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Alcohol-dependent molecular adaptations of the NMDA receptor system

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Abstract

Phenotypes such as motivation to consume alcohol, goal-directed alcohol seeking and habit formation take part in mechanisms underlying heavy alcohol use. Learning and memory processes greatly contribute to the establishment and maintenance of these behavioral phenotypes. The N-methyl-d-aspartate receptor (NMDAR) is a driving force of synaptic plasticity, a key cellular hallmark of learning and memory. Here, we describe data in rodents and humans linking signaling molecules that center around the NMDARs, and behaviors associated with the development and/or maintenance of alcohol use disorder (AUD). Specifically, we show that enzymes that participate in the regulation of NMDAR function including Fyn kinase as well as signaling cascades downstream of NMDAR including calcium/calmodulin-dependent protein kinase II (CamKII), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and the mammalian target of rapamycin complex 1 (mTORC1) play a major role in mechanisms underlying alcohol drinking behaviors. Finally, we emphasize the brain region specificity of alcohol's actions on the above-mentioned signaling pathways and attempt to bridge the gap between the molecular signaling that drive learning and memory processes and alcohol-dependent behavioral phenotypes. Finally, we present data to suggest that genes related to NMDAR signaling may be AUD risk factors.

Keywords

Addiction; alcohol; AMPA; amygdala; CaMKII; Fyn; kinase; mTOR; NMDA; nucleus accumbens; phosphatase; PTPalpha; signaling; STEP; striatum

A prominent theory of AUD is the alcohol-dependent ‘hijacking’ of learning and memory processes which leads to pathological alcohol seeking and taking (Hyman *et al*. 2006; Torregrossa *et al*. 2011). Repeated exposure to alcohol promotes synaptic plasticity in key brain regions contributing to the formation of strong memories related to the rewarding experience of alcohol consumption (Torregrossa *et al*. 2011). In parallel, learning of the adverse consequences associated with harmful alcohol use may be attenuated (Torregrossa *et al*. 2011). As a result, patients suffering from AUD exhibit excessive alcohol intake, escalation of consumption and uncontrolled seeking and taking behaviors, which are key characteristics of AUD (Apa 2013; Koob & Volkow 2010). Thus, a better understanding of

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the molecular events underlying the aberrant form of learning and memory associated with AUD will enhance the discovery of new potential therapeutic targets.

The NMDAR is a critical factor of learning and memory functions (Mayford *et al.* 2012), and a primary target of alcohol (Ron and Wang 2009). In recent years, the molecular mechanisms that drive (the GO pathways) or that prevent (the STOP pathways) the development of AUD have started to unravel (Ron & Barak 2016), and we focus here on the contribution of the NMDAR signaling to the GO pathways in studies that used chronic voluntary consumption of alcohol in rodents. Specifically, we provide a description of the normal function of the NMDAR in learning and memory processes. We then detail the molecular events that promotes NMDAR activity, in particular Fyn kinase-dependent mechanisms, as well as signaling downstream of the receptor, including CaMKII, which are both critically affected by excessive alcohol drinking. Next, we review the potential link between alcohol-induced NMDAR inhibition and downstream signaling involving mTORC1. Finally, we review evidence from human genetic studies suggesting that variants in NMDAR-related genes contribute to the risk of developing AUD.

The NMDAR system

Glutamate, the major excitatory neurotransmitter in the mammalian brain, contributes to various crucial brain functions, including learning and memory (Mayford *et al.* 2012). Glutamate signaling is mediated through the activation of two families of transmembrane receptors: the G protein-coupled metabotropic receptors (mGluRs) and the ionotropic receptors, NMDA, AMPA and Kainate receptors (Traynelis *et al.* 2010). The NMDAR is a tetramer comprised of an obligatory subunit GluN1, and the regulatory subunits GluN2A-D and GluN3A-B (Traynelis *et al.* 2010). The most common regulatory subunits are GluN2A and GluN2B that differentially control the electrophysiological properties of the channel such as conductance and open probability (Traynelis *et al.* 2010). Each GluN2 subunit is composed of a long intracellular C-terminal sequence which contains binding sites for scaffolding proteins as well as kinase phosphorylation sites that control channel trafficking, membranal localization and activity (Traynelis *et al.* 2010). The NMDARs are enriched in the postsynaptic density (PSD), which is a dense network of hundreds of proteins including kinases, phosphatases, small G proteins and scaffolding proteins that mediate functional and structural plasticity of the excitatory synapses (Kennedy 2000).

The NMDAR contributes to the strengthening of synaptic connections (synaptic plasticity), that are essential to the formation of long-term memory (Mayford *et al.* 2012). Short-term synaptic plasticity is achieved through transient synaptic transmission changes (milliseconds to minutes), which then quickly return back to baseline levels, whereas long-term plasticity is achieved by repeated stimulation that causes persistent changes (hours to days) in the synaptic connections (Mayford *et al.* 2012). The NMDAR-dependent long-term potentiation (LTP) is the basis of synaptic plasticity (Malenka 2003; Mayford *et al.* 2012), and is associated with (1) the forward trafficking of AMPARs, (Malinow & Malenka 2002), (2) *de novo* protein synthesis (Kasai *et al.* 2010) and (3) enlargement of existing spines in addition to the formation of new spines (Matsuzaki *et al.* 2005). The NMDAR-dependent LTP was initially described in the hippocampus but was later observed in various brain regions

important for the development of AUD, including the ventral tegmental area, the nucleus accumbens (NAc), the dorsal striatum, the prefrontal cortex (PFC) and amygdala (Bernier *et al.* 2011; Britt *et al.* 2012; Cho *et al.* 2012; Taylor *et al.* 2016; Wang *et al.* 2012).

Src family tyrosine kinases and the NMDAR

This section focuses on modulation of NMDAR activities through tyrosine phosphorylation and dephosphorylation. Kinase phosphorylation of the GluN2A and GluN2B subunits plays a major role in the localization, activation state and physiological properties of the NMDARs (Traynelis *et al.* 2010). Kinases including protein kinase C, CaMKII, and cyclin-dependent kinase 5 phosphorylate the GluN2 subunits on serine and threonine sites (Ron 2004), while the Src family of tyrosine kinases (PTKs), Fyn and Src phosphorylates the GluN2 subunits on tyrosine sites. (Ohnishi *et al.* 2011; Ron 2004; Trepanier *et al.* 2012). Fyn and Src are the best characterized kinases modulating the NMDAR function. As such, the kinases play an important role in synaptic plasticity, learning and memory (Ohnishi *et al.* 2011; Salter & Kalia, 2004; Trepanier *et al.* 2012).

Fyn and Src are composed of regulatory and catalytic domains (Engen *et al.* 2008). In their inactive conformation, Fyn and Src are phosphorylated on tyrosine 528 (mouse)/531 (rat/human) (PhosphoTyr$^{528/531}$[Fyn/Src]) enabling the formation of an intramolecular bond with the SH2 domain that keeps the kinases in a closed inactive conformation (Engen *et al.* 2008). Dephosphorylation of this site results in a conformational change, allowing the kinases to undergo autophosphorylation at tyrosine 417 (mouse)/420 (rat and human) (PhosphoTyr$^{417/420}$[Fyn/Src]), which is the hallmark of the active kinase (Engen *et al.* 2008). Conversely, dephosphorylation of PhosphoTyr$^{417/420}$ inhibits Fyn and Src activity (Engen *et al.* 2008). The phosphatase responsible for dephosphorylating the inactive site PhosphoTyr$^{527/530}$[Fyn/Src], and thus that enables the activation of the kinases is the protein tyrosine phosphatase alpha (PTPalpha) (Bhandari *et al.* 1998; Ponniah *et al.* 1999; Su *et al.* 1999; Vacaresse *et al.* 2008; Vacaru & den Hertog 2010; Zheng *et al.* 2000). The termination of Fyn activation in the brain is mediated via the dephosphorylation of the active phosphorylation site Tyr$^{417/}$[Fyn] by the striatal-enriched tyrosine phosphatase (STEP) (Goebel-Goody *et al.* 2012). Although studies have mainly focused on STEP dephosphorylating Fyn thereby inhibiting kinase activity (Goebel-Goody *et al.* 2012), it is plausible that similar mechanisms hold true for Src. Finally, the activity of STEP is controlled in part, by protein kinase A (PKA) (Goebel-Goody *et al.* 2012). Specifically, PKA phosphorylates STEP resulting in an inhibition of the activity of the phosphatase thereby facilitating kinase activity (Goebel-Goody *et al.* 2012).

Once active, Fyn phosphorylates tyrosine residues within the cytoplasmic tail of GluN2B subunits (Ohnishi *et al.* 2011; Salter & Kalia 2004; Trepanier *et al.* 2012). The consequence of Fyn-mediated phosphorylation of GluN2B is the enhancement of channel function (Trepanier *et al.* 2012; Yaka *et al.* 2002, 2003a), which is due, at least in part, of increased membranal retention of GluN2B (Dunah *et al.* 2004; Nakazawa *et al.* 2001; Prybylowski *et al.* 2005). Both Fyn (Yaka *et al.* 2002) and PTPalpha (Lei *et al.* 2002) are part of the NMDAR complex, and the close proximity of Fyn to GluN2B allows the efficient phosphorylation of the subunit (Sato *et al.* 2008; Tezuka *et al.* 1999; Yaka *et al.* 2002; Yaka

et al. 2003a). Finally, STEP was reported to dephosphorylate GluN2B leading to channel internalization (Snyder *et al.* 2005). How alcohol co-opts the Fyn signaling-dependent regulation of NMDAR to drive alcohol-related phenotypes is reviewed herein.

CamKII and the NMDAR

At its resting state, the pore of the NMDAR is blocked by Mg^{2+} ions (Traynelis *et al.* 2010). Presynaptic stimulation causes a strong postsynaptic depolarization mediated by AMPAR which enables the removal of Mg^{2+} and, together with glutamate binding, leads to channel opening resulting in the entry of calcium (Ca^{2+}) and sodium (Na^{+}) ions (Traynelis *et al.* 2010). Ca^{2+} entering the NMDAR pore binds calmodulin, which associates with, and activates CaMKII (Irvine *et al.* 2006). CaMKII is a serine and threonine kinase composed of 12 independent subunits (Irvine *et al.* 2006). Once Ca^{2+} is bound, CaMKII is autophosphorylated by neighboring subunits of the holoenzyme, a mechanism that keeps the kinase active even after Ca^{2+} levels decrease and calmodulin dissociates from the kinase (Irvine *et al.* 2006). The autonomous activity of the holoenzyme is considered to be a molecular transducer of synaptic plasticity and long-term memory (Coultrap & Bayer 2012; Herring & Nicoll 2016; Muller *et al.* 2016). Specifically, NMDAR-dependent LTP is mediated by CaMKII phosphorylation and forward trafficking of AMPARs to the synaptic membrane (Herring & Nicoll 2016; Malinow & Malenka 2002). How alcohol promotes signaling activation downstream of the NMDAR is discussed herein.

Alcohol and NMDAR phosphorylation

Although the inhibition of NMDARs by acute alcohol exposure has been most extensively studied (Lovinger *et al.* 1989, Ron and Wang 2009), chronic alcohol increases NMDAR function in numerous brain regions (Abraham 2008; Floyd *et al.* 2003; Grover *et al.* 1998; Gulya *et al.* 1991; Iorio *et al.* 1992; Smothers *et al.* 1997; Wang *et al.* 2007, 2010). This section focuses on mechanisms underlying enhanced NMDAR signaling by repeated cycles of alcohol drinking and withdrawal.

Alcohol and Fyn signaling

Over the past decade, we have generated data to suggest that the Fyn signaling pathway is activated in the striatum of rodents in response to alcohol exposure. The striatum can be divided into ventral (NAc), dorsomedial (DMS) and dorsolateral (DLS) subregions based on neuroanatomical and functional divergences (Everitt & Robbins 2013). We found that the level of $PhosphoTyr^{417}$[Fyn] and thus the activity of the kinase is increased in the dorsal striatum in response to alcohol exposure (Wang *et al.* 2007). Furthermore, we found that contingent and non-contingent activation of Fyn by alcohol is localized to the DMS and was not observed in the DLS or in the NAc (Darcq *et al.* 2014; Gibb *et al.* 2011; Wang *et al.* 2007, 2010). Although both Src and Fyn are expressed throughout the brain, we generated data to suggest that alcohol specifically activates Fyn in the dorsal striatum. Specifically, we used an immunoprecipitation assay to pull down the kinase and detected the specific Fyn phosphorylation by alcohol (Wang *et al.* 2007). In contrast, Src was not activated by alcohol in the dorsal striatum (Wang *et al.* 2007). Interestingly, the activation of Fyn by alcohol is long-lasting. Specifically, repeated daily systemic administration of a non-hypnotic dose of

alcohol or a 7-week intermittent access (IA) to 20% alcohol in a two-bottle choice paradigm (IA20%-2BC) led to Fyn activation in the DMS of mice and rats that was maintained even after 16–24 h of withdrawal (Darcq *et al*. 2014; Gibb *et al*. 2011; Wang *et al*. 2010). We further showed that Fyn activation by alcohol is not due to alterations in the levels of the kinase (Wang *et al*. 2011) but instead resulted from the lateral movement of PTPalpha in close proximity to Fyn (Gibb *et al*. 2011), and through PKA-mediated phosphorylation of STEP, which in turn prevented the ability of STEP to dephosphorylate the kinase (Darcq *et al*. 2014). The PKA has been shown to be an upstream transducer of many of alcohol's actions in the brain (Ron & Barak 2016), and Xu *et al*. recently reported that the alcohol-mediated PKA-dependent phosphorylation and thus inhibition of STEP prevented the phosphatase from dephosphorylating and inactivating PTPalpha (Xu *et al*. 2015), thus enabling PTPalpha to translocate into lipid rafts and activate Fyn (Gibb *et al*. 2011; Xu *et al*. 2015). Curiously, similar to the activation profile of Fyn in response to alcohol exposure, PKA-dependent phosphorylation of STEP was localized to the DMS and was not detected in other striatal regions, (Darcq *et al*. 2014; Gibb *et al*. 2011; Wang *et al*. 2010; Xu *et al*. 2015). Finally, moderate consumption of alcohol modeled by a continuous access to 10% alcohol in a 2BC paradigm (CA10%-2BC) did not activate Fyn in the DMS of mice (Fig. 1, original data), suggesting that Fyn is activated only in response to heavy drinking of alcohol.

As described in above, one of the major substrates of Fyn in the brain is GluN2B, and as expected, the pattern of GluN2B phosphorylation in response to both contingent and non-contingent alcohol exposure resembles the pattern of Fyn activation by alcohol. Specifically, repeated daily systemic administration of alcohol for 7 days, or 7–8 weeks of IA20%-2BC resulted in a long-lasting phosphorylation of GluN2B at the synaptic membrane (Ben Hamida *et al*. 2013; Darcq *et al*. 2014; Wang *et al*. 2010), and similar to Fyn, the phosphorylation was localized to the DMS and was not detected in the DLS or NAc (Darcq *et al*. 2014; Gibb *et al*. 2011; Wang *et al*. 2010). Finally, GluN2B phosphorylation by alcohol led to increased membranal stabilization of GluN2B and a long-lasting facilitation of NMDAR activity (Wang *et al*. 2007, 2010, 2011). Together, these studies suggest that repeated cycles of alcohol exposure and withdrawal activate Fyn in the DMS, which in turn phosphorylates GluN2B resulting in the enhancement of channel activity (Fig. 2). This conclusion is supported by experiments in which lentivirus gene delivery was used to infect the DMS with short hairpin RNA (shRNA) sequence to knockdown PTPalpha or STEP mRNA. As predicted, knockdown of PTPalpha in the DMS prevented alcohol-dependent Fyn activation and GluN2B phosphorylation (Ben Hamida *et al*. 2013). Conversely, downregulation of STEP in the DMS potentiated the alcohol-mediated Fyn activation and GluN2B phosphorylation (Darcq *et al*. 2014). Curiously, Hicklin *et al*. reported that *ex vivo* exposure of hippocampal neurons to alcohol increased STEP-mediated dephosphorylation of GluN2B, which the authors suggested contributes to the inhibitory actions of alcohol of the NMDAR activity (Hicklin *et al*. 2011; Wu *et al*. 2011). These data are opposite to the finding that GluN2B is phosphorylated in response to *ex vivo* exposure of hippocampal neurons to alcohol (Yaka *et al*. 2003b). It is important to note however that the high basal level of GluN2B phosphorylation described by Hicklin *et al*. could be masking the actions of alcohol.

Nevertheless, compelling evidence has been generated to indicate that Fyn plays a critical role in alcohol-related behaviors. Pioneer studies showed that genetic deletion of Fyn (Fyn−/−) resulted in higher sensitivity of mice to the acute sedative hypnotic actions of alcohol, which required GluN2B phosphorylation (Boehm *et al.* 2003; Miyakawa *et al.* 1997; Yagi 1999; Yaka *et al.* 2003c). The early data regarding the role of Fyn in alcohol drinking in the 2BC paradigm have been controversial. For instance, we did not find differences in alcohol intake between wild-type (WT) and Fyn−/− mice even when mice were bred on two different backgrounds; C57BL/6J or 129SVJ (Yaka *et al.* 2003c), whereas Boehm *et al.* (2003) observed a reduction of alcohol drinking in Fyn−/− mice compared to WT mice (Boehm *et al.* 2003). More intriguing was the observation that alcohol consumption and preference were reduced in Fyn overexpression transgenic mice (Boehm *et al.* 2004). Fyn−/− mice display abnormal arrangements of cells in specific brain regions as evident by undulations in granule cell layer in the hippocampus (Grant *et al.* 1992). Thus, possible compensatory mechanisms during development may be the explanation of these discrepancies. Moreover, it is important to note that these early studies used the CA10%-2BC paradigm, which, as we show herein, does not produce an activation of Fyn in the DMS (Fig. 1), and possibly in other brain regions, thus providing an explanation to the lack of changes in the consumption of 10% alcohol intake in the Fyn−/− mice vs. WT littermates (Yaka *et al.* 2003c).

Numerous studies link the Fyn signaling pathway to the scaffolding protein postsynaptic density protein (PSD-95). PSD-95 associates with Src PTKs (Kalia and Salter, 2003), and the phosphorylation of GluN2B enhances the binding of PSD-95 to the subunit (Rong *et al.*, 2001). PSD-95 also associates with STEP and the association destabilizes STEP leading to its degradation (Won *et al.*, 2016). Interestingly, PSD-95 KO consume less alcohol compared to WT (Camp *et al.*, 2011), however, PSD-95 KO mice consume more alcohol compared to baseline after deprivation (Camp *et al.*, 2011).

More recently, models of operant self-administration of 20% alcohol have been proven useful to study multiple aspects of AUD including heavy alcohol intake, alcohol seeking and relapse (Carnicella *et al.* 2014). In this paradigm, rats undergo a period of 7 weeks of IA20%-2BC followed by an operant self-administration training period during which rats press on a designated ‘active’ lever to obtain a drink of alcohol (Carnicella *et al.* 2014). After acquisition of self-administration of 20% alcohol, cannula is be implanted in specific brain regions to pharmacologically manipulate the activity of an enzyme within a signaling pathway. Infusion of the Fyn inhibitor, PP2, in the dorsal striatum (Wang *et al.* 2007) and specifically into the DMS of rats (Wang *et al.* 2010) prior to a self-administration session reduced the number of presses on the active lever and the amount of alcohol consumed (Wang *et al.* 2007, 2010). In contrast, PP2 infusion into the NAc or DLS did not alter alcohol self-administration, indicating that the role of Fyn on alcohol intake is brain region-specific (Wang *et al.* 2007, 2010). PP2 infusion in the dorsal striatum did not affect the inter-response interval distribution, thus ruling out general locomotor alterations (Wang *et al.* 2007). To test whether the contribution of Fyn in the DMS to self-administration is generalized to other reinforcing substances, Wang *et al.* trained rats to self-administer sucrose, a natural reward, and showed that Fyn inhibition in the DMS did not affect sucrose self-administration, suggesting that Fyn specifically drives alcohol drinking behavior (Wang

et al. 2010). Furthermore, Wang *et al*. also tested the contribution of Fyn in the DMS to relapse to alcohol seeking. To model relapse, rats trained to self-administer alcohol undergo an extinction training in which the active lever is not reinforced (i.e. no alcohol is delivered upon pressing the active lever) (Carnicella *et al*. 2014; Epstein *et al*. 2006). Once the operant behavior is extinguished, the reinstatement test is carried out in which a small drop of alcohol is placed in the reward port and lever presses previously associated with alcohol are recorded (Carnicella *et al*. 2014; Epstein *et al*. 2006). The sensory cues (i.e. odor + taste) of the alcohol prime trigger a robust reinstatement of alcohol seeking (Carnicella *et al*. 2014). Using this model, Wang *et al*. found that Fyn inhibition in the DMS reduces the reinstatement of alcohol seeking, showing a key role for Fyn in mechanisms underlying relapse to alcohol use (Wang *et al*. 2010). Together, these data strongly suggest that Fyn in the DMS plays a role in intake of alcohol as well as relapse to alcohol seeking.

Upstream of Fyn is PTPalpha, which is required for the activation of the kinase (Bhandari *et al*. 1998). In line with the molecular findings described in this section, PTPalpha in the DMS plays an important role in mechanisms underlying excessive alcohol drinking. Specifically, PTPalpha knockdown in the DMS reduced alcohol intake and preference in an IA20%-2BC paradigm in rats without affecting water or sucrose consumption (Ben Hamida *et al*. 2013). Furthermore, to test whether PTPalpha contributes to the development of excessive alcohol drinking, the DMS of naïve mice was infected with a lentivirus expressing an shRNA sequence targeting PTPalpha prior to the beginning of the IA20%-2BC paradigm. Downregulation of PTPalpha reduced the consumption of, and preference for, 6%, 10% and 20% alcohol solution (Ben Hamida *et al*. 2013), suggesting that PTPalpha in the DMS is required for the developmental phase of excessive alcohol drinking. Conversely, infection of DMS neurons with a lentivirus expressing an shRNA sequence targeting STEP enhanced alcohol drinking and preference (Darcq *et al*. 2014). STEP was shown to contribute to NMDAR-mediated fear memory in the amygdala (Paul *et al*. 2007) and Hicklin *et al*. found that STEP is required for alcohol attenuation of fear conditioning (Hicklin *et al*. 2011). Furthermore, STEP−/− mice displayed increased level of alcohol intake in the 2BC paradigm as compared with WT mice (Legastelois *et al*. 2015). While WT and STEP−/− mice drink similar amounts of the sweet rewarding solution, saccharin, STEP−/− mice consume more of bitter tasting solutions of quinine or denatonium, that typically trigger aversion in WT mice (Legastelois *et al*. 2015). To test whether genetic deletion of STEP abolished the sensitivity to aversive stimulus, Legastelois *et al*. used a conditioned place aversion paradigm in which mice are conditioned to receive an injection of lithium chloride, an malaise-inducing agent, in a specific compartment. Following conditioning, WT but not STEP−/− mice avoided spending time in the lithium chloride-paired compartment (Legastelois *et al*. 2015). These results suggest that STEP controls the consumption of alcohol in part by attenuating its aversive bitter taste.

As described above, the PTPalpha activation and STEP inhibition by excessive alcohol drinking converge to promote Fyn activation, which in turn, phosphorylates GluN2B resulting in the enhancement of NMDAR activity. Similar to the reduction of alcohol consumption in response to Fyn inhibition in the DMS, GluN2B-containing NMDAR blockade by infusion of ifenprodil in the dorsal striatum (Wang *et al*. 2007) and more specifically in the DMS (Wang *et al*. 2010) reduced alcohol operant self-administration. The

latter effect was brain region-specific as intra-NAc (Wang *et al.* 2007) or intra-DLS (Wang *et al.* 2010) infusion of ifenprodil was ineffective at reducing alcohol self-administration. Similar to PP2, intra-DMS administration of ifenprodil did not alter sucrose self-administration (Wang *et al.* 2010). Moreover, intra-DMS infusion of ifenprodil was sufficient to reduce alcohol-priming-induced reinstatement of alcohol seeking similar to Fyn inhibition (Wang *et al.* 2010). Thus, NMDAR activity in the DMS is crucial to alcohol intake and relapse.

Alcohol and CaMKII signaling

The molecular transducer of NMDAR activation is CaMKII (Irvine *et al.* 2006), and a direct link between CaMKII and alcohol-dependent behavioral phenotypes has been established by two studies (Easton *et al.* 2013; Salling *et al.* 2014). Specifically, Salling *et al.* reported that moderate consumption of alcohol increases the protein level of CaMKIIalpha in the amygdala, and that the administration of the CaMKII inhibitors KN-93, or a CamKII inhibitory peptide into the mouse Central Amygdala (CeA) reduces self-administration of a sweetened solution of 10% alcohol (Salling *et al.* 2014). A second study by Easton *et al.* used transgenic mice expressing a mutant form of the kinase in which threonine at position 286 in the alpha subunit of the kinase has been mutated to alanine (αCaMKII^{T286A}) thereby preventing the autophosphorylation and thus the autonomous activation of the kinase (Easton *et al.* 2013). The authors showed that αCaMKII^{T286A} mice exhibited a delay in the onset of alcohol consumption as compared with WT mice (Easton *et al.* 2013), indicating that the CaMKII mediates the development of alcohol drinking behaviors.

Downstream of NMDAR-dependent activation of CaMKII is the phosphorylation and forward trafficking of the AMPAR. Salling *et al.* further reported that moderate consumption of alcohol increases the protein levels as well as the phosphorylation of the GluA1 subunit of AMPARs in the CeA (Salling *et al.* 2014). In addition, we showed that excessive alcohol intake initiates the forward trafficking of AMPAR subunits in the DMS suggesting that CaMKII may be activated in the DMS in response to consumption of excessive amounts of alcohol (Wang *et al.* 2012). Furthermore, the contribution of AMPAR to the behavioral effects of alcohol was shown by Corbit *et al.* (2014), Salling *et al.* (2014) and Wang *et al.* (2012). Specifically, intra-DMS infusion of the AMPAR antagonist, NBQX into the DMS of rat, reduced self-administration of a 20% alcohol solution (Wang *et al.* 2012), whereas intra-amygdala infusion of NBQX reduced self-administration of a sweetened solution of 10% alcohol in mice (Salling *et al.* 2014). Finally, intra-DLS infusion of NBQX in the DLS blocked the expression of alcohol habits (Corbit *et al.* 2014).

Alcohol and mTORC1 signaling

As detailed above, the NMDAR is an essential mediator of synaptic plasticity and learning and memory (Malenka 2003), and alcohol addiction is thought to be a maladaptive form of learning and memory (Hyman 2005; Nestler 2001). It is well established that *ex vivo* acute application of alcohol in various types of neurons inhibits the activity of NMDAR (Lovinger *et al.* 1989; Ron & Wang 2009). These findings raise an interesting question: how can alcohol on one hand inhibit the activity of the channel and on the other hand also contribute

to alcohol-dependent learning and memory phenotypes? A clue for solving this puzzle stemmed from studies reporting that the inhibition of the NMDAR function activates mTORC1 (Dwyer & Duman 2013), a kinase responsible for the initiation of the translational machinery at dendrites (Buffington *et al.* 2014), that plays an essential role in synaptic plasticity and learning and memory (Hoeffer & Klann 2010; Santini *et al.* 2014). Specifically, a single systemic administration the NMDAR inhibitors ketamine or Ro25-6981 was shown to produce a rapid activation of mTORC1 in the medial PFC (mPFC), which, in turn, produced an increase in the protein levels of the synaptic proteins PSD-95 and the GluA1 subunit of AMPAR, as well as to an increase in the protein levels of the presynaptic protein, synapsin I (Li *et al.* 2010). These molecular changes lead to increased number of spines and to an increase in synaptic strength of mPFC neurons (Li *et al.* 2010). Thus, it is plausible that the acute inhibition of the NMDAR by alcohol initiates mTORC1-mediated synaptic and structural plasticity that in turn drives initial alcohol associated learning. In fact, we obtained data to suggest that this mechanism may be responsible, at least in part to alcohol-associated reward learning. The NAc is a key component of the brain reward system (Sesack & Grace 2010), which orchestrates the acquisition (learning) and expression (memory retrieval) of the rewarding and reinforcing properties of alcohol (Koob 2003). We previously reported that unlike in the DMS in which alcohol both inhibits and enhances NMDAR function, alcohol's actions in the NAc are exclusively inhibitory (Wang *et al.* 2007, 2010, 2011). Interestingly, we found that alcohol also activates mTORC1 in the NAc (Neasta *et al.* 2010). Specifically, we showed that acute systemic administration of alcohol as well as episodes of heavy alcohol drinking activates mTORC1 in the NAc shell of rodents (Laguesse *et al.* 2016; Neasta *et al.* 2010). We further showed that mTORC1 activation is detected in dopamine D1 receptor (D1R) expressing NAc neurons during the first session of alcohol drinking in naïve mice resulting in increases in synaptic strength of NAc D1R neurons (Beckley *et al.* 2016). Furthermore, similar to the actions of ketamine and Ro25-6981 (Li *et al.* 2010), excessive alcohol consumption initiated the activation of the translational machinery leading to the translation of synaptic proteins including the collapsin response mediator protein 2 (CRMP-2), HOMER, PSD-95, Arc and GluA1 (Beckley *et al.* 2016; Liu *et al.* 2016; Neasta *et al.* 2010). Furthermore, the increase in CRMP-2 levels in the NAc in response to long-term heavy alcohol drinking promotes microtubules assembly (Liu *et al.* 2016). Importantly, we showed that systemic administration of the selective mTORC1 inhibitor, rapamycin, decreased rodents ' alcohol seeking and drinking as well as alcohol place preference (Beckley *et al.* 2016; Neasta *et al.* 2010). Together, these data suggest an intriguing possibility that the inhibition of the activity of NMDAR by alcohol activates the mTORC1-dependent signaling pathway (Fig.3), which in turn drives the memory of alcohol reward. Interestingly, we recently found that long-term excessive drinking of alcohol activates the mTORC1 signaling pathway in the orbitofrontal cortex (Laguesse *et al.* 2016). It would therefore be of interest to determine whether the NMDAR is inhibited by alcohol in this brain region as well and if so, whether it is linked to the activation of mTORC1 and/or to increases in synaptic plasticity and alcohol-dependent behavioral phenotypes.

The NMDAR signaling and AUD risk factors

The preclinical studies described above point to the critical role of NMDAR signaling in the development of AUD. Accordingly, several components of the NMDAR signaling pathway have been identified in human studies to be associated with AUD phenotypes. Specifically, single nucleotide polymorphisms (SNPs) within the Fyn gene were shown to be associated with increased risk of developing AUD and in increased severity of the disorder (Ishiguro *et al.* 2000; Pastor *et al.* 2009; Schumann *et al.* 2003). Furthermore, Gelernter *et al.* utilized data from the genome-wide association studies together with data from the Study of Addiction: Genetics and Environment and the Collaborative Study on the Genetics of Alcoholism and found that Fyn is localized within a gene network that was enriched for genes associated with alcohol dependence in both European Americans and African Americans (Han *et al.* 2013). Furthermore, several SNPs within the CaMKII gene including one located in the autophosphorylation site of the kinase were also found to be associated with the severity of AUD (Easton *et al.* 2013), and with increased frequency of alcohol consumption (Meyers *et al.* 2013). Finally, mutations within the AMPAR subunits as well as mTOR, and HOMER were also shown to be associated with increased alcohol use (Meyers *et al.* 2015). Although more studies are needed, these studies strongly suggest that the NMDAR network could be viewed as a potential AUD risk factor.

Summary and future directions

In this review, we summarized preclinical data showing that excessive alcohol drinking alters NMDAR signaling, which in turn contributes to the expression of alcohol-related behaviors such as alcohol reward, intake, seeking and relapse. In addition, human studies identified genetic variants within the NMDAR signaling pathway as risk factors for AUD. Together, findings in humans and rodents converge to support the important role of NMDAR signaling in the disease. Thus, targeting the NMDAR-associated molecular pathways affected by alcohol may represent a novel strategy to prevent and treat AUD in humans.

We described signaling mechanisms which center on the NMDAR, that may account, in part, for both the molecular (Figs. 2 3) and behavioral adaptations that ultimately drive alcohol-related phenotypes. We focused on alterations of NMDAR signaling in three brain regions, e.g. the CeA, the DMS and the NAc. However, such signaling mechanisms may occur in other brain regions. For instance, enhanced GluN2B-containing NMDAR currents has been observed in the basolateral amygdala (BLA) and the ventral bed nucleus of the stria terminalis (vBNST) following early cessation from chronic passive alcohol exposure using alcohol liquid diet or vapor administration, respectively (Floyd *et al.* 2003; Kash *et al.* 2009). Thus, it would be of interest to determine whether voluntary consumption of alcohol enhances the activity of the NMDARs in the BLA and vBNST, and if so, whether alcohol does so via the activation of the above-mentioned signaling pathways. However, it is plausible that non-physiological long-term exposure of rodents to alcohol (e.g. liquid diet and vapor exposure) produces different molecular outcomes compared with a voluntary drinking paradigm.

An intriguing question is the rather striking brain region selectivity of alcohol's effects. For example, the Fyn signaling pathway is activated by alcohol in the DMS but not in the DLS or NAc, whereas mTORC1 is activated in the NAc shell and the OFC, but not in other striatal or cortical regions. An intriguing possibility is that alcohol, by altering the lipid membrane fluidity (Chin *et al.* 1978, 1979), changes the composition of signaling proteins within lipid rafts. Lipid rafts are detergent-insoluble membrane microdomains which are enriched in glycosphingolipids, glycophosphatidylinositol-anchored proteins and cholesterol that localize signaling molecules into one membranal site (Allen *et al.* 2007). Signaling molecules are known to move in or out of lipid rafts depending on the activation signal (Allen *et al.* 2007). PTPalpha was shown to regulate the activity of Fyn in rafts (Maksumova *et al.* 2005; Vacaresse *et al.* 2008) and we previously showed that alcohol induces the association of PTPalpha and Fyn in lipid rafts in the DMS (Gibb *et al.* 2011). Interestingly, cholesterol content varies across brain regions (Svenningsson *et al.* 2004), and thus it is plausible that differences in cholesterol content between brain regions of similar neuronal composition such as the DMS and the NAc determine which signaling cascades will be activated in response to alcohol exposure.

Finally, we focused herein on NMDAR-dependent signaling molecules in the DMS, NAc and CeA. The 3 brain regions play a different role in learning and memory-dependent mechanisms such as goal-directed (Yin & Knowlton 2006), reward learning (Sesack & Grace 2010) and anxiety-related behaviors (Janak & Tye 2015), thus, future directions will be required to link these signaling cascades to specific circuitries that contribute to the behavior.

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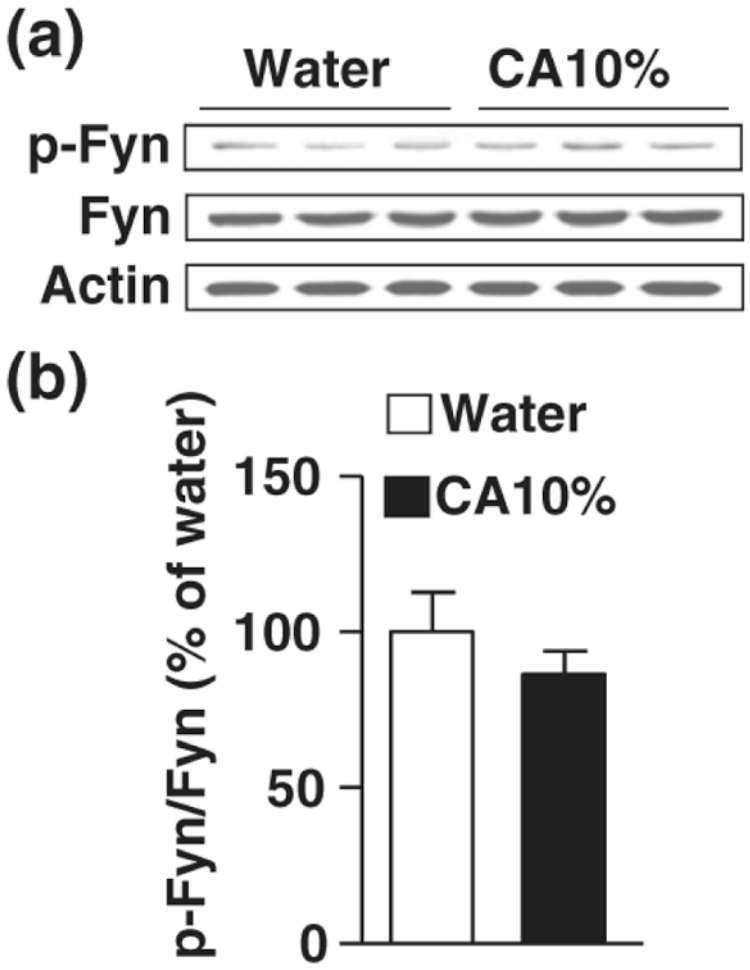

Figure 1. Moderate consumption of alcohol does not trigger Fyn activation in the DMS

Mice had CA10% (black) or water only (white) in a 2BC paradigm for 21 days, and the DMS was dissected immediately after the last 24-h alcohol drinking session. (a) $pY^{417/420}$[Fyn/Src] as well as the total protein level of Fyn and actin, which was used as a loading control, were measured by western blot analysis. (b) Histogram shows the mean ratio of $pY^{417/420}$[Fyn/Src] to total Fyn ± SEM, expressed as percentage of water controls. Data analysis indicates that CA10% does not affect Fyn activation in the DMS ($t_{10} = 0.92$, $P = 0.38$, unpaired Student's *t*-test). $n = 6$.

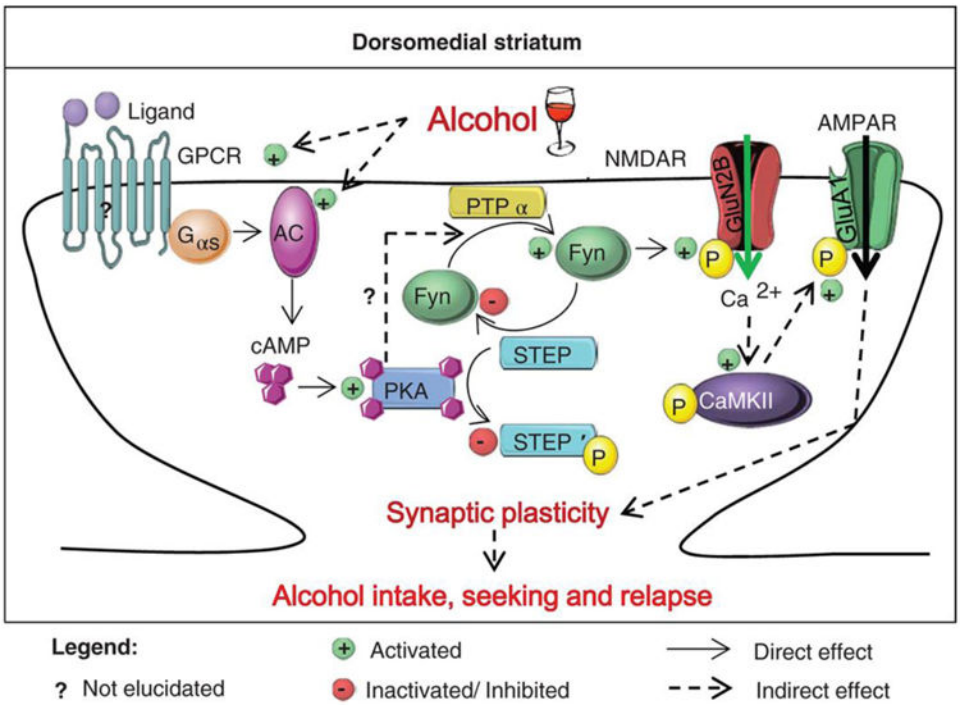

Figure 2. Molecular pathways transducing alcohol's signal in the DMS

Alcohol activates PKA, which phosphorylates STEP inhibiting the activity of the phosphatase. Inhibition of STEP allows for the long-lasting activation of Fyn. Alcohol also enables the membranal colocalization of Fyn with its activator PTPalpha. Active Fyn phosphorylates GluN2B, which enhances the activity of the channel. Calcium entry through the GluN2B-containing NMDARs enables the activation of CaMKII. CaMKII activation promotes the forward trafficking of the AMPAR subunits, which in turn contributes to synaptic plasticity and alcohol dependent behavioral phenotypes.

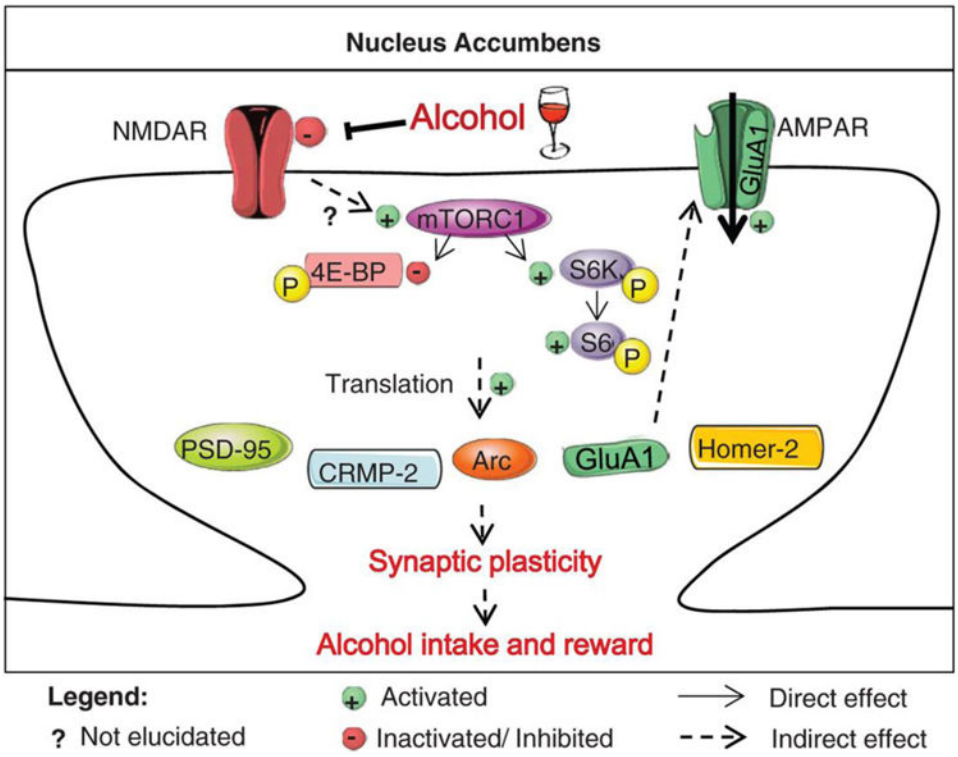

Figure 3. Molecular pathways transducing alcohol's signal in the NAc

Alcohol inhibits the activity of the NMDARs, which may contribute to the activation of mTORC1. mTORC1 phosphorylates its downstream substrates 4-eukaryote binding protein (4-EBP) and the ribosomal protein S6 kinase (S6K) resulting in the induction of the translational machinery and in the translation of the microtubule-binding protein (CRMP-2), the scaffolding proteins HOMER and PSD-95 as well as the GluA1 subunit of AMPAR, all of which play a major role in synaptic plasticity and alcohol-related phenotypes.