**Differential Roles for L-Type Calcium Channel Subtypes in Alcohol Dependence**

# Differential roles for L-type calcium channel subtypes in alcohol dependence

Running title: **Role of Cav1.2 in alcoholism**

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**ABSTRACT**

It has previously been shown that the inhibition of L-type calcium channels (LTCCs) decreases alcohol consumption, although the contribution of the central LTCC subtypes Cav1.2 and Cav1.3 remains unknown. Here, we determined changes in Cav1.2 (*Cacna1c*) and Cav1.3 (*Cacna1d*) mRNA and protein expression in alcohol dependent rats during protracted abstinence and naïve controls using *in situ* hybridization and Western Blot analysis. Functional validation was obtained by electrophysiological recordings of calcium currents in dissociated hippocampal pyramidal neurons. We then measured alcohol self-administration and cue-induced reinstatement of alcohol-seeking in dependent and non-dependent rats after intracerebroventricular (i.c.v.) injection of the LTCC antagonist verapamil, as well as in mice with a conditional knockout (KO) of Cav1.2 in $Ca^{2+}$/Calmodulin-dependent protein kinase II α (CaMKIIα)-expressing neurons.

Our results show that *Cacna1c* mRNA levels were increased in the amygdala and hippocampus of alcohol dependent rats after 21 days of abstinence, with no changes in *Cacna1d* mRNA. This was associated with increased Cav1.2 protein levels and L-type calcium current amplitudes. Further analysis of *Cacna1c* mRNA in the CA1, basolateral amygdala (BLA) and central amygdala (CeA) revealed a dynamic regulation over time during the development of alcohol dependence. The inhibition of central LTCCs via i.c.v. administration of verapamil prevented cue-induced reinstatement of alcohol-seeking in alcohol dependent rats. Further studies in conditional Cav1.2-KO mice showed a lack of dependence-induced increase of alcohol-seeking behavior. Together, our data indicate that central Cav1.2 channels, rather than Cav1.3, mediate alcohol-seeking behavior. This finding may be of interest for the development of new anti-relapse medications.

## INTRODUCTION

Although alcohol consumption is widely socially accepted and legal, excessive and compulsive alcohol use has serious consequences for the health, social environment, and ability to work of the individual. The World Health Organization estimates that in 2012, approximately 5.9% of all global deaths were directly or indirectly attributable to alcohol consumption (World Health Organization, 2014). A large proportion of alcohol-related harm is due to alcohol use disorders (AUD), including addiction. One major concern is relapse, which can occur even after years of abstinence, as the repeated cycles of alcohol use and abstinence lead to persistent alterations in neurotransmitter systems and brain activity. After withdrawal symptoms have ceased, relapse can be triggered by internal and external factors such as stress or environmental cues (Spanagel, 2009;Heilig et al., 2010). Studying the alterations in the brain underlying the increased propensity to relapse after dependence has developed is therefore of great importance for the development of new and improved therapeutic approaches.

Recently, variation at the *CACNA1C* gene locus was identified as an important contributor to the risk for psychiatric disorders (Strohmaier et al., 2013;Erk et al., 2014), which in turn has sparked renewed interest in the function of L-type calcium channels (LTCCs) in the brain (Berger and Bartsch, 2014). Two subtypes, Cav1.2 and Cav1.3 (with the respective gene loci *CACNA1C* and *CACNA1D*), are expressed in the central nervous system (Hell et al., 1993), and both have been implicated in regulating the effects of, and dependence to, alcohol (Pucilowski et al., 1989;Littleton et al., 1990;Rezvani and Janowsky, 1990;Fadda et al., 1992;Colombo et al., 1995;De Beun et al., 1996a;1996b;Gardell et al., 1997;1999).
Until recently, little effort has been made to distinguish between LTCC subtypes in the investigation of addictive behaviors. This is largely due to the lack of selective pharmacological tools. Many studies on alcohol drinking behavior have used systemic

administration of LTCC ligands, affecting LTCCs not only in the brain but also in various other organs, including the heart (Rezvani and Janowsky, 1990;Fadda et al., 1992;De Beun et al., 1996a;de Beun et al., 1996b). Substantial side effects were demonstrated in these studies, 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). Furthermore, previous studies on the role of LTCCs have primarily used free choice paradigms in which alcohol was presented as an alternative drinking solution to laboratory rodents, which typically does not result in clinically relevant brain alcohol exposure levels or neuroadaptive processes important for the development of addiction.

In this study we examined whether the central LTCC subtypes Cav1.2 and Cav1.3 play different roles in alcohol dependence. We used an established animal model in which dependence is induced by chronic intermittent intoxication with alcohol vapor, leading to persistent neuronal and behavioral adaptations (Rimondini et al., 2002;Meinhardt and Sommer, 2015). Although this post-dependent state is based on experimenter controlled intoxication, it has been invaluable in identifying the mechanisms underlying high relapse propensity in addicted subjects, such as chronic hyperactivity of central stress systems, a hyperdopaminergic state during protracted abstinence, and long-lasting alterations of forebrain neurogenesis (Sommer et al., 2008;Hansson et al., 2010;Hirth et al., 2016). Here, we used this model to investigate mRNA and protein expression of LTCC subtypes in several brain regions over the course of intoxication, acute withdrawal, and into protracted abstinence. Functional validation of these findings was obtained using patch-clamp recordings

of LTCC currents in CA1 hippocampal neurons. We then tested the effect of central verapamil injection on alcohol self-administration and seeking behavior in alcohol dependent and non-dependent rats. Finally, using transgenic mice with a time- and cell-type specific Cav1.2 knockout (KO), we established the role of Cav1.2 in cue-induced reinstatement of alcohol-seeking.

## METHODS AND MATERIALS

Male Wistar rats weighing 210 – 300 g at the beginning of the experiment were used. Alcohol dependence was induced by exposure to chronic intermittent cycles of alcohol inhalation, as previously described (Rimondini et al., 2002;Sommer et al., 2008;Bjork et al., 2010;Hansson et al., 2010;Meinhardt et al., 2013;Hirth et al., 2016). After seven weeks of alcohol exposure, rats were sacrificed for *Cacna1c* and *Cacna1d* mRNA analysis by *in situ* hybridization at different time points of abstinence (0, 1, 3, 7 and 21 days after the last vapor exposure) according to Hansson et al (2010) and Hermann et al. (2012). Further analysis on protein and functional level were performed in 3-week abstinent rats by Western Blotting and electrophysiological patch-clamp recordings of LTCC currents in dissociated hippocampal pyramidal neurons, respectively. Additionally, behavioral analyses (alcohol self-administration and cue-induced reinstatement to alcohol seeking behavior after i.c.v. injection of verapamil or vehicle (artificial cerebral spinal fluid, aCSF) were performed in 3-week abstinent rats. Alcohol self-administration and cue-induced reinstatement to alcohol seeking was also analyzed in mice with a conditional knockout of Cav1.2 in $Ca^{2+}$/Calmodulin-dependent protein kinase II α (CaMKIIα)-expressing cells ($Cav1.2^{KO}$, with $Cav1.2^{fl/fl}$ mice as controls) to establish the role of this LTCC subtype in dependence. All drugs, experimental procedures, stereotactic surgeries and statistics are described in detail in Supplemental Information (SI). The 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 (Regierungspräsidium Karlsruhe, Germany, license numbers 35-9185.81/G-163/13 and 35-9185.81/G-301/14).

## RESULTS

### *Cacna1c* mRNA and protein is increased in alcohol dependent rats after 3 weeks of abstinence

Alcohol dependence was induced in rats by exposure to intermittent cycles of alcohol vapor intoxication. Average blood alcohol concentrations per daily cycle were 266.2 ± 12.6 mg/dl. Transcriptional levels of *Cacna1c* and *Cacna1d* were measured after 3 weeks of abstinence to investigate long-term changes in mRNA expression in different reward-related forebrain regions (Noori et al., 2012): 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)], hypothalamic paraventricular nucleus (PVN), and dorsal hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1 and CA3] (see Fig.1 A, B and Table 1).

*Cacna1c* mRNA was significantly increased in the hippocampus (CA1: 76%, CA3: 24%, and CA4: 33% increase) and amygdala (CeA: 40%, and basolateral amygdala, BLA: 70% increase, Fig.1 A). *Cacna1c* expression in other analyzed regions remained unchanged. *Cacna1d* mRNA was unchanged in all investigated regions. For absolute values and statistical values, see Table 1.

The increase in *Cacna1c* mRNA in the hippocampus was confirmed on the protein level by Western Blot analysis (Fig.1 C). Alcohol dependent rats showed a 26% increase of Cav1.2 protein compared to non-dependent controls ($p<0.05$).

**Dynamic changes of *Cacna1c* mRNA during acute alcohol intoxication, withdrawal and extended abstinence**

*Cacna1c* mRNA levels were investigated over time, from acute intoxication to withdrawal and following extended abstinence, in the CA1, CeA, and BLA (Fig. 2, Table 2). Rats were sacrificed immediately after vapor exposure, and after 1, 3, 7 or 21 d of abstinence (Hansson et al., 2010;Hermann et al., 2012). We found highly significant effects of Treatment (CA1: $F[1,75]=49.48$, $p<0.001$; CeA: $F[1,73]=137.4$, $p<0.001$; BLA: $F[1,77]=46.94$, $p<0.001$), Time (CA1: $F[5,75]=13.46$, $p<0.001$; CeA: $F[5,73]=43.3$, $p<0.001$; BLA: $F[5,77]=19.29$, $p<0.001$), and 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: $F[5,77]=19.29$, $p<0.001$).

In the CA1, *Cacna1c* mRNA was increased compared to controls when the animals were still intoxicated (0 d; $p<0.05$). After 1 d, there was a decrease that did not reach significance. Transcriptional levels returned to control levels on day 3 (p=not significant, n.s.) before increasing slightly, but not significantly, on day 7. We also found a strong increase in *Cacna1c* mRNA in alcohol dependent rats after 21 d of abstinence ($p<0.001$). In the amygdala, we observed a similar dynamic. On day 0, *Cacna1c* mRNA was strongly increased (CeA: $p<0.001$; BLA: $p<0.001$), on day 1 decreased (CeA: $p<0.01$; BLA: $p<0.01$), and on day 3 it normalized again (CeA: p=n.s.; BLA: p=n.s.). In the CeA, there was a trend toward an increase in *Cacna1c* mRNA on day 7 ($p=0.06$), and a significant increase on day 21 ($p<0.001$). There was no difference between alcohol dependent and control rats on day 7 in the BLA (p=n.s.), but a significant increase in alcohol dependent rats on day 21 ($p<0.001$). For absolute values, see Table 2.

Furthermore, the expression of a panel of non-LTCC channels (i.e. *Cacna1A* as P/Q-type channel, *Cacna1B* as N-type channel, *Cacna1G* as T-type channel) was analyzed by qRT-

PCR and showed a significant decrease in brain tissue of the hippocampus and amygdala region of 3 weeks abstinent rats (see a summary of the results in Suppl. Table S2).

**Increased Cav1.2 current in alcohol dependent rats**

For functional validation of the increased Cav1.2 expression in alcohol dependent rats, we conducted voltage-clamp recordings of L-type currents in acutely dissociated CA1 neurons (Fig. 3), in which the majority (~80%) of LTCC currents are attributable to Cav1.2 (Hell et al., 1993). Although this allows for 20% of Cav1.3 contribution, the notably larger Cav1.2 expression in combination with the different activation thresholds of the two subtypes enabled the approximate distinction between Cav1.2 and Cav1.3 current. The dependence increased Cav1.2 expression in the hippocampus described earlier and the generally high expression in hippocampal subregions (Liebmann et al., 2008;Bernardi et al., 2014) also supported the choice of the CA1 region for electrophysiological measurements. Total whole-cell-$Ca^{2+}$ currents were recorded on step depolarizations of 200 ms from -60 to +50 mV with −70 mV holding potential (Vh). The currents started activating at the expected voltages (> −40 mV in 2 mM $Ca^{2+}$), had progressive fast activation with increasing voltages between -40 and 0 mV, and displayed the typical slowly inactivating time course due to the presence of 5 mM EGTA inside the pipette (Fig. 3A). Space- and dynamic-clamp control of the recordings was of high quality as shown in the Suppl. Fig. 1 and discussed in the SI and Methods. The high-voltage activated calcium currents had a clear trend to be larger in alcohol dependent animals, although the increase did not reach significance. Normalized $Ca^{2+}$ channel conductances ($G_{norm}(V)$) were identical between CA1 neurons from control and alcohol dependent animals, indicating no changes in the voltage-dependence of calcium channel activation. The potential of half-maximal activation ($V_{1/2}$) (-15.2 ± 0.9 mV vs. -15.23 ± 1 mV for control and alcohol dependent rats, respectively) and the maximal slope (k) of $G_{norm}(V)$ (6 ± 0.4 mV vs. 5.3 ± 0.4 mV for control and alcohol dependent rats, respectively) remained unchanged (Fig.3 C), in

good agreement with values typical for high-voltage activated calcium channels (Baldelli et al., 2000;Marcantoni et al., 2010;Mahapatra et al., 2011). Since we were interested in the contribution of the L-type component to the total $Ca^{2+}$- current, we consequently applied nifedipine (3 μM), which is nearly saturating for Cav1.2 and Cav1.3 channels when applied on neurons held at Vh = −70 mV (Mahapatra et al., 2011). At 0 mV, during which L-type currents reach their peak in 2 mM extracellular calcium, the nifedipine block was significantly larger in CA1 neurons from alcohol dependent animals as compared to controls ($p<0.05$) (Fig.3 D). The L-type component contributed to 22.4 ± 3.1 % and 36.6 ± 3.6 % in control and alcohol dependent animals, respectively ($p<0.05$) (Fig.3 D, right) . The high degree of expression of Cav1.2 channels in CA1 neurons and the relatively high $V_{1/2}$ derived from the Boltzmann fits of $G_{norm}(V)$ indicate that the contribution of Cav1.3 channels to the DHP-sensitive L-type currents is likely to be a small percentage (<20%) of the alcohol effects on Cav1 channels. We thus conclude that alcohol exposure leads to an increase of functional L-type calcium channels with typical high-voltage activated channel features, as reported for Cav1.2. However, we should note that since the total calcium current amplitude was not significantly altered (Fig. 3B), the possibility that a history of dependence may also act on non-L type calcium currents by reducing them cannot be excluded.

**Central blockade of LTCCs prevents alcohol seeking but not taking in dependent rats**

Verapamil, a non-selective LTCC antagonist, which is suitable for i.c.v. injections because of its water solubility (Jacob and de la Torre, 2009), was injected into the lateral ventricle of alcohol dependent or non-dependent rats immediately prior to a self-administration session (120 μg/5 μl aCSF). Two-way ANOVA showed a significant effect of the alcohol vapor exposure ($F[1,23]=5.35$, $p<0.05$), but no treatment or alcohol*treatment interaction effect. There was no difference between the individual groups according to the Newman Keuls post-hoc test (Fig. 4). However, in the cue-induced reinstatement test verapamil attenuated the

seeking response in alcohol dependent rats, while all other groups displayed a significant increase in active lever responses compared to extinction sessions (Non-dependent + aCSF: $p<0.001$; Non-dependent + verapamil: $p<0.001$; Alcohol dependent + aCSF: $p<0.001$). There was also a significant difference between verapamil-treated alcohol dependent and non-dependent rats ($p<0.001$) and between verapamil-treated and aCSF-treated alcohol dependent rats ($p<0.001$). Additionally, aCSF-treated alcohol dependent rats showed stronger reinstatement compared to aCSF-treated non-dependent rats ($p<0.01$) (Fig. 4).

**Lack of Cav1.2 in CaMKII$\alpha$-positive neurons blocks cue-induced reinstatement in alcohol dependent mice**

Because verapamil can act on Cav1.3, we validated the role of Cav1.2 for alcohol seeking behavior using a transgenic model in which a knockout of the gene is induced in forebrain neurons of adult mice. Three weeks after KO induction by tamoxifen, $Cav1.2^{KO}$ mice and control littermates ($Cav1.2^{fl/fl}$) were trained to self-administer alcohol followed by induction of dependence as described together with the verification of the knockout in Suppl. Information and Suppl. Fig. S2 and S3.

Two-way ANOVA for Genotype*Treatment did not reveal significant overall effects on alcohol self-administration (Genotype: $F[1,27]=0.38$, p=n.s.; Treatment: $F[1,27]=0.15$, p=n.s.). A trend for a Genotype*Treatment interaction was seen ($F[1,27]=4.07$, $p=0.05$), that was due to a significant dependence induced increase in active lever presses of control mice ($p<0.05$, dependent vs non-dependent $Cav1.2^{fl/fl}$ mice), which was not observed in $Cav1.2^{KO}$ mice (see Fig. 5A).

Cue-induced reinstatement of alcohol-seeking was analyzed by repeated measures two-way ANOVA (Genotype*Treatment*Session, with Session consisting of extinction and cue-induced reinstatement). Significant main effects were found for Genotype*Treatment and Session (Genotype*Treatment: $F[1,27]=6.48$, $p<0.05$; Session: $F[1,27]=27.76$, $p<0.001$).

Other main effects were not significant (Genotype: F[1,27]=0.36; Treatment: F[1,27]=0.75; Genotype*Session: F[1,27]=0.22; Treatment*Session: F[1,27]=0.32; Genotype*Treatment*Session: F[1,27]=3.43; all p=n.s.). Importantly, $Cav1.2^{KO}$ mice displayed a significant reduction of cue-induced reinstatement only under alcohol dependent conditions ($p<0.05$, nondependent vs. dependent $Cav1.2^{KO}$ mice; $p<0.001$ extinction vs. reinstatement in nondependent $Cav1.2^{KO}$ mice, Fig. 5B), which is in line with the verapamil experiment in rats. In contrast, $Cav1.2^{fl/fl}$ mice showed an opposite pattern, i.e. significant cue-induced reinstatement only in the alcohol dependent group ($p<0.05$, extinction vs. reinstatement in nondependent $Cav1.2^{fl/fl}$ mice), although this result was less robust.

No changes in locomotor activity and anxiety related behavior were detected as assessed in an Open Field test between naïve $Cav1.2^{KO}$ mice and their littermate controls (Suppl. Fig. S4).

## DISCUSSION

Given the wide-spread clinical use of calcium antagonists that can influence the activity of LTCCs, the most salient message from the present study is that Cav1.2 is strongly upregulated in the alcoholic brain during abstinence and plays an important role in relapse behavior. Furthermore, 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, with no changes in *Cacna1d* mRNA. This was accompanied by elevated Cav1.2 protein levels in the hippocampal CA1 subregion of alcohol dependent rats. These findings were functionally validated by an increase in Cav1.2 currents in hippocampal CA1 neurons. Importantly, pharmacological blockade of central LTCCs prevented cue-induced alcohol-seeking in dependent, but not control rats. Likewise, genetic knockout of Cav1.2 in forebrain neurons prevented dependence-induced increase in alcohol self-administration and seeking. Together, our findings suggest an involvement of Cav1.2 in alcohol-seeking and relapse behavior, with substantial neuroadaptations taking place during abstinence.

To our knowledge, no studies have yet been published investigating the involvement of central LTCC subtypes on alcohol-related behavior. There have been efforts, however, to determine the individual roles of Cav1.2 and Cav1.3 in the abuse of other drugs such as nicotine (Bernardi et al., 2014), morphine (Shibasaki et al., 2011), and cocaine and amphetamine (Giordano et al., 2010;Schierberl et al., 2011;Schierberl et al., 2012). The findings in these studies are in line with our results on alcohol-seeking, showing different contributions of the two subtypes during distinct stages following exposure to drugs of abuse. It appears that Cav1.2 is especially involved in the effects of long-term exposure to drugs of abuse and prolonged abstinence, as our current data, our study on nicotine sensitization (Bernardi et al., 2014), and the findings of Giordano et al. (2006) on long-term amphetamine and cocaine exposure (Giordano et al., 2010), suggest.

To further establish the involvement of Cav1.2 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 (LeDoux, 2003;Kelley, 2004;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 relapse to drug intake. All three regions showed a consistent pattern of *Cacna1c* mRNA increase during acute intoxication, followed by a decrease during early withdrawal (1 day of abstinence), and then again an increase during the following extended abstinence. These dynamic changes underline the possibility of differential involvement of this subunit over time. Cav1.2 may not be relevant during early withdrawal, as *Cacna1c* expression decreased immediately after the last alcohol vapor exposure, and rapidly normalized during the following days of abstinence. The strong increase after 21 days of abstinence indicates a role for Cav1.2 in long-term abstinence and possibly relapses. Similar fluctuations over time have recently been found in dopaminergic and glutamatergic systems during abstinence (Hermann et al., 2012;Hirth et al., 2016).

Using a Cav1.2-specific antibody, we were able to confirm the increased *Cacna1c* mRNA on the protein level. Because of the lack of subtype-specific pharmacological tools, we turned to electrophysiology for functional validation of our expression findings. Patch-clamp recordings from isolated neurons provide sufficient sensitivity and specificity to distinguish Cav1.2 from Cav1.3- mediated currents. Cav1.2 channels open at membrane potentials of about -30 mV and reach their half maximal activation point at -5 mV, whereas Cav1.3 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 Cav1.2/Cav1.3 currents in the CA1 region of the hippocampus in alcohol dependent and control rats, as 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 Cav1.2 channels (Hell et al., 1993;Clark et al., 2003), and possess four times as much *Cacna1c* mRNA compared to *Cacna1d* (Nunez-Santana et al., 2014). This suggests that, despite nifedipine being a nonselective Cav1 channel blocker, most of the measured L-type currents in neurons of control and alcohol dependent rats are mainly carried by Cav1.2. This is supported by the $G_{norm}(V)$ curves (Fig.3 C), in which the voltage-dependence matches more the Cav1.2 than Cav1.3 activation range. Our electrophysiological data are in very good agreement with the findings on the mRNA and protein levels, as nifedipine has a stronger blocking effect on calcium currents in neurons of alcohol dependent than control rats. It is of course possible that non-LTCCs are also involved in the measured response, considering the limited selectivity of available LTCC blockers. Therefore, the possibility of a compensatory decrease of either Cav1.3 or non-LTCCs to achieve an unchanged overall current cannot be excluded. A previous study from N'Gouemo and Morad (2003) has shown that both L- and P-type calcium channels are increased in the inferior colliculus of rats after alcohol withdrawal from 4 d of intragastric administration (N'Gouemo and Morad, 2003), indicating effects of alcohol on non-LTCCs. In this paradigm, Cav1.3 appears to be the most relevant channel (N'Gouemo et al., 2015), however the paradigm is very different from our model of alcohol dependence as well as the analyzed brain regions. Furthermore, there are reports depicting a role of non-LTCCs, i.e. N-type (Newton et al., 2008;Newton and Messing, 2009) and T-type calcium channels (Carden et al., 2006;Nordskog et al., 2006), in alcohol-related behavior. Therefore, we analyzed the expression of P/Q-, N-type and T-type non-LTCCs in three weeks abstinent rats by qRT-PCR. Unlike *Cacna1c*, mRNA levels of non-LTCCs were significantly decreased in the amygdala and hippocampus (see Suppl. Table S2), thus suggesting opposite regulation of Cav1.2 and non-LTCCs. The results may further explain the unchanged total calcium current observed in our dependent rats.

Pharmacological validation was provided in alcohol dependent and control rats trained to self-administer alcohol in an operant conditioning experiment. The non-specific LTCC antagonist verapamil, which has previously been used to block LTCCs in alcohol-related (Rezvani et al., 1990;De Beun et al., 1996a) and other research (Zhu and Herbert, 1997;Blackburn-Munro et al., 2000), was injected into the lateral ventricle, thus inhibiting only central LTCCs. Centrally injection of verapamil had no effect on alcohol self-administration in either group, but prevented cue-induced reinstatement of alcohol-seeking only in alcohol dependent rats. Previous studies have demonstrated a reduction of alcohol intake and preference by i.p. administration of verapamil in alcohol-preferring rat strains (Rezvani and Janowsky, 1990;Fadda et al., 1992;De Beun et al., 1996a). These differences could be explained by the peripheral effects of systemic verapamil administration. The dissociation of verapamil's action on alcohol self-administration and seeking provides additional assurance for the behavioral specificity of the treatment inasmuch as there is no general suppression of behavior.

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 noted above, based on the reviewed literature, that many compounds lack distinct 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 characteristic of the development of dependence (for review, see (Meinhardt and Sommer, 2015)). To date, only a few neurochemical systems have been identified that are 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 corticotropin-releasing hormone

(CRH) system (Hansson et al., 2006;Hansson et al., 2007;Sommer et al., 2008), but similar dependence-specific adaptations have been observed in other brain stress systems, such as dynorphin (Walker et al., 2011) and vasopressin (Edwards et al., 2012) systems. Whether or not Cav1.2 mediates the actions of these stress systems and provides a distinct pharmacological access point into peptide systems proven difficult to target directly warrants further investigation. Such a prospect would justify the development of LTCC ligands with brain specific pharmacodynamic properties, such as those acting via Cav1.2.

As verapamil might also affect Cav1.3 channels, we used a transgenic mouse model to establish a specific role of Cav1.2 in alcohol dependence related behaviors. Specifically, deletion of the *Cacna1c* gene in forebrain neurons attenuated alcohol seeking in alcohol-dependent mice. Together with our verapamil data from rats these findings provide converging evidence for increased sensitivity of Cav1.2 mediated effects in alcohol dependence. Also, alcohol self-administration is reduced in dependent Cav1.2$^{KO}$ mice compared to their Cav1.2$^{fl/fl}$ counterparts. This result is not in line with the rat data, and thus may indicate opposite effects of Cav1.2 and Cav1.3 on alcohol self-administration.

In addition to alcohol dependence, Cav1.2 has been implicated in other mental disorders. Genetic studies have consistently associated variation in the *CACNA1C* gene locus with risk for schizophrenia, bipolar disorder, major depression, and autism (Casamassima et al., 2010;Strohmaier et al., 2013;Berger and Bartsch, 2014;Erk et al., 2014). Considering that there is a strong comorbidity between alcohol and other mental disorders, it seems plausible that Cav1.2, rather than Cav1.3, should play a role in the relapse to alcohol drinking.

In conclusion, we provide consistent evidence for increased Cav1.2 function in protracted abstinence. This hyperfunction is associated with increased alcohol-seeking. We suggest

Cav1.2 to be a new target for relapse prevention. 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.

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## TABLES

**Table 1. *Cacna1c* and *Cacna1d* mRNA expression in alcohol dependent and non-dependent rats after 3 weeks of abstinence.** Data were measured in nCi/g, shown as Mean ± SEM. Statistical analysis was performed by region-wise one-way ANOVA followed by Bonferroni's correction; n.s. = not significant; corrected p values: *p<0.05; **p<0.01; ***p<0.001vs non-dependent rats, N=5-7 rats per group, alcohol dependent (Alc. dep.). For details on treatment, see Materials and Methods.

| mRNA | Region | Control (nCi/g) | Alc. dep. (nCi/g) | F | p |
|---|---|---|---|---|---|
| ***Cacna1c*** | Cing | 2.01 ± 0.3 | 1.96 ± 0.1 | 0.03 | n.s. |
| | PreL | 2.77 ± 0.3 | 3.54 ± 0.2 | 4.15 | n.s. |
| | IL | 4.41 ± 0.3 | 3.84 ± 0.2 | 2.51 | n.s. |
| | OFC | 2.71 ± 0.2 | 2.66 ± 0.1 | 0.05 | n.s. |
| | AcbC | 1.62 ± 0.2 | 1.34 ± 0.2 | 0.98 | n.s. |
| | AcbS | 6.03 ± 0.8 | 6.18 ± 0.2 | 0.03 | n.s. |
| | CPu | 0.93 ± 0.2 | 0.87 ± 0.1 | 0.07 | n.s. |
| | BNST | 0.76 ± 0.1 | 1.33 ± 0.2 | 11.13 | n.s. |
| | CeA | 3.56 ± 0.1 | 4.97 ± 0.3 | 23.91 | <0.01** |
| | MeA | 4.82 ± 0.4 | 6.15 ± 0.3 | 6.85 | n.s. |
| | BLA | 2.96 ± 0.2 | 5.04 ± 0.3 | 34.72 | <0.01** |
| | CA1 | 16.8 ± 0.1 | 29.5 ± 0.2 | 27.27 | <0.01** |
| | CA3 | 15.65 ± 0.5 | 19.44 ± 0.8 | 16.93 | <0.05* |
| | CA4 | 13.26 ± 0.1 | 17.68 ± 0.4 | 151.98 | <0.001*** |
| | DG | 16.39 ± 0.6 | 18.29 ± 0.6 | 4.73 | n.s. |
| | M1 | 1.63 ± 0.1 | 1.51 ± 0.1 | 0.64 | n.s. |

| | | | | | |
|---|---|---|---|---|---|
| | PVN | 6.56 ± 0.5 | 6.73 ± 0.4 | 0.06 | n.s. |
| ***Cacna1d*** | Cing | 7.89 ± 0.5 | 8.36 ± 0.3 | 0.70 | n.s. |
| | PreL | 10.26 ± 0.5 | 9.68 ± 0.2 | 1.23 | n.s. |
| | IL | 10.65 ± 0.2 | 10.49 ± 0.5 | 0.10 | n.s. |
| | OFC | 9.27 ± 0.3 | 8.81 ± 0.2 | 1.87 | n.s. |
| | AcbC | 5.14 ± 0.2 | 5.23 ± 0.1 | 0.13 | n.s. |
| | AcbS | 7.84 ± 0.3 | 7.85 ± 0.2 | 0.001 | n.s. |
| | CPu | 3.87 ± 0.3 | 3.77 ± 0.1 | 0.11 | n.s. |
| | BNST | 7.29 ± 0.3 | 6.76 ± 0.2 | 1.76 | n.s. |
| | CeA | 10.06 ± 0.7 | 8.17 ± 0.3 | 6.77 | n.s. |
| | MeA | 7.98 ± 0.4 | 8.15 ± 0.2 | 0.11 | n.s. |
| | BLA | 8.93 ± 0.2 | 8.72 ± 0.2 | 0.45 | n.s. |
| | CA1 | 11.71 ± 0.2 | 12.03 ± 0.7 | 0.18 | n.s. |
| | CA3 | 11.82 ± 0.3 | 13.27 ± 0.2 | 12.116 | n.s. |
| | CA4 | 12.96 ± 0.6 | 14.58 ± 0.8 | 2.72 | n.s. |
| | DG | 40.61 ± 2.7 | 39.67 ± 3.3 | 0.05 | n.s. |
| | M1 | 8.28 ± 0.4 | 8.21 ± 0.2 | 0.03 | n.s. |
| | PVN | 14.73 ± 0.6 | 15.23 ± 0.9 | 0.24 | n.s. |

Cingulate cortex (Cing), prelimbic cortex (PreL), infralimbic region (IL), and orbitofrontal cortex (OFC), primary 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)], hypothalamic paraventricular nucleus (PVN), and dorsal hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1 and CA3].

**Table 2. *Cacna1c* mRNA expression in alcohol dependent and non-dependent rats after 0, 1, 3, 7, and 21 d of abstinence.** Data were measured in nCi/g, shown as Mean ± SEM. Central amygdala (CeA), basolateral amygdala (BLA), dorsal hippocampal Cornus Ammon (CA) region CA1, alcohol dependent (Alc. dep.). For details on treatment, see Materials and Methods.

| Region | Days of abstinence | Control (nCi/g) | Alc. dep. (nCi/g) | N Control | N Alc. dep. |
|---|---|---|---|---|---|
| **CA1** | 0 | 11.28 ± 1.2 | 13.75 ± 1.0 | 8 | 6 |
| | 1 | 13.69 ± 0.7 | 10.38 ± 0.9 | 8 | 8 |
| | 3 | 10.66 ± 0.5 | 11.04 ± 1.0 | 6 | 7 |
| | 7 | 10.63 ± 0.6 | 13.05 ± 1.5 | 8 | 8 |
| | 21 | 7.54 ± 0.1 | 13.33 ± 1.0 | 7 | 8 |
| **CeA** | 0 | 15.62 ± 0.7 | 21.45 ± 0.5 | 8 | 6 |
| | 1 | 18.17 ± 0.9 | 13.70 ± 0.4 | 7 | 8 |
| | 3 | 18.70 ± 0.6 | 16.78 ± 0.7 | 6 | 8 |
| | 7 | 13.57 ± 0.6 | 16.71 ± 1.4 | 8 | 7 |
| | 21 | 12.44 ± 0.4 | 15.99 ± 0.6 | 7 | 8 |
| **BLA** | 0 | 13.33 ± 0.66 | 17.65 ± 0.3 | 8 | 6 |
| | 1 | 15.47 ± 0.6 | 12.88 ± 0.3 | 8 | 8 |
| | 3 | 15.76 ± 0.6 | 15.47 ± 0.8 | 6 | 8 |
| | 7 | 15.12 ± 0.5 | 16.73 ± 1.6 | 8 | 7 |
| | 21 | 11.86 ± 0.4 | 13.76 ± 0.6 | 7 | 8 |

**FIGURE LEGENDS**

**Fig. 1. *Cacna1c* mRNA and protein are upregulated in alcohol dependent rats. A.** Changes of *Cacna1c* and *Cacna1d* mRNA expression in alcohol dependent rats measured by *in situ* hybridization. Bar graphs show 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, corrected p values: *p<0.05, **p<0.01, ***p<0.001, alcohol dependent (Alc. dep.). Absolute values of all measured regions are listed in Table 1. ISH, *in situ* hybridization. **B.** Schematic representation of measured areas and mRNA expression pattern of *Cacna1c* and *Cacna1d* at Bregma -0.3 mm and -2.3 mm. Scale bar = 2.5 mm. 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)], hypothalamic paraventricular nucleus (PVN), and dorsal hippocampal formation [dentate gyrus (DG) and Cornus Ammon (CA) regions CA1 and CA3], scale bar = 2 mm. **C.** Western Blot analysis of micro-punches from the hippocampal CA1 region revealed increased Cav1.2 protein levels in alcohol dependent rats compared to controls. Bar graphs 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 bottom.

**Fig. 2. *Cacna1c* mRNA levels change over time during abstinence. A.** Schematic showing established anatomical connections of the dorsal hippocampal CA1 region and the basolateral (BLA) and central (CeA) amygdala. Between CA1 and BLA, there are connections in both directions. The BLA then relays the signal to the CeA (LeDoux, 2003;Kelley,

2004;Mandyam, 2013). **B. – D.** *In situ* hybridization shows similar dynamic regulation of *Cacna1c* mRNA during acute intoxication, withdrawal and prolonged abstinence in the CA1 (**B**), BLA (**C**) and CeA (**D**). Bar graphs show the percentage of regulation relative to control rats. Statistical analysis was performed by region-wise two-way ANOVA (time, treatment) followed by Bonferroni's correction, n=6-8/group, p values: *p<0.05, **p<0.01, ***p<0.001, alcohol dependent (Alc. dep.). Absolute values are listed in Table 2. For a detailed description of the *in situ* hybridization see Materials and Methods.

**Fig. 3. Alcohol exposure results in long-term upregulation of functional L-type $Ca^{2+}$ channels. A.** Representative traces of whole-cell $Ca^{2+}$ currents from -60 to 0 mV (left) and from +10 to +50 mV (right) recorded from neurons of alcohol dependent (red) and control rats (black). Color code is valid for all panels. **B.** Current-voltage relationship of calcium currents measured in 135 mM extracellular TEA and 300 nM TTX. **C.** Normalized conductance, $G = I/(V-E_{Ca})$ of whole cell calcium currents. $E_{Ca}$ is the reversal potential for calcium and set to +50 mV. Data were fit by Boltzmann equations with $V_{1/2}$ = -15.2 mV and 15.23 mV, and k = 6.5 mV and 5.3 mV for neurons of control and alcohol dependent rats (continuous curve). **D.** Representative traces of calcium current block by the LTCC antagonist nifedipine (3 µM) and summarizing bar graph; *p<0.05, t-test, alcohol dependent (Alc. dep.).

**Fig. 4. LTCC antagonist verapamil i.c.v. administration blocks cue-induced reinstatement of alcohol-seeking in alcohol dependent, but not control rats, with no effect on alcohol self-administration. A.** Experimental outline: Rats were trained to self-administer alcohol, and vapor- or air-exposed. During the following abstinence, guide cannulas for i.c.v. injections were implanted. After recovery rats were re-trained, then tested for self-administration (SA) following i.c.v. injection of verapamil (Ver, 120 µg/5 µl) or vehicle (aCSF, 5 µl). This was followed by one week of extinction, after which cue-induced

reinstatement was tested following i.c.v. injection of verapamil (120 µg/5 µl) or aCSF (5 µl). For details, see Materials and Methods. **B.** 1) Graph shows lever presses during 6 weeks of self-administration training, with active lever (AL) presses steadily increasing and inactive lever presses (IL) at an expected low level. 2) As AL presses were followed by a 3s timeout period during which additional lever presses did not result in alcohol delivery, rats received less reinforcers, which is shown here. Neither AL and IL presses nor number of reinforcers differ between control and alcohol dependent groups. 3) Self-administration following i.c.v. administration of verapamil or aCSF did not result in significant differences between groups. IL presses (not shown) were below 10 for all groups (range: 3.57 – 6.66 IL presses) 4) Cue-induced reinstatement of alcohol-seeking following i.c.v. injection of verapamil was blocked in alcohol dependent rats. All other groups show significant reinstatement compared to extinction, and alcohol dependent rats receiving aCSF show significantly higher reinstatement than control rats receiving aCSF. N=5-8/group. IL (not shown) were below 10 for all groups (range: 1.42 – 2.27 IL presses in extinction, 3.5 – 6.8 IL presses in cue-induced reinstatement. Statistical analysis was performed by repeated measures ANOVA, followed by Newman-Keuls post-hoc test, where appropriate, p values: **p<0.01, ***p<0.001 alc.-dep. vs. control; ###p<0.001 verapamil vs. CSF; Alc. – alcohol, Alc. dep. - alcohol dependent.

**Figure 5. Cav1.2 knockout reduces alcohol self-administration and cue-induced reinstatement of alcohol-seeking in alcohol dependence.** Alcohol dependent and non-dependent CaMKIIα-Cav1.2 knockout ($Cav1.2^{KO}$) and littermate $Cav1.2^{fl/fl}$ control mice were trained to self-administer 10% alcohol in operant chambers, followed by extinction and cue-induced reinstatement. **A.** Alcohol dependence significantly increased active lever presses in $Cav1.2^{fl/fl}$ mice as compared to non-dependent $Cav1.2^{fl/fl}$ mice. This increase was prevented in $Cav1.2^{KO}$ mice; *p<0.05. **B.** Alcohol dependent $Cav1.2^{fl/fl}$ mice and non-dependent $Cav1.2^{KO}$

mice show significant reinstatement, *p<0.05, ***p<0.001 vs. respective extinction group. Reinstatement behavior is attenuated in alcohol dependent Cav1.2$^{KO}$ mice, #p<0.05, dependent vs. non-dependent Cav1.2$^{KO}$ mice, N=6-10/group, Alc. – alcohol, Alc. dep. - alcohol dependent.

**Figure 1**

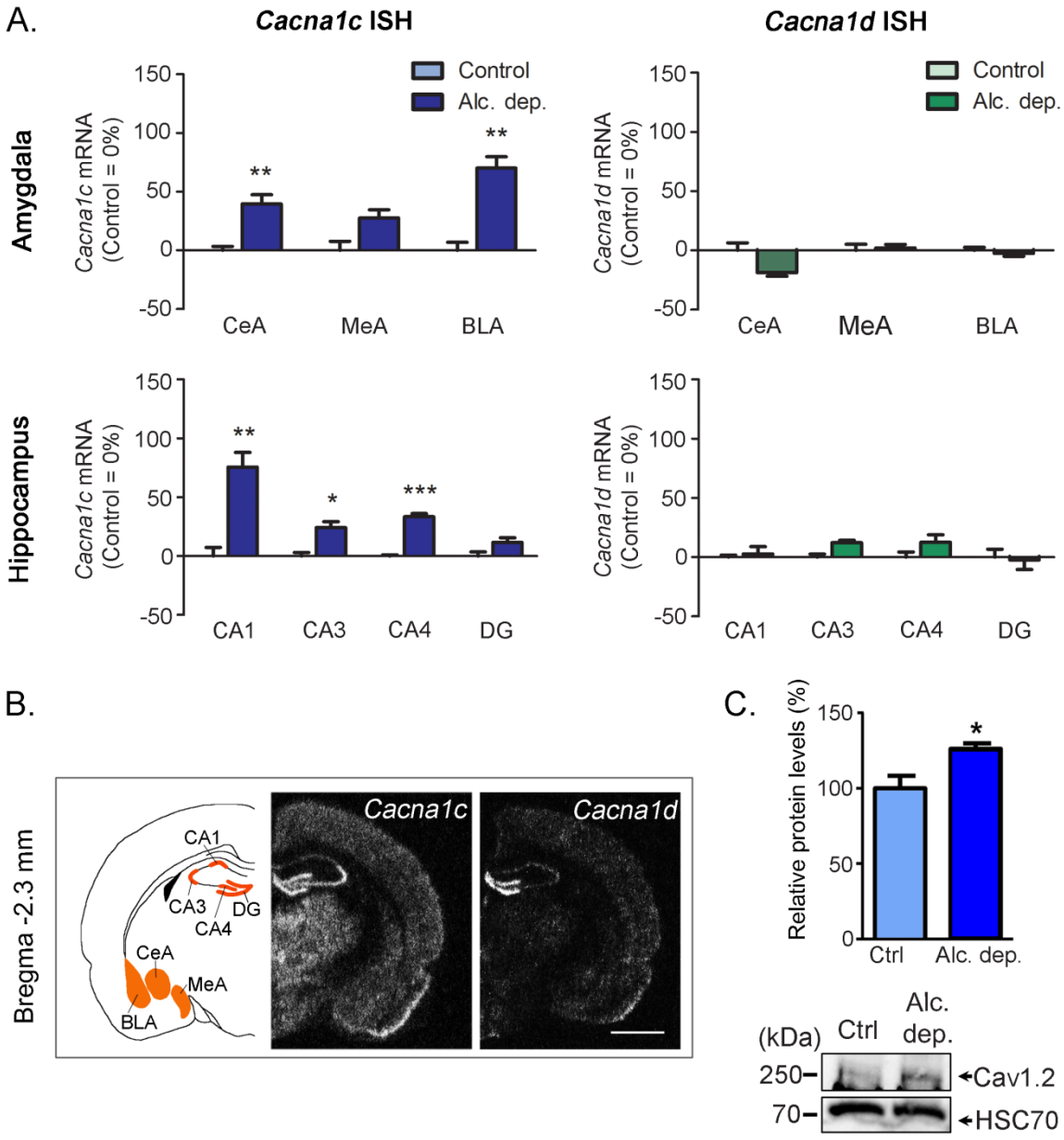

A.
Cacna1c ISH
Cacna1d ISH
Amygdala
Hippocampus
Control
Alc. dep.
Cacna1c mRNA (Control = 0%)
Cacna1d mRNA (Control = 0%)
150
100
50
0
-50
**
**
*
***
CeA
MeA
BLA
CA1
CA3
CA4
DG
B.
Bregma -2.3 mm
Cacna1c
Cacna1d
C.
Relative protein levels (%)
Ctrl
Alc. dep.
(kDa)
250
70
Cav1.2
HSC70

**Figure 2**

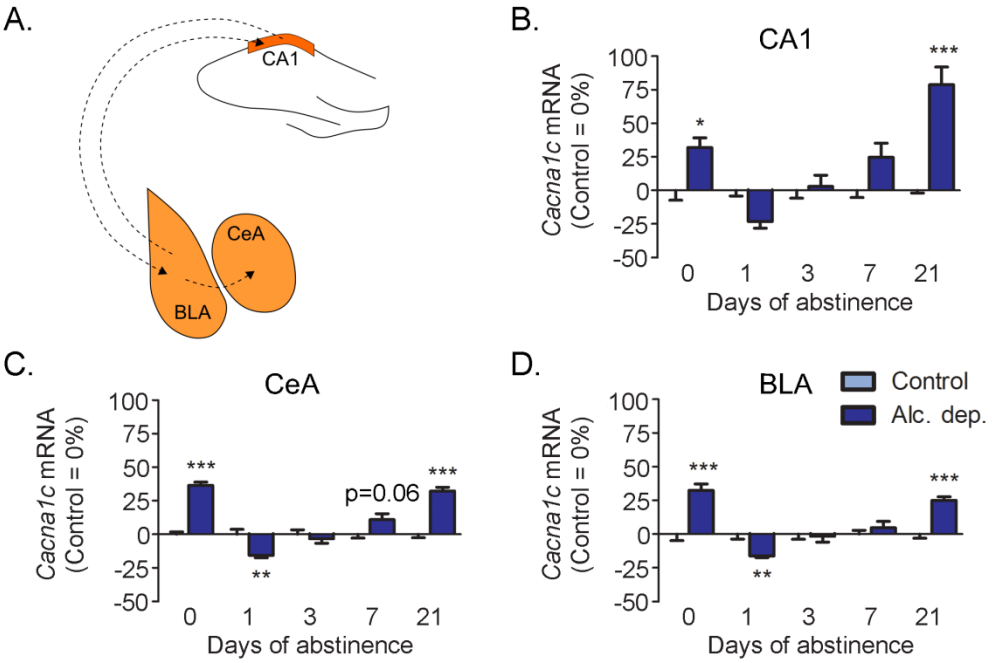

A.
CA1
CeA
BLA
B.
CA1
Cacna1c mRNA (Control = 0%)
100
75
50
25
0
-25
-50
*
***
0
1
3
7
21
Days of abstinence
C.
CeA
Cacna1c mRNA (Control = 0%)
***
**
p=0.06
***
Days of abstinence
D.
BLA
Control
Alc. dep.
Cacna1c mRNA (Control = 0%)
***
**
***
Days of abstinence

**Figure 3**

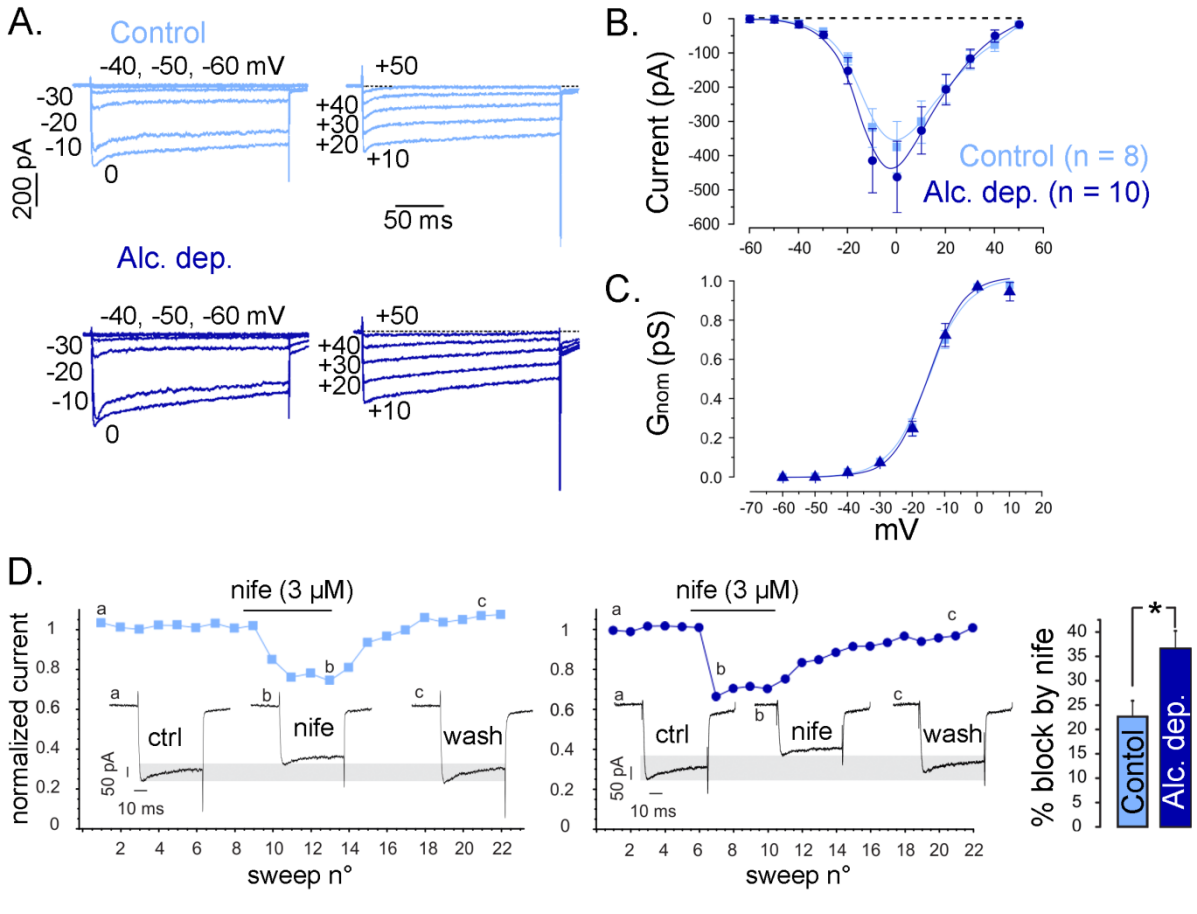

A.
Control
-40, -50, -60 mV
-30
-20
-10
0
+50
+40
+30
+20
+10
200 pA
50 ms
Alc. dep.
B.
Current (pA)
Control (n = 8)
Alc. dep. (n = 10)
C.
Gnom (pS)
mV
D.
nife (3 µM)
normalized current
ctrl
nife
wash
50 pA
10 ms
sweep n°
% block by nife
Contol
Alc. dep.
*

**Figure 4**

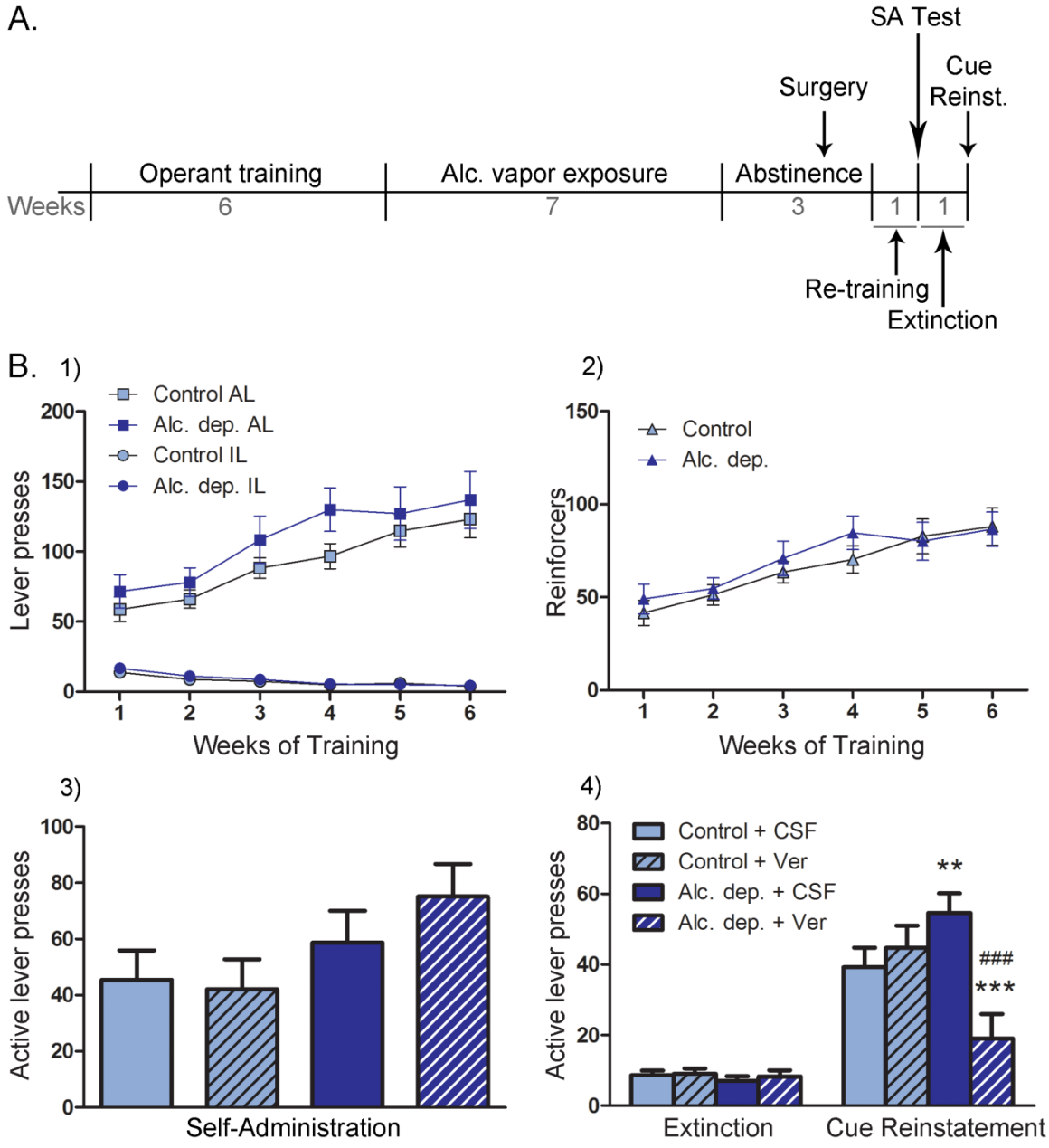
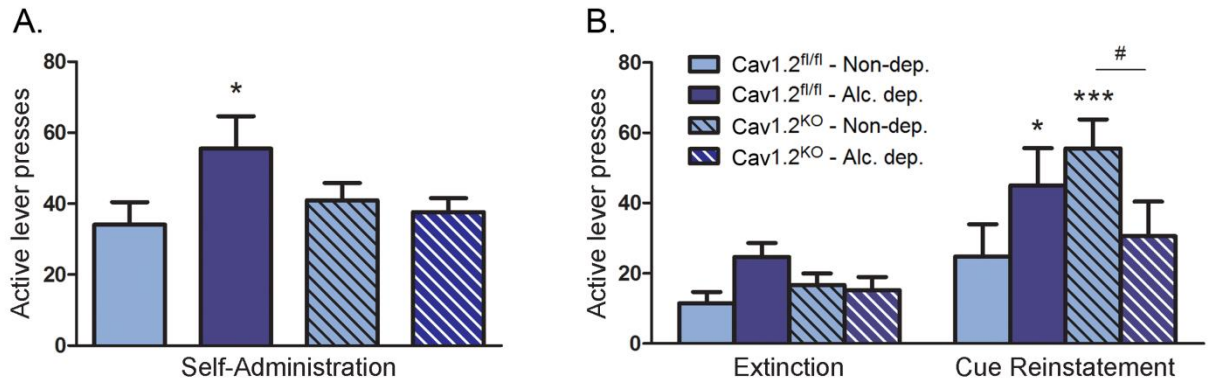

A.
SA Test
Surgery
Cue
Reinst.
Operant training
Alc. vapor exposure
Abstinence
Weeks
6
7
3
1
1
Re-training
Extinction
B. 1)
Control AL
Alc. dep. AL
Control IL
Alc. dep. IL
Lever presses
Weeks of Training
2)
Control
Alc. dep.
Reinforcers
Weeks of Training
3)
Active lever presses
Self-Administration
4)
Control + CSF
Control + Ver
Alc. dep. + CSF
Alc. dep. + Ver
**
###
***
Active lever presses
Extinction
Cue Reinstatement

**Figure 5**


A.
Active lever presses
80
60
40
20
0
*
Self-Administration
B.
Cav1.2fl/fl - Non-dep.
Cav1.2fl/fl - Alc. dep.
Cav1.2KO - Non-dep.
Cav1.2KO - Alc. dep.
#
***
*
Extinction
Cue Reinstatement