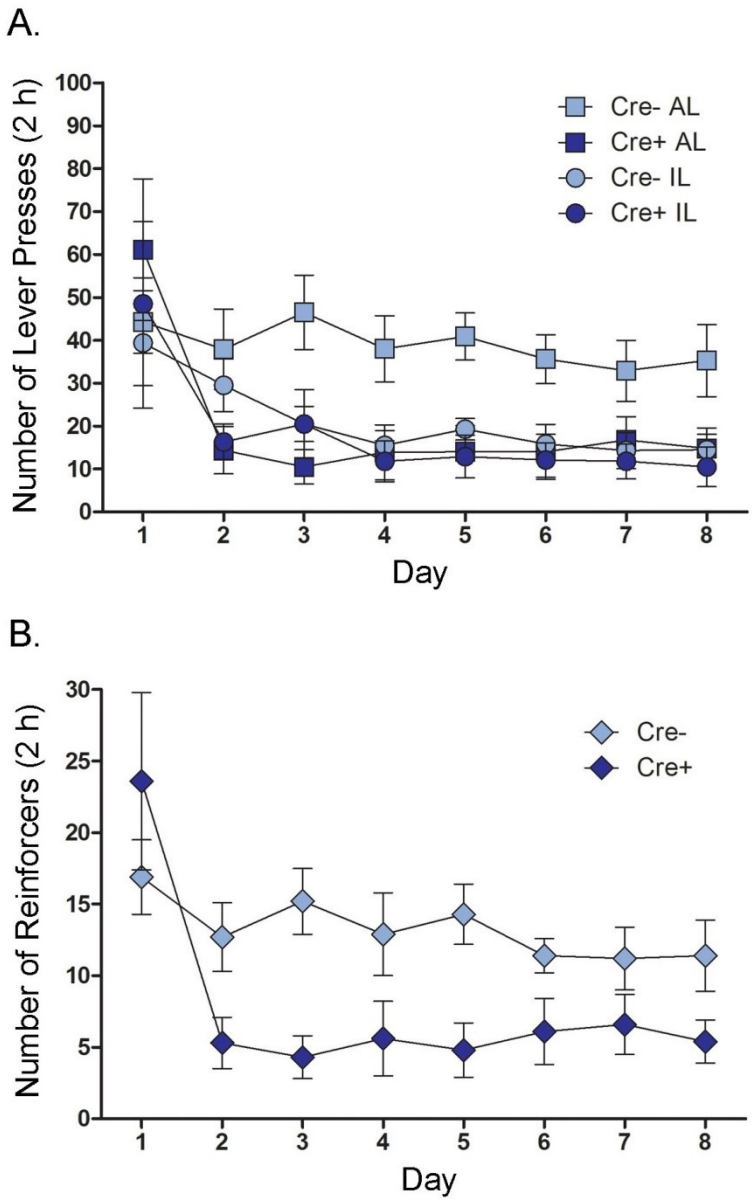

Figure 33. Nicotine self-administration is decreased in $Ca_V1.2$ mutants. Active (AL) and inactive (IL) lever presses (A.) and number of reinforcers (B.) during 8 consecutive days of 2h self-administration sessions. Data are expressed as mean ± SEM. N=8-10. The data was produced by Dr. Rick E. Bernardi.

4.6. Summary of Study IV

We found a differential regulation of *Cacna1c* and *Cacna1d* mRNA in response to nicotine. *Cacna1d* mRNA was strongly upregulated in several brain regions after acute nicotine, with little changes in *Cacna1c* mRNA levels. After chronic nicotine, *Cacna1c* was down-regulated in some regions, but after an additional period of prolonged abstinence, there was a strong upregulation of *Cacna1c* mRNA in almost all regions. Both after chronic nicotine administration and prolonged abstinence, *Cacna1d* mRNA was mostly at control levels. On a behavioral level, nicotine-induced increase in locomotor activity was inhibited by nifedipine. A decrease of *Cacna1c* mRNA in CAMKII-positive neurons of transgenic mice furthermore reduced nicotine SA behavior compared to controls, implicating $Ca_V1.2$ as the influential LTCC in nicotine-related behavior.

5. Study V: Analysis of L-type calcium channel subtype expression in human postmortem samples of patients with mental disorders

Several single nucleotide polymorphisms (SNPs) in *CACNA1C* have been identified as risk indicators for psychiatric disorders such as schizophrenia, major depressive disorder (MDD) and bipolar disorder. However, the changes in *CACNA1C* expression taking place in these disorders are not well understood. In *CACNA1D*, so far no SNPs have been detected which indicate a risk of developing mental disorders, although it may still be of importance. Considering our findings in Study I, II and III, changes of *CACNA1C* mRNA might also be found in alcohol dependent subjects.

To address the changes in *CACNA1C* and *CACNA1D* expression, we measured the mRNA in postmortem samples of alcoholic patients, schizophrenia patients, and suicide completers with major depression, compared to their respective age-matched controls by qRT-PCR, also considering their genotype in the SNP rs1006737.

5.1. No changes in *CACNA1C* mRNA expression in the striatum of human alcoholics

CACNA1C and *CACNA1D* mRNA were measured in the nucleus caudatus (NC) and ventral striatum (VS) in postmortem brain tissue of human alcoholics and control subjects from the New South Wales Tissue Resource Centre at the University of Sydney, Australia, by qRT-PCR. As alcoholic samples included some patients with blood alcohol at the time of death, three conditions were considered: non-intoxicated alcoholics, intoxicated alcoholics, and controls.

No significant changes for either LTCC subtype were found for condition, and there was no correlation with the appearance of the risk alleles AA or AG of the *CACNA1C* SNP rs1006737. dCt values are given in Suppl. Table 15.

Confounding factors such as age or the use of nicotine could influence the analysis. Therefore we performed an analysis of covariants. After corrections for multiple analyses, the only significant confounding factor was the PMI in the analysis of *CACNA1D* mRNA in the NC ($p=0.032$).

Table 3. Clinical characteristics of alcoholic patients and control subjects.

	Control		Alcoholic		Intoxicated alcoholic	
	VS (n = 49)	NC (n = 45)	VS (n = 28)	NC (n = 32)	VS (n = 14)	NC (n = 15)
Genotype (GG/AG/AA)	27/16/6	24/15/6	15/9/4	16/11/5	5/9/0	5/10/0
Gender (female/male)	11/38	11/34	9/19	9/23	2/12	3/12
Age (years)	56.4 ± 1.6	56.2 ± 1.7	60.2 ± 2.0	58.3 ± 2.0	54.1 ± 4.2	55.0 ± 3.8
RIN values	8.5 ± 0.1	8.6 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	8.6 ± 0.2	8.5 ± 0.1
Brain pH	6.6 ± 0.04	6.6 ± 0.04	6.4 ± 0.1	6.4 ± 0.1	6.6 ± 0.1	6.5 ± 0.1
PMI	29.3 ± 2.1	29.1 ± 2.3	34.1 ± 3.1	34.8 ± 3.0	28.4 ± 3.3	28.4 ± 2.9
Current smokers	15	14	19	24	6	6

5.2. Decreased *CACNA1C* and *CACNA1D* mRNA in forebrain regions of schizophrenia patients

A small set of postmortem samples of schizophrenia patients (n=7) and control subjects (n=6) from the Department of Neuropathology, Mental Hospital Wiesloch, Germany, was used for qRT-PCR with LTCC subtype-specific primers to determine *CACNA1C* and *CACNA1D* mRNA levels.

Table 4. Clinical characteristics of alcoholic patients and control subjects.

	Controls	Schizophrenia patients
Gender (female/male)	1/5	4/5
Age (years)	61.8 ± 6.8	68.2 ± 5.0
PMI (hours)	15.7 ± 2.3	20.6 ± 3.6
Brain pH	6.8 ± 0.1	6.7 ± 0.1
RIN values BA10	7.32 ± 0.2	7.86 ± 0.3
RIN values BA21	7.18 ± 0.4	6.8 ± 0.2
RIN values NC	7.76 ± 0.2	8.46 ± 0.2
RIN values vermis	6.48 ± 1.1	7.84 ± 0.3

Two forebrain regions, the anterior prefrontal cortex Brodmann Area 10 (BA) and the medial temporal gyrus BA21, as well as the NC and vermis of the cerebellum were analyzed.

Schizophrenia patients exhibited decreased *CACNA1C* mRNA expression in both the BA10 (8.1% decrease) and BA21 (21.8% decrease). *CACNA1D* mRNA was equally decreased in the BA10 (12.2%) and the BA21 (17.1% decrease). No changes in mRNA expression for either *CACNA1C* or *CACNA1D* were found in the NC and the vermis. dCt values, ddCt values, F and p values are listed in Suppl. Table 16.

Due to the limited sample numbers it was not possible to perform an analysis of covariants. The results therefore have to be considered with caution.

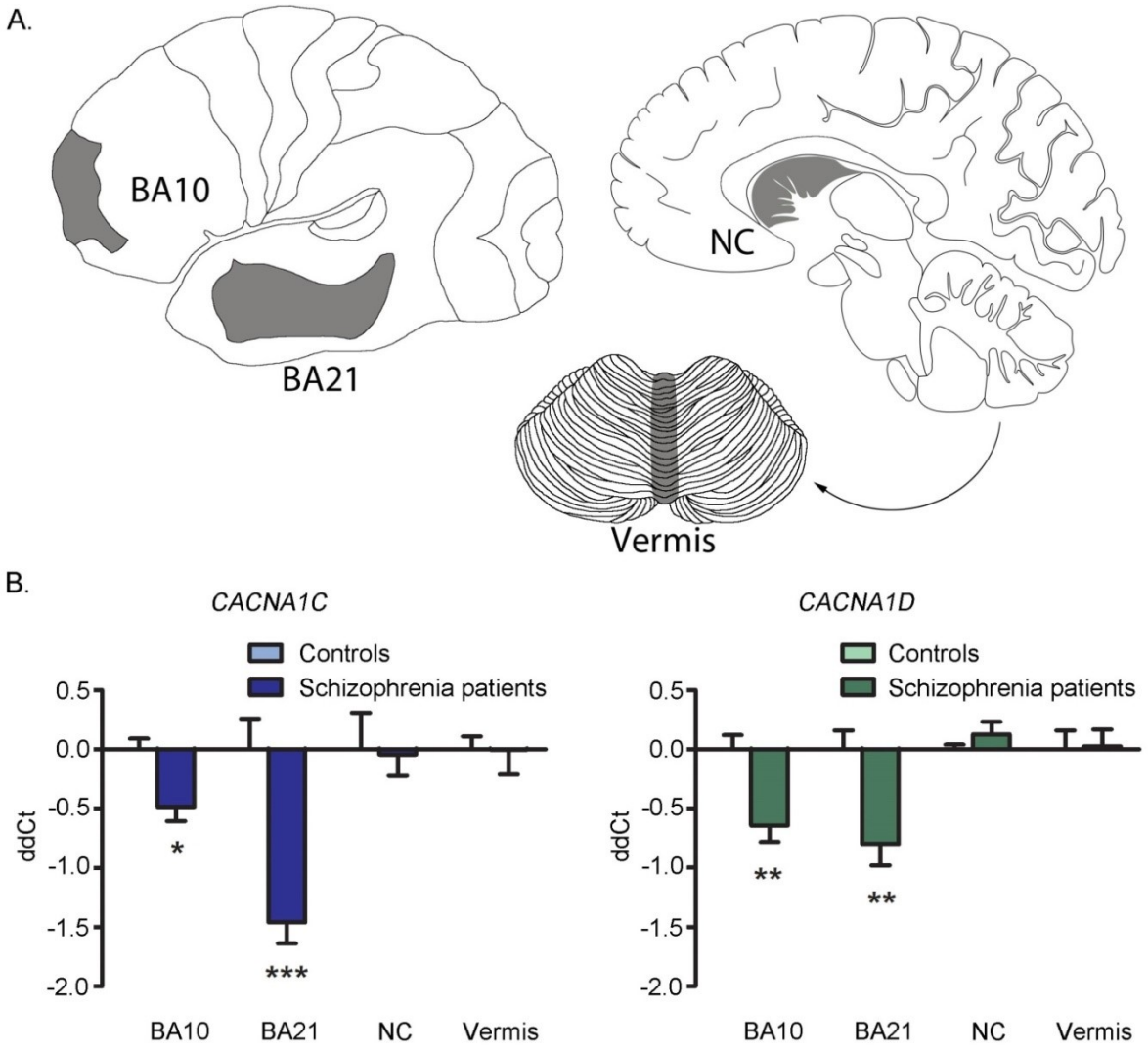

Figure 34. qRT-PCR revealed decreased *CACNA1C* and *CACNA1D* mRNA expression in forebrain regions of schizophrenia patients compared to control subjects. A. Schematic representation of measured areas. B. Bars show ddCt values, with *GAPDH* as internal normalizer, as mean ± SEM. Brodmann Area, BA; *p<0.05, **p<0.01, ***p<0.001, n=5-7.

5.3. No effect of haloperidol and clozapine on *Cacna1c* and *Cacna1d* mRNA expression

Schizophrenia patients are often treated with medication that might itself alter the expression of *CACNA1C* and *CACNA1D* mRNA. We therefore treated rats with two commonly used antipsychotics, haloperidol and clozapine, for 12 weeks, and analyzed *Cacna1c* and *Cacna1d* mRNA levels in several brain regions, to determine whether these medications are a confounding factor in our human postmortem analysis.

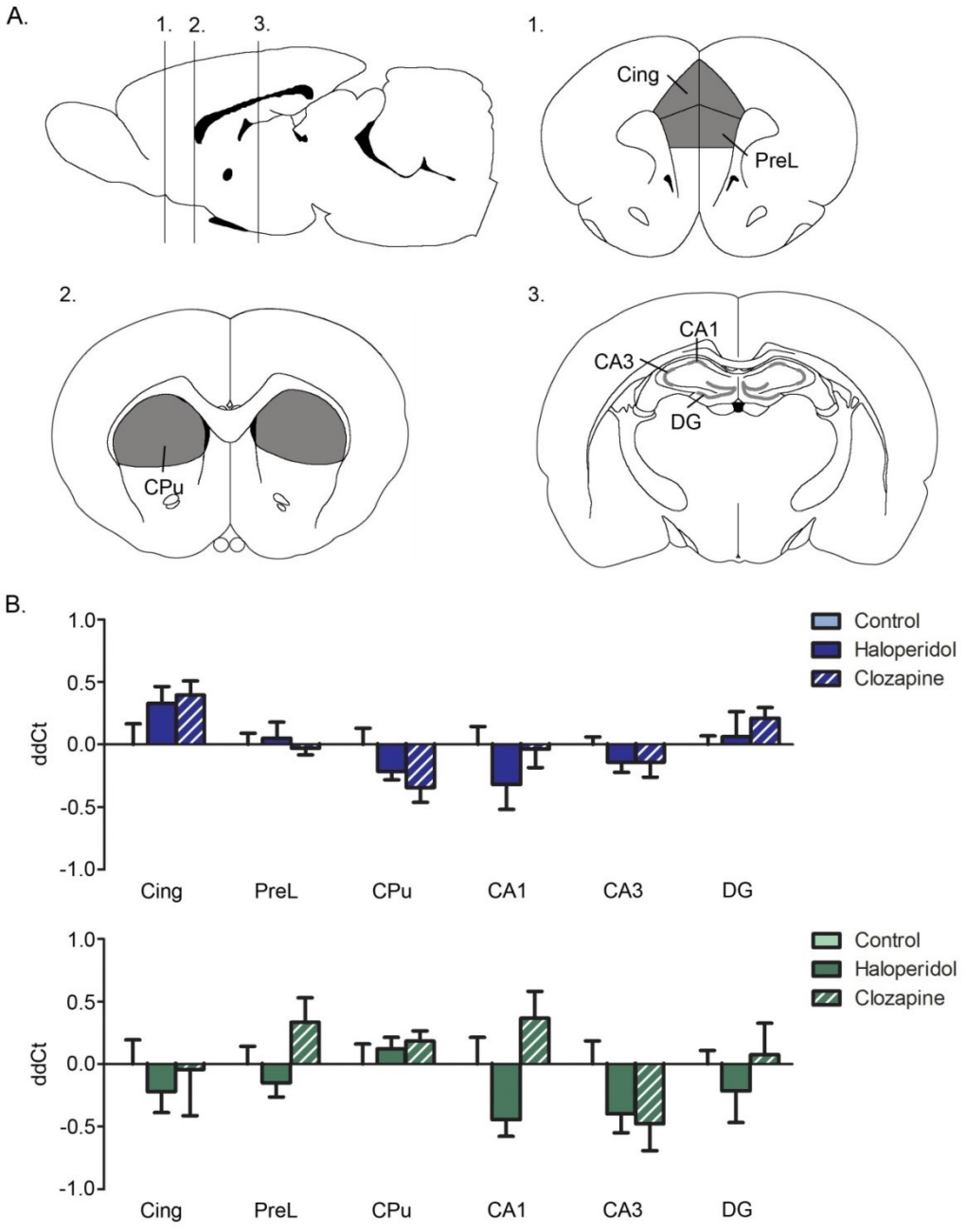

Figure 35. 12 week-treatment with haloperidol or clozapine does not affect *Cacna1c* and *Cacna1d* mRNA expression. A. Schematic representation of analyzed regions. B. Bars show ddCt values, with *Gapdh* as internal normalizer, as mean ± SEM. Region-wise one-way ANOVA did not reveal significant differences, n=6-9.

However, we did not find any significant differences between *Cacna1c* and *Cacna1d* mRNA expression in rats treated with an antipsychotic compared to controls. dCt values of *Cacna1c* and *Cacna1d*, and Ct values of *Gapdh* are shown in Suppl. Table 17.

5.4. mRNA expression of LTCC subtypes is not altered in the dorsolateral prefrontal cortex of suicide completers with major depression

For interpretation of qRT-PCR data on postmortem samples of the dorsolateral prefrontal cortex (BA9) of suicide completers with a diagnosis of major depression and controls from the Human Brain Tissue Bank, Budapest, an analysis of covariance was conducted for diagnosis and gender, also considering the possible influences of age and postmortem interval.

No significant main effects were found for diagnosis, gender, or the interaction between these factors. Analysis of covariance did not reveal any potential confounding factors.

Table 5. Clinical characteristics of suicide completers with major depression and control subjects.

	Female control	Male control	Female suicide	Male suicide
Genotype (GG/AG/AA)	10/20/1	21/15/7	3/7/0	6/6/2
Age (years)	70.3 ± 3.5	60.2 ± 2.1	50.8 ± 5.4	46.0 ± 3.2
PMI (hours)	3.8 ± 0.4	3.1 ± 0.3	5.4 ± 0.8	5.4 ± 1.0

5.5. Summary of Study V

CACNA1C and *CACNA1D* mRNA were analyzed in postmortem samples of alcoholic patients compared to controls, but no changes were detected for either subtype. However, both *CACNA1C* and *CACNA1D* mRNA are decreased in forebrain regions of schizophrenia patients. Analysis of rats treated with the antipsychotics haloperidol and clozapine did not show a difference in LTCC mRNA expression, excluding these medications as confounding factors in the analysis of human postmortem samples.
In addition, we investigated *CACNA1C* and *CACNA1D* mRNA levels in postmortem samples of depressive patients and controls, but did not find a significant difference in the BA9.

V. Discussion

1. Study I: Differential roles for L-type calcium channel subtypes in alcohol dependence

In the effort to improve treatments for alcohol dependence, new targets have to be considered. As manipulation of LTCCs has been shown to influence alcohol consumption in rodents, we investigated the changes in the expression of central LTCC subtypes as well as the impact of antagonism of central LTCCs in alcohol dependence.

The most salient message from the present study is the strong upregulation of $Ca_V1.2$ in the alcoholic brain during long-term abstinence and the important role $Ca_V1.2$ appears to play in relapse behavior. These findings encourage the efforts to implicate $Ca_V1.2$ as a potential target for drug development.

In our study, we demonstrate the dynamic regulation of *Cacna1c* mRNA in the rat brain during abstinence from alcohol dependence. After 21 d of abstinence, *Cacna1c* mRNA was strongly increased in the amygdala and hippocampus, with no changes in *Cacna1d* mRNA. This was accompanied by elevated $Ca_V1.2$ protein levels in the hippocampal CA1 subregion of alcohol dependent rats. These findings were functionally validated by an increase in $Ca_V1.2$ currents in hippocampal CA1 neurons. Importantly, blocking central LTCCs prevented cue-induced alcohol-seeking in dependent, but not control rats. Together, our findings suggest an involvement of $Ca_V1.2$ in relapse behavior, with substantial neuroadaptations taking place during abstinence.

Until recently, little effort has been made to distinguish between LTCC subtypes in the investigation of addictive behaviors. Many studies on alcohol drinking behavior used systemic administration routes for administering LTCC ligands, affecting not only neuronal subtypes but LTCCs in many organs (De Beun *et al*, 1996a; de Beun *et al*, 1996b; Fadda *et al*, 1992; Rezvani *et al*, 1990). These studies suffered from substantial side effects, having been performed at high doses to compensate for the low brain penetrance of most calcium channel blockers. For example, i.p. administration of nifedipine decreases locomotor activity for up to 12 h (Bernardi *et al*, 2014), also increasing plasma corticosterone levels (Waltereit *et al*, 2008), while systemic verapamil administration appears to augment stress-induced impairment of memory retrieval (Rashidy-Pour *et al*, 2009). This effect is of particular importance as alcohol dependent rats display an increased sensitivity to stress (Sommer *et al*, 2008).

To our knowledge no studies have yet been published investigating the involvement of central LTCCs on alcohol-related behavior. There have been efforts, however, to determine the individual roles of $Ca_V1.2$ and $Ca_V1.3$ in the abuse of other psychostimulants such as nicotine (Bernardi *et al*, 2014), morphine (Shibasaki *et al*, 2011), or cocaine and amphetamine (Giordano *et al*, 2010; Schierberl *et al*, 2012; Schierberl *et al*, 2011). The findings in these studies are in line with our results on alcohol-seeking, showing different contributions of the two subtypes to distinct stages within the use and abuse of psychostimulants. It appears that $Ca_V1.2$ is especially involved in the effects of long-term exposure to psychostimulants and prolonged abstinence, as our current data, our study (Study IV) on nicotine sensitization (Bernardi *et al*, 2014), and the findings of (Giordano *et al*, 2010) on long-term amphetamine and cocaine exposure suggest.

To further establish the involvement of $Ca_V1.2$ in different stages of the development of alcohol dependence, we performed a time course analysis of *Cacna1c* mRNA levels over time in the CA1, BLA and CeA. Not only did these regions show the most pronounced effects after 21 days of abstinence, they are also strongly interconnected (Kelley, 2004; LeDoux, 2003; Mandyam, 2013) and involved in the reconsolidation or retrieval of stimulus-associated memory (Otis *et al*, 2014; Shi *et al*, 2015). As such, these regions appear to play important roles in the relapse to drug intake. All three regions showed a consistent pattern of *Cacna1c* mRNA increase during acute intoxication, decrease during early withdrawal (1 day of abstinence), and then again increase during the following extended abstinence. These dynamic changes underline the possibility of different roles of this subunit over time. This pattern of dynamic changes is reminiscent of neuroadaptations in dopaminergic and glutaminergic systems during abstinence (Hermann *et al*, 2012; Hirth *et al*, 2016a), indicating also an interaction with neurotransmitters.

Because of the lack of subtype-specific pharmacological tools, we used patch clamp recordings from isolated neurons to validate the expression data. This method provides sufficient sensitivity and specificity to distinguish $Ca_V1.2$- from $Ca_V1.3$-mediated currents, compensating for the lack of selectivity of LTCC antagonists through the differential electrophysiological properties of the two subtypes. $Ca_V1.2$ channels open at membrane potentials of about -30 mV and reach their half maximal activation point at -5 mV, whereas $Ca_V1.3$ has been shown to open at a much lower membrane potential (approximately -55 mV), with a half maximal activation point at -30 mV (Lipscombe, 2002).

We measured $Ca_V1.2$/$Ca_V1.3$ currents in the CA1 region of the hippocampus in alcohol dependent and control rats, since we found a strong increase of *Cacna1c* mRNA in this region after 21 days of abstinence. The CA1 region of adult rats also express high densities of $Ca_V1.2$ channels (Clark *et al*, 2003; Hell *et al*, 1993), and possess four times more *Cacna1c* mRNA compared to *Cacna1d* (Nunez-Santana *et al*, 2014). Together with the $G_{norm}(V)$ curves, which match the $Ca_V1.2$ activation range, this suggests that most of the measured L-type currents in neurons of control and alcohol dependent rats are carried by $Ca_V1.2$. Our electrophysiological data are in very good agreement with the findings on mRNA and protein level, as nifedipine has a stronger blocking effect on calcium currents in neurons of alcohol dependent than control rats. Considering that the overall currents are not altered in alcohol dependent rats, it is possible that other calcium channel currents are decreased as a compensatory mechanism. N- and P/Q-type calcium channels are also regulated by ethanol and may be altered after long-term alcohol exposure (Newton and Messing, 2009; Simasko *et al*, 1999; Solem *et al*, 1997), although this has not been investigated thoroughly. Analysis of changes of other calcium channel types during alcohol dependence would be necessary to determine potential compensatory actions or interactions between the channel types.

Pharmacological validation was provided in alcohol dependent and control rats trained to self-administer alcohol in an operant conditioning experiment. The non-specific antagonist verapamil was injected into the lateral ventricle, thus inhibiting only central LTCCs. Most interestingly, verapamil had no effect on alcohol self-administration in either group, but prevented cue-induced reinstatement of alcohol-seeking specifically in alcohol dependent rats. One explanation for the lack of efficacy on alcohol self-administration might be the simultaneous block of $Ca_V1.3$ by verapamil. Although *Cacna1d* mRNA expression is not altered after 3 weeks of abstinence and may not be specifically involved in this behavior, a decrease from normal activation levels by the antagonist may still have measurable consequences.

The most encouraging finding in terms of treatment development is the fact that verapamil did not alter cue-induced reinstatement in control rats, while completely blocking it in alcohol dependent animals. We have previously noted, based on the reviewed literature, that many compounds lack distinctive effects on alcohol behaviors between alcohol dependent and non-dependent rats, or in other words, may not specifically target the excessive component of alcohol responding added during the development of dependence (for review, see (Meinhardt *et al*, 2015)). Only a few neurochemical systems have been identified so far that seem

additionally recruited in the control of alcohol behaviors in alcohol dependent animals in a "between-systems" adaptation mode as proposed by (Koob and Le Moal, 2008). The best studied in this respect is the amygdala CRH system (Hansson *et al*, 2007; Hansson *et al*, 2006a; Sommer *et al*, 2008), but similar dependence specific adaptations have been observed in other brain stress systems, such as dynorphin (Walker *et al*, 2011), or vasopressin (Edwards *et al*, 2012). Whether or not $Ca_V1.2$ mediates the actions of these stress systems and provides a distinct pharmacological access point into peptide systems that have proven to be difficult to target directly warrants further investigation. Such a prospect would justify the development of LTCC ligands with brain specific pharmacodynamic properties, e.g. acting via $Ca_V1.2$.

In conclusion, we provide consistent evidence for increased $Ca_V1.2$ function in protracted abstinence, leading to an increase in alcohol-seeking during abstinence. Further studies to distinctively determine the function of $Ca_V1.2$ in alcohol-seeking behavior are warranted, for example using transgenic mice. However, considering the evidence presented here, $Ca_V1.2$ might already be considered a new target for relapse prevention. As existing antagonists, when administered systemically, have multiple unfavorable side effects, efforts in medicinal chemistry to develop centrally acting LTCC compounds are required to further explore the utility of this target for the treatment of alcohol use disorders.

2. Study II: Characterization of L-type calcium channel subtype expression in animal models of alcoholism

Animal models, although imperative parts of research, can only model aspects of complex disorders such as alcohol dependence. It is therefore of great importance to choose the most suitable animal model for each question. In Study I, the alcohol vapor intoxication model was used to investigate the contributions of central LTCCs in long-term abstinence from alcohol. Differential alterations of gene expression of *Cacna1c* and *Cacna1d* helped to distinguish between these subtypes, and behavioral experiments provided a basis for the hypothesis that $Ca_V1.2$, rather than $Ca_V1.3$, plays an important role in long-term abstinence and relapse-like behavior. However, there are other animal models of alcohol dependence, both genetic and functional, which might prove equally valuable.

In this study, we determined changes in *Cacna1c* and *Cacna1d* mRNA expression in several animal models of alcoholism. An increase of both LTCC subtypes in the amygdala and hippocampus of msP rats with a genetic preference for alcohol consumption has been shown previously (Uhrig, 2012). Comparison of alcohol preferring AA to Wistar rats also showed increased *Cacna1c* mRNA in the amygdala and both *Cacna1c* and *Cacna1d* mRNA are increased in the hippocampus. However, measurements of *Cacna1c* mRNA in non-preferring ANA rats compared to the Wistar controls did not yield such a clear picture, as mRNA levels were increased in the BLA, but decreased in the CA4. *Cacna1d* mRNA was decreased in the amygdala and hippocampus of ANA rats. Unlike msP and AA/ANA rats, the functional ADE model did not show any changes in *Cacna1c* or *Cacna1d* mRNA expression. Although the vapor intoxication model proved valuable in Study I and other studies, e.g. (Hansson *et al*, 2008; Hirth *et al*, 2016b; Meinhardt *et al*, 2015; Pfarr *et al*, 2015), dependence is induced involuntarily. Analysis of alcohol dependent and non-dependent rats with additional voluntary alcohol consumption revealed decreased *Cacna1c* mRNA in the amygdala and hippocampus of dependent rats, while *Cacna1d* mRNA was increased by voluntary alcohol consumption in many regions. The value of alcohol vapor intoxication itself was underlined by comparing a shorter (4 week) exposure followed by 3 weeks of abstinence with the usual 7 week exposure and 3 weeks of abstinence. Although previous studies have determined the neuroadaptations to take place at some point between week 4 and week 7 of exposure ("temporal threshold") (Rimondini *et al*, 2003), there was an even stronger *Cacna1c* mRNA increase in the amygdala and hippocampus of 3 weeks abstinent 4 weeks exposed rats than after 7 weeks of exposure. *Crhr1* and *Crh* mRNA, which are crucial for the "postdependent phenotype" with its

heightened sensitivity to stress (Sommer *et al*, 2008), were increased only after 7 weeks of vapor intoxication.

To interpret the different findings, the animal models have to be carefully considered. msP rats have shown an increased voluntary alcohol consumption and preference compared to Wistar rats, but they also exhibit a higher sensitivity to stress (Ciccocioppo *et al*, 2006). The increase of *Cacna1c* mRNA might therefore be a component of the alcohol consumption behavior, while our own data showing the regulation of *Cacna1d* by stress might explain the alterations in its expressions in msP rats.

Changes in AA rats resemble the findings in msP rats, although *Cacna1d* is increased in the hippocampus, rather than the amygdala. It was shown that AA rats, in contrast to other models of alcoholism, do not display increased anxiety-like behavior (Sommer *et al*, 2006), in some tests they even showed anxiolytic-like properties (Moller *et al*, 1997). If $Ca_V1.3$ is increased in the amygdala of msP rats to mediate anxiety-like behavior, the anxiolytic-like behavior of AA rats may explain the unchanged *Cacna1d* mRNA expression in the amygdala of AA rats. On the other hands, ANA rats display increased anxiety (Sommer *et al*, 2006), and here we find a decrease in *Cacna1d* mRNA in the MeA and hippocampal regions. The explanation might therefore be more complex. Overall, it appears that in most regions the regulation of both LTCC subtypes is opposite in AA and ANA rats. However, direct comparisons between AA and ANA rats have to be considered with care. Selective breeding for a specific trait, in this case preference for alcohol, leads to pronounced changes not only in neurotransmission but also metabolic activity, and thereby may affect the entire animal, even with regard to health and memory functions (Sommer *et al*, 2006). Comparison to a control strain, such as Wistar, is already complicated by this, but in comparing two selectively bred strains to each other, the unwanted differences might be even more pronounced. For example, ANA rats show an increased anxiety-like behavior compared to AA or Wistar rats (Moller *et al*, 1997), which in itself may compromise data on drug dependence. Additionally, AA and ANA rats were derived from a foundation stock including Wistar and Sprague-Dawley rats, and were later crossed with F1 hybrids from Lewis and Brown Norwegian rats. Choosing one strain as a control therefore does not reflect an accurate picture, which is also true for msP rats. However, the drinking behavior of Wistar rats is described well, and this strain has been used as controls in other studies in this thesis, as well as in other studies investigating genetically selected rat models (Caberlotto *et al*, 2001; Hansson *et al*, 2006a; Sommer *et al*, 2001; Sommer *et al*, 2006).

Genetic models can provide good insights, especially considering the *CACNA1C* SNP rs1006737 which indicates as a genetic component the increased risk to develop schizophrenia or depression. However, functional models are more suited to investigate changes caused by the consumption of, or exposure to, alcohol. As we did not find any alterations in the expression of either LTCC subtype in the ADE rat model, these channels may not be involved in binge drinking behavior or the craving of alcohol, which is the focus of this model (Vengeliene *et al*, 2014). Although the ADE is also aimed at modelling relapse behavior, the dependence in this model differs from the dependence after intermittent cycles of alcohol vapor exposure. Rodents do not voluntarily consume intoxicating amounts of alcohol. Through repeated cycles of access to alcohol and abstinence, some rat strains may be persuaded to consume enough alcohol to significantly increase their blood alcohol levels, but in mice this has not yet been successful (Vengeliene *et al*, 2014). During alcohol vapor exposure, on the other hand, alcohol is not taken up voluntarily, but is controlled by the experimenter and can therefore be kept at a constant high level throughout vapor exposure, leading to withdrawal signs after the end of exposure (Hansson *et al*, 2008; Rimondini *et al*, 2002; Sommer *et al*, 2008).

For the analysis of LTCC subtypes, alcohol vapor intoxication appears to be the most suitable model, as mainly *Cacna1c* mRNA expression is altered (Study I) and the contributions of $Ca_V1.2$ and $Ca_V1.3$ can therefore be better distinguished than in the genetic animal models of alcohol dependence. It also allows for a detailed timeline, since LTCC subtype expression can be measured at any time during abstinence (Study I). Alternatively, the number cycles of exposure to alcohol vapor can be varied. The CRH system, which is a key neuropeptide system in alcohol dependence to the increased sensitivity to stress (Sommer *et al*, 2008), has already been shown to activate only 3 weeks after a 7 week vapor exposure time. Four weeks of vapor exposure are not enough to alter *Crhr1* expression, indicating that this system is recruited at some point between the 4 and 7 weeks of exposure. *Cacna1c*, however, is even more strongly increased after only 4 weeks of exposure than it is after 7 weeks. This suggests that $Ca_V1.2$ may be involved in the recruitment of other systems, such as the CRH system and the dopamine system, which is also crucial after 7 weeks of exposure and during varying times of abstinence (Hirth *et al*, 2016b; Liu *et al*, 2014b). As our findings only describe changes on the expression level, the recruitment of the CRH system by $Ca_V1.2$ remains speculative, and validation would only be possible through functional studies.

In the comparison of the *Crhr1* mRNA expression after 4 and 7 weeks of alcohol vapor exposure and 3 weeks of abstinence, there is also a significant difference between the two control groups. This may be due to the fact that 4 weeks- and 7-weeks-groups were sacrificed 3 weeks apart. The CRH system is highly sensitive to external stimuli, therefore minor changes in the environment and the daily routine might already cause the differences observed between control groups. However, in this study the comparison between the alcohol dependent rats and their respective control groups is the main concern, and these groups were treated equally, housed in the same room, and sacrificed on the same day, to prevent any mRNA differences caused by these external stimuli.

It appears that $Ca_V1.3$ might play a role in the voluntary consumption of alcohol. After 3 weeks of free access an alcoholic solution, rats showed increased *Cacna1d* mRNA in the MeA and the CA1. In other regions, the increase did not reach significance, which might be due to the limited number of rats per group. In voluntary alcohol consumption, a larger variation amongst animals has to be expected, as some rats drink more alcohol than others. In alcohol dependent rats, the effect of subsequent access to alcohol was varied across regions. As alcohol dependence itself does not appear to alter *Cacna1d* mRNA expression, the differences may be caused by the interaction with other systems regulated by dependence. Therefore, the interplay of $Ca_V1.3$ with neurotransmitter or hormone systems should be investigated more thoroughly.

LTCCs are regulated by chronic stress (Maigaard *et al*, 2012), but the effects of acute stress are more relevant for the interpretation of experiments on alcohol or nicotine dependence. We therefore measured *Cacna1c* and *Cacna1d* mRNA expression in rats 4 h after they had been subjected to restraint stress for 1 h. This time point was chosen because it appears to be the most crucial time for the stress reaction, with corticosteroid receptors and neurotrophic factors (e.g. glucocorticoid receptor, mineralcorticoid receptor, BDNF) altered most strongly 4 h after restraint stress (Hansson and Fuxe, 2002; Hansson *et al*, 2001; Hansson *et al*, 2003; Hansson *et al*, 2006b). However, both LTCC subtypes showed only little changes as a response to restraint stress, with *Cacna1c* mRNA downregulated mostly in the striatum and *Cacna1d* mRNA upregulated in the hippocampus. As *Cacna1c* mRNA expression is also altered after 4 weeks of alcohol vapor exposure, a time point where *Crhr1* and *Crh* mRNA are still comparable to control levels, the 4 h time point might not be the best to measure LTCC subtype expression. LTCC expression might always be changed at a time point before the CRH system is recruited. In this case, an earlier time might yield more pronounced effects

throughout investigated regions, which could also support the hypothesis of a recruitment of the CRH system by LTCCs.

LTCCs are regulated mostly through alternative splicing, calcium-dependent inactivation, and C-terminal auto-regulation (Hulme *et al*, 2006; Tang *et al*, 2004; Zhang and Shaw, 2013). The often contrary regulation of *Cacna1c* and *Cacna1d* mRNA expression might be caused by the calcium-induced inactivation, setting in earlier or later for one channel, depending on the actions of the other. However, other mechanisms such as receptor-mediated second messenger cascades, involving protein kinase A or G (Mahapatra *et al*, 2012), may also play a role, and detailed experiments would be necessary to fully determine the molecular mechanisms behind the differential regulation of the LTCC subtypes.

In summary, *Cacna1c* and *Cacna1d* mRNA expression is altered in genetic rat models of alcohol dependence, but the differentiation of their individual roles in these models is challenging. The ADE model of binge drinking, craving, and relapse behavior may not be suited to investigate the role of LTCC subtypes in alcohol dependence, as no changes in *Cacna1c* and *Cacna1d* mRNA were found. As a functional model, which can be adapted to fit the specific questions, the alcohol vapor intoxication model already used in Study I was confirmed as the most prudent animal model to study the contributions of the LTCC subtypes to alcohol dependence. The increased *Cacna1c* mRNA after only 4 weeks of alcohol vapor exposure hints at the possibility that $Ca_V1.2$ may be responsible for the recruitment of other important neurotransmitter or hormone systems such as the CRH system.

3. Study III: L-type calcium channel subtype $Ca_V1.2$ mediates dependence-induced increase in alcohol self-administration

In Study I we provided indirect evidence for a role of the subtype $Ca_V1.2$ in relapse-like behavior, a finding based on the increase of *Cacna1c* mRNA, as well as $Ca_V1.2$ protein and current, in 3 weeks abstinent rats, and on the prevention of cue-induced reinstatement of alcohol-seeking by the unspecific LTCC antagonist verapamil. The alcohol vapor exposure model was also confirmed as the most interesting animal model to study LTCCs in alcohol dependence, with pronounced changes in *Cacna1c* mRNA expression after 4 and 7 weeks of vapor exposure followed by 3 weeks of abstinence, and a clear differentiation between *Cacna1c* and *Cacna1d* mRNA expression.

Here, we used a transgenic mouse line with a conditional knockout of $Ca_V1.2$ in CaMKII-positive neurons ($Ca_V1.2$flox x CaMKII^{CreERT2}) to directly determine the function of $Ca_V1.2$ in alcohol dependence. The knockout was demonstrated by PCR on genomic DNA and cDNA, as well as region-specific by *in situ* hybridization. Mutant mice exhibited normal locomotor activity comparable to controls. The mice were trained to self-administer alcohol, then alcohol dependence was induced in half of the controls and mutants, respectively, through cycles of alcohol vapor intoxication. Withdrawal signs and elevated BACs confirmed the alcohol dependence in exposed mice. Control mice showed an increase in alcohol self-administration as a response to the induction of alcohol dependence. This increase was completely blocked in mutants, suggesting a critical role for $Ca_V1.2$ in dependence-induced alcohol-seeking.

Several studies have investigated the effects of systemic LTCC antagonism on alcohol consumption (De Beun *et al*, 1996a; de Beun *et al*, 1996b; Fadda *et al*, 1992; Rezvani *et al*, 1990). Other studies determined differential functions for the LTCC subtypes $Ca_V1.2$ and $Ca_V1.3$ in the effects and use of other stimulants (Bernardi *et al*, 2014; Giordano *et al*, 2010; Schierberl *et al*, 2012; Schierberl *et al*, 2011; Shibasaki *et al*, 2011). However, Study I was the first to show the specific involvement of central LTCCs in alcohol dependence in rats. As there are no subtype-specific LTCC antagonists, the use of a transgenic mouse line was now warranted to determine the specific role of the LTCC subtype $Ca_V1.2$. We chose an inducible $Ca_V1.2$ KO in CaMKII-positive neurons, through the expression of the Cre recombinase under the CaMKII promotor in homozygous $Ca_V1.2$flox mice. The KO was induced before the start of the experiment by injection of tamoxifen, with an additional six weeks of recovery time to account for the unfavorable effects of the tamoxifen itself. This was done to exclude developmental effects of the change in $Ca_V1.2$ expression and to avoid neuroadaptations in

other calcium channels or neurotransmitter systems. The CaMKII promotor was chosen for this study for several reasons. The CaMKII is an important part of the LTCC signaling pathway (Ebert *et al*, 2013; Jenkins *et al*, 2010; Lee *et al*, 2009; Wheeler *et al*, 2008), and has itself been implicated in the mediation of the reinforcing effects of alcohol (Salling *et al*, 2014). It also interacts with a number of other molecules which in turn regulate alcohol-related behaviors (Menard *et al*, 2015; Pandey, 2004; Schroeder *et al*, 2008), and therefore appears to be crucial for many neuroadaptations in alcohol dependence. In addition, CaMKII is highly expressed in the hippocampus (Erondu *et al*, 1985), a region in which $Ca_V1.2$ is strongly increased in alcohol dependence, as demonstrated in Study I, and which shows the highest expression of both LTCC subtypes (Liebmann *et al*, 2008). CaMKII-dependent $Ca_V1.2$ KO in mice has also successfully been used in other studies (Lee *et al*, 2012; McKinney *et al*, 2008).

Validation of the KO was provided by PCR using genomic DNA and cDNA, yielding results comparable to the original study on these $Ca_V1.2$flox mice (Seisenberger *et al*, 2000). In addition, we could show a significant decrease in *Cacna1c* mRNA in the hippocampus of mutants compared to control mice via *in situ* hybridization. *Cacna1c* mRNA is only knocked out in cells expressing CaMKII. Therefore *in situ* hybridization measurements can only show a decrease instead of a complete KO, since other cells in the same brain region still express *Cacna1c* mRNA, and it is not possible to distinguish between cell types.
We next measured locomotor activity in mutants and controls, as a change in locomotion would have implications for other behavioral experiments, and has been observed in other genetic mouse models such as the $Ca_V1.2$ KO in Nestin-Cre mice (Dr. Dusan Bartsch, personal communication). However, there was no difference between the locomotor activity of mutants and controls, excluding altered locomotion as confounding factor.

Our main focus was the impact of $Ca_V1.2$ on alcohol self-administration. Alcohol dependence was induced in half of the control and mutant mice, and alcohol self-administration was tested. Dependence was shown by measurement of Withdrawal Scores, which were pronounced in alcohol dependent mice 4-12 hours after the last vapor exposure. At this time, BACs had dropped below intoxicating levels. During subsequent alcohol self-administration sessions, we found a strong increase of lever pressing in alcohol dependent control mice compared to their non-dependent littermates. This increased alcohol self-administration is often seen after induction of alcohol dependence by chronic intermittent alcohol vapor exposure (Meinhardt *et al*, 2015; Rimondini *et al*, 2002). Here, the dependence-induced

increase of self-administration in control mice is indeed very notable, with a 2.5fold enhanced lever pressing activity in alcohol dependent mice. Most interestingly, mutant alcohol dependent did not increase their lever pressing compared to mutant non-dependent mice. Both groups exhibited almost identical lever pressing activity. This strongly indicates that $Ca_V1.2$ function is crucial for the dependence-induced increase of alcohol self-administration. In rats (Study I) self-administration was not influenced by LTCC antagonism via central verapamil injection. However, as noted earlier, verapamil acts on $Ca_V1.2$ as well as $Ca_V1.3$, and the effects of decreased $Ca_V1.2$ function might be masked by the blockage of $Ca_V1.3$. It would now be interesting to analyze the impact of a conditional $Ca_V1.2$ KO on cue-induced reinstatement of alcohol-seeking. Unfortunately, our attempt to measure cue-induced reinstatement in these mice was unsuccessful, as the alcohol dependent control mice did not extinguish the alcohol seeking behavior even in the absence of reward. Nevertheless, the involvement of $Ca_V1.2$ in alcohol self-administration in itself is an indication that this particular LTCC subtype is critically involved in alcohol-related behavior.

In summary, we have provided evidence of a $Ca_V1.2$ KO in CaMKII-positive neurons, which did not impair normal locomotor activity. Alcohol dependence, induced through repeated cycles of alcohol vapor intoxication and withdrawal, leads to increased alcohol-seeking behavior in control, but not mutant mice. These data suggest a role for $Ca_V1.2$ in alcohol-related behavior, confirming this LTCC subtype as interesting target for anti-relapse/craving medication development.

4. Study IV: Functions of L-type calcium channel subtypes $Ca_V1.2$ and $Ca_V1.3$ in nicotine-related behavior

Nicotine and alcohol dependence are highly comorbid substance abuse disorders (Dawson, 2000; Grant *et al*, 2004). Both substances act on the reward system, and the development of dependence engages the same neurotransmitter and hormone systems. It stands to reason that LTCCs are involved in nicotine-related behavior, considering their role in the development of alcohol dependence (Studies I, II, and III).

After investigation of *Cacna1c* and *Cacna1d* transcription following acute and repeated nicotine exposure, we report a differential regulation of the two subtypes, indicating a distinct role in nicotine-related behavior. 24 h after an acute nicotine injection, *Cacna1d* was increased in a number of brain areas, with no significant changes in *Cacna1c* transcription. *Cacna1d* mRNA did not differ from saline-injected mice at 24 h and 7 d after repeated nicotine injections. *Cacna1c*, on the other hand, was decreased at 24 h after repeated nicotine. After 7 d without nicotine administration, *Cacna1c* transcript levels were strongly increased in brain areas involved in nicotine dependence-related regions. In addition, the non-selective LTCC antagonist nifedipine impaired nicotine-induced locomotor sensitization, and a $Ca_V1.2$ KO in CaMKII-positive neurons blocked nicotine self-administration.

Our findings on the differential regulation of *Cacna1c* and *Cacna1d* transcription as a response to acute and repeated exposure to nicotine are in line with a study on cocaine and amphetamine. There, a molecular switch from $Ca_V1.3$ to $Ca_V1.2$ during the development of neuroadaptations was reported (Giordano *et al*, 2010). Together with our study I and III, implicating $Ca_V1.2$, rather than $Ca_V1.3$ in the mediation of long-term effects of alcohol dependence, these data suggest a common mechanism for the actions of alcohol, nicotine, and other psychostimulants.

A specific trait of nicotine is the binding of nicotinic acetylcholine receptors (nAChRs). These receptors, amongst other functions, mediate calcium influx through LTCCs (Chang and Berg, 2001; Dickinson *et al*, 2007; Stevens *et al*, 2003). Research into this interaction revealed that nifedipine impairs a nicotine-induced increase in calcium influx over an extended period of exposure to nicotine in cultured mouse cerebral neurons (Katsura *et al*, 2002). In addition, 72 h of nicotine exposure led to increased α1C ($Ca_V1.2$) and α1D ($Ca_V1.3$) subunits, and α1C and α1D protein levels were increased in cerebral cortical tissue after 7 d of repeated high-dose nicotine administration (Hayashida *et al*, 2005; Katsura *et al*, 2002). Our results on *Cacna1c*

and *Cacna1d* mRNA expression during withdrawal from acute and chronic nicotine complement these data by differentiating in more detail the individual contributions of the two subtypes.

To evaluate the effect of the changes in LTCC subtype expression on a behavioral level we measured locomotor sensitization, which occurs after repeated drug administration and is mediated by neuroadaptations taking place during chronic drug intake (Biala and Budzynska, 2010; Biala and Staniak, 2010). Here, locomotor activity was measured after the first acute nicotine administration (Day 1), after 14 d of repeated nicotine or saline administration (Day 14) and after 7 d of abstinence, during which either nifedipine or vehicle were injected (Day 21). While vehicle-treated mice exhibited a clear locomotor sensitization to the nicotine which was given prior to the test, nifedipine blocked this sensitization effect. As there was no effect of nifedipine on saline-treated mice, we can assume that the findings are specific to nicotine-induced neuroadaptations. Our results are also in line with findings on cocaine and amphetamine sensitization (Giordano *et al*, 2010; Schierberl *et al*, 2011), as well as morphine sensitization (Zhang *et al*, 2003). The effect appears to be robust, as (Biala, 2003) reached similar conclusions using a different protocol.

Previous studies have analyzed changes in locomotor sensitization by nifedipine, when the LTCC antagonist was administered in parallel to nicotine. In our paradigm, however, nifedipine was given during the abstinence phase, in the absence of nicotine. This enabled sensitization to nicotine during the 14 d of repeated nicotine injections, but blocked further neuroadaptations induced during abstinence. The enhanced locomotor sensitization in mice which had received vehicle during the 7 d of abstinence is in line with previous studies on cocaine showing an increased sensitization effect after a period of abstinence (Hammer and Cooke, 1996; Todtenkopf *et al*, 2002). The neuroadaptations occurring during abstinence from repeated nicotine may reflect those changes in the brain of human smokers that induce the longing (craving) for nicotine (Kalivas *et al*, 1998; Robinson and Berridge, 1993, 2001). The attenuation of increased locomotor sensitization after abstinence by nifedipine suggests LTCC antagonists as a possible treatment to prevent craving and relapse. These findings, although using a very different approach than in Study I, support the data obtained in analyses during alcohol dependence. Given the high comorbidity of alcohol and nicotine dependence and the involvement of similar neurotransmitter and hormone systems, it is not surprising that the findings coincide. For example, the dopamine system, which, as an essential part of the reward system in the brain, is crucial for alcohol dependence ((Hirth *et al*, 2016a), see

Discussion of Study I) is also altered during the development of sensitization (Anderson and Pierce, 2005).

Nifedipine, as a non-selective LTCC antagonist, blocked both $Ca_V1.2$ and $Ca_V1.3$ during nicotine sensitization. The individual contributions of the subtypes could be to an extent inferred from the mRNA expression data obtained at different time points, as mentioned before. However, it is not certain that the changes in expression are also reflected in the amount of functional calcium channels. Conclusive evidence of the functions of $Ca_V1.2$ and $Ca_V1.3$ could therefore only be obtained using transgenic mouse lines. As in Study III, we used a mouse line with an inducible KO in $Ca_V1.2$ in CaMKII-positive neurons. Although alcohol self-administration (SA) and nicotine SA cannot be directly compared due to the difference in protocols and strength of the effects of the psychostimulants, we performed nicotine SA experiments with this mouse line. The results, a complete lack of SA in mutant mice, indicate a crucial role for $Ca_V1.2$ in the rewarding effects of nicotine which would motivate the animals to self-administer the psychostimulant. However, our findings can only be considered with caution. For more conclusive evidence, a group of mice receiving saline instead of nicotine would have to be directly compared to a nicotine self-administering group. Nevertheless, the results encourage further investigation of the role $Ca_V1.2$ in nicotine sensitization and dependence.

In this study, we have comprehensively shown a differential regulation of *Cacna1c* and *Cacna1d* mRNA by acute and repeated nicotine as well as abstinence. *Cacna1d* was mainly altered by a single nicotine injection, while *Cacna1c* transcription was increased most significantly after a period of abstinence from repeated nicotine exposure. The non-selective LTCC antagonist nifedipine, when administered during said abstinence phase, abolished the increase in locomotor sensitization on Day 21. Together with data implicating $Ca_V1.2$ as the primary LTCC subtype involved in the rewarding effects of nicotine during self-administration, our findings suggest $Ca_V1.2$ in particular as an interesting target for relapse-prevention.

5. Study V: Analysis of L-type calcium channel subtype expression in human postmortem samples of patients with mental disorders

In Studies I – IV, central LTCCs, and especially $Ca_V1.2$, have been consistently linked to drug dependence. Additionally, $Ca_V1.2$ seems to be implicated in other mental disorders. Genetic studies have associated variation in the *CACNA1C* gene locus with risk for schizophrenia, bipolar disorder, major depression, and autism (Berger and Bartsch, 2014; Casamassima *et al*, 2010; Erk *et al*, 2014; Strohmaier *et al*, 2013), in particular the SNP rs1006737. Considering that there is a strong comorbidity between alcohol and other mental disorders, it seems plausible that $Ca_V1.2$, rather than $Ca_V1.3$, should play a role in the relapse to alcohol drinking. *CACNA1C* has therefore been the focus of several studies in this context, but there are ambiguous reports on the alterations in the expression of the LTCC subtype in schizophrenia or depression (Bigos *et al*, 2010; Gershon *et al*, 2014; Roussos *et al*, 2014; Yoshimizu *et al*, 2015), and expression changes of both subtypes in human alcoholics are also unknown.

In our study we investigated *CACNA1C* and *CACNA1D* mRNA expression in postmortem samples of alcoholic and schizophrenia patients, as well as suicide completers with a history of major depression.

Analysis of human postmortem samples of alcoholic patients and control subjects did not reveal any changes of *CACNA1C* or *CACNA1D* mRNA expression in the striatum, and there was no effect of the genotype concerning the SNP rs1006737. In Study I, alcohol dependent rats did not show any changes of *Cacna1c* or *Cacna1d* mRNA in this brain region either. Although the striatum is extensively involved in alcohol dependence (Hirth *et al*, 2016b), LTCC expression is very limited in this region (Liebmann *et al*, 2008). Our findings in human postmortem samples are in line with the results of Study I. It would be interesting to analyze *CACNA1C* and *CACNA1D* mRNA expression in the amygdala and hippocampus of human alcoholic patients and controls, as *Cacna1c* was strongly increased in these regions in alcohol dependent rats.

Analysis of covariants did also not reveal any influence of smoking on the results. As nicotine alters *Cacna1c* and *Cacna1d* mRNA expression (Study IV), an effect of the smoking status of the patients and controls might have been expected. On the other hand, these patients and controls were selected for their alcohol drinking behavior, and information on their smoking habits are limited. It is unknown how many cigarettes per day current smokers consumed, and for how long they have done so. For subjects who have quit smoking, it is equally unclear for

how long and how many cigarettes they had consumed, and since when they quit smoking. Additionally, for some subjects the smoking habits are completely unknown. Therefore, the evaluation of the effects of smoking on *CACNA1C* and *CACNA1D* mRNA expression is not very reliable in this sample set.

In schizophrenia patients, both *CACNA1C* and *CACNA1D* mRNA levels were decreased in the prefrontal cortex, Brodmann area 10 (BA10), and the anterior temporal cortex, BA21. No changes were found in the nucleus caudatus and in the vermis of the cerebellum.

Schizophrenia is a very complex disorder, and involves the dysfunction of many brain regions. Neuroadaptations also depend on the manifestation of positive, negative or cognitive symptoms, and their relative severity. For this study we chose four brain regions which are structurally altered in schizophrenia: The BA10, a region with a role in cognitive functioning, shows a reduced connectivity with the anterior limb of the internal capsule that is associated with increased symptom severity in schizotypal personality disorder (Hazlett *et al*, 2012). The temporal cortex with BA21 is important for language and auditory processing, which may be important for positive symptoms (e.g. auditory hallucinations) and negative symptoms (e.g. incoherent speech). Structural changes in the temporal lobe are also associated with suicide in schizophrenia patients (Lee *et al*, 2016). As for the nucleus caudatus, white matter is reduced in schizophrenia (Takase *et al*, 2004), which has also been linked to an impairment of working memory (Levitt *et al*, 2002). Lastly, the cerebellum with its importance for cognition has been studied to understand its role in symptoms of schizophrenia, e.g. (Bernard and Mittal, 2015; Guo *et al*, 2015; Parker, 2015). The vermis especially appears to be altered, as a study found a decrease in vermis volume, with unchanged cerebellar hemispheres and total volume (Ichimiya *et al*, 2001; Okugawa *et al*, 2003). Although it would be interesting to also analyze *CACNA1C* and *CACNA1D* expression in other brain regions associated with schizophrenia symptoms, our results already provide evidence for changes in LTCC expression in schizophrenia symptoms, warranting further research into this topic.

As mentioned above, previous studies have provided opposing findings on the expression regulation of *CACNA1C* in schizophrenia. Some have found an increase (Bigos *et al*, 2010), others a decrease (Gershon *et al*, 2014; Yoshimizu *et al*, 2015) in *CACNA1C* expression associated with the risk SNP rs1006737. This ambiguity in findings can be explained by the analysis of different regions, the different cell types found in the respective areas, and the broad spectrum of schizophrenia disorders and symptoms. Accordingly, any potential

medication targeted at LTCCs must be carefully chosen depending on the symptoms presented in each patient.

In contrast to our findings of decreased *CACNA1C* and *CACNA1D* mRNA in schizophrenia, our animal models of alcoholism show an increase in LTCC subtype expression. This might be surprising considering the strong comorbidity between alcohol dependence and schizophrenia. However, the above mentioned different findings across human studies again stress the importance of careful consideration of the regions which are investigated. Unfortunately, we do not have a direct comparison of brain regions between alcoholic patients, where the striatum did not reveal any significant changes in LTCC subtype expression, and schizophrenia patients. Furthermore, no data is available on the alcohol consumption behavior of our schizophrenia patients and controls.

Depression, as drug addiction and schizophrenia, is a highly diverse disorder for which the *CACNA1C* SNP rs1006737 indicates an increased risk. Postmortem samples of suicide completers with major depression and controls have been successfully used to study a SNP of the human neuropeptide Y gene (Sommer *et al*, 2010). However, in the BA9 region of these samples we could not find significant differences for either *CACNA1C* or *CACNA1D*. This might be due to the limited number of samples per group (n=10-43, see Table 5), but the more likely explanation is that central LTCCs are not altered in this particular region, which is relevant mostly for cognitive functions (MacLeod *et al*, 1998). Analysis of brain regions involved in the emotional components of depression, such as the amygdala, could therefore yield promising results, especially as SNPs of *CACNA1C* interact with adverse life events to increase the risk of developing psychiatric disorders (personal communication from Dr. Jan Deussing).

In conclusion, *CACNA1C* and *CACNA1D* mRNA expression were not altered in the striatum of alcoholic patients. In postmortem samples of schizophrenia patients, we showed decreased *CACNA1C* and *CACNA1D* expression in the BA10 and BA21, while no changes were found in the nucleus caudatus and the cerebellar vermis. These findings underline a role for the LTCC subtypes $Ca_V1.2$ and $Ca_V1.3$ in schizophrenia, and stress the fact that these channels may be important for some symptoms, while having no impact on others. Further studies are necessary to distinctly define the involvement of the LTCCs in positive, negative and cognitive symptoms, also considering the different types of schizophrenia. No alterations of LTCC subtype expression in the BA9 of suicide completers with major depression could be found, but other brain regions might yield different results. Nonewithstanding the negative

findings in postmortem samples of alcoholic patients and suicide completers, and considering the findings in human schizophrenia patients, $Ca_V1.2$ and $Ca_V1.3$ remain promising targets for medication development.

6. General discussion: LTCC involvement in mental disorders

The involvement of $Ca_V1.2$ and $Ca_V1.3$ was investigated in alcohol dependence (Studies I, II and III), acute and chronic nicotine administration and abstinence (Study IV) and in human postmortem samples of alcoholic and schizophrenia patients, and suicide completers with major depression (Study V). Our results suggest that increased $Ca_V1.2$ is found in abstinent alcohol dependent rats and during abstinence from chronic nicotine. CaMKII-specific KO of $Ca_V1.2$ ameliorates dependence-induced increase of alcohol self-administration as well as on nicotine self-administration. In schizophrenia, the expression of *CACNA1C* and *CACNA1D* is decreased at least in some brain regions.

It appears that the functions of $Ca_V1.2$ and $Ca_V1.3$ may be consistent across different psychostimulants. In this thesis, we found increased *Cacna1d* mRNA after voluntary alcohol consumption and after a single nicotine injection, while *Cacna1c* mRNA was increased during abstinence from alcohol dependence and from chronic nicotine. A previous study on cocaine and amphetamine sensitization also found a switch from $Ca_V1.3$ during the acute effects of the psychostimulant to $Ca_V1.2$ during abstinence from chronic administration (Giordano *et al*, 2010). Although different psychostimulants act on the brain in different ways and on different receptors, similarities between the mechanisms during acute and chronic drug administration and abstinence are not surprising.

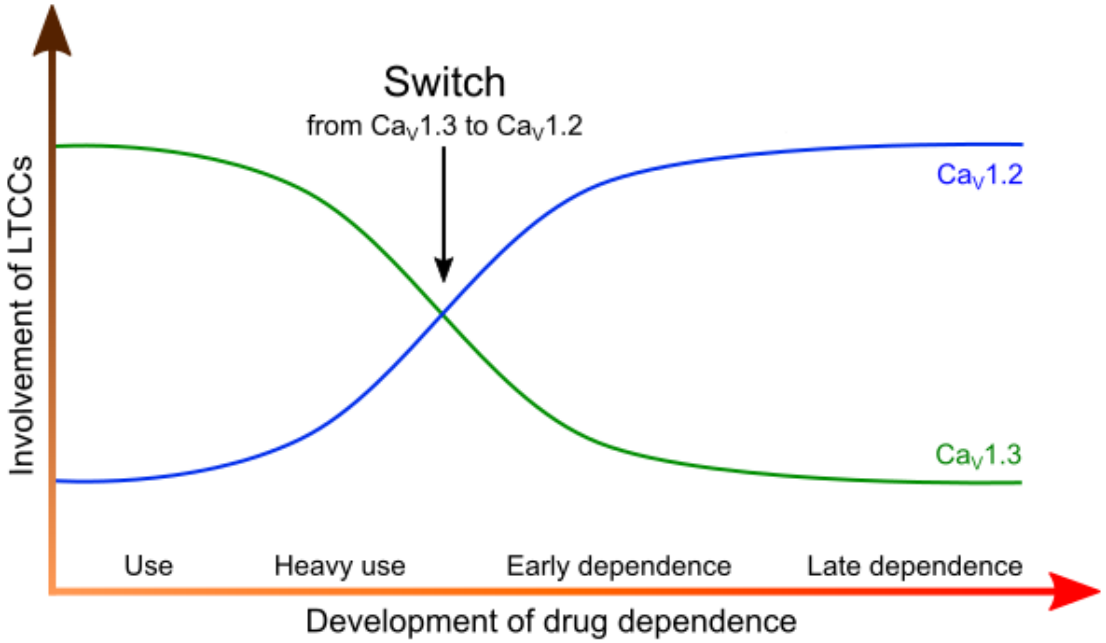

Figure 36. Hypothesis of a switch from $Ca_V1.3$ to $Ca_V1.2$ involvement during the development of drug dependence: $Ca_V1.3$ appears to play a role during drug use and excessive consumption. As dependence develops, the relevance of $Ca_V1.3$ decreases, while $Ca_V1.2$ expression and activity increase (switch). Total calcium current remains stable over time, indicating compensatory regulation of the other subtype.

For example, the reward system and the stress circuitry are affected by any psychostimulant, albeit to a different extent, and play a significant role in addiction in general (Rimondini *et al*, 2002; Spanagel *et al*, 2014a; Spanagel and Weiss, 1999; Vengeliene *et al*, 2008). The LTCC subtypes have been implicated in both the reward and stress systems, regulating the firing of dopamine and corticotropin-releasing hormone neurons (Krishnan *et al*, 2010; Liu *et al*, 2014b). The findings of this thesis, together with the role of $Ca_V1.2$ and $Ca_V1.3$ in the effects of acute and chronic cocaine and amphetamine (Giordano *et al*, 2010), lead to the hypothesis of a switch from $Ca_V1.3$ to $Ca_V1.2$ during the development of drug dependence.

In alcohol dependence, the alcohol vapor exposure model appears to be the most relevant for changes in LTCC subtype expression, as it clearly shows the differential regulation of *Cacna1c* and *Cacna1d*. The increase in *Cacna1c* mRNA in this rat model during abstinence was restricted to the amygdala and hippocampus. In Study IV, however, we found increased *Cacna1c* expression during the abstinence from chronic nicotine in several brain regions, including the prefrontal cortex, striatum and hippocampus. This disparity might be explained by the different methods of psychostimulant administration. While in Study I, II and III, alcohol dependence was induced through repeated cycles of alcohol vapor intoxication and withdrawal, nicotine was only administered once a day for 14 days. This reliably induces nicotine sensitization behavior during locomotor experiments, but the animals may not be considered to be dependent, as evidenced by the lack of withdrawal symptoms.

Mice with a conditional KO of $Ca_V1.2$ in CaMKII-positive neurons were used both to evaluate alcohol self-administration behavior in dependent and non-dependent animals and nicotine self-administration behavior. Here again, the methodological processes are very different. In addition, nicotine is a stronger reinforcer than alcohol, which can be seen for example in the amount of time needed to encourage nicotine and alcohol self-administration. This might explain why the conditional $Ca_V1.2$ KO completely blocks nicotine self-administration, while there is no significant difference between non-dependent controls and mutants in alcohol self-administration. Here, differences are only evident in alcohol dependent mice, where mutants do not increase their alcohol intake compared to non-dependent mice. Most likely, alcohol dependence increases the value of alcohol as a reinforcer almost to a level which nicotine already provides on its own, without dependence.

It is not easy to compare the results of our psychostimulant studies with the postmortem samples of human patients. We did not find any differences between human alcoholics and control subjects, probably due to the areas which were investigated. However, we have

consistently shown an important role for $Ca_V1.2$ in alcohol dependence, and in abstinence from chronic nicotine, in rodents, encouraging further analysis of human subjects.

Alcohol dependence shows a strong comorbidity with nicotine dependence (Dawson, 2000; Falk *et al*, 2006; Grant *et al*, 2004) as well as schizophrenia (Cantor-Graae *et al*, 2001; Koskinen *et al*, 2009; Mueser *et al*, 1990; Nesvag *et al*, 2015) and depression (Regier *et al*, 1990). As mentioned earlier, there are strong similarities between the mechanisms of the abuse of different psychoactive substances. People with a susceptibility to one drug therefore are likely to be easily influenced by other substances, as well. With schizophrenia, there are more differences in the regions involved and the kind of dysfunction. However, there are also similarities, for example the involvement of the amygdala. Many schizophrenia patients show flattened affect (Lindner *et al*, 2016) and there are structural changes in the amygdala (Williams *et al*, 2016), which is also dysregulated in alcohol dependence (Hansson *et al*, 2007; Hansson *et al*, 2006a). It would be interesting to compare the expression of *CACNA1C* in the amygdala and hippocampus of schizophrenia patients and control subjects, as well as of alcoholics and controls, to have a more direct comparison to our measurements in alcohol dependent rats, and to more comprehensively determine the contribution of $Ca_V1.2$ to the comorbidity of alcohol dependence and schizophrenia.

Considering the results we obtained, it is obvious that $Ca_V1.2$ function is involved in alcohol dependence, as well as abstinence from chronic nicotine, and in some aspects of schizophrenia, and should therefore be studied in more detail, to possibly warrant the development of centrally acting LTCC antagonists as a medication for any of these disorders.

VI. Summary and Outlook

This thesis was aimed to disentangle the role of $Ca_V1.2$ and $Ca_V1.3$ in alcohol dependence, as well as comorbid disorders.

Indeed, the two central LTCC subtypes are differentially involved in alcohol dependence, with an increase of *Cacna1c* mRNA in the amygdala and hippocampus, as well as $Ca_V1.2$ protein and current during protracted abstinence. In Study I we also show that $Ca_V1.2$ is likely responsible for cue-induced reinstatement of alcohol seeking in alcohol dependent rats. Study II attempts to determine which animal model of alcohol dependence, genetic or functional, is the most appropriate for the investigation of LTCC subtype contributions to alcohol dependence. In Study III, mice with a KO of $Ca_V1.2$ in CaMKII-positive neurons lack the dependence-induced increase in alcohol seeking found in their control littermates. Our results on *Cacna1c* and *Cacna1d* expression changes due to nicotine (Study IV) show a similar pattern as in alcohol dependence, as *Cacna1d* was mainly increased by a single nicotine injection, while an increase in *Cacna1c* mRNA was found after chronic nicotine administration and subsequent 7 d abstinence. Abstinence from chronic nicotine augments nicotine sensitization in mice, but the administration of nifedipine during the abstinence period completely blocks this effect. Additionally, a KO of $Ca_V1.2$ in CaMKII-positive neurons completely blocked nicotine self-administration. In Study V, we investigated postmortem samples of alcoholic and schizophrenia patients, as well as suicide completers with major depression, and found a decrease of *CACNA1C* and *CACNA1D* mRNA in schizophrenia patients compared to control subjects in the BA10 and BA21. Although there was no difference in alcoholic or depressive patients and their respective control subjects, this may be due to the investigated regions.

On the expression level, different regions should now be analyzed for a better comparison between rat and human experiments. Another interesting aspect would be the downstream effect of $Ca_V1.2$ increase. As LTCCs activate gene transcription via the transcription factor CREB (Wheeler *et al*, 2008; Zhang *et al*, 2006), the induction of the CREB-regulated immediate early gene *c-fos* (Gruol *et al*, 2005) by foot shock stress in alcohol dependent and non-dependent mice with a conditional $Ca_V1.2$ KO could be analyzed by *in situ* hybridization. *C-fos* has already been linked to alcohol dependence (Hansson *et al*, 2008), and it would be expected that mutant mice show a decrease in *c-fos* mRNA after acute stimulation, while alcohol dependent controls display increased *c-fos* expression.

At the same time, the regulation of *Cacna1c* and *Cacna1d* themselves might be equally interesting. Similar mechanisms such as calcium-dependent inactivation, C-terminal auto-regulation, and alternative splicing have been determined as regulators of both LTCC subtypes (Hulme *et al*, 2006; Tang *et al*, 2004; Zhang *et al*, 2013). With respect to mental disorders it would be interesting to investigate at which point the differences in *Cacna1c* and *Cacna1d* mRNA expression are defined. This might help to disentangle the mechanisms behind the proposed switch from $Ca_V1.3$ to $Ca_V1.2$ during the development on drug dependence.

Expression analysis, even when translated into protein level (Study I), can only provide a basis for functional experiments. In alcohol dependence, we have indirectly shown the importance of $Ca_V1.2$ for relapse behavior, and using transgenic mice provided direct evidence of the involvement of $Ca_V1.2$ in craving in alcohol dependence. As the prevention of relapse is the most crucial goal in the search for new treatments for alcohol dependence, the role of $Ca_V1.2$ in relapse behavior should be further investigated, before clinical studies may be warranted. The increased *Cacna1d* mRNA after voluntary alcohol consumption and a single nicotine injection suggests the use of another mouse model with conditional $Ca_V1.3$ KO, to further differentiate between $Ca_V1.2$ and $Ca_V1.3$ and to elaborate on the role of $Ca_V1.3$ after short-term psychostimulant exposure or voluntary consumption.

In order to investigate the role of the *CACNA1C* SNP rs1006737, a mouse line with the human *CACNA1C* gene could also be created. Studies on the alcohol- and nicotine-related behavior in these animals could give further insight into the impact of the AA and AG risk alleles.

In schizophrenia, it would be interesting to investigate the contributions of the LTCC subtypes to the individual symptoms, for example using animal models of specific aspects of the schizophrenia spectrum.

Considering the difficulties of blocking only central LTCCs, or even one specific subtype, the pathways through which LTCCs exert their effects should be further investigated. Possibly, another target along the pathways may be better accessible for manipulations or yield fewer side effects. Additionally, the interactions of LTCCs with other systems such as the dopamine and the CRH system should be examined more closely in the context of drug dependence and schizophrenia.

In summary, this thesis provides insight into the involvement of LTCCs in mental disorders. The results give a comprehensive foundation for further studies into this topic, and support the hypothesis of a switch from an involvement of $Ca_V1.3$ during acute and short-term drug consumption to $Ca_V1.2$ in drug dependence and abstinence. Moreover, it implicates central LTCCs as viable targets for the development of new and improved treatments for drug dependence and schizophrenia, to prevent relapse into drug use and counteract specific positive, negative or cognitive symptoms of schizophrenia.

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IX. Appendix

1. Supplementary Tables

Suppl. Table 1. Demographic data and tissue characteristics of postmortem samples of human alcoholics and controls subjects.

DSMIV Alcohol class	Geno-type	Gender	Age	PMI	Brain pH	COD	Toxicology	Smo-king
Control	AG	Female	56	23,0	6,65	Pulmonary thromboembolus	-	Ex
Control	GG	Female	33	24,0	6,77	Cardiac arrythmia; myocardial fibrosis	-	No
Control	GG	Female	71	16,0	6,20	Adenocarcinoma of the pancreas	-	Ex
Control	AG	Female	78	11,0	6,30	Respiratory Failure due to pulmonary fibrosis	-	No
Control	AG	Female	49	15,0	6,93	Cardiac Tamponade, acute myocardial infarction	chloride ion 118 mmol/L	?
Control	GG	Female	52	11,0	6,21	Ischaemic Heart Disease, coronary atherosclerosis	-	Yes
Control	GG	Female	72	25,0	7,00	Cardiac	-	No
Control	GG	Female	51	37,5	6,92	acute myocardial infarction	-	No
Control	AA	Female	63	42,0	7,02	a)Coronary artery thrombosis b)Coronary artery atherosclerosis.	-	Yes
Control	AG	Female	52	43,0	6,33	Cardiac	Amiodarone 1.9 mg/L Paracetamol <3 mg/L	Yes
Control	GG	Female	63	50,0	6,46	Cardiac	Codeine: <0.05mg/L	No
Control	AA	Male	38	13,5	6,26	Atherosclerotic cardiovascular disease	-	Yes
Control	AG	Male	55	7,5	6,90	Atherosclerotic cardiovascular disease	Amphetamines positive THC positive	?
Control	AG	Male	37	21,0	6,64	Ischaemic Heart Disease	-	?
Control	AG	Male	50	29,0	6,68	Ischaemic Heart Disease	-	No
Control	AA	Male	59	20,0	6,56	Coronary Thrombosis	-	Yes
Control	AG	Male	43	13,0	6,43	Thrombotic Coronary Artery Occlusion	-	Ex
Control	GG	Male	51	20,0	5,88	Cardiac tamponade	-	?
Control	AG	Male	46	25,0	6,65	Mitral valve prolapse	-	?
Control	GG	Male	58	12,0	6,46	Ischaemic Heart Disease	-	Yes
Control	GG	Male	50	19,0	6,26	Ischaemic heart disease	-	Ex
Control	AA	Male	53	16,0	6,84	Dilated cardiomyopathy	Lignocaine: 0.9 mg/L Sotalol: 3.8 umol/L	No
Control	GG	Male	48	24,0	6,73	Ischaemic heart disease	-	Yes
Control	GG	Male	44	50,0	6,60	Ischaemic heart disease	-	Ex
Control	GG	Male	56	37,0	6,76	Left ventricular scarring , hypertension, and cardiomegaly	-	Yes
Control	GG	Male	63	72,0	6,90	Severe coronary artery atherosclerosis	-	Ex
Control	GG	Male	69	16,0	6,60	Atherosclerotic cardiovascular disease	paracetamol 23 mg/L 1% blood saturation of CO (low)	Yes
Control	GG	Male	57	18,0	6,60	Ischaemic Heart Disease	-	Ex
Control	GG	Male	24	43,0	6,27	Idiopathic cardiac arrhythmia	-	Yes
Control	GG	Male	60	28,0	6,80	Ischaemic Heart Disease Coronary Artery Atherosclerosis	-	No

Suppl. Table 1 (continued)

DSMIV Alcohol class	Geno-type	Gender	Age	PMI	Brain pH	COD	Toxicology	Smo-king
Control	GG	Male	60	25,0	6,70	Bacterial Peritonitis, ascites, carcinomatosis, gastrointestinal stomach tumor	-	No
Control	AG	Male	63	24,0	6,94	Atherosclerotic coronary heart disease	Atenolol: <1mg/L Irbesartan: 0.8mg/L Lignocaine: 1.7mg/L	Yes
Control	AG	Male	73	48,0	6,80	Dilated cardiomyopathy, ischaemic heart disease.	-	Yes
Control	GG	Male	64	9,5	6,94	Ischaemic heart disease	-	Yes
Control	GG	Male	73	51,0	6,82	congestive cardiac failure, atrial fibrillation, ischaemic heart disease	-	Yes
Control	AG	Male	53	27,0	6,64	acute myocardial infarct of the anterolateral wall of the left ventricle, atherosclerotic coronary artery disease with 90% stenosis of the left marginal artery.	-	?
Control	AG	Male	55	39,0	6,89	Coronary artery atherosclerosis	Irbesartan 0.4mg/L.	No
Control	GG	Male	64	39,5	6,68	Coronary artery thrombosis	-	No
Control	AG	Male	59	43,0	6,69	atherosclerotic cardiovascular disease	-	Yes
Control	AG	Male	68	45,5	6,12	Ischaemic Heart Disease	Amiodarone 0.7 mg/L Paracetamol 3 mg /L	No
Control	GG	Male	69	40,0	6,53	Ischaemic Heart Disease. Coronary athersclerosis	Amiodarone 1.9 mg/L	Ex
Control	GG	Male	56	19,0	6,90	Atherosclerotic coronary artery disease.	-	No
Control	AA	Male	55	12,0	6,39	Hypertensive heart disease	-	No
Control	AG	Male	73	38,5	6,28		-	Ex
Control	GG	Male	66	63,0	6,91		-	No
Control	AA	Male	62	46,0	6,95		-	Ex
Control	GG	Male	36	34,0	6,67		-	Ex
Control	GG	Male	54	28,0		Cardiac Arrest TBC	-	Ex
Control	GG	Male	60	21,5	6,66	Ischaemic heart disease	-	No
C. Substance Abuse (alcohol)	GG	Female	73	60,0	6,68	Cardiomyopathy	paroxetine 0.3mg/L zolpidem <0.1mg/L paracetamol <3mg/L	
C. Substance Abuse (alcohol)	AG	Female	75	9,0	6,00	hepatic encephalopathy, spontaneous bacterial peritonitis, liver failure, liver cirrhosis, renal impairment, diabetis mellitis	-	No
B: Substance Dependence (alcohol)	AG	Female	47	16,0	6,19	Lobar Pneumonia; Right Lung	Alcohol: not detected codeine: 0.48 mg/L Diazepam: 0.34 mg/L Morphine: <0.05 mg/L Nordiazepam: 0.29 mg/L Oxazepam: 0.02 mg/L Paracetamol: 5 mg/L Quinine: 1 mg/L Temazepam: 0.07 mg/L	Yes
B: Substance Dependence (alcohol)	AG	Female	67	18,0	5,89	Hepatic	Lignocaine: 0.1mg/l Metoclopramide: <0.1 mg/ll Midazolam: <0.1mg/l	Yes
B: Substance Dependence (alcohol)	GG	Female	45	41,5	6,8	Cardiac	Metoclopramide: <0.1 mg/L	Yes

Suppl. Table 1 (continued)

DSMIV Alcohol class	Geno-type	Gender	Age	PMI	Brain pH	COD	Toxicology	Smo-king
B: Substance Dependence (alcohol)	GG	Female	62	39,0	6,78		-	Yes
B: Substance Dependence (alcohol)	AA	Female	61	49,0	6,75	Respiratory	-	Yes
C. Substance Abuse (alcohol)	GG	Female	85	23,0	6,44	Pulmonary Thromboembolus	Codeine (free) 0.20 mg/L; Diazepam 0.5 mg/L; Ibuprofen 9.0 mg/L;Irbesartan 0.6 mg/L; Morphine (free) <0.05 mg/L;Nordiazepam 1.2 mg/L; Oxazepam 0.2 mg/L;Temazepam 0.1 mg/L;	Yes
B: Substance Dependence (alcohol)	GG	Female	48	49,0	6,57	Hepatic/Blood loss	-	No
C. Substance Abuse (alcohol)	GG	Male	66	11,5	6,14	Pneumonia	-	Yes
C. Substance Abuse (alcohol)	GG	Male	70	32,0	6,05	Sepsis, alcoholic Liver Disease	-	?
C. Substance Abuse (alcohol)	GG	Male	65	32,0	5,66	Complications of Chronic Alcoholism	Moclobemide: 17mg/L Codeine: 0.07mg/L Phenytoin: 6mg/L# Paracetamol: 7mg/L# Quinine: 0.4mg/L	?
	GG	Male	39	24	6,56	Aortic stenosis	-	Yes
C. Substance Abuse (alcohol)	GG	Male	56	15,0	6,66	Ischaemic heart disease and emphysema	Nordiazepam <0.1mg/L	?
C. Substance Abuse (alcohol)	GG	Male	50	17,0	6,30	Ischaemic heart disease		?
C. Substance Abuse (alcohol)	AG	Male	67	48,0	6,40	Acute Bronchopneumonia due to Chronic Airways Disease Other-Morphine toxicity	Morphine: 3mg/L Nordiazepam: 0.2 mg/L Paracetamol: 5mg/L	Yes
C. Substance Abuse (alcohol)	AG	Male	52	45,5	6,78	Lobar pneumonia and chronic alcoholism	-	Yes
C. Substance Abuse (alcohol)	AG	Male	53	57,0	6,75	Chronic airflow limitation - Pending	-	Yes
C. Substance Abuse (alcohol)	GG	Male	41	54	6,7	Epilepsy and chronic alcoholism	-	Yes
C. Substance Abuse (alcohol)	AA	Male	60	51,0	6,70	hepatic cirrhosis and its consequences, clinical history of alcoholism	paracetamol 22 mg/L	No
C. Substance Abuse (alcohol)	GG	Male	58	20,0	6,64	Ischaemic heart disease, cirrhosis	guaiphenesis 8.5 mg/L, ibuprofen 3.5 mg/L and paracetamol 16 mg/L	Yes
C. Substance Abuse (alcohol)	GG	Male	43	29,0	6,29	Intra-abdominal haemorrhage, complications of sepsis and multiple abdominal surgeries, massive hepatic necrosis, chronic hepatitis, chronic cholecystitis	-	Yes
C. Substance Abuse (alcohol)	AA	Male	73	43,5	6,59	Coronary artery atheroma	-	No
C. Substance Abuse (alcohol)	AG	Male	58	21,5	6,65	focal acute and chronic pancreatitis	-	Yes
C. Substance Abuse (alcohol)	AA	Male	63	25,5	6,21	Combined effects of ischaemic heart disease and chronic lung disease	-	Yes

Suppl. Table 1 (continued)

DSMIV Alcohol class	Geno-type	Gender	Age	PMI	Brain pH	COD	Toxicology	Smo-king
C. Substance Abuse (alcohol)	GG	Male	73	19,0	6,84	Ischaemic bowel. Atherrosclerotic cardiovascular disease	-	Yes
B: Substance Dependence (alcohol)	AA	Male	61	27,5	5,87	Combination of liver failure and Ischaemic heart disease	Metoclopramide <0.1 mg/L Morphine <0.05 mg/L	Yes
C. Substance Abuse (alcohol)	AG	Male	56	67	6.47		-	Yes
B: Substance Dependence (alcohol)	AG	Male	45	18,5	6,57		-	Yes
C. Substance Abuse (alcohol)	GG	Male	54	27,0	6,16	Ischaemic heart disease	delta-9-THC acid 0.01mg/L; amiodarone 5.0 umol/L; marijuana breakdown product	Yes
C. Substance Abuse (alcohol)	GG	Male	58	44,5	6,47		-	Yes
B: Substance Dependence (alcohol)	AG	Male	65	72,0	6,88	Acute Inteacerebral haemorrhage; Cerebral vascular malformation	-	Yes
C. Substance Abuse (alcohol)	AG	Male	41	38.5	6.55	Alcohol related	-	Yes
C. Substance Abuse (alcohol)	GG	Female	27	36,0	6,31	Combined effects of Moclobemide,Dothiepin and Alcohol intoxication	Blood alcohol: 0.132g/100ml Moclobemide 2.9mg/L Dothiepin 1mg/L	?
C. Substance Abuse (alcohol)	AG	Female	59	22,0	6,63	Ischaemic Heart Disease	Blood alcohol: 0.362g/100ml Cialopram: 0.2mg/L	Ex
C. Substance Abuse (alcohol)	AG	Female	49	48	6,11	Chronic alcoholism	-	No
C. Substance Abuse (alcohol)	AG	Male	54	17,0	6,41	Chest and Abdominal injury, IHD, Chronic alcoholism	Blood alcohol 0.016g/100ml	Yes
C. Substance Abuse (alcohol)	AG	Male	46	24,0	6,51	Alcohol Toxicity	Blood alcohol: 0.315g/100mL Nordiazepam 0.2mg/L	?
C. Substance Abuse (alcohol)	AG	Male	50	24,0	6,59	Upper gastrointestinal haemorrhage/ alcoholic liver disease/cirrhosis	Blood alcohol 0.241g/100mL	Yes
C. Substance Abuse (alcohol)	AG	Male	73	24,0	6,30	Consequences of Cirrhosis	blood alcohol 0.118g/100mL	No
C. Substance Abuse (alcohol)	AG	Male	56	45,0	6,51	Bleeding Oesophageal varices	Blood alcohol 0.283g/100mL	?
C. Substance Abuse (alcohol)	AG	Male	37	17,0	6,33	Acute Alcohol poisoning	blood alc.0.479g/100mL Carbamazepine 1mg/L	No
C. Substance Abuse (alcohol)	GG	Male	61	21,0	6,93	Combined effect of hypertensive heart disease and chronic alcoholism	Blood Alcohol: 0.020g/100ml Metoprolol 0.5mg/L	Yes
C. Substance Abuse (alcohol)	GG	Male	41	54,0	6,70	Epilepsy and chronic alcoholism	Delta-9-THC acid: 0.016mg/l Delta-9-THC: 0.005 mg/l Phenytoin: 0.1 mg/l	Yes
C. Substance Abuse (alcohol)	GG	Male	42	41,0	6,50	Combined bromoxynil and alcohol toxicity	CNS Drugs DL:01mg/l); Alcohol: 0.174g per 100ml; Bromoxynil:1.5mg/l	No

Suppl. Table 1 (continued)

DSMIV Alcohol class	Geno-type	Gender	Age	PMI	Brain pH	COD	Toxicology	Smo-king
C. Substance Abuse (alcohol)	GG	Male	60	16,5	6,48	Alcoholic liver cirrhosis and drug toxicity	Blood alcohol: (0.017g/100mL), Codeine (0.31 mg/L), Ibuprofen 14mg/L, Morphine 0.10 mg/L; Bile: Codeine 3mg/L, Morphine 4mg/L.	Yes
B: Substance Dependence (alcohol)	AG	Male	64	39,0	6,76	Acute alcohol toxicity	Alcohol 0.293 g/100ml	Yes
C. Substance Abuse (alcohol)	AG	Male	88	17,0	6,85	Asphyxia due to choking with food	BAL: 0.206 g/100ml; Amiodarone 1.2 mg/L; Nordiazepam0.1 mg/L; Paracetamol 4 mg/L; Valproic acid <10 mg/L;	No
A: Harmful Use (Alcohol)	GG	Male	59	35	6,57	Coronary artery thrombosis. Antecedent cause Coronary artery thrombosis.	-	Yes

Suppl. Table 2. Description of schizophrenic patients and control subjects. Schizophrenia (SCZ), Postmortem Interval (PMI), Electroconvulsive shock (ECS), chlorpromazine equivalents (CPE).

Diagnosis	Gender	Age (y)	PMI (h)	Brain pH	COD	Last medication	Alcohol	ECT	Age at onset (y)	Hospitalization (y)	Duration of medication (y)	CPE last dose (g)	CPE last 10 years (kg)
Control	Male	41	7	7.2	Heart infarction		No						
Control	Female	91	16	6.8	Cardio-pulmonary insufficiency		No						
Control	Male	57	24	6.5	Heart infarction		No						
Control	Male	53	18	7.1	Heart infarction		No						
Control	Male	63	13	6.9	Heart infarction		No						
Control	Male	66	16	6.5	Heart infarction		No						
SCZ	Female	64	23	6.6	Heart infarction	Zotepine 150 mg, Olanzapine 10 mg	No	Yes	24	5	40	54.4	4.6
SCZ	Male	73	20	6.9	Heart infarction	Perphenazine 32 mg, Promethazine 150 mg	No	No	30	33	40	507.4	1.7
SCZ	Male	43	18	6.4	Heart infarction	Zuclopethixol 40 mg, Valproate 1200 mg, Tiapride 300 mg	No	No	20	13	20	464	2.6
SCZ	Female	76	17	7.1	Cardio-pulmonary insufficiency	Perazine 300 mg	No	Yes	27	30	47	300	4.9
SCZ	Female	63	31	6.3	Heart infarction	Olanzapine 15 mg	No	Yes	24	30	30	75	1.8
SCZ	Male	51	7	6.7	Heart infarction	Flupenthixol 15 mg	No	Yes	19	20	25	174	0.6
SCZ	Male	81	4	6.8	Corpulmonale, heart insufficiency	Haloperidol 4 mg, Prothipendyl 80 mg	No	No	19	48	50	92.8	1.4
SCZ	Male	92	37	6.6	Heart infarction	Prothipendyl 160 mg, Perazine 100 mg	No	No	41	51	48	100	3.4
SCZ	Male	71	28	6.5	Heart infarction	Haloperidol 32 mg, Pipamperone 40 mg	No	No	30	12	35	782.4	10

Suppl. Table 3 Demographic data, genotype, and tissue characteristics of postmortem samples of suicide completers and controls subjects. Postmortem interval, PMI.

Diagnosis	Genotype	Gender	Age (y)	PMI (h)	Cause of death
Control	AG	Female	71	6,5	stroke - cardiovascular-pulmonary insufficiency
Control	GG	Female	93	6,5	Alzheimer's disease, cardiovascular-respiratory insufficiency
Control	AG	Female	38	2	acute cardiac and respiratory insufficiency, chronic myocardial infarction, chronic heart failure
Control	GG	Female	58	1	acute myocardial infarction, arterosclerosis
Control	AG	Female	86	4,5	Alzheimer's disease, cardiovascular-respiratory insufficiency
Control	AG	Female	78	5,5	brain hemorrhage
Control	AA	Female	79	4,5	cardiac and respiratory insufficiency
Control	AG	Female	68	3	pneumonia, respiratory insufficiency
Control	GG	Female	76	2,5	traffic accident
Control	AG	Female	76	6	heart failure
Control	GG	Female	81	5	dementia vasculosa globalis
Control	AG	Female	8	5,5	diffuse Lewy disease
Control	AG	Female	93	5,5	cardio-respiratory insufficiency, pulmonary embolism
Control	AG	Female	94	6	dementia
Control	GG	Female	89	1,5	cardiac insufficiency, coronary arteriosclerosis
Control	GG	Female	56	6	cardiac and respiratory insufficiency
Control	GG	Female	60	3	acute cardiac insufficiency
Control	GG	Female	33	0,5	acute myocardial infarction
Control	AG	Female	64	1,5	heart failure
Control	AG	Female	80	1	acute respiratory insufficiency, senile, hypertensive arteriosclerosis
Control	GG	Female	44	5	myocardial infarction
Control	AG	Female	74	6	cardio-respiratory insufficiency
Control	GG	Female	78	1,5	chronic myocardial infarction, pulmonary embolism
Control	AG	Female	87	6	dementia, myocardial insufficiency
Control	AG	Female	63	0,5	heart failure, cardiac and respiratory insufficiency, chronic myocardial infarction
Control	AG	Female	89	1,5	atherosclerosis cerebri, dementia
Control	AG	Female	62	6	schizophrenia chronica, Parkinson syndrome, bronchopneumonia
Control	AG	Female	72	3,5	acute myocardial infarction, earlier heart failure, arterosclerosis
Control	AG	Female	86	4	dementia, myocardial insufficiency
Control	AG	Female	56	5	myocardial infarction
Control	AG	Female	88	2,5	cardiac insufficiency, heart failure
Control	AG	Male	40	3,5	toxication of smoke
Control	AG	Male	83	0,5	acute cardiac and acute respiratory insufficiency, chronic heart failure, coronary stenosis
Control	GG	Male	73	6	acute cardiac failure
Control	AG	Male	59	1	acute cardiac insufficiency, coronary stenosis, acute myocardial infarction
Control	GG	Male	80	5,5	stroke
Control	AG	Male	55	3	cardiac insufficiency, coronary stenosis
Control	AG	Male	58	2	myocardial infarction, earlier chronic myocardial infarction, coronary sclerosis
Control	AA	Male	74	3	acute myocardial infarction

Suppl. Table 3 (continued)

Diagnosis	Genotype	Gender	Age (y)	PMI (h)	Cause of death
Control	GG	Male	71	2	acute heart failure, arterosclerosis
Control	GG	Male	68	2	chronic heart failure, coronary sclerosis
Control	AG	Male	68	2,5	acute myocardial infarction; senile, hypertensive arteriosclerosis
Control	AG	Male	63	2	cardiac insufficiency, coronary sclerosis, earlier myocardial infarction
Control	GG	Male	57	1	acute cardiac insufficiency, coronary stenosis, fatty (Laënnec's) cirrhosis
Control	AA	Male	81	5	heart failure, cardiogen shock
Control	GG	Male	55	1	acute myocardial infarction, earlier myocardial infarction
Control	AG	Male	35	6	suicide (hanging - acute respiratory insufficiency)
Control	GG	Male	47	2	acute cardiac insufficiency, chronic myocardial infarction, chronic heart failure, coronary sclerosis
Control	GG	Male	62	5,5	respiratory and cardiac insufficiency
Control	AG	Male	83	6	respiratory and cardiac insufficiency
Control	GG	Male	56	2	acute heart failure, acute myocardial infarction
Control	GG	Male	64	4	pneumonia
Control	GG	Male	83	5	Alzheimer-like dementia
Control	AA	Male	50	0,5	peritonitis, cardiac and respiratory insufficiency, atrophic cirrhosis
Control	GG	Male	50	4	acute heart failure, senile, hypertensive arteriosclerosis
Control	AG	Male	64	6	myocardial infarction
Control	GG	Male	42	2,5	suicide (hanging - asphyxia, acute paralytic stroke)
Control	AG	Male	52	4,5	myocardial infarction
Control	GG	Male	47	2	cardiac insufficiency, pneumonia, respiratory insufficiency, earlier myocardial infarction, fatty (Laënnec's) cirrhosis
Control	GG	Male	37	8	electric shock
Control	AA	Male	52	3,5	acute myocardial infarction
Control	AG	Male	67	1	respiratory and cardiac insufficiency, pneumonia, senile, hypertensive arteriosclerosis, multilobular cirrhosis
Control	AG	Male	42	3,5	acute respiratory insufficiency
Control	AG	Male	45	3	respiratory insufficiency, acute myocardial infarction, coronary stenosis
Control	GG	Male	53	5	pulmonary embolism
Control	GG	Male	64	1	acute heart failure, acute cardiac insufficiency, coronary stenosis
Control	AG	Male	74	1	acute cardiac insufficiency, chronic heart failure
Control	AA	Male	47	1	heart failure, coronary sclerosis
Control	GG	Male	50	2	myocardial infarction
Control	AA	Male	65	1	heart failure, cardiac and respiratory insufficiency
Control	GG	Male	75	5	bipolar affective psychosis
Control	AA	Male	85	3	heart failure
Control	GG	Male	46	4	senile, hypertensive arteriosclerosis; acute myocardial infarction
Control	GG	Male	65	1	pulmonary embolism, arterosclerosis, heart failure
Suicide	AG	Female	42	3	suicide (hanging - asphyxia)
Suicide	GG	Female	42	11	suicide (drug overdose)
Suicide	AG	Female	83	6	suicide (hanging - asphyxia)
Suicide	AG	Female	28	6	suicide (drug overdose + alcohol, acute heart failure)

Suppl. Table 3 (continued)

Diagnosis	Genotype	Gender	Age (y)	PMI (h)	Cause of death
Suicide	GG	Female	36	2	suicide (drug overdose - aspiration pneumonia, asphyxia)
Suicide	AG	Female	48	6,5	suicide (drug overdose)
Suicide	GG	Female	65	5	suicide (hanging - asphyxia)
Suicide	AG	Female	72	3,5	suicide (drug overdose)
Suicide	AG	Female	49	6	suicide (drug overdose)
Suicide	AG	Female	43	4,5	suicide (hanging - asphyxia)
Suicide	AG	Male	71	1	suicide (jumping from a height)
Suicide	GG	Male	36	6	suicide (hanging - asphyxia)
Suicide	AG	Male	48	6	suicide (hanging - asphyxia)
Suicide	AG	Male	66	6	suicide (hanging - asphyxia)
Suicide	AA	Male	47	6	suicide (hanging - asphyxia, acute paralytic stroke)
Suicide	GG	Male	43	3	suicide (hanging - asphyxia)
Suicide	GG	Male	48	3	suicide (drug overdose + alcohol)
Suicide	AA	Male	45	4	suicide (drug overdose)
Suicide	GG	Male	42	4	suicide (hanging –acute paralytic stroke, acute respiratory insufficiency)
Suicide	AG	Male	31	8	suicide (hanging - asphyxia)
Suicide	AG	Male	43	4	suicide (hanging)
Suicide	AG	Male	57	16	suicide (hanging - asphyxia)
Suicide	GG	Male	32	6	suicide (hanging - asphyxia)
Suicide	GG	Male	35	2	suicide (drug overdose)

Suppl. Table 4. *Cacna1c* and *Cacna1d* mRNA expression in alcohol-dependent and non-dependent rats after 3 weeks of abstinence. Statistical analysis was performed by region-wise one-way ANOVA followed by Bonferroni's correction; n.s. = not significant; p values: *p<0.05; **p<0.01; ***p<0.001vs non-dependent rats, n=5-7/group. Cingulate cortex (Cing), prelimbic cortex (PreL), infralimbic region (IL), and orbitofrontal cortex (OFC), motor cortex M1, nucleus accumbens [core (AcbC) and shell (AcbS)], caudate putamen (CPu), extended amygdala [bed nucleus of the stria terminalis (BNST), central amygdala (CeA), medial amygdala (MeA), and basolateral amygdala (BLA)], paraventricular nucleus (PVN), and hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1 and CA3].

mRNA	Region	Control (nCi/g; mean ± SEM)	Alc. dep. (nCi/g; mean ± SEM)	F	p
Cacna1c	Cing	2.01 ± 0.3	1.96 ± 0.1	[1,10]0.03	n.s.
	PreL	2.77 ± 0.3	3.54 ± 0.2	[1,11]4.15	n.s.
	IL	4.41 ± 0.3	3.84 ± 0.2	[1,10]2.51	n.s.
	OFC	2.71 ± 0.2	2.66 ± 0.1	[1,12]0.05	n.s.
	AcbC	1.62 ± 0.2	1.34 ± 0.2	[1,11]0.98	n.s.
	AcbS	6.03 ± 0.8	6.18 ± 0.2	[1,12]0.03	n.s.
	CPu	0.93 ± 0.2	0.87 ± 0.1	[1,11]0.07	n.s.
	BNST	0.76 ± 0.1	1.33 ± 0.2	[1,11]11.13	n.s.
	CeA	3.56 ± 0.1	4.97 ± 0.3	[1,11]23.91	0.008143**
	MeA	4.82 ± 0.4	6.15 ± 0.3	[1,11]6.85	n.s.
	BLA	2.96 ± 0.2	5.04 ± 0.3	[1,12]34.72	0.001241**
	CA1	1.68 ± 0.1	2.95 ± 0.2	[1,12]27.27	0.003638**
	CA3	15.65 ± 0.5	19.44 ± 0.8	[1,12]16.93	0.024395*
	CA4	13.26 ± 0.1	17.68 ± 0.4	[1,11]151.98	0.000000***
	DG	16.39 ± 0.6	18.29 ± 0.6	[1,12]4.73	n.s.
	M1	1.63 ± 0.1	1.51 ± 0.1	[1,10]0.64	n.s.
	PVN	6.56 ± 0.5	6.73 ± 0.4	[1,11]0.06	n.s.

Suppl. Table 4. (continued)

mRNA	Region	Control (nCi/g; mean ± SEM)	Alc. dep. (nCi/g; mean ± SEM)	F	p
Cacna1d	Cing	7.89 ± 0.5	8.36 ± 0.3	[1,12]0.70	n.s.
	PreL	10.26 ± 0.5	9.68 ± 0.2	[1,11]1.23	n.s.
	IL	10.65 ± 0.2	10.49 ± 0.5	[1,12]0.10	n.s.
	OFC	9.27 ± 0.3	8.81 ± 0.2	[1,12]1.87	n.s.
	AcbC	5.14 ± 0.2	5.23 ± 0.1	[1,12]0.13	n.s.
	AcbS	7.84 ± 0.3	7.85 ± 0.2	[1,12]0.001	n.s.
	CPu	3.87 ± 0.3	3.77 ± 0.1	[1,12]0.11	n.s.
	BNST	7.29 ± 0.3	6.76 ± 0.2	[1,12]1.76	n.s.
	CeA	10.06 ± 0.7	8.17 ± 0.3	[1,10]6.77	n.s.
	MeA	7.98 ± 0.4	8.15 ± 0.2	[1,11]0.11	n.s.
	BLA	8.93 ± 0.2	8.72 ± 0.2	[1,11]0.45	n.s.
	CA1	11.71 ± 0.2	12.03 ± 0.7	[1,10]0.18	n.s.
	CA3	11.82 ± 0.3	13.27 ± 0.2	[1,10]12.116	n.s.
	CA4	12.96 ± 0.6	14.58 ± 0.8	[1,11]2.72	n.s.
	DG	40.61 ± 2.7	39.67 ± 3.3	[1,11]0.05	n.s.
	M1	8.28 ± 0.4	8.21 ± 0.2	[1,11]0.03	n.s.
	PVN	14.73 ± 0.6	15.23 ± 0.9	[1,11]0.24	n.s.

Suppl. Table 5. *Cacna1c* mRNA expression in alcohol-dependent and non-dependent rats after 0, 1, 3, 7, and 21 d of abstinence. Central amygdala (CeA), basolateral amygdala (BLA), Cornus Ammon (CA) regions CA1. N=6-8/group

Region	Days of abstinence	Control (nCi/g; mean ± SEM)	Alc.-dep. (nCi/g; mean ± SEM)
CA1	0	11.28 ± 1.2	13.75 ± 1.0
	1	13.69 ± 0.7	10.38 ± 0.9
	3	10.66 ± 0.5	11.04 ± 1.0
	7	10.63 ± 0.6	13.05 ± 1.5
	21	7.54 ± 0.1	13.33 ± 1.0
CeA	0	15.62 ± 0.7	21.45 ± 0.5
	1	18.17 ± 0.9	13.70 ± 0.4
	3	18.70 ± 0.6	16.78 ± 0.7
	7	13.57 ± 0.6	16.71 ± 1.4
	21	12.44 ± 0.4	15.99 ± 0.6
BLA	0	13.33 ± 0.66	17.65 ± 0.3
	1	15.47 ± 0.6	12.88 ± 0.3
	3	15.76 ± 0.6	15.47 ± 0.8
	7	15.12 ± 0.5	16.73 ± 1.6
	21	11.86 ± 0.4	13.76 ± 0.6

Suppl. Table 6. *Cacna1c* and *Cacna1d* mRNA expression in AA, ANA, and Wistar rats. Statistical analysis was performed by region-wise one-way ANOVA followed by Bonferroni's correction; n.s. = not significant; p values: *p<0.05; **p<0.01; ***p<0.001 overall effect, n=5-7/group. Cingulate cortex, Cing; Prelimbic cortex, PreL; Infralimbic cortex, IL; caudate putamen, CPu; nucleus accumbens core, AcbC; nucleus accumbens shell, AcbS; Central amygdala, CeA; medial amygdala, MeA; basolateral amygdala, BLA; hippocampal formation [dentate gyrus, DG;and Cornus Ammon (CA) regions CA1, CA3, and CA4].

mRNA	Region	ANA (nCi/g; mean ± SEM)	Wistar (nCi/g; mean ± SEM)	AA (nCi/g; mean ± SEM)	F	p
Cacna1c	Cing	12.77 ± 0.5	11.75 ± 0.3	11.83 ± 0.7	[2,13]1.19	n.s.
	PreL	18.67 ± 0.7	17.56 ± 0.4	18.47 ± 0.7	[2,13]1.19	n.s.
	IL	24.35 ± 1.2	21.00 ± 0.4	22.67 ± 0.7	[2,11]5.27	n.s.
	CPu	17.65 ± 0.4	16.98 ± 0.5	16.96 ± 0.1	[2,15]0.96	n.s.
	AcbC	20.62 ± 1.0	20.04 ± 0.3	19.49 ± 0.4	[2,13]0.85	n.s.
	AcbS	23.10 ± 1.0	21.84 ± 0.5	23.27 ± 0.5	[2,15]1.28	n.s.
	CeA	15.61 ± 0.4	15.46 ± 0.3	16.09 ± 0.2	[2,12]1.16	n.s.
	MeA	17.64 ± 0.3	18.00 ± 0.3	18.95 ± 0.4	[2,15]4.32	n.s.
	BLA	15.66 ± 0.4	13.48 ± 0.2	15.86 ± 0.3	[2,15]17.38	0.00087***
	CA1	11.12 ± 0.2	9.92 ± 0.6	12.62 ± 0.5	[2,11]8.47	0.041601*
	CA3	74.29 ± 2.2	78.55 ± 1.6	79.65 ± 1.4	[2,15]2.49	n.s.
	CA4	53.72 ± 1.0	59.84 ± 1.8	68.28 ± 0.4	[2,15]37.19	0.00001***
	DG	68.13 ± 2.1	66.01 ± 1.4	80.65 ± 1.3	[2,13]20.16	0.00073***

Suppl. Table 6. (continued)

mRNA	Region	ANA (nCi/g; mean ± SEM)	Wistar (nCi/g; mean ± SEM)	AA (nCi/g; mean ± SEM)	F	p
Cacna1d	Cing	32.86 ± 1.3	34.95 ± 0.8	34.35 ± 1.4	[2,13]0.87	n.s.
	PreL	37.39 ± 1.0	40.87 ± 0.8	41.82 ± 0.6	[2,12]7.63	n.s.
	IL	42.98 ± 2.5	42.09 ± 1.0	44.40 ± 0.8	[2,13]0.58	n.s.
	CPu	12.07 ± 0.2	13.53 ± 0.3	13.83 ± 0.2	[2,15]14.99	0.00265**
	AcbC	21.44 ± 1.1	21.69 ± 0.9	22.95 ± 0.4	[2,14]0.96	n.s.
	AcbS	29.18 ± 1.6	27.96 ± 0.7	31.31 ± 0.6	[2,15]2.44	n.s.
	CeA	31.31 ± 0.4	32.79 ± 0.7	34.43 ± 0.6	[2,15]7.42	0.040362*
	MeA	27.77 ± 0.5	35.57 ± 1.1	33.58 ± 0.3	[2,15]32.97	0.00002***
	BLA	29.56 ± 0.4	30.09 ± 0.2	31.26 ± 0.7	[2,15]3.26	n.s.
	CA1	36.73 ± 0.4	42.23 ± 0.7	45.20 ± 1.7	[2,15]15.32	0.001666**
	CA3	49.33 ± 0.6	51.49 ± 0.4	50.03 ± 0.5	[2,15]4.55	n.s.
	CA4	44.05 ± 0.6	52.19 ± 1.3	57.53 ± 0.9	[2,15]46.90	0.00000***
	DG	130.54 ± 2.3	136.28 ± 2.6	174.33 ± 5.7	[2,15]38.43	0.00001***

Suppl. Table 7. *Cacna1c* and *Cacna1d* mRNA expression in ADE and control rats. Statistical analysis was performed by region-wise one-way ANOVA followed by Bonferroni's correction; n.s. = not significant; p values: *p<0.05; **p<0.01; ***p<0.001 overall effect, n=5-7/group. Central amygdala (CeA), medial amygdala (MeA), basolateral amygdala (BLA), hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1 and CA3].

mRNA	Region	Control (nCi/g; mean ± SEM)	ADE (nCi/g; mean ± SEM)	F	p
Cacna1c	CeA	14.48 ± 0.8	14.66 ± 0.6	[1,8]0.03	n.s.
	MeA	15.54 ± 1.0	14.77 ± 0.7	[1,8]0.39	n.s.
	BLA	15.64 ± 1.2	13.52 ± 0.5	[1,8]2.77	n.s.
	CA1	12.21 ± 0.7	14.29 ± 1.0	[1,8]2.89	n.s.
	CA3	57.5 ± 0.8	56.92 ± 0.4	[1,8]0.49	n.s.
	DG	52.24 ± 1.9	59.49 ± 3.7	[1,8]3.04	n.s.
Cacna1d	CeA	14.74 ± 0.2	13.73 ± 0.6	[1,5]3.17	n.s.
	MeA	10.79 ± 0.7	12.24 ± 0.7	[1,5]1.89	n.s.
	BLA	12.74 ± 0.4	12.47 ± 0.2	[1,6]0.20	n.s.
	CA1	18.60 ± 1.2	15.68 ± 0.8	[1,6]2.84	n.s.
	CA3	18.66 ± 0.5	18.51 ± 1.0	[1,6]0.02	n.s.
	DG	64.52 ± 1.4	59.78 ± 0.2	[1,6]6.04	n.s.

Suppl. Table 8. *Cacna1c* and *Cacna1d* mRNA in alcohol dependent and non-dependent rats with or without 3 weeks of free access to alcohol. Data are expressed as nCi/g, n=5-7/group. Cingulate cortex, Cing; Prelimbic cortex, PreL; Infralimbic cortex, IL; caudate putamen, CPu; nucleus accumbens core, AcbC; nucleus accumbens shell, AcbS; Central amygdala, CeA; medial amygdala, MeA; basolateral amygdala, BLA; hippocampal formation [dentate gyrus, DG;and Cornus Ammon (CA) regions CA1, CA3, and CA4].

mRNA	Region	Control (nCi/g; mean ± SEM)	Control + Drinking (nCi/g; mean ± SEM)	Alc. dep. (nCi/g; mean ± SEM)	Alc. dep. + Drinking (nCi/g; mean ± SEM)
Cacna1c	Cing	2.01 ± 0.3	0.58 ± 0.1	1.96 ± 0.1	0.66 ± 0.1
	PreL	2.77 ± 0.3	1.06 ± 0.1	3.54 ± 0.2	1.56 ± 0.2
	IL	4.41 ± 0.3	2.61 ± 0.3	3.84 ± 0.2	2.74 ± 0.2
	CPu	0.93 ± 0.2	0.88 ± 0.1	0.88 ± 0.1	1.30 ± 0.1
	AcbC	1.62 ± 0.2	0.82 ± 0.1	1.34 ± 0.2	0.92 ± 0.1
	AcbS	6.03 ± 0.8	3.77 ± 0.2	6.18 ± 0.2	3.88 ± 0.3
	CeA	3.56 ± 0.1	3.81 ± 0.4	4.97 ± 0.3	2.76 ± 0.2
	MeA	4.82 ± 0.4	4.78 ± 0.2	6.15 ± 0.3	3.55 ± 0.2
	BLA	2.96 ± 0.2	3.76 ± 0.2	5.04 ± 0.3	2.61 ± 0.4
	CA1	1.68 ± 0.1	2.38 ± 0.3	2.95 ± 0.2	2.27 ± 0.2
	CA3	15.65 ± 0.5	18.68 ± 0.8	19.44 ± 0.8	13.83 ± 0.8
	DG	16.39 ± 0.6	15.64 ± 0.4	18.29 ± 0.6	11.40 ± 1.0

Suppl. Table 8. (continued)

mRNA	Region	Control (nCi/g; mean ± SEM)	Control + Drinking (nCi/g; mean ± SEM)	Alc. dep. (nCi/g; mean ± SEM)	Alc. dep. + Drinking (nCi/g; mean ± SEM)
Cacna1d	Cing	7.89 ± 0.5	9.61 ± 0.4	8.36 ± 0.3	9.82 ± 0.2
	PreL	10.26 ± 0.5	11.29 ± 0.6	9.68 ± 0.2	11.81 ± 0.5
	IL	10.65 ± 0.2	12.21 ± 0.5	10.49 ± 0.5	14.19 ± 0.6
	CPu	3.87 ± 0.3	4.52 ± 0.2	3.77 ± 0.1	4.63 ± 0.2
	AcbC	5.14 ± 0.2	5.64 ± 0.3	5.23 ± 0.1	6.96 ± 0.1
	AcbS	7.84 ± 0.3	8.67 ± 0.4	7.85 ± 0.2	10.57 ± 0.8
	CeA	9.62 ± 0.7	10.99 ± 0.7	8.17 ± 0.3	10.51 ± 0.3
	MeA	7.98 ± 0.4	9.87 ± 0.3	8.15 ± 0.2	8.75 ± 0.5
	BLA	8.93 ± 0.2	10.23 ± 0.7	8.72 ± 0.2	6.92 ± 0.4
	CA1	11.71 ± 0.2	14.75 ± 0.8	12.03 ± 0.7	14.11 ± 0.9
	CA3	11.82 ± 0.3	13.44 ± 0.5	13.27 ± 0.2	13.64 ± 0.8
	DG	40.61 ± 2.7	50.61 ± 4.5	39.67 ± 3.3	55.68 ± 4.6

Suppl. Table 9. *Cacna1c* and *Cacna1d* mRNA in alcohol dependent and non-dependent rats with or without 3 weeks of free access to alcohol. Statistical analysis was performed as two-way ANOVA for Treatment (alcohol vapor exposure vs. air exposure) and Access to alcohol (3 weeks of freely available alcohol v.s no access) followed by Bonferroni's correction; n=5-7/group. p values: *p<0.05; **p<0.01; ***p<0.001. Central amygdala (CeA), medial amygdala (MeA), basolateral amygdala (BLA), hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1 and CA3].

mRNA	Region	Treatment		Access to alcohol		Treatment*Access	
		F	p	F	p	F	p
Cacna1c	Cing	[1,21]0.01	n.s.	[1,21]90.57	0.0000***	[1,21]0.21	n.s.
	PreL	[1,22]8.52	n.s.	[1,22]72.35	0.0000***	[1,22]0.41	n.s.
	IL	[1,18]0.78	n.s.	[1,18]32.39	0.0002***	[1,18]1.92	n.s.
	CPu	[1,22]1.95	n.s.	[1,22]2.00	n.s.	[1,22]3.26	n.s.
	AcbC	[1,22]0.27	n.s.	[1,22]13.26	0.0144*	[1,22]1.29	n.s.
	AcbS	[1,23]0.08	n.s.	[1,23]24.80	0.0005***	[1,23]0.00	n.s.
	CeA	[1,23]0.46	n.s.	[1,23]13,51	0.0075**	[1,23]21.11	0.0008***
	MeA	[1,21]0.03	n.s.	[1,21]19.16	0.0016**	[1,21]18.05	0.0022**
	BLA	[1,23]2.82	n.s.	[1,23]8.62	0.0445*	[1,23]33.90	0.0000***
	CA1	[1,21]7.86	n.s.	[1,21]0.01	n.s.	[1,21]11.28	0.0179*
	CA3	[1,22]0.56	n.s.	[1,22]3.39	n.s.	[1,22]37.99	0.0000***
	DG	[1,22]2.92	n.s.	[1,22]31.17	0.0001 ***	[1,22]20.15	0.0011**

Suppl. Table 9. (continued)

mRNA	Region	Treatment		Access to alcohol		Treatment*Access	
		F	p	F	p	F	p
Cacna1d	Cing	[1,23]0.77	n.s.	[1,23]16.84	0.00434**	[1,23]0.11	n.s.
	PreL	[1,22]0.01	n.s.	[1,22]11.89	0.02294*	[1,22]1.45	n.s.
	IL	[1,22]3.87	n.s.	[1,22]32.52	0.0001***	[1,22]5.44	n.s.
	CPu	[1,24]0.00	n.s.	[1,24]13.12	0.01359*	[1,24]0.25	n.s.
	AcbC	[1,23]11.83	0.02238*	[1,23]29.58	0.0002***	[1,23]8.95	n.s.
	AcbS	[1,24]0.07	n.s.	[1,24]12.35	0.01776*	[1,24]3.50	n.s.
	CeA	[1,23]2.95	n.s.	[1,23]10.96	0.0183*	[1,23]0.74	n.s.
	MeA	[1,23]1.38	n.s.	[1,23]9.39	0.0329*	[1,23]2.51	n.s.
	BLA	[1,22]14.3	0.0062**	[1,22]0.30	n.s.	[1,22]11.07	0.0184*
	CA1	[1,21]0.05	n.s.	[1,21]13.16	0.0095**	[1,21]0.45	n.s.
	CA3	[1,20]2.16	n.s.	[1,20]3.16	n.s.	[1,20]1.22	n.s.
	DG	[1,22]0.28	n.s.	[1,22]11.13	0.0180*	[1,22]0.60	n.s.

Suppl. Table 10. *Cacna1c* mRNA expression in alcohol dependent rats after 4 weeks or 7 weeks of alcohol vapor exposure and 3 weeks of abstinence. Values are expressed in nCi/g, n=5-8/group. Central amygdala (CeA), medial amygdala (MeA), basolateral amygdala (BLA), hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1 and CA3].

mRNA	Region	Control (nCi/g; mean ± SEM)	4 weeks exposed (nCi/g; mean ± SEM)	7 weeks exposed (nCi/g; mean ± SEM)
Cacna1c	CeA	12.45 ± 0.4	23.03 ± 1.4	16.00 ± 0.6
	MeA	11.66 ± 0.4	23.24 ± 1.4	14.59 ± 1.1
	BLA	11.86 ± 0.4	18.99 ± 0.6	13.76 ± 0.6
	CA1	7.54 ± 0.1	15.56 ± 1.9	13.34 ± 1.0
	CA3	49.60 ± 1.7	70.91 ± 1.6	62.52 ± 2.5
	DG	49.64 ± 0.5	63.83 ± 1.7	54.83 ± 3.1

Suppl. Table 11. *Crhr1* mRNA expression in alcohol dependent rats after 4 weeks or 7 weeks of alcohol vapor exposure and 3 weeks of abstinence. Values are expressed in nCi/g, n=3-7/group. Central amygdala (CeA), medial amygdala (MeA), basolateral amygdala (BLA).

mRNA	Region	Control 4 weeks (nCi/g; mean ± SEM)	4 weeks exposed (nCi/g; mean ± SEM)	Controls 7 weeks (nCi/g; mean ± SEM)	7 weeks exposed (nCi/g; mean ± SEM)
Crhr1	CeA	2.29 ±0.2	2.59 ± 0.3	3.47 ± 0.2	4.22 ± 0.3
	MeA	2.31 ± 0.1	3.05 ± 0.2	3.44 ± 0.2	5.02 ± 0.3
	BLA	4.28 ± 0.3	5.22 ± 0.4	6.23 ± 0.3	8.45 ± 0.6

Suppl. Table 12. *Cacna1c* and *Cacna1d* mRNA expression in Wistar rats exposed to 1 h of restraint stress. Statistical analysis was performed by region-wise one-way ANOVA followed by Bonferroni's correction; n.s. = not significant; *p<0.05; overall effect, n=4-6/group. Central amygdala (CeA), medial amygdala (MeA), basolateral amygdala (BLA), hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1 and CA3].

mRNA	Region	Non-stressed (nCi/g; mean ± SEM)	Stressed (nCi/g; mean ± SEM)	F	p
Cacna1c	Cing	11.75 ± 0.3	11.70 ± 1.1	[1,8]0.00	n.s.
	PreL	17.56 ± 0.4	18.84 ± 0.7	[1,8]3.31	n.s.
	IL	21.00 ± 0.4	23.19 ± 0.8	[1,7]7.90	n.s.
	CPu	16.98 ± 0.5	18.12 ± 0.5	[1,10]2.31	n.s.
	AcbC	20.04 ± 0.3	15.92 ± 0.5	[1,10]45.09	0.00053***
	AcbS	21.84 ± 0.5	19.40 ± 0.3	[1,10]15.52	0.02775*
	CeA	15.46 ± 0.3	14.79 ± 0.4	[1,10]2.05	n.s.
	MeA	17.99 ± 0.3	15.64 ± 0.5	[1,10]15.08	0.03238*
	BLA	13.48 ± 0.2	13.94 ± 0.2	[1,10]2.85	n.s.
	CA1	9.92 ± 0.6	11.18 ± 0.5	[1,4]2.34	n.s.
	CA3	78.55 ± 1.6	74.45 ± 2.05	[1,10]2.47	n.s.
	CA4	59.84 ± 1.8	58.18 ± 1.5	[1,10]0.52	n.s.
	DG	66.01 ± 1.4	67.1 ± 1.7	[1,9]0.24	n.s.

Suppl. Table 12. (continued)

mRNA	Region	Non-stressed (nCi/g; mean ± SEM)	Stressed (nCi/g; mean ± SEM)	F	p
Cacna1d	Cing	34.95 ± 0.8	31.80 ± 0.7	[1,10]8.88	n.s.
	PreL	40.87 ± 0.8	36.21 ± 0.5	[1,10]24.68	0.00563**
	IL	42.09 ± 1.0	39.53 ± 0.1	[1,8]4.36	n.s.
	CPu	13.53 ± 0.3	16.30 ± 0.4	[1,10]31.03	0.00237**
	AcbC	21.69 ± 0.9	20.66 ± 0.4	[1,10]0.98	n.s.
	AcbS	27.96 ± 0.7	226.40 ± 0.9	[1,10]1.77	n.s.
	CeA	32.79 ± 0.7	36.19 ± 0.8	[1,10]9.9	n.s.
	MeA	35.57 ± 1.1	32.18 ± 0.3	[1,10]9.32	n.s.
	BLA	30.09 ± 0.2	31.86 ± 0.6	[1,10]7.34	n.s.
	CA1	42.23 ± 0.7	45.86 ± 0.5	[1,10]18.48	0.01099*
	CA3	51.5 ± 0.4	56.86 ± 2.1	[1,9]7.72	n.s.
	CA4	52.19 ± 1.3	53.96 ± 2.0	[1,10]0.54	n.s.
	DG	136.28 ± 2.6	151.69 ± 2.5	[1,10]17.8	0.01239*

Suppl. Table 13. *Cacna1c* and *Cacna1d* mRNA expression levels at different time points in forebrain regions of C57/BL6 mice. Nicotine treatment: 0.175 mg/kg, IP; Saline treatment: 0.9% saline IP. Data are expressed as nCi/g (means ± SEM), n = 4-8/group.

		1d after a single injection		1d after chronic treatment		7d after chronic treatment	
mRNA	**Region**	**Saline (nCi/g; mean ± SEM)**	**Nicotine (nCi/g; mean ± SEM)**	**Saline (nCi/g; mean ± SEM)**	**Nicotine (nCi/g; mean ± SEM)**	**Saline (nCi/g; mean ± SEM)**	**Nicotine (nCi/g; mean ± SEM)**
Cacna1c	**Cing**	7.3 ± 0.2	5.7 ± 0.6	9.6 ± 0.1	8.4 ± 0.2	8.5 ± 0.2	8.4 ± 0.2
	PreL	11.4 ± 0.4	11.0 ± 0.2	15.7 ± 0.4	13.1 ± 0.4	11.7 ± 0.3	14.9 ± 0.2
	IL	8.9 ± 0.3	6.6 ± 0.3	12.6 ± 0.1	12.4 ± 0.3	9.8 ± 0.2	11.6 ± 0.3
	OFC	8.7 ± 0.3	8.8 ± 0.1	11.2 ± 0.2	10.2 ± 0.3	9.1 ± 0.1	11.2 ± 0.3
	CPu	11.3 ± 0.2	9.6 ± 0.2	12.8 ± 0.2	11.7 ± 0.1	10.9 ± 0.1	12.6 ± 0.2
	AcbC	10.7 ± 0.2	9.7 ± 0.4	13.7 ± 0.2	13.8 ± 0.4	10.3 ± 0.3	11.5 ± 0.1
	AcbS	11.7 ± 0.5	13.4 ± 0.2	18.9 ± 0.2	19.4 ± 0.5	13.7 ± 0.5	17.8 ± 0.2
	VTA	10.1 ± 0.2	8.9 ± 0.3	11.2 ±0.3	9.5 ± 0.2	9.5 ± 0.2	10.7 ± 0.4
	CA1	14.5 ± 0.3	12.6 ± 0.4	17.4 ± 0.6	17.7 ± 0.3	13.4 ± 0.2	16.7 ± 0.4
	CA3	59.3 ± 1.0	63.8 ± 0.7	68.3 ± 1.0	56.6 ± 0.6	59.6 ± 0.4	68.0 ± 1.0
	DG	46.0 ± 0.8	43.8 ± 0.6	54.6 ± 0.3	51.7 ± 0.5	41.3 ± 1.3	52.5 ± 0.8
Cacna1d	**Cing**	9.0 ± 0.8	13.7 ± 0.1	12.5 ± 0.2	13.3 ± 0.7	16.5 ± 0.3	16.4 ± 0.3
	PreL	13.6 ± 1.2	16.6 ± 0.3	17.2 ± 0.6	16.5 ± 0.8	18.1 ± 0.2	19.4 ± 0.1
	IL	8.8 ± 0.5	10.7 ± 0.2	11.7 ± 0.3	12.0 ± 0.4	14.2 ± 0.1	12.7 ± 0.4
	OFC	9.3 ± 0.3	14.8 ± 0.3	15.6 ± 0.6	14.0 ± 0.5	16.4 ± 0.5	17.1 ± 0.4
	CPu	7.7 ± 0.4	9.0 ± 0.2	9.4 ± 0.1	8.5 ± 0.3	9.4 ± 0.3	10.7 ± 0.2
	AcbC	10.3 ± 0.2	10.8 ± 0.1	10.9 ± 0.2	11.2 ± 0.3	11.6 ± 0.2	12.2 ± 0.3
	AcbS	11.8 ± 0.5	17.1 ± 0.2	16.6 ± 0.4	15.0 ± 0.5	16.9 ± 0.2	17.3 ± 0.2
	VTA	11.1 ± 0.9	11.8 ± 0.4	11.2 ± 0.5	11.1 ± 0.4	12.7 ± 0.2	13.1 ± 0.4
	CA1	24.1 ± 1.3	27.5 ± 0.4	26.4 ± 0.3	25.6 ± 0.3	31.7 ± 0.3	33.5 ± 0.5
	CA3	33.2 ± 2.1	36.2 ± 0.3	33.4 ± 0.2	32.2 ± 0.3	36.4 ± 0.2	36.8 ± 0.3
	DG	48.9 ± 3.7	52.9 ± 0.4	48.9 ± 0.8	53.9 ± 1.0	55.7 ± 0.4	55.9 ± 0.3

Suppl. Table 14. Regions with statistically significant effects on *Cacna1c* and *Cacna1d* mRNA expression 1d after a single nicotine injection (0.175 mg/kg, IP), or 1d and 7d after chronic nicotine treatment (14 daily injections of 0.175 mg/kg, IP). Control mice received 14 daily vehicle (0.9% saline IP) injections followed by a 1d or 7d abstinence period. Statistical analysis was performed by region-wise one-way ANOVA in brain regions associated with the reward circuitry. Bonferroni's corrected p-values: *p<0.05; **p<0.01; ***p<0.001; n.s. = not significant; n=4-8/group.

		1d after a single injection		1d after chronic treatment		7d after chronic treatment	
mRNA	**Region**	**F**	**P**	**F**	**P**	**F**	**P**
Cacna1c	**Cing**	[1,13]7.0	0.020236	[1,14]13.0	0.002834*	[1,13]1.4	n.s.
	PreL	[1,11]1.8	n.s.	[1,14]18.3	0.000766**	[1,13]43.1	0.000018***
	IL	[1,11]34.3	0.000110**	[1,14]0.1	n.s.	[1,13]20.5	0.000563**
	OFC	[1,10]0.3	n.s.	[1,14]6.2	0.026205	[1,12]38.4	0.000046***
	CPu	[1,12]5.2	0.041982	[1,14]17.5	0.000927*	[1,13]50.2	0.000008***
	AcbC	[1,12]3.8	n.s.	[1,11]0.0	n.s.	[1,13]10.2	0.006983
	AcbS	[1,9]10.9	0.009238	[1,12]0.2	n.s.	[1,10]39.0	0.000096**
	VTA	[1,10]5.1	0.047360	[1,11]21.6	0.000704**	[1,13]5.5	0.035111
	CA1	[1,10]15.7	0.002667*	[1,9]0.2	n.s.	[1,12]32.6	0.000098**
	CA3	[1,10]13.1	0.004719	[1,12]87.4	0.000001***	[1,11]63.8	0.000007***
	DG	[1,6]5.1	n.s.	[1,10]6.7	0.026866	[1,10]58.9	0.000017***
Cacna1d	**Cing**	[1,11]52.4	0.000017***	[1,11]0.2	n.s.	[1,14]0.1	n.s.
	PreL	[1,13]4.8	0.047064	[1,13]0.4	n.s.	[1,14]5.2	0.039285
	IL	[1,13]6.2	0.026685	[1,12]0.4	n.s.	[1,12]1.9	n.s.
	OFC	[1,12]37.1	0.000054***	[1,13]0.5	n.s.	[1,14]0.2	n.s.
	CPu	[1,14]12.1	0.003644*	[1,13]5.4	0.037387	[1,13]12.4	0.003725*
	AcbC	[1,12]6.2	0.028847	[1,12]0.7	n.s.	[1,13]2.9	n.s.
	AcbS	[1,10]154.8	0.000000***	[1,11]9.0	0.012195	[1,14]0.7	n.s.
	VTA	[1,9]1.3	n.s.	[1,10]0.0	n.s.	[1,12]3.2	n.s.
	CA1	[1,9]10.9	0.009204	[1,11]3.5	n.s.	[1,14]5.1	0.041224
	CA3	[1,12]1.5	n.s.	[1,11]12.4	0.004820	[1,14]0.6	n.s.
	DG	[1,13]1.9	n.s.	[1,12]17.9	0.001168*	[1,14]0.0	n.s.

Suppl. Table 15. *CACNA1C* and *CACNA1D* mRNA levels in postmortem samples of alcoholic patients and control subjects were determined by qRT-PCR. dCt values ($Ct_{CACNA1C/D} - Ct_{GAPDH}$) and Ct_{GAPDH} values are shown as means ± SEM. Ct_{GAPDH} values do not differ significantly between groups.

mRNA	Region	Genotype	Control (dCt; mean ± SEM) (n)	Alcoholic (dCt; mean ± SEM) (n)	Intoxicated alcoholic (dCt; mean ± SEM) (n)
CACNA1C	VS	GG	6.71 ± 0.1 (27)	6.62 ± 0.2 (15)	6.75 ± 0.3 (5)
		AG	6.77 ± 0.1 (16)	7.02 ± 0.3 (9)	7.01 ± 0.1 (9)
		AA	6.20 ± 0.2 (6)	5.18 ± 1.5 (4)	-
	NC	GG	6.75 ± 0.2 (24)	6.71 ± 0.3 (16)	7.44 ± 0.2 (5)
		AG	7.20 ± 0.2 (15)	7.80 ± 0.4 (11)	7.51 ± 0.2 (10)
		AA	6.87 ± 0.3 (6)	7.62 ± 0.3 (5)	-
CACNA1D	VS	GG	5.25 ± 0.1 (27)	5.24 ± 0.2 (15)	5.71 ± 0.2 (5)
		AG	5.52 ± 0.1 (16)	5.63 ± 0.2 (9)	5.86 ± 0.1 (9)
		AA	5.06 ± 0.1 (6)	3.81 ± 1.6 (4)	-
	NC	GG	5.66 ± 0.2 (24)	5.50 ± 0.3 (16)	5.98 ± 0.2 (5)
		AG	6.01 ± 0.1 (15)	6.12 ± 0.3 (11)	6.11 ± 0.1 (10)
		AA	5.72 ± 0.2 (6)	6.05 ± 0.1 (5)	-
mRNA	**Region**	**Genotype**	**Control (Ct; mean ± SEM) (n)**	**Alcoholic (Ct; mean ± SEM) (n)**	**Intoxicated alcoholic (Ct; mean ± SEM) (n)**
GAPDH	VS	GG	19.37 ± 0.5 (27)	19.86 ± 0.7 (15)	19.01 ± 0.4 (5)
		AG	18.72 ± 0.2 (16)	19.72 ± 0.7 (9)	18.52 ± 0.1 (9)
		AA	20.90 ± 1.3 (6)	20.22 ± 1.7 (4)	-
	NC	GG	19.70 ± 0.5 (24)	20.35 ± 1.0 (16)	18.95 ± 0.3 (5)
		AG	18.50 ± 0.2 (15)	20.09 ± 0.9 (11)	18.61 ± 0.2 (10)
		AA	19.60 ± 0.8 (6)	18.87 ± 0.5 (5)	-

Suppl. Table 16. Quantitative RT-PCR for *CACNA1C* and *CACNA1D* in postmortem brain samples of schizophrenia patients and controls. *GAPDH* was used as normalizer to determine dCt values, statistical analysis was performed by region-wise one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001; n.s. = not significant; n=5-7/group.

mRNA	Region	Controls (dCt; mean ± SEM)	Schizophrenia patients (dCt; mean ± SEM)	ddCt	F	p
CACNA1C	BA10	6.000 ± 0.09	6.485 ± 0.12	-0.485	[1,10]8.518	0.015337*
	BA21	6.684 ± 0.26	8.143 ± 0.18	-1.459	[1,10]22.661	0.000768***
	NC	8.105 ± 0.31	8.147 ± 0.18	-0.042	[1,10]0.016	n.s.
	Vermis	6.801 ± 0.11	6.803 ± 0.21	-0.002	[1,9]0.000	n.s.
CACNA1D	BA10	5.257 ± 0.12	5.900 ± 0.14	-0.643	[1,10]10.780	0.008242**
	BA21	4.354 ± 0.16	5.155 ± 0.18	-0.800	[1,10]10.181	0.009641**
	NC	6.104 ± 0.04	5.979 ± 0.11	0.125	[1,10]0.837	n.s.
	Vermis	4.554 ± 0.16	4.527 ± 0.14	0.027	[1,9]0.014	n.s.

Suppl. Table 17. *Cacna1c* and *Cacna1d* mRNA were measured in rats treated with haloperidol or clozapine to determine whether treatment in human postmortem samples could confound the results. dCt values of *Cacna1c* and *Cacna1d* mRNA are given as mean ± SEM. *Gapdh* was used as internal normalizer, Ct values are given below. N=8-9/group.

mRNA	Region	Controls (dCt; mean ± SEM)	Haloperidol (dCt; mean ± SEM)	Clozapine (dCt; mean ± SEM)
Cacna1c	Cing	7.67 ± 0.2	7.34 ± 0.1	7.27 ± 0.1
	PreL	7.51 ± 0.1	7.46 ± 0.1	7.54 ± 0.1
	CPu	7.89 ± 0.1	8.10 ± 0.1	8.23 ± 0.1
	CA1	8.01 ± 0.1	8.33 ± 0.2	8.05 ± 0.1
	CA3	6.83 ± 0.1	6.97 ± 0.1	6.97 ± 0.1
	DG	7.68 ± 0.1	7.61 ± 0.2	7.46 ± 0.1
Cacna1d	Cing	11.84 ± 0.2	12.06 ± 0.2	11.88 ± 0.4
	PreL	11.90 ± 0.1	12.05 ± 0.1	11.57 ± 0.2
	CPu	12.23 ± 0.2	12.10 ± 0.1	12.04 ± 0.1
	CA1	11.96 ± 0.2	12.40 ± 0.1	11.59 ± 0.2
	CA3	12.43 ± 0.2	12.83 ± 0.2	12.91 ± 0.2
	DG	10.96 ± 0.1	11.17 ± 0.3	10.88 ± 0.3
	Region	**Controls (Ct; mean ± SEM)**	**Haloperidol (Ct; mean ± SEM)**	**Clozapine (Ct; mean ± SEM)**
Gapdh	Cing	19.85 ± 0.2	20.82 ± 0.9	20.16 ± 0.2
	PreL	19.81 ± 0.2	21.90 ± 2.3	20.62 ± 0.9
	CPu	19.69 ± 0.2	19.35 ± 0.2	19.65 ± 0.1
	CA1	19.73 ± 0.3	19.78 ± 0.2	20.01 ± 0.1
	CA3	19.82 ± 0.2	19.88 ± 0.3	19.67 ± 0.2
	DG	20.07 ± 0.1	19.71 ± 0.3	20.15 ± 0.2

Suppl. Table 18. *CACNA1C* and *CACNA1D* mRNA levels in human postmortem samples of suicide completers and controls were determined by qRT-PCR. dCt values ($Ct_{CACNA1C/D}$ – Ct_{GAPDH}) and Ct_{GAPDH} values are shown as means ± SEM. Ct_{GAPDH} do not differ significantly between groups and are listed below.

mRNA	Genotype	Female control (dCt; mean ± SEM)	Male control (dCt; mean ± SEM)	Female suicide (dCt; mean ± SEM)	Male suicide (dCt; mean ± SEM)
CACNA1C	GG	6.34 ± 0.2 (10)	5.97 ± 0.1 (17)	6.02 ± 0.3 (3)	5.67 ± 0.1 (4)
	AG	6.11 ± 0.2 (12)	5.77 ± 0.2 (14)	6.27 ± 0.2 (5)	5.91 ± 0.1 (6)
	AA	5.56 (1)	5.85 ± 0.3 (3)	-	5.64 ± 0.4 (2)
CACNA1D	GG	5.44 ± 0.2 (4)	4.64 ± 0.1 (17)	5.31 ± 0.4 (3)	4.28 ± 0.3 (5)
	AG	4.51 ± 0.2 (17)	4.43 ± 0.2 (10)	5.03 ± 0.3 (3)	5.12 ± 0.3 (2)
	AA	4.14 (1)	4.33 ± 0.3 (4)	-	4.55 ± 0.8 (2)
	Genotype	**Female control (Ct; mean ± SEM)**	**Male control (Ct; mean ± SEM)**	**Female suicide (Ct; mean ± SEM)**	**Male suicide (Ct; mean ± SEM)**
GAPDH	GG	18.90 ± 0.4 (10)	19.27 ± 0.2 (20)	20.04 ± 0.4 (3)	19.60 ± 0.6 (6)
	AG	19.64 ± 0.2 (20)	19.31 ± 0.3 (15)	19.34 ± 0.4 (7)	18.43 ± 0.3 (6)
	AA	20.01 (1)	19.81 ± 0.8 (6)	-	18.88 ± 0.5 (2)

Corrigendum

Figure 8 on page 31 is not entirely correct and should be replaced by the following figure:

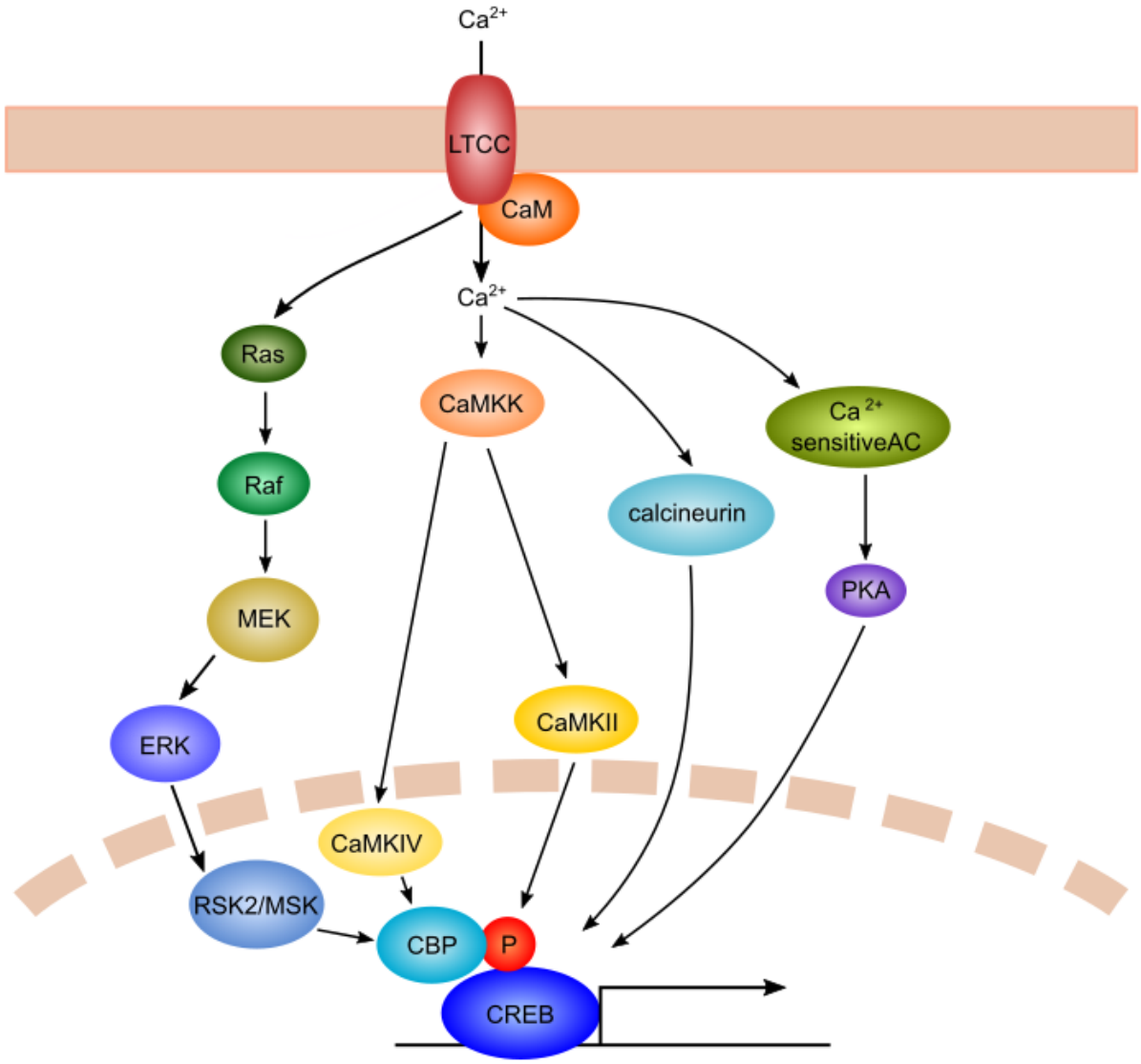

Figure 37. LTCC signaling regulates gene expression. The RAS-ERK-RSK/MSK pathway is activated upon LTCC stimulation. Calcium influx through the LTCCs additionally triggers signaling cascades. CaMKK activates CaMKII and CaMKIV, which then propagates the signal into the nucleus to change transcription of various genes. The calcium dependent phosphatase calcineurin and the calcium-sensitive adenylate cyclase (AC), which acts via protein kinase A (PKA) are also activated by calcium influx to modulate gene expression. CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CBP, CREB-binding protein; CREB, cAMP regulatory element-binding protein; MEK, mitogen-activated protein kinase kinase; MSK, mitogen and stress activated kinase; RSK, ribosomal S6 kinase. Adapted from (Ebert *et al*, 2013).