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Titration tipsy targets: the neurobiology of low-dose alcohol

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Abstract

Limited attention has been given to our understanding of how the brain responds to low-dose alcohol (ethanol) and what molecular and cellular targets mediate these effects. Even at concentrations lower than 10 mM (0.046 g% blood alcohol concentration, BAC), below the legal driving limit in the USA (BAC 0.08 g%), alcohol impacts brain function and behavior. Understanding what molecular and cellular targets mediate the initial effects of alcohol and subsequent neuroplasticity could provide a better understanding of vulnerability or resilience to developing alcohol use disorders. We review here what is known about the neurobiology of low-dose alcohol, provide insights into potential molecular targets, and discuss future directions and challenges in further defining targets of low-dose alcohol at the molecular, cellular, and circuitry levels.

Keywords

Alcohol; ethanol; low-dose; molecular targets; intoxication; neurobiology

LOW-DOSE ALCOHOL IMPACTS BRAIN FUNCTION AND BEHAVIOR

Alcohol (ethanol) induces intoxication in humans in a time- and dose-dependent manner [1, 2]. Low doses of alcohol commonly consumed during social drinking (1 – 3 drinks) produce mild intoxication: euphoria, stimulation, disinhibition, talkativeness, loss of social fear, relaxation, and sedation (depending on the individual), and impacting cognition, attention, motor behavior and emotional processing (Figure 1) [3, 4, 5, 6, 7]. The the BAC legal limit for driving in the USA is 0.08 g/dl, which corresponds to a brain alcohol concentration of 17.4 mM. However, even at concentrations <10 mM, which are below the legal driving limit and equivalent to 1 – 2 drinks for a 70 kg man, alcohol impacts brain function and behavior [3, 6, 8, 9], suggesting potential societal consequences even with the consumption of low-dose alcohol. Human brain imaging studies show that selected brain regions are sensitive to

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Conflict of Interest

The authors have no conflicts of interest to declare.

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alcohol at concentrations of 10 mM or below, reflected by changes in glucose metabolism, as well as dopamine and opioid peptide release [10, 11, 12]. Sustained progress has been made in understanding how low-dose alcohol might impact neuronal activity and synaptic function. However, precisely what molecular targets mediate such effects and which brain neurocircuits convey mild intoxication at these low doses remain uncertain. Studies using expression systems, such as human embryonic kidney (HEK) cells and *Xenopus oocytes* have shown that some subtypes of receptors, channels, and signaling molecules are sensitive to alcohol at 10 mM or less, and can be hypothesized to be the molecular targets mediating the actions of low-dose alcohol *in vivo*. In the following sections, we present the neurobiology of low-dose (10 mM) alcohol with an integrated review of current knowledge on neurotransmitter release, synaptic activity, and molecular targets that are sensitive to low-dose alcohol, followed by the discussion of future directions and challenges in further defining targets of low-dose alcohol at the molecular, cellular, and circuitry levels. Understanding what molecular and cellular targets mediate the initial effects of alcohol provides a missing link that bridges the neuroplasticity associated with mild intoxication to vulnerability or resilience to developing alcohol use disorders. For the purposes of this review, we use the term low-dose to refer to alcohol – ethanol in the context of this review – at concentrations of 10 mM or below. BACs (g%) are also presented in mM calculated by: $\text{mM} = (\text{g\%/46}) \times 10000$.

NEUROTRANSMITTER RELEASE AND SYNAPTIC ACTIVITY

The Dopaminergic System

The dopaminergic system has a key role in mediating the rewarding effects of alcohol and conveying incentive salience to cues linked to alcohol ingestion [13], and is sensitive to low-dose alcohol. In freely moving rats, intraperitoneal injection of 0.25 – 0.5 g/kg alcohol (estimated BAC 0.025 – 0.05 g%; 5.4 mM – 10.9 mM) stimulates dopamine release in the nucleus accumbens (NAc) and causes behavioral stimulation characterized by increased grooming, locomotion, and rearing [14]. In another *in vivo* study, intravenous injection of alcohol produced dose-dependent increases in the tonic firing rate of ventral tegmental area (VTA) dopaminergic neurons, which are more sensitive to low-dose alcohol (0.1 – 0.5 g/kg, estimated BAC 1.2 mM – 6.2 mM) than those in the substantia nigra [15]. Later studies have confirmed that the increased dopamine release in the NAc by alcohol is most likely due to the action of alcohol on dopaminergic neurons in the VTA, and is not due to its action on nerve terminals in the NAc to regulate release or reuptake. Thus, increased firing of VTA dopamine neurons may contribute to low-dose alcohol-induced dopamine release.

Numerous studies have examined how alcohol modulates VTA dopamine neuron activity. These studies used different experimental systems, such as acutely dissociated VTA dopamine neurons, brain slices, and *in vivo* recording, and found that multiple targets may contribute to alcohol's effects on dopamine neurons [16]. However, only a few of these targets, including L-type Ca^{2+} channels, G protein-gated inwardly rectifying potassium (GIRK) channels, and the glutamatergic, GABAergic and glycinergic systems, are sensitive to low-dose alcohol, and may contribute to dopamine release and the behavioral effects observed previously in freely moving rats [17, 18, 19, 20, 21, 22], discussed further in the

section addressing molecular targets. The exact targets responsible for increased dopaminergic neuron firing elicited by low-dose alcohol *in vivo* remain to be determined.

The Opioid Peptide Systems

Both human and animal studies have shown that alcohol stimulates the endogenous opioid system in brain regions that are associated with reward and reinforcement, and opioid receptor antagonists are well documented to blunt the rewarding effects of alcohol in animals and humans [23]. Furthermore, μ -opioid receptor knockout mice do not self-administer alcohol [24]. A PET imaging study in humans using the μ -opioid receptor selective agonist [^{11}C] carfentanil showed that one standardized drink of alcohol (resulting in $\text{BAC} < 0.05\text{ g\%} \sim 10.8\text{ mM}$) increased the release of endogenous opioid peptides that act at μ -opioid receptors in the NAc and the orbital frontal cortex [11]. However, the exact mechanisms by which low-dose alcohol selectively stimulates endogenous opioid release in these two brain regions are not clear. Animal studies using radioimmunoassay demonstrate that 10 mM alcohol stimulates the release of β -endorphin in the hypothalamus isolated from mice or rats [25, 26]. In addition, it has been shown that the action of alcohol on β -endorphin release in the hypothalamus is biphasic, and low to moderate concentrations of alcohol (10 – 40 mM) produce significant release, whereas higher concentrations of alcohol (60 – 120 mM) produce a less pronounced release of β -endorphin. Further studies will be necessary to understand how low-dose alcohol elicits endogenous opioid release in a brain region dependent manner and what molecular targets mediate the effects of low-dose alcohol. One hypothesis is that endogenous opioid peptide activation in turn drives the release of dopamine, but note that there are both dopamine dependent and dopamine independent actions of alcohol [27, 28]. Thus, the endogenous opioid system may play an important role in the rewarding effect of alcohol, possibly independent of dopamine release.

Synaptic Effects of Low-dose Alcohol

Studies have shown that low concentrations of alcohol modulate synaptic activity in several brain regions, including the NAc [29], the central nucleus of the amygdala (CeA) [30], the lateral habenula (LHB) [31], the dorsal striatum [32], and the cerebellum [33]. For example, 5 mM alcohol inhibits spike-timing dependent long-term potentiation (tLTP) in the NAc of mouse brain slices [29]. Deletion of the $\beta 4$ subunit of large-conductance calcium-gated potassium channels (BK channels) abolished the inhibitory effect of alcohol, suggesting that BK channels contribute to alcohol inhibition on tLTP in the NAc [29]. In addition, NMDA-type glutamate receptors, which are crucial in controlling tLTP in the NAc [34] and are inhibited by low-dose alcohol, may also be involved in alcohol inhibition of tLTP. Whether other molecular targets, such as L-type calcium channels, play a role in high alcohol sensitivity of tLTP in the NAc remains to be determined.

Acute alcohol exposure also inhibits glutamatergic transmission notably in the CeA and the cerebellum. Low-dose alcohol (even at 5 mM) decreased the amplitudes of compound glutamatergic excitatory postsynaptic potentials (EPSPs) and currents (EPSCs) with a half maximal dose of 14 mM in a subset of CeA neurons in brain slices from alcohol naïve rats [30]. The inhibitory effect of alcohol on these excitatory synaptic responses may be mediated primarily by the reduction of a non NMDAR-mediated component (AMPA and

kainate), because a NMDA receptor antagonist (AP5) did not affect alcohol inhibition [30]. In contrast, NMDA receptor-mediated currents are responsible for the high alcohol sensitivity of Purkinje cells in the cerebellum. For example, 10 mM alcohol inhibited AP-5 sensitive NMDA-mediated EPSCs of Purkinje cells at the climbing fiber synapse and impaired cerebellar long-term depression (LTD) [33].

Low-dose alcohol also enhanced glutamatergic transmission and firing of neurons in the lateral habenula (LHB) in brain slices [31]. Alcohol at concentrations less than 10 mM significantly enhanced excitatory synaptic transmission by facilitating glutamate release, such that 1.4 mM alcohol increased the frequency of spontaneous EPSCs (sEPSCs) by 50% [31]. A subsequent study showed that low-dose alcohol also potentiated GABAergic transmission (inhibitory postsynaptic currents, IPSCs) in LHB neurons with an EC₅₀ of 1.5 mM. Alcohol increased glutamatergic EPSCs and GABAergic IPSCs in most of the LHB cells, but there was a greater percentage of cells with a higher enhancement of EPSCs than IPSCs resulting in a net increase in LHB activity [35].

Synaptic effects of low-dose alcohol have also been demonstrated in human motor cortex by paired associative transcranial magnetic stimulation (PAS). Low-dose alcohol intake resulting in BAC less than 5 mM suppressed PAS-LTP-like plasticity and enhanced PAS-LTD-like plasticity in the human motor cortex [36, 37]. The alteration of PAS-induced plasticity, either LTP or LTD, was only observed by administration of low-dose alcohol but not by administration of alprazolam and zolpidem, two drugs that cause sedative effects by enhancing neurotransmission through $\alpha 1$ containing GABA_A receptors [36, 37]. This suggests that the effects of low-dose alcohol were most likely mediated by non- $\alpha 1$ containing GABA_A receptor mechanisms in these studies and that NMDA receptors and extrasynaptic GABA_AR-mediated tonic currents may have a role in the effects of low-dose alcohol on synaptic plasticity in the human motor cortex [36, 37].

Thus, the synaptic effects of low-dose alcohol may arise from presynaptic, postsynaptic, or extrasynaptic actions of alcohol, triggered by diverse molecular targets that are sensitive to low-dose alcohol. Further understanding of the molecular target(s) and synaptic site(s) of the actions of low-dose alcohol will provide key insights into the neurocircuitry altered by low-dose alcohol.

MOLECULAR TARGETS SENSITIVE TO LOW-DOSE ALCOHOL

Alcohol allosterically modulates many receptors, channels, and signaling molecules in the brain via diverse mechanisms [38, 39]. Although numerous studies have used relatively high concentrations of alcohol to obtain maximum and consistent alcohol effects, some studies, using different experimental systems, have demonstrated that multiple neurotransmitter receptors, ion channels, and signaling molecules are modulated by alcohol at concentrations of 10 mM or below (Table 1 and Figure 2, Key Figure). Significant progress has been made in defining potential alcohol binding sites in targeted proteins through the structural and functional analysis of alcohol interactions with receptors, and through molecular modeling based on high resolution crystal structures of relevant receptors. These studies have revealed that alcohol may bind to extracellular, transmembrane, or intracellular domains of receptors,

channels or signaling molecules (Figure 3). It remains to be determined which of these sites mediate the actions of low-dose alcohol. In the following discussion, we review low-dose alcohol sensitive targets from the perspective of alcohol sensitivity, mechanisms of alcohol action, and the potential alcohol binding sites in the targets.

Neurotransmitter Receptors – Ligand-gated Ion Channels

Glutamate receptors—Alcohol inhibits all three subtypes (AMPA, kainate, and NMDA) of ionotropic glutamate receptors. As one of the primary targets of alcohol, NMDA receptors are inhibited by a wide range of alcohol concentrations. For example, 5 – 100 mM alcohol dose-dependently inhibits NMDA receptor-mediated currents in cultured hippocampal neurons, and 10 mM alcohol inhibited the amplitudes of NMDA currents by 30% [40]. Further studies have demonstrated that the alcohol sensitivity of NMDA receptors is subunit dependent, and GluN2B subunit-containing receptors are more sensitive to alcohol than those containing the GluN2A subunit in HEK cell recombinant system [41]. This is consistent with the observation that the inhibitory effects of alcohol on NMDA-mediated EPSCs mainly arise from GluN2B-containing NMDA receptors in brain slices of the ventral bed nucleus of the stria terminalis (BNST) and CeA [30, 42]. However, non-GluN2B containing NMDA receptors may also contribute to the effects of low-dose alcohol, such as that observed in brain slices of the orbital frontal cortex [43]. In addition, 10 mM alcohol inhibits NMDA-mediated currents in Purkinje cells and impairs cerebellar synaptic plasticity, and these actions may contribute to the high sensitivity of cerebellar circuits to alcohol [33]. Thus, the sensitivity of NMDA receptors to low-dose alcohol (< 10 mM) has been demonstrated in recombinant systems, in cultured neurons and in slice preparations. To define further the sites of alcohol action on NMDA receptors, various studies have analyzed how mutations of amino acid residues in GluN1 and GluN2 subunits may affect alcohol inhibition. These structural and functional analyses coupled with molecular modeling show that residues in the transmembrane domain 3 (TM3) of the GluN1 subunit and in the TM3/TM4 of the GluN2 may contribute to the physical site where alcohol interacts in the receptor (Figure 3C) [44, 45, 46]. In addition to NMDA receptors, low-dose alcohol also inhibits non-NMDA (AMPA and kainate) glutamate receptor-mediated currents in hippocampal neurons. For example, 4.6 mM alcohol inhibited kainate receptor-mediated enhancement of GABAergic neuron excitability by 50% in the CA1 region of hippocampal slices [47]. Furthermore, 10 mM alcohol reduced the amplitudes of AMPA receptor-mediated currents by 12% in neurons from hippocampal slices of neonatal rats [48]. The mechanisms underlying the inhibition of AMPA and kainate glutamate receptors by low-dose alcohol remain to be determined.

GABA_A receptors—A few studies have shown that δ containing GABA_A receptors, which give rise to tonic extrasynaptic currents, are highly sensitive to alcohol potentiation at low millimolar concentrations (1 – 3 mM) when the receptors are expressed in *Xenopus* oocytes [49, 50]. Receptors containing the δ subunit, $\alpha 4\beta 3\delta$ and $\alpha 6\beta 3\delta$, are more sensitive to alcohol than are γ -containing GABA_A receptors [50]. However, some controversy exists over the alcohol sensitivity of delta subunit containing GABA_A receptors and the direct action of alcohol at extrasynaptic sites [51, 52]. Similar to other ligand-gated receptors, the alcohol sensitivity of GABA_A receptors is subject to modulation by intracellular signaling

molecules. Evidence suggests that protein kinase C ϵ (PKC ϵ) regulates alcohol (3 – 10 mM) potentiation of GABA currents mediated by $\alpha 1\beta 2\gamma 2L$ receptors expressed in HEK cells, such that activation of PKC ϵ suppresses, while inhibition of PKC ϵ enhances, the potentiation effect of alcohol on $\gamma 2$ -containing GABA $_A$ receptors [53]. To determine how molecular targets contribute to the effects of low-dose alcohol in vivo, one approach is to create knock-in mice with mutations that render the receptor insensitive to alcohol modulation, but retain otherwise normal function. For example, mice with S270H and L277A mutations in the GABA $_A$ $\alpha 2$ subunit show weaker conditioned taste aversion and complete loss of the motor stimulation effect of 1 g/kg alcohol (but note this knock-in potentiated alcohol drinking), suggesting that $\alpha 2$ containing GABA $_A$ receptors may contribute to some of the behavioral effects of alcohol [54].

Significant progress has been made in identifying potential alcohol binding sites in GABA $_A$ receptors through the structural and functional analysis of alcohol actions and high-resolution crystal structures of the bacterial pentameric ligand-gated ion channel GLIC, which is a structural homolog of Cys-loop receptors (Figure 3A, B)[55, 56]. Crystal structures of the alcohol-sensitized variant of GLIC reveal the inter-subunit cavity as the common structural feature for alcohol potentiation of human glycine and GABA $_A$ receptors [55]. In addition, it has been demonstrated that multiple amino acid residues located at the TM domains may serve as potential alcohol binding sites [56]. For example, analysis of $\alpha 1\beta 2\gamma 2$ GABA $_A$ R expressed in oocytes have identified N265 in TM2 and M286 in TM3 of the $\beta 2$ subunit as forming an intra-subunit cavity where alcohol interacts to potentiate GABA $_A$ receptor-mediated responses [57].

Glycine receptors—Alcohol at concentrations less than 10 mM potentiates glycine receptor (GlyR)-mediated currents, and $\alpha 1$ -containing glycine receptors are more sensitive to alcohol than $\alpha 2$ containing receptors when expressed in the *Xenopus oocytes* [58]. In addition, 1 – 10 mM alcohol significantly potentiates $\alpha 1$ containing human GlyR mediated currents in HEK cells [59]. This high sensitivity of $\alpha 1$ GlyR to alcohol arises from an alcohol effect on single-channel open probability, such that 10 mM alcohol enhances channel open probability by 21% without affecting channel conductance in HEK cells [59]. Alcohol at 1 mM also potentiates the amplitudes of glycine-elicited currents in cultured spinal neurons by 12% and potentiates peaks of glycine currents by 20 % in acutely dissociated VTA neurons (Table 1) [20, 60]. In addition, the high sensitivity of GlyRs to alcohol may be responsible for the inhibitory effect of alcohol (11 mM, 44%) on the excitability of neurons in brain slices of the lateral orbitofrontal cortex [43]. Multiple structural domains of GlyRs may contribute to the high alcohol sensitivity. Amino acid residues in the N-terminal domain (Ala52) and in TM1 – 4 are crucial for alcohol sensitivity and may contribute to the actions of alcohol on GlyRs [61, 62]. In addition, the intracellular loop domain (316RFRRK320,385KK386) that mediates G-protein modulation of GlyRs through G $\beta\gamma$ may be responsible for the low-dose alcohol sensitivity of $\alpha 1$ containing GlyRs [59, 63]. The role of G proteins and other factors, such as Zn²⁺ [64], in modulating alcohol sensitivity of GlyRs suggests that intracellular signaling and extracellular environments have a significant impact on the degree of receptor modulation by alcohol. Thus, it is important to identify low-dose targets in the native environment.

Nicotinic acetylcholine receptors (nAChRs)—nAChRs in the VTA and the lateral tegmental nucleus (LDTg) contribute to motor control, motor learning and reward. Alcohol has differential effects on nAChRs depending on the receptor subtype. Expression of different combinations of nAChR subunits in *Xenopus oocytes* reveals that homomeric $\alpha 7$ nAChRs are more sensitive to alcohol than nAChRs composed of $\alpha 2\beta 4$, $\alpha 4\beta 4$, $\alpha 2\beta 2$, $\alpha 4\beta 2$, $\alpha 3\beta 2$ or $\alpha 3\beta 4$ [65]. Alcohol at concentrations of 5 – 100 mM dose dependently inhibits $\alpha 7$ nAChRs expressed in *Xenopus oocytes*, and 10 mM alcohol inhibits the amplitudes of $\alpha 7$ currents by 15% [66]. In addition, 10 mM alcohol significantly inhibits currents mediated by $\alpha 7$ nAChRs by about 20% from neurons in brain slices of the LDTg [67]. Alcohol suppressed $\alpha 7$ nAChR-mediated currents by facilitating receptor desensitization in a protein kinase A dependent manner, suggesting that the phosphorylation state of $\alpha 7$ nAChRs influences its alcohol sensitivity [67]. This is consistent with the hypothesis that desensitization is one of the mechanisms underlying acute alcohol modulation of receptors and channels. In addition, co-administration of 5 mM alcohol and 100 nM nicotine synergistically increases the alcohol-induced enhancement of AMPA receptor-mediated currents in VTA dopamine neurons in brain slices, which requires $\alpha 6$ nAChR function, suggesting that $\alpha 6$ nAChRs may contribute to low-dose alcohol and nicotine co-use [68].

Other Ion Channels

Alcohol either inhibits or potentiates a variety of ion channels that are important in the regulation of intrinsic neuronal properties and neurotransmitter release. Among many ion channels modulated by alcohol, BK channels and L-type voltage-gated calcium channels are particularly sensitive to alcohol. At concentrations equal to or less than 10 mM, alcohol potentiated BK channels in different expression systems (i.e. *Xenopus oocytes*, HEK293 cells), and in terminals and neurons of different brain regions (hypothalamic - neurohypophyseal axis and the nucleus accumbens) [69, 70, 71]. For example, 1 mM and 10 mM alcohol increased BK channel open probability by 1.45 and 2.5 fold, respectively, in excised patches from the soma of NAc neurons [70]. The sensitivity of BK channels to alcohol is tightly controlled by β auxiliary subunits, such that β subunits significantly reduce amplitudes of BK channels and that $\beta 4$ containing BK channels are more sensitive to alcohol than those containing the $\beta 1$ subunit [70]. In addition to their role in mediating alcohol tolerance [72], BK channels may also play an important role in the effects of alcohol on pacemaker activity in the external globus pallidus (GPe) [73]. Alcohol at 10 mM decreased the firing rate of low-frequency firing GPe neurons in GPe slices in a BK channel-dependent manner, an observation that is supported by alcohol - induced enhancement of BK channel open probability recorded in cell-attached mode of Npas1-expressing neurons [73]. Multiple amino acid residues in the cytosolic tail domain of the α subunit of BK channels have been implicated in forming the potential binding pocket for alcohol, among which K361 forms a hydrogen bond with the alcohol molecule (Figure 3D) [74]. Importantly, mutation of T352 completely eliminated alcohol potentiation while only minimally affecting basal channel function in *C. elegans* and human BK channels, suggesting that T352 either coordinates alcohol actions and/or mediates alcohol-induced changes in the conformation of the channel protein [75]. Alcohol has differential effects on subtypes of calcium channels, which play important roles in a variety of neurophysiological functions, such as neurotransmitter and hormone release as well as gene transcription. L-type calcium channels are particularly

sensitive to alcohol. For instance, 5 mM alcohol significantly inhibits peak amplitudes of L-type calcium channel-mediated currents in PC 12 cells by 33.6% [76]. In addition, 10 mM alcohol decreased the mean open probability of L-type channels in the nerve terminal of the rat neurohypophysis [21].

Other Signaling Molecules

In addition to neurotransmitter receptors and ion channels, low-dose alcohol targets other signaling molecules, such as L1 cell adhesion molecule (L1-CAM) and Insulin-Like Growth Factor-I (IGF1). Alcohol has high affinity for the L1-CAM and may directly interact with the extracellular domain of L1 at the interface of the immunoglobulin (Ig)4 and Ig1 domains (Figure 3E) [77]. At concentrations below 10 mM, alcohol significantly inhibited L1 adhesion in 3T3 cells, such that 5.1 mM alcohol reduced the number of adherent cells by 50% [78]. L1-CAM plays a crucial role in the developing nervous system [79], and several studies have focused on understanding the interactions between alcohol and L1-CAM in the fetal alcohol spectrum disorders (FASD). In the adult brain, L1 CAM is also expressed in various regions and plays an important role in structural and synaptic plasticity, as well as in neurological diseases [79]. Whether low-dose alcohol targets L1-CAM in the adult brain and how such interactions may contribute to low-dose alcohol effects remain to be determined.

IGF-1, which plays an important role in energy homeostasis and neural plasticity [80], is sensitive to low-dose alcohol. Alcohol at 10 mM inhibited the IGF-1-mediated proliferation of 3T3 Cells by 30% and depressed tyrosine phosphorylation of the IGF-1 receptor [81]. This inhibitory action of alcohol may be due to its direct interaction with the carboxyl terminal of the receptor protein [82]. In the adult brain, the IGF1- receptor is expressed in neurons and most abundantly in the neocortex, thalamus, and choroid plexus [80]. Whether alcohol interacts with IGF-1 in neurons at concentrations below 10 mM remains to be determined.

Concluding Remarks

Human and animal studies have demonstrated that low doses of alcohol, at concentrations that can be achieved by 1 – 3 drinks in humans, impact many aspects of brain function and elicit the behavioral effects of alcohol that are colloquially defined as mild intoxication. It remains to be determined what molecules, cells, and neurocircuits are targeted by low concentrations of alcohol in the brain to elicit these behavioral changes and how these targets orchestrate neuroadaptive responses to low-dose alcohol.

What we currently know is that alcohol allosterically modulates a variety of targets with a wide range of sensitivities. Studies using various experimental systems have revealed that several subtypes of receptors, channels, and signaling molecules are sensitive to alcohol at 10 mM and below (Table 1, Figure 2). Nevertheless, it remains unclear which of these are crucial for mediating mild intoxication. It is also possible that the key brain proteins that are very sensitive to low-dose alcohol *in vivo* have yet to be discovered (see question 1 in the Outstanding Questions). Unlike other drugs, alcohol has relatively low affinity for its molecular targets. It diffuses through membranes, which enables alcohol to concurrently interact with targets in the extracellular, transmembrane, and intracellular domains. This

property of alcohol broadens its potential targets beyond classic transmembrane proteins, which may set apart alcohol targets from those of other drugs (Question 3). It is important to keep this unique property of alcohol in consideration when identifying low-dose alcohol targets.

Outstanding Questions Box

- How do multiple molecular targets orchestrate low-dose alcohol effects in vivo in a concentration-dependent manner? Are there key brain proteins that are very sensitive to alcohol that remain to be discovered?
- For any given neurocircuit, how do heterogeneous sub-populations of neurons respond to alcohol? How does one identify the subtypes of neurons and associated neuropathways that are sensitive to low-dose alcohol?
- What are the obstacles to identifying molecular targets of low-dose alcohol? How does the unique property of alcohol broaden its potential targets beyond classic transmembrane proteins and set apart the targets of alcohol from those of other drugs?
- How does alcohol at low concentrations (< 10 mM) interact with putative alcohol binding sites revealed in several receptors, channels, and signaling molecules? How do alcohol concentrations alter its interaction with the identified molecular targets?

To understand how alcohol interacts with molecular targets, several studies have identified specific amino acid residues that are crucial for the actions of alcohol and have revealed multiple potential alcohol binding sites (Figure 3). How exactly alcohol at low concentrations (< 10 mM) interacts with these sites remains to be determined (Question 4). In addition, given that diverse subunit compositions exist in different sub-populations of neurons, and the modulation of receptors and channels by a variety of endogenous factors, the native environment will be crucial for accurately identifying the most sensitive alcohol targets. It is also possible that the receptors and channels affected only by high concentrations of alcohol in vitro may be sensitive to low-dose alcohol in the native environment. Furthermore, the multiple potential molecular targets of low-dose alcohol (Table 1, Figure 2) suggest that the effects of low-dose alcohol on neuronal activities may arise from the alcohol induced modulation of multiple targets. These considerations, together with the membrane diffusion properties of alcohol, provide significant challenges in identifying the most sensitive target(s) of alcohol.

It is also known that several neurobiological/neurochemical systems and circuits are highly sensitive to alcohol, including, the dopaminergic system in the VTA, the cholinergic system in the LDTg, the opioid system in the NAc and the orbital frontal cortex, and the glutamatergic system in the CeA, LHB, and cerebellum, as well as the GABAergic system in the LHB, cerebellum, and dorsal striatum. However, it is not clear exactly how low doses of alcohol, even at sub 10 mM concentrations, regulate neurotransmitter release and neuronal firing or modify synaptic plasticity within systems that mediate mild intoxication. Although

we have a basic knowledge of the role of the VTA, NAc and CeA in the reinforcing effects of alcohol in animal studies, much more knowledge is needed for any given neurocircuit to be able to understand how low-dose alcohol affects the heterogeneous populations of neurons within that circuit and which inputs and outputs connecting various brain regions to that circuit to mediate low-dose effects (Outstanding Question 2). Such circuitry level regulation will be unlikely detected by studying isolated cells or in recombinant systems, and may partly account for some of the differences in alcohol sensitivity detected between different experimental systems and *in vivo*. In addition to pharmacological approaches, genetic approaches using either mutation knock-in or gene knock-out, coupled with circuitry and behavioral analyses may help determine how different molecular targets of alcohol contribute to the behavioral effects of low-dose alcohol and which circuits mediate such effects. Rapid advances in research techniques in the coming years will provide new opportunities to overcome these challenges to permit the identification of the targets of low-dose alcohol and to achieve a mechanistic understanding of actions of low-dose alcohol at the molecular, cellular and circuitry levels. Novel neuroscience techniques that will allow one to detect and manipulate neuronal activities *in vivo* with high temporal and spatial resolution, such as those developed through the BRAIN initiative, may be a rich source of tools for this endeavor (www.braininitiative.nih.gov).

Alcohol is a ubiquitous intoxicant derived from the product of the “saccharine principle” [2], which refers to the conversion of any carbohydrate to glucose and then subsequent fermentation, and alcohol has been used by humans for thousands of years. A looming question is what exactly alcohol does to the brain to produce such highly valued intoxicating effects that have been celebrated by Bacchus (the Roman god of agriculture), MacDuff (in the play MacBeth by Shakespeare), and the songs of Jimmy Buffet. Our argument here in “titrating a tipsy target” is that a focus on the science of low-dose alcohol will allow us to solve one of human culture’s most mysterious questions: – why alcohol is so highly valued for its intoxicating effects – and determine at what point on the inverted U-shaped alcohol dose effect curve the neuroadaptations begin to turn the effects of alcohol to the “dark side” of the motivational systems of the brain [83]. The knowledge of the molecular targets, cellular actions and neurocircuitry modulation of low-dose alcohol, summarized above, combined with new advances in neuroscience techniques give promise that one of the great neuropharmacological mysteries of human experience will be solved in the near future.

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Trends Box

- Low doses of alcohol at concentrations that can be achieved by social drinking (1 – 3 drinks) impact brain function and behavior.
- Low-dose alcohol impacts neuronal activity and synaptic function, however, there is a lack of understanding precisely what molecular targets mediate such effects and what brain neurocircuits respond to low-dose alcohol.
- Alcohol allosterically modulates a variety of receptors, channels, and signaling molecules with a wide range of sensitivities.
- Understanding what molecular and cellular targets mediate the initial effects of alcohol will provide a window into the early stages of neuroplasticity that contribute to vulnerability and resilience to developing alcohol use disorders.

Figure 1.

Effects of alcohol (ethanol) related to blood alcohol levels. Alcohol has a biphasic dose-effect curve in humans. At lower concentrations, such as at 0.05 g% (10.9 mM) and below, alcohol causes disinhibition and relaxation. At higher concentrations, alcohol impairs motor function (at 0.1 – 0.2 g% or 22 – 44 mM) and causes stupor (at 0.2 – 0.3 g% or 44 – 65 mM), coma (at 0.3 – 0.4 g% or 65 – 87 mM), and even death (at 0.4 – 0.5 g% or 87 mM – 109 mM). The legal limit of blood alcohol concentration (BAC) for driving in the USA is 0.08 g% corresponding to 17.4 mM.

Figure 2.

Sensitivity of Molecular Targets to Alcohol at Concentrations 10 mM. Activity changes of multiple receptors, channels, and signaling molecules responding to alcohol at concentrations 10 mM. The graph provides an overview of sensitivities of different molecular targets to alcohol based on data from expression systems and isolated neurons and is not intended to demonstrate strict comparisons. References to the data are listed in Table 1. Abbreviations: BK, large-conductance calcium-gated potassium channel; HEK, human embryonic kidney cell line; Hipp, hippocampus; NAc, nucleus accumbens; Neurohyp., neurohypophysis; 3T3, mouse fibroblast 3T3 cell line (for other targets see text).

Figure 3.

Putative Alcohol Binding Sites Identified in Diverse Molecular Targets of Alcohol. (A) Alcohol (orange and red spheres) in the inter-subunit cavity shown in the crystal structure of an alcohol sensitized GLIC F14'A mutant co-crystallized with alcohol [55]. (B) Alcohol modulation sites of pentameric ligand-gated ion channels, including the GABA_A, glycine (Gly), and nicotinic acetylcholine (ACh) receptor subtypes, mapped onto the crystal structure of two neighboring subunits of the prokaryotic GLIC protein (depicted by ribbons). Putative binding sites are indicated by colored ribbons. Residues implicated in the interactions of alcohol with multiple subtypes of GABA_A, glycine (Gly), and nicotinic acetylcholine (ACh) receptors, are labeled by notation [56]. (C) Molecular model of the alcohol site in the NMDA receptor formed by the transmembrane (TM)3 domain of the GluN1 subunit (grey) and the TM4 domain of GluN2B subunit (blue). The labeled residues in intersubunit interfaces of the TM3 and TM4 domain interact to regulate alcohol action [46]. (D) Discrete alcohol sensing regions in the cytosolic tail domain (CTD) of the BK channel depicted using the mSlo1 CTD homology model based on the crystal structure of hSlo1. The hydrogen bond between alcohol and K361 is shown as a dotted line in the region 2 [74]. (E) Potential alcohol binding sites in L1 adhesion molecules identified by photo-labeling with azilalcohol. Potential alcohol binding pocket (E33 and Y418) and a disease causing residue (L120) are mapped onto horseshoe-shaped immunoglobulin (Ig)1-4 domains of human L1 structure modeled based on the crystal structure of axonin Ig1-4 [77].

Table 1

Molecular Targets that are Sensitive to Low-dose Alcohol (10 mM)

Molecular Target	Experimental System	Ethanol Concentration (mM)	Effect	Specificity	References
NMDA receptors	Hippocampal neurons (cultured)	5 – 50	Inhibition	~32% inhibition at 10 mM	[40]
NMDA currents (EPSC)	Purkinje cells (in slices)	10 – 50	Inhibition	11.4% – 45.7% inhibition at 10 mM	[33]
NMDA receptors	HEK cells	10 – 50	Inhibition	~13% inhibition at 10 mM (GluN1A/GluN2B)	[41]
$\alpha 4\beta 2$ GABA receptors	Oocytes	1 – 3	potentiation	45% potentiation at 1 mM	[49]
$\alpha 4\beta 3\delta$, $\alpha 6\beta 3\delta$ GABA receptors	Oocytes	1 – 300	potentiation	15.6% potentiation at 3 mM	[50]
Glycine receptors $\alpha 1 > \alpha 2$	Oocytes	5 – 200	Potentiation	~14% for $\alpha 1$ and ~6% for $\alpha 2$ at 10 mM	[58]
Glycine current	Spinal neurons (cultured)	1 – 400	potentiation	~20% potentiation at 10 mM	[60]
Human GlyR $\alpha 1$	HEK cells	1 – 200	potentiation	21% potentiation at 10 mM	[59]
$\alpha 7$ nicotinic receptors	Oocytes	5 – 100	inhibition	~15% inhibition at 10 mM	[66]
Nicotinic receptors	PC 12 cells	0.01 – 10	inhibition	~30% inhibition at 10 mM	[84]
5HT3 receptors	Oocytes	1 – 200	potentiation	~10% potentiation at 10 mM	[66]
BK channels	neurohypophysial terminals	10 – 50	potentiation	50% potentiation at 10 mM	[69]
BK channels	Acute isolated neurons in the NAC	1 – 50	potentiation	45% potentiation at 1 mM; 150% potentiation at 10 mM	[70]
L-type Calcium channels	PC12 cells	5 – 50	inhibition	33.6% inhibition at 5 mM	[76]
Insulin-like growth factor-1 (IGF-1)	3T3 cells	10 – 100	inhibition	30% inhibition at 10 mM	[81]
L1 adhesion molecule	3T3 cells	1 – 100	inhibition	~40% inhibition at 5 mM	[78]