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SNARE complex-associated proteins and alcohol

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Abstract

Alcohol addiction causes major health problems throughout the world, causing numerous deaths and incurring a huge economic burden to society. To develop an intervention for alcohol addiction, it is necessary to identify molecular target(s) of alcohol and associated molecular mechanisms of alcohol action. The functions of many central and peripheral synapses are impacted by low concentrations of ethanol. While the postsynaptic targets and mechanisms are studied extensively, there are limited studies on the presynaptic targets and mechanisms. This article is an endeavor in this direction, focusing on the effect of ethanol on the presynaptic proteins associated with the neurotransmitter release machinery. Studies on the effects of ethanol at the levels of gene, protein, and behavior are highlighted in this article.

Keywords

ethanol; alcoholism; neurotransmitter; presynaptic; postsynaptic; neuron; volatile anesthetics; exocytosis; SNARE complex

Introduction

Alcohol is one of the most harmful drugs of abuse (Nutt et al., 2010) and alcohol addiction is a major health problem throughout the world, causing numerous deaths and incurring a huge economic burden to society (Sacks et al., 2015). Defining the molecular mechanism of alcohol (ethanol) action is key to developing an intervention to alcohol addiction. To this end, it is important to understand ethanol's role in the changes of behavior and the brain during the descent into addiction. At relatively low concentrations, ethanol affects the function of many central and peripheral synapses (Liu and Hunt, 1999) and modulates synaptic plasticity (McCool, 2011, Lovinger and Roberto, 2013, Roberto and Varodayan, 2017). Although clear effects of ethanol have been identified in postsynaptic compartments, its effects on the presynaptic compartments is less studied. Ethanol has multiple targets (Harris et al., 2008, Howard et al., 2011) and affects many neuronal circuitries in the brain (Abrahao et al., 2017). While ethanol's effects on postsynaptic receptors, such as $GABA_A$ (Olsen and Liang, 2017), glycine (Soderpalm et al., 2017) and glutamate (Rao et al., 2015)

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Conflict of interest statement

Nothing declared.

are well-established, there has been increasing evidence of a significant effect of ethanol on presynaptic function at concentrations well below 100 mM, but above 17 mM, which is the legal threshold for intoxication in human (Siggins et al., 1987, Roberto et al., 2003, Nie et al., 2004, Diamond and Gordon, 1997). Recent studies suggest that ethanol may be directly affecting synaptic transmission by altering vesicle fusion and neurotransmitter release (Barclay et al., 2010), possibly by interacting with the proteins associated with the neurotransmitter release machinery. The objective of the present article is to critically review relevant data from the literature and provide a future perspective of the current research. The effects of ethanol described here are at the level of gene, protein or behavioral changes. Effects of volatile anesthetics on these proteins, wherever available, are also included, as there is a notion that alcohol and volatile anesthetics may share a common mechanism of action (Franks and Lieb, 1984, Franks and Lieb, 1985, Homanics et al., 2002, Stubbs and Rubin, 1993) and are believed to share the same binding site in ligand-gated ion channels, such as glycine and $GABA_A$ receptors (Sauguet et al., 2013).

Neurotransmitter release machinery

The neurotransmitters are released by Ca^{2+}-triggered synaptic vesicle exocytosis. Involvement of the central component proteins of the release machinery leads to membrane fusion and neurotransmitter release (Rizo, 2018, Ramakrishnan et al., 2012). Major proteins that constitute this machinery are syntaxin, Munc18, synaptobrevin, SNAP-25, synaptotagmin, Munc13, and complexin (Sudhof, 2013). First, vesicles are attached to the protein complexes at the presynaptic membrane, facilitating the contact between v-SNARE (soluble N-ethyl maleimide sensitive factor attachment protein receptor) and t-SNARE proteins. Synaptobrevin is a v-SNARE because of its vesicle localization, while syntaxin 1 and SNAP-25 are called t-SNAREs because they reside on the target membrane. Syntaxin 1, SNAP-25, and synaptobrevin-2 bind tightly with each other through their α-helices to form a core structure and bring the vesicle and plasma membrane together. Synaptotagmin 1 (Hui et al., 2011) acts as the major Ca^{2+} sensor by triggering neurotransmitter release upon its binding to membrane phospholipids and to the SNAREs and causing conformational changes within the SNARE proteins. Recycling of the SNARE and another round of vesicle fusion occurs by the dissociation of N-ethyl maleimide sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs) from the SNARE complex. The roles of Munc18–1 and Munc13–1 are to orchestrate the SNARE complex formation. Munc18–1 binds to synaptobrevin and to syntaxin 1 in its self-inhibited “closed” conformation. Munc13–1 facilitates the assembly formation by bridging the vesicle and plasma membranes. Complexins (Yang et al., 2010) play major roles in regulating this interactions. In addition, DoC2 proteins (Groffen et al., 2010) and synapsin (Pan et al., 2009) are also recognized for their involvement in the regulation of Ca^{2+}-triggered exocytosis. A simplified picture showing the involvement of several proteins are shown in Figure 1 (Maximov et al., 2009, Kaeser et al., 2011).

Syntaxin 1A

Syntaxin 1A is a member of the nervous system-specific syntaxin superfamily of proteins consisting of fifteen members in human (Teng et al., 2001). The proteins vary in their

cellular localization, tissue distributions and functions. For example, syntaxin 1(A/B) is located at the presynaptic plasma membrane of neuronal and secretory cells, but syntaxin 12 is localized in endosomes and has ubiquitous tissue distribution. While syntaxin 1(A/B) is involved in neuronal exocytosis and regulates secretion, syntaxin 12 is involved in the recycling of surface protein and early endosome fusion (Teng et al., 2001). Syntaxin is a key protein in the synaptic exocytosis process and in ion channel regulation (Vardar et al., 2016).

Syntaxin consists of a single C-terminal transmembrane domain (TMD), an α-helical SNARE domain (known as H3), a short linker region, and the N-terminal Habc domain. The SNARE domain plays an important role as both SNAP-25 and synaptobrevin dock at this domain in order to form the SNARE complex. The Habc domain, which consists of three α-helical regions, serves as an autoinhibitory domain. It associates with the SNARE domain of syntaxin inducing a “closed” state, thereby creating a physical barrier to the formation of the SNARE motif (Fernandez et al., 1998, Khvotchev et al., 2007). These domains are conserved in *Drosophila* and *C. elegans* and their sequence identity is about 66–70% with rat syntaxin 1A.

Previous studies suggest that syntaxin proteins are involved in mechanisms of alcohol action. At the gene level, a mutation in the *Syntaxin 1A* gene disrupts the capacity to acquire ethanol tolerance in *Drosophila* (Krishnan et al., 2012). Syntaxin 12, another member of the syntaxin family, is a potential contributor to ethanol preference in mice (Treadwell et al., 2004, Weng et al., 2009). The conclusion was based upon the expression and ethanol preference studies in C57BL/6J and DBA/2J inbred mouse strains using a two-bottle choice drinking paradigm. It was shown that *Stx12* mRNA levels were elevated in the brains of D2 mice as compared to B6 strain mice. Upon acute alcohol treatment, expression was reduced in D2, but not in B6. Further, *Stx12* mRNA expression was higher in the ethanol-avoiding F2 mice than the ethanol-preferring F2 mice. In a subsequent study, the authors showed that Syntaxin 12 c.*1370G>A polymorphism segregated with alcohol preference in the B6D2F2 population and syntaxin 12 expression and alcohol preference in the selected B6D2F2 males were correlated (Weng et al., 2009). Syntaxin 12 is also responsible for the recycling of AMPA receptors that mediate fast excitatory synaptic neurotransmission in the brain (Lee et al., 2001), suggesting a connection between AMPA receptor and ethanol preference. While studying the expression levels in the prefrontal cortex of human chronic alcoholics, elevated expression was observed for a synaptic vesicle protein, synaptophysin 1, but there were no significant changes in the expression levels of syntaxin 1A, SNAP-25 or VAMP (Henriksson et al., 2008).

The role of syntaxin 1 in mediating the action of general anesthetics is well-studied *in vivo* and *in vitro* (Zalucki et al., 2015, Nagele et al., 2005, Hawasli et al., 2004). The expression of a syntaxin 1A truncation mutant in the H3 domain, md130A, blocked isoflurane-mediated inhibition of neurotransmitter release in permeabilized PC12 cells (Herring et al., 2009). This was also reflected in behavioral sensitivity tests where there was significant reduction in the locomotion of *C. elegans* md130A heterozygotes upon isoflurane treatment (van Swinderen et al., 1999). This suggests that the isoflurane-syntaxin interaction influences the inhibition of the release machinery. In *Drosophila*, similar, but not identical, syntaxin mutations in the H3 domain result in strong resistance to the effects of isoflurane (Zalucki et

al., 2015). The binding of volatile anesthetics with syntaxin 1A has been characterized by NMR and CD spectroscopy and some of these anesthetics have been shown to cause structural alterations of the protein (Nagele et al., 2005).

In summary, *Syntaxin 1A* in *Drosophila* and *Stx12* gene in mice are associated with ethanol preference. In *Drosophila*, *C. elegans* and PC12 cells, syntaxin 1A is shown to mediate actions of general anesthetics.

SNAP-25

Synaptosomal-associated protein 25 kDa (SNAP-25) is a major protein of the neural SNARE complexes. Its role has been implicated in the pathology of various neurological disorders, such as Alzheimer's disease, schizophrenia, attention deficient hyperactivity disorder and epilepsy (Corradini et al., 2009, Noor and Zahid, 2017). In forming the SNARE complex, synaptobrevin, syntaxin 1 and SNAP-25 wrap around each other and form a coiled-coil quaternary structure with their α-helices.

A recent proteomic analysis of orbitofrontal cortical samples from adult male monkeys following long-term alcohol drinking showed significant increases in SNAP-25 protein expression (Nimitvilai et al., 2017). In mouse cortical neurons, treatment of 60 mM ethanol for 1 h and heat shock up-regulate *Snap25* gene expression (Varodayan et al., 2011). A more recent study found a significant decrease in SNAP-25 protein in the lateral amygdala of a male macaque with a history of heavy drinking (Alexander et al., 2018). No significant changes were observed in females. A long-term ethanol drinking paradigm (Grant et al., 2008) was used in this study.

In studying the effects of general anesthetic isoflurane on SNAP-25, it was found that isoflurane's ability to inhibit neurotransmitter release in PC12 cells was dependent on the levels of SNAP-25 and SNAP-23 (Xie et al., 2013). NMR studies show that isoflurane binds to SNAP-25 at clinical concentrations (Nagele et al., 2005).

In summary, ethanol affects the expression of SNAP-25 at the mRNA and protein levels. Proteomic analysis data indicate that protein expression is brain region- and gender-specific. Cellular experiments suggest that the effect of general anesthetics is dependent on SNAP-23/25.

VAMP

Synaptobrevins belong to the vesicle-associated membrane protein (VAMP) family of proteins. There are eight members in this family in human, VAMP 1–8. VAMP1 and VAMP2 are known as synaptobrevins and are expressed in the brain. VAMP8 is known as endobrevin and is expressed in pancreatic acinar cells. Synaptobrevins are small integral membrane proteins of secretory vesicles. Out of four α-helices of the core SNARE complex, synaptobrevin contributes one, syntaxin contributes one and the remaining two are contributed by SNAP-25.

Using culture of mouse cortical neurons treated with 10–150 mM ethanol, it was found that ethanol activated the transcription factor heat shock factor 1 (HSF1) to induce *Vamp2* mRNA expression, while *Vamp1* mRNA levels remained unaffected (Varodayan and Harrison, 2013). Ethanol and heat shock also increased VAMP2 protein levels, without affecting VAMP1 expression, which is consistent with observations at the mRNA levels (Varodayan et al., 2011). The authors suggested that these differences could be due to different location of the alcohol response element (ARE) sequence in the *Vamp1* and *Vamp2* genes and differential expression of the corresponding proteins in the CNS. *Vamp2* is expressed throughout the mouse brain, particularly in the cortex, whereas *Vamp1* predominates in regions of the diencephalon and midbrain (Varodayan and Harrison, 2013).

Using a mouse model of alcoholic pancreatitis, it has been shown that VAMP8 mediates basolateral exocytosis and its deletion induces alcoholic pancreatitis (Cosen-Binker et al., 2008).

Rab3

Rab3 is the major isoform of the Rab family of proteins known to regulate presynaptic exocytosis (Fukuda, 2008). There are seventy different Rab proteins identified in human to date. These proteins vary in their localization, membrane trafficking pathways and effector proteins (Hutagalung and Novick, 2011). For example, while Rab3A is localized in secretory vesicles and plasma membrane, Rab6 is localized in the Golgi and Rab11 is localized in the Golgi, recycling endosomes and early endosomes. While Rab3A is involved in exocytosis and neurotransmitter release, Rab6 is involved in intra-Golgi transport, endosome to Golgi and Golgi to ER trafficking. Rabs are GTPases and act as molecular switches. In the GTP-bound state, they produce docking/fusion-competent vesicles by associating with the membrane. However, upon GTP hydrolysis, the production of the competent vesicles are switched off (Grosshans et al., 2006). *Rab3*-null mice show increased synaptic depression, implicating its role in vesicle recruitment (Geppert et al., 1994a).

In *C. elegans, rab-3*-null mutants show decreased ethanol sensitivity (Kapfhamer et al., 2008, Davies et al., 2012). In the dispersion assay, in the presence of 400 mM exogenous ethanol (internal concentration, 15–20 mM), the mutant worms dispersed to a greater extent, as compared to wild-type. Also, multiple *rab-3* loss-of-function mutants moved significantly faster than wild-types. This phenotype is replicated in the loss-of-function mutant of its GTP exchange factor, *aex-3* (Kapfhamer et al., 2008). These results indicate that the reduction in ethanol sensitivity was specific to the GTP-bound state of the Rab3 protein and not just due to the loss of Rab3. Further, the role of Rab3 in ethanol resistance was confirmed by measuring the voluntary ethanol consumption of the knockout mice (Kapfhamer et al., 2008). In a two-bottle choice drinking paradigm, *Rab3A+/−*mice voluntarily consumed significantly more ethanol than wild-type or *Rab3A−/−*mice and ethanol preference was not dependent on ethanol concentration in *Rab3A+/−*mice, establishing the role of this protein in modulating the drinking behavior. The authors concluded that this resistance to ethanol sensitivity may reduce recruitment of vesicles from the reserve to the releasable pool. This study also showed that reduction of Rab3 in both *C. elegans* and mice altered the behavioral response to ethanol.

Recent proteomic analysis studies found a significant decrease in Rab3c protein in the lateral amygdala of a female macaque with a history of heavy drinking (Alexander et al., 2018) using a long-term drinking paradigm (Grant et al., 2008). No significant changes were observed in males.

To test if ethanol-impaired secretion of pituitary FSH and LH is associated with Rab proteins, Ren et al (Ren et al., 2005) studied the effect of ethanol in diet on the expression of Rab6, Rab3B, Rab11 and Rab1B in rats. It was shown that the decrease in the expression of these proteins were dependent on the time of ethanol exposure (5–60 days). However, mRNA levels of these Rab proteins were unaffected by such ethanol exposure. The authors concluded that this reduction in key Rab proteins may cause alteration in vesicle trafficking and ethanol-induced disruption of pituitary FSH and LH secretion (Ren et al., 2005).

In rat ethanol-damaged livers, Rab2's association with a Golgi compartment was significantly reduced as compared with controls, but no changes with Rab6 was observed (Larkin et al., 1996).

The effect of volatile anesthetic halothane on *rab-3* gene was studied in *C. elegans*. The results show that the worms with mutants of the *rab-3* displayed resistance to halothane (Davies et al., 2012).

In summary, Rab3 modulates drinking behaviors in mice and ethanol sensitivity in *C. elegans*. Ethanol alters expression of several Rab isoforms in rodents at the protein level. In *C. elegans*, *rab-3* regulates the actions of general anesthetics.

Munc18

Munc18–1 is a member of the Sec1/Munc18 (SM) protein family, which is critically involved in most types of intracellular membrane trafficking (Carr and Rizo, 2010, Sudhof and Rothman, 2009). There are six members in this family of proteins in humans. Total abrogation of neurotransmitter release observed in Munc18–1 knockout mice illustrates the importance of Munc18–1 in this process (Verhage et al., 2000). Munc18–1 binds tightly to syntaxin 1 in its closed conformation (Misura et al., 2000), to the syntaxin 1 N-terminal region through both the N-peptide and the Habc domain, and to the four-helix bundle of the SNARE complex (Dulubova et al., 2007, Shen et al., 2007, Xu et al., 2010).

The syntaxin binding protein 1 gene (*Stxbp1*) encodes the Sec1/Munc18-type protein and is a candidate for an ethanol preference drinking locus on mouse chromosome 2 (Fehr et al., 2005). The genetic study of two mouse strains, C57BL/6J and DBA/2J, with the former having higher ethanol preference than the latter in a two-bottle choice assays indicated a correlation with a polymorphism (D216N) in Munc18–1 (Fehr et al., 2005). The orthologous mutation (D214N) in the *C. elegans* Unc18 resulted in resistance to both the stimulatory (21 mM) and sedative effects (400 mM) of ethanol (Graham et al., 2009). These *unc-18*-null transgenics are rescued by both D214N and wild-type, producing phenotypically similar locomotion, suggesting no significant effect of this mutation in the vesicle fusion. Amperometric recording revealed that this missense mutation lengthened the duration of

quantum release and slowed the frequency of release, suggesting ethanol's interference in synaptic vesicle exocytosis (Graham et al., 2009).

D216N mutation in Munc18 protein affects its binding to syntaxin. This is neither through a closed conformation mode of interaction nor through binding to the syntaxin N-terminus. Rather, this mutant has a specific impairment in binding the assembled SNARE complex. Analysis of an alternative Munc18–1 mutation (I133V), which also affects syntaxin binding and shows similar ethanol sensitivity to the D216N, revealed the link between reduced SNARE complex binding and ethanol resistance (Figure 2). Transcriptome analysis of human brain tissue identified *STXBP1* as a hub gene in a co-expression module corresponding to lifetime ethanol consumption in humans (Farris et al., 2015).

The Rab3 binding residues in Munc18 were also studied for their alcohol sensitivity in *C. elegans* (Johnson et al., 2013). Expressing the orthologous E466K mutation (Unc-18 E465K) enhanced alcohol sensitivity, which is independent of Rab3. On the other hand, Unc-18 R39C, which decreases syntaxin binding, enhanced sensitivity to alcohol in a Rab3-dependent manner. It was also shown that the overexpression of R39C could partially suppress the reduction in neurotransmitter release in Rab3 mutant worms, whereas wild-type or E465K mutants did not show such rescue (Figure 2). The conclusion was that the epistatic interactions between Unc18 and Rab3 in modulating sensitivity to alcohol are not similar to their interactions that affect neurotransmitter release (Johnson et al., 2013).

A significant increase in the levels of Munc18 was observed in the orbitofrontal cortical samples of adult male monkeys following long-term ethanol drinking (Nimitvilai et al., 2017).

In alcoholic pancreatitis, pre-incubation with ethanol enabled low-dose cholecystokinin to displace Munc18c from basolateral plasma membrane, leading to SNARE complex assembly in the basolateral plasma membrane (Lam et al., 2007). Further, it was shown that 20 mM ethanol or submaximal or supramaximal cholecystokinin stimulation caused PKCα-mediated activation of Munc18c and triggered pathologic basolateral exocytosis in pancreatic acinar cells (Cosen-Binker et al., 2007).

In summary, the D214N polymorphism in Munc18–1 is correlated with differential drinking behavior in B6 and D2 mice. The orthologous mutation in Unc-18 develops resistance to both stimulatory and sedative effects of ethanol in *C. elegans*. Several sites of interaction between Munc18 and Rab3 and between Munc18 and syntaxin that related to neurotransmitter release are not correlated with ethanol sensitivity. Ethanol drinking increases Munc18 protein levels in the orbitofrontal cortical region of monkey brain.

Munc13–1 and Munc13–2

Munc13–1 belongs to a family of evolutionarily-conserved presynaptic active zone proteins that are essential for vesicle fusion (Betz et al., 1997, Sassa et al., 1999) and neurotransmitter release (Betz et al., 1998, Brose et al., 2000, Varoqueaux et al., 2002) in mammals. Its orthologs in *Drosophila* and *C. elegans* are called Dunc13 and Unc13, respectively. Munc13–1 is essential for fusion competence of predominantly glutamatergic

synaptic vesicles (Augustin et al., 1999). Glutamatergic neurons from Munc13–1 knockout mice show a 90% reduction in the readily releasable vesicle pool (RRP) and evoked transmitter release, even though they form an ultrastructurally normal number of synapses. This deficit of transmitter release competence in Munc13–1 knockout mice was due to a complete shutdown of the majority of glutamatergic synapses.

Munc13–1 protein interacts with both syntaxin and Munc18 proteins during synaptic vesicle fusion (Betz et al., 1997, Sassa et al., 1999). In addition to helping to open syntaxin 1, Munc13–1 also bridges synaptic vesicles and plasma membranes. Recent structure and function studies on Munc13–1 suggests that it is a master regulator of neurotransmitter release (Dittman, 2019).

Munc13–1 is a member of the Munc13 family of proteins. Munc13–2, Munc13–3 and Munc13–4 are other members known to date (Chen et al., 2013). Munc13–1 is expressed predominantly in the hippocampus, cerebellum, cortex, and striatum regions of rat brain (Augustin et al., 1999a) and modulates short-term presynaptic plasticity (Das et al., 2013, Lipstein et al., 2013) and long-term potentiation through its interactions with an active zone protein, RIM. On the other hand, Munc13–2 is expressed in rostral brain regions, including cerebral cortex and the CA region of the hippocampus. Munc13–3 is expressed exclusively in the cerebellum (Augustin et al., 1999a). Munc13–1 and 13–2 double- knockout mice result in complete abolishment of neurotransmitter release (Augustin et al., 1999, Varoqueaux et al., 2002, Aravamudan et al., 1999, Richmond et al., 1999).

Munc13–1 is a large peripheral membrane protein with a molecular weight of ~200 kDa. It consists of three C2 domains, one C1 domain, and a MUN domain. The high-affinity diacylglycerol (DAG)/phorbol ester-binding C1 domain is in between the N-terminal C2A domain and a Ca^{2+} binding C2 domain (C2B). The characteristic MUN domain connects the C2B and the C-terminal C2 domain (C2C) (Aravamudan et al., 1999, Sturm et al., 2013, Basu et al., 2005). DAG is the endogenous ligand for Munc13–1 and its binding to the C1 domain lowers the energy barrier for vesicle fusion, facilitating neurotransmitter release (Basu et al., 2007, Rhee et al., 2002). The H567K point mutation in the C1 domain abolishes the augmentation in vesicle fusion in mice and leads to death of the pups within hours after birth, despite no alteration in Ca^{2+}-evoked neurotransmitter release. These observations illustrate the importance of the C1 domain in the regulation of release and survival (Rhee et al., 2002). DAG/phorbol ester binding to the Munc13–1 C1 domain is hindered by a tryptophan side chain that blocks the phorbol ester-binding site. This tryptophan residue modulates both phorbol ester and membrane binding (Das et al., 2018).

Munc13–1 binds to ethanol and deficits in *Drosophila* ortholog Dunc13 lead to defects in alcohol sensitivity, tolerance and self-administration in *Drosophila* (Das et al., 2013, Xu et al., 2018). Using a two-choice CAFÉ assay, it was shown that Dunc13$^{P84200/+}$ heterozygotes display increased ethanol preference compared to the wild type. Synaptic vesicle release in excitatory neurons downstream of Ca^{2+} influx into the active zone in *Drosophila* is impaired by ethanol, and a reduction in *Dunc13* in Dunc13$^{P84200/+}$ produces resistance to the sedative effects of ethanol both behaviorally and physiologically. It was shown that the binding of ethanol to the C1 domain of Munc13–1 at intoxicating concentrations reduces the binding of

its endogenous ligand, diacylglycerol (DAG), which will reduce the activity of Munc13–1 and should lead to presynaptic inhibition. The authors argued that this reduction of Munc13–1/Dunc13 activity will be similar to the genetic reduction of *Dunc13*, suggesting a possible role of Dunc13 in the development of ethanol tolerance (Xu et al., 2018). However, these effects were not observed in mice that were treated with ethanol using the drinking-in-the-dark paradigm (Wooden et al., 2019), suggesting that the effects of ethanol on Munc13–1/Dunc13 were species-dependent. Alcohols bind to the Glu-582 residue of the C1 domain of Munc13–1 (Figure 3). Glutamic acid is also known to bind alcohol in proteins, such as Glu-262 in the acetylcholine receptor (Pratt et al., 2000); Glu-33 in the L1 cell adhesion molecule (Arevalo et al., 2008); Glu-163 and Glu-193 in the Rho GDP dissociation inhibitor (Ho et al., 2008); Glu-146 in lignin peroxidase (Ambert-Balay et al., 1998); and Glu-13 in pepsin (Andreeva et al., 1984). The glutamate residue is likely to form a hydrogen bond with the ethanol molecule and mutating this residue with alanine reduces the alcohol binding (Das et al., 2013, Xu et al., 2018). Structural studies of the LUSH protein–alcohol complex highlighted the importance of hydrogen bonding between the alcohol molecule and the alcohol binding residue. In LUSH, the odorant-binding protein in *Drosophila*, the hydroxyl group of alcohol forms hydrogen bonds with Thr-57, the replacement of which by alanine completely abolished alcohol binding (Kruse et al., 2003, Thode et al., 2008). The alcohol-binding residue in PKCδ C1B, a structural homolog of Munc13–1 C1 is Tyr-236 (Das et al., 2004, Shanmugasundararaj et al., 2012).

In Munc13–1 C1, the Glu-582 residue is about 4.8Å from the nearest Zn^{2+} that coordinates with three cysteine and one histidine residues, indicating a possible role of Zn^{2+} in the alcohol binding to this protein (Figure 3). It has been demonstrated that 25 mM ethanol inhibited binding of DAG to the Munc13–1 C1 domain in the presence of 5 mM $ZnSO_4$. A similar role of Zn^{2+} has also been implicated in the alcohol binding for GABA (Aguayo and Alarcon, 1993, Frye et al., 1996) and Glycine receptors (Laube et al., 2000, McCracken et al., 2010, McCracken et al., 2013b, McCracken et al., 2013a).

The McCool group studied the role of Munc13–2 on ethanol-anxiety interaction by measuring glutamate release in the basolateral amygdala, which contributes to both the anxiolytic effect of ethanol intoxication and the anxiogenic effects of ethanol withdrawal. In comparing two different strains of mice, first they found that C57BL/6J mice expressed substantially higher levels of Munc13–2 as compared with the DBA/2J strain, whereas expression of several release-related proteins, including Munc13–1, was equivalent (Gioia et al., 2016). Using shRNA, they knocked down the expression of Munc13–2 in the mPFC terminal within the BLA of C57BL/6J, whose glutamate terminals are normally ethanol-insensitive. This manipulation made the glutamate terminals ethanol-sensitive, which is generally seen for the glutamate terminals of DBA/2J mice. Ethanol inhibition of vesicle recycling, releasable pool recovery and post-tetanic potentiation following high-frequency stimulation were dependent on the levels of Munc13–2, suggesting that Munc13–2 is involved in the ethanol-anxiety interactions (Gioia et al., 2017).

In a drinking-in-the-dark paradigm, Munc13–1 was found to be upregulated in the hippocampus, which is associated with memory (Ghosh et al., 2017). Recent proteomic

analysis studies found a significant decrease in Munc13–2 in the lateral amygdala of macaque with a history of heavy drinking (Alexander et al., 2018).

The effect of general anesthetic, isoflurane on Unc13 was studied using locomotion assays and global level of neurotransmitter assays on adult *C. elegans*. It was found that *unc-13* loss-of-function mutants were highly resistant to general anesthetic isoflurane. It was also found that isoflurane decreased DAG-mediated synaptic localization of Unc13 (Metz et al., 2007).

In summary, alcohol binds to the C1 domain of Munc13–1. Reduction of *Dunc13* increases ethanol self-administration and develops tolerance in *Drosophila*. Reduction of *munc13–1* in mice, however did not affect these behaviors. In *C. elegans*, Unc13 mediates the action of general anesthetics.

Complexin 2

Complexins are small, soluble proteins that play major roles in neurotransmitter release (McMahon et al., 1995). A marked decrease in evoked release was observed upon reducing the levels of complexin 1 and complexin 2, the two major mammalian isoforms (Huntwork and Littleton, 2007, Martin et al., 2011, Maximov et al., 2009, Reim et al., 2001). Also, a decrease in vesicle priming was observed upon knockout or knockdown of these two isoforms (Xue et al., 2010, Yang et al., 2010). Complexin's role has also been implicated in the etiology or pathogenesis of several CNS diseases, such as schizophrenia, Huntington's disease, depression, bipolar disorder, Parkinson's disease, Alzheimer's disease, and also in fetal alcohol syndrome disorders (Brose, 2008).

Proteomic analysis suggests a significant decrease in complexin 2 in the lateral amygdala of a male macaque with a history of very heavy drinking (Alexander et al., 2018). A long-term ethanol self-administration method of drinking was used in this study (Grant et al., 2008).

Synaptotagmin 1

Synaptotagmin 1 is a member of the synaptotagmin family of proteins and acts as a Ca^{2+} sensor in synaptic exocytosis and other types of Ca^{2+}-evoked secretion (Brose et al., 1992). There are fifteen members in this family. Synaptotagmin 1 is expressed in the forebrain, midbrain, and in most brainstem and spinal cord neurons (Xu et al., 2007). Impairment in the fast synchronous component of evoked excitatory postsynaptic currents (EPSCs) was observed in hippocampal neurons of synaptotagmin 1-deficient mice (Geppert et al., 1994b). However, the reduction of inhibitory post synaptic currents (IPSCs) was observed in cortical neurons of the same animals (Xu et al., 2007). In mouse hippocampal cultures, overexpression of synaptotagmin 1 increases the probability of evoked vesicle release (Han et al., 2004). Synaptotagmin has been shown to be a novel biomarker for Alzheimer's disease (Ohrfelt et al., 2016).

In a microarray screen, *Syt1*, encoding synaptotagmin 1 protein, was identified as an alcohol-responsive gene (Pignataro et al., 2007). Ethanol induces synaptotagmin 1 expression in mouse neurons via activation of heat shock factor 1 (Varodayan et al., 2011).

Cortical neurons were exposed to varying concentrations of ethanol for a specified time period (15 min-24 h) and upregulation of both mRNA and protein was observed in a rapid and robust manner. Ethanol also altered the distribution of the synaptotagmin 1 protein along neuronal processes as shown by the increase in the number and size of the synaptotagmin 1-positive puncta per 100 μm of neurite length as compared to the control. The authors concluded that this may be a mechanism by which ethanol could affect neurotransmitter release.

Synapsin

Synapsins are phosphoproteins that bind to synaptic vesicles and to actin. They also bind to ATP, and show structural similarity to ATP-utilizing enzymes (Song and Augustine, 2015, Hilfiker et al., 1999). The mammalian genome contains three synapsin genes encoding synapsin I, synapsin II and synapsin III, each having two different isoforms. *Drosophila*, however, have a single copy of the gene. Numerous *in vitro* studies suggest synapsins' involvements in several neuronal processes, such as neurite elongation, synaptogenesis, synaptic maturation, regulation of synaptic plasticity and neurotransmitter release (Ferreira et al., 1995, Han and Greengard, 1994).

Using a *Drosophila* knockout for all synapsin isoforms, the role of these proteins have been implicated in the development of tolerance to ethanol (Godenschwege et al., 2004). In this study, flies were exposed to 50% ethanol vapor in a vertical tube until the loss of postural control was observed. Initially, both the knock-outs and the wild-type flies displayed similar sensitivity to ethanol. However, upon a second exposure to intoxicating levels of ethanol, the knock-outs of synapsins took longer time for the loss of postural control, suggesting the role of synapsins in ethanol tolerance.

Dynamin

Dynamin is a ~100 kDa GTPase responsible for endocytosis in the eukaryotic cell (Jimah and Hinshaw, 2018) and is implicated in membrane vesicle scission including synaptic vesicle recycling (Praefcke and McMahon, 2004). During clathrin-mediated endocytosis, dynamin binds to and assembles around the neck of the endocytic vesicle (Morlot and Roux, 2013). In mammals, three different dynamins are known to date. While Dynamin-2 is ubiquitous, Dynamin-1 is predominantly expressed in the presynaptic compartment of the neurons. Dynamin-3, on the other hand, is expressed in the postsynaptic compartment and in testicular tissue.

Microarray analysis of 5000 genes in the dorsal hippocampus of rats treated with 12% ethanol (v/v) for fifteen months showed down-regulation of the dynamin-1 gene (Saito et al., 2002). In *Drosophila*, it has been found that functional dynamin protein activity is required for acquisition of tolerance during ethanol intoxication, although ethanol did not alter the expression level of the single copy of the dynamin gene, *shibire* (Krishnan et al., 2012).

Summary and future perspectives

Ethanol's impact on the proteins involved in the neurotransmitter release machinery has been summarized in Table 1 and the key points are: (1) syntaxin 1A, Dunc13, synapsins and Dynamin 1 contribute to the development of tolerance. (2) protein-protein interactions involving syntaxin, Unc18 and Rab3 are important for ethanol sensitivity. (3) Syntaxin 1A, Dunc13, Rab3 and Munc18 mediate alcohol drinking behavior. (4) Ethanol upregulates expression of syntaxin 1b, synaptophysin 1, SNAP-25, synaptotagmin 1, Munc13–1, Vamp-2 and Munc18 either at the mRNA or protein level. (5) Ethanol downregulates the expression of Rab3b, Rab3c, Rab6, Rab11, Munc13–2 and complexin 2 either at the mRNA or protein level. (6) Ethanol's effect on protein expression are brain region- and gender-specific. (7) Ethanol sensitivity is species-dependent. (8) Ethanol's effects are protein isoform-specific, as in Munc13–1/2 and VAMP-1/2.

Now, based on these findings, will it be possible to draw some sort of general principles on ethanol's effect on presynaptic function? Clearly, ethanol has effects (expression, localization and function) on several proteins involved in vesicle priming, release and recycling. However, the major issue here is the heterogeneity of the data, meaning that some effects are species-specific, gender-specific, brain region-specific or protein isoform-specific. For example, while Dunc13 heterozygous flies showed higher ethanol preference as compared to wild-type, there were no differences in drinking preference between Munc13–1 heterozygous and wild-type mice. For Dynamin-1, ethanol down-regulated the gene in rats, but it did not do so in flies. For Rab3, reduction of the protein increased ethanol sensitivity in *rab-3* heterozygous *C. elegans* and increased ethanol preference in heterozygous mice, but not in homozygous mice. At the level of protein-protein interactions, both E466K and R39C mutants of Unc18 that affect Rab3 binding are ethanol-sensitive. Whereas ethanol sensitivity in E466K is independent of Rab3, ethanol sensitivity in the R39C requires Rab3. Therefore, ethanol's effect on presynaptic function at an organismal level is complex and will reflect the time-dependent integration of all these effects. In mammals, added complexities arise from gender-differences and the presence of multiple isoforms of a single protein having differential effects. However, the identification of genes important for ethanol responses should be the focus of newer studies, rather than the directionality or magnitude of an ethanol phenotype.

Studies on the structure and function of the exocytosis machinery proteins by itself is a fascinating area of current research, wherein the exact role of some of these proteins are still under investigation. Elucidating the impact of ethanol on these proteins bears additional significance because it may provide a mechanism by which a relatively low affinity drug molecule like ethanol could bring about changes in the neurotransmitter concentrations in the presynaptic compartment, leading to the changes in the brain and behavior. The microarray analysis identified some of the alcohol-sensitive genes; proteomic analysis identified expression levels of some of these proteins; electrophysiology measurements quantified the changes in neurotransmitter release; mutational analysis provided important information on the protein-protein interactions; and knockouts were used for studying the behavioral changes. While these studies establish ethanol's role in the neurotransmitter release in the presynaptic compartments, literature data are very diverse, sketchy and

piecemeal. Therefore, more systematic studies are required to understand the mechanism of ethanol's action in a region-specific, neuron-specific, and neuro-circuitry-specific manner in the brain. Most of the studies reported in this article are based on invertebrate model systems, such as *Drosophila* and *C. elegans*. It is critical to conduct these experiments in the vertebrate system, in a gender-specific manner, to correlate the findings from model systems to human alcoholism. The binding affinity of ethanol for the proteins that directly bind it, should be measured in isolation and in the milieu (in association with other components of the SNARE complex), which will provide insights as to how protein-protein interaction can modulate alcohol affinity. Some of these proteins are shown to exist in different isoforms and each isoform should be studied for its role in ethanol action. Furthermore, elucidating the structure of the ethanol-protein complex can provide useful knowledge on the microenvironment of the alcohol binding site that could be useful in designing drugs for alcohol addiction.

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Abbreviations:

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BLA	basolateral amygdala
CA	Cornu Ammonis
CD	circular dichroism
DAG	diacylglycerol
DoC2	double C2 domain protein
EPSC	excitatory postsynaptic current
FRET	fluorescence resonance energy transfer
FSH	follicle stimulating hormone
GABA	gamma amino butyric acid
GTP	guanosine triphosphate
H3	syntaxin SNARE domain
Habc	syntaxin regulatory domain
Hsc70	heat shock 70 kDa protein 8
IPSC	inhibitory postsynaptic current

LH	luteinizing hormone
NMR	nuclear magnetic resonance
PKC	protein kinase C
PFC	prefrontal cortex
SNARE	soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
v-SNARE	vesicle SNARE
t-SNARE	target SNARE
NSF	N-ethylmaleimide sensitive fusion protein
SNAP	soluble N-ethylmaleimide sensitive fusion protein attachment protein
SNAP-25	synaptosomal-associated protein of 25 kDa molecular mass
SNAP-23	synaptosomal-associated protein of 23 kDa molecular mass
SM	Sec1/Munc-18 proteins
RRP	readily-releasable pool of vesicles
TMD	trans membrane domain

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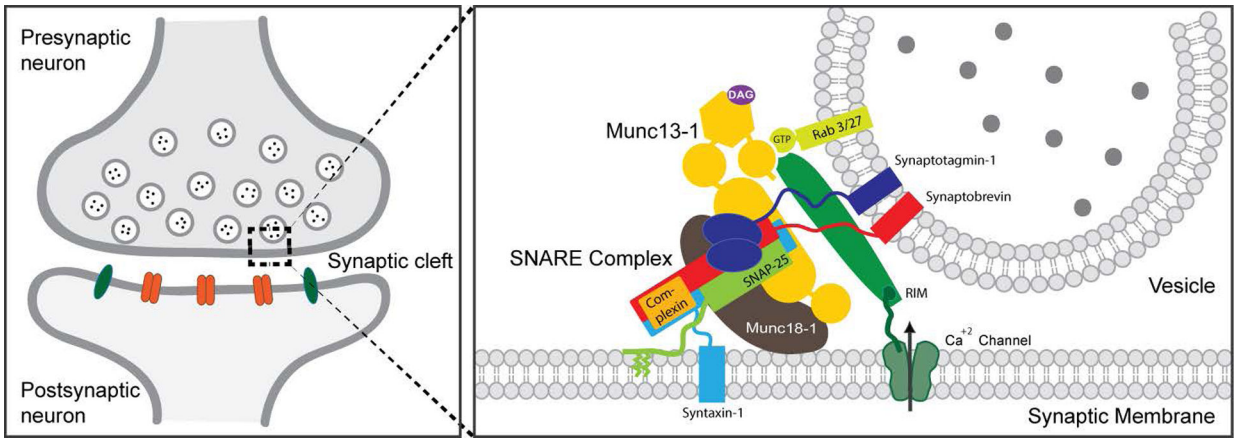

Figure 1:

A simplified model showing the active zone proteins involved in vesicle priming and fusion in mammals. Some of the proteins shown are RIM, Munc13, Ca^{2+} channels, SNAP-25, syntaxin 1, Munc18–1, complexin, Rab3 and synaptotagmin 1. Munc13 is mammalian homolog of Unc13 in nematode worm *C. elegans* and Dunc13 in *Drosophila*. Munc18 is the mammalian homolog of Unc18 in *C. elegans* and *Drosophila*.

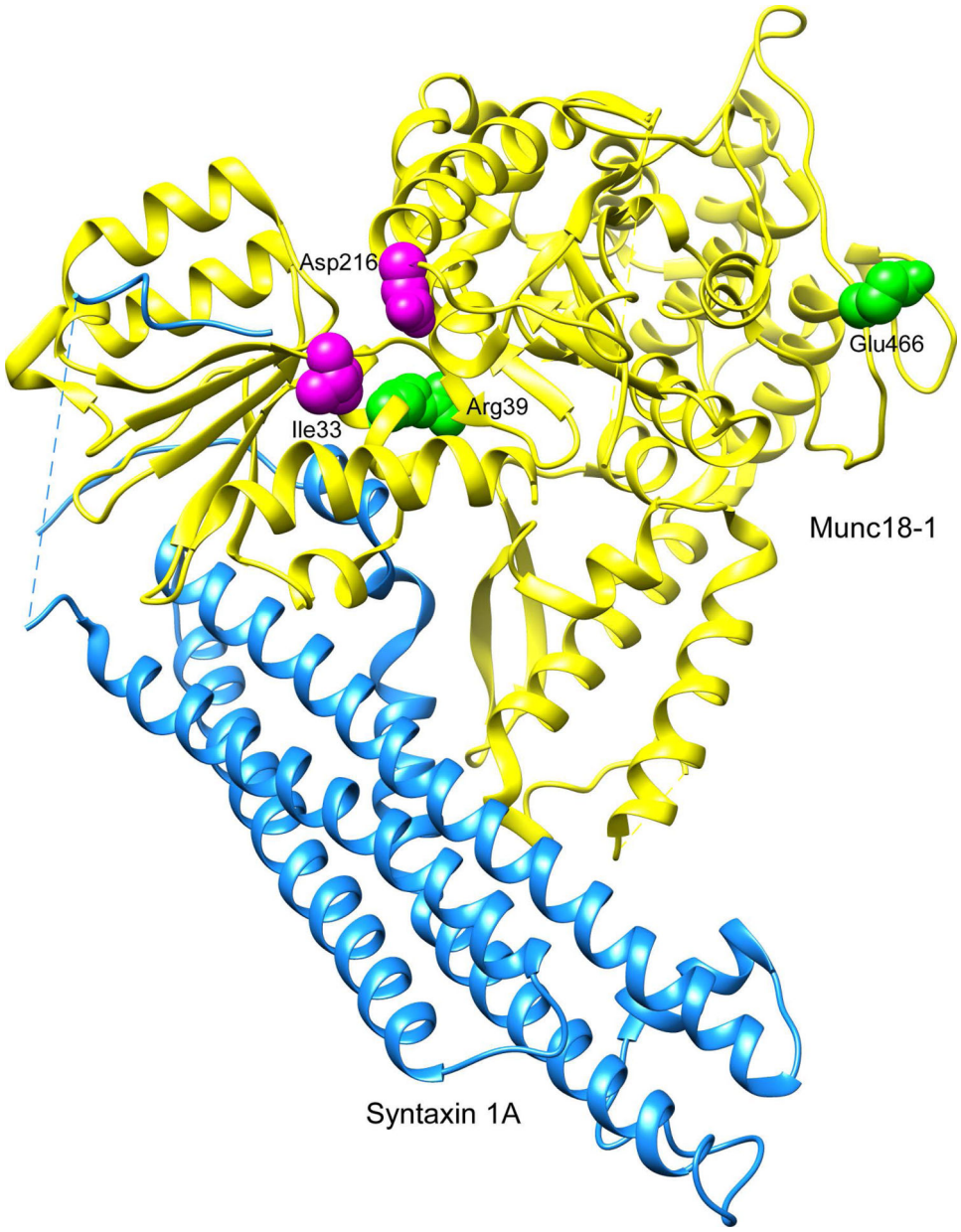

Figure 2:

Location of Munc18–1 residues responsible for ethanol sensitivity. Asp216 and Ile133 shown in magenta interact with syntaxin and mutations at these sites in *C. elegans* make them resistant to the stimulatory and sedative effects of acute ethanol. Arg39 and Glu466 (465 in Unc18) shown in green interact with Rab3. In comparison to wild-type Unc18, both the R39C and E465K mutants show increased sensitivity to acute ethanol. The residues between Arg9 and Arg27 are represented as a dashed line to reflect the disorder in the crystal structure. The model was built using the crystal structure of the Munc18–1/syntaxin 1A complex (PDB ID: 3C98).

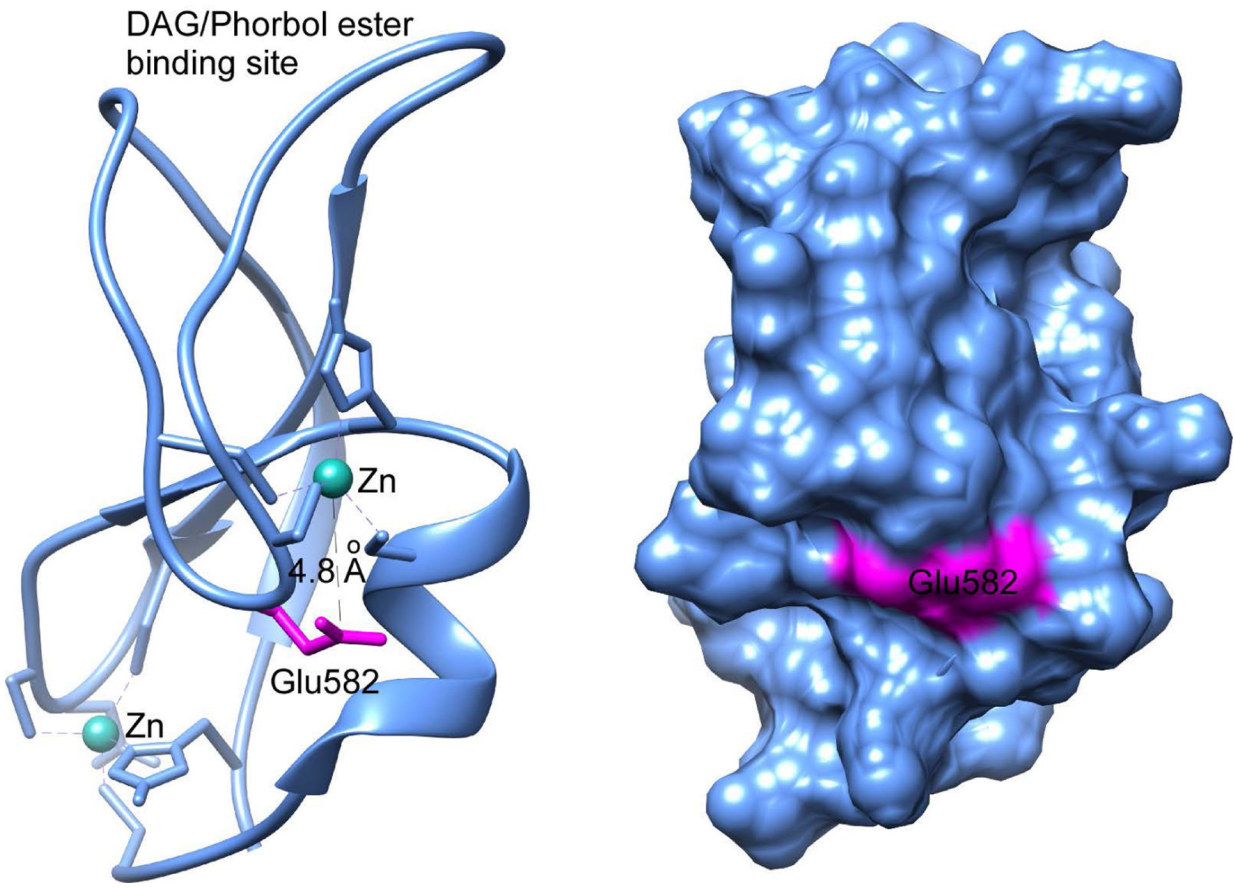

Figure 3:

Location of the alcohol binding residue in Munc13–1 C1. Photolabeling and mass spectrometric analysis identified Glu582 as the alcohol-binding residue, which is shown in magenta. The ribbon (left) and surface (right) diagrams were generated using the NMR structure of Munc13–1 C1 (PDB ID: 1Y8F).

Table 1:

Effect of ethanol/anesthetics on the SNARE complex associated proteins

SNARE Protein	Gene	Alcohol/ anesthetic	Mode of action	Reference
Syntaxin 1A/Syntaxin 12	*Syntaxin 1A*	Ethanol	Tolerance to ethanol in *Drosophila*	Krishnan et al., 2012
	Stx12	Ethanol	Ethanol preference in mice	Treadwell et al., 2004; Weng et al., 2009
	Stx12	Ethanol	Protein expression level in mice	Weng et al., 2009
	Unc-64	Isoflurane and halothane	Resistance to anesthesia in *C. elegans*	van Swinderen et al., 1999
	Syntaxin 1A	Isoflurane	Resistance to anesthesia in *Drosophila*	Zalucki et al., 2015
	Stx1a	Isoflurane and halothane	*In vitro* protein binding	Nagele et al., 2005
SNAP-25	*Snap25*	Ethanol	Protein expression in monkey brain	Nimitvilai et al., 2017; Alexander et al., 2018
	Snap25	Ethanol	Gene expression in mice neuron	Varodayan et al., 2011
	Snap25	Isoflurane	Protein expression and neurotransmitter release in PC12 cells	Xie et al., 2013
		Isoflurane	*In vitro* protein binding	Nagele et al., 2005
VAMP2 (Synaptobrevin)	*Vamp2*	Ethanol	Protein and gene expression level in mice cortical neuron	Varodayan et al., 2011; Varodayan and Harrison, 2013
Rab3	*RAB3C*	Ethanol	Protein expression in monkey brain	Alexander et al., 2018
	rab-3	Ethanol	Changes in locomotion in *C. elegans*	Kapfhamer et al., 2008; Johnson et al., 2013; Davies et al., 2012
	RAB3A	Ethanol	Drinking behavior in mice	Kapfhamer et al., 2008
	rab-3	Halothane	Changes in locomotion in *C. elegans*	Davies et al., 2012
Unc18/Munc18	*unc-18*	Ethanol	Protein-protein interaction in behavioral changes in *C. elegans*	Graham et al., 2009; Johnson et al., 2013
	Stxbp1	Ethanol	Changes in gene expression in mice	Fehr et al., 2005
	Munc-18	Ethanol	Changes in protein expression in monkey brain	Nimitvilai et al., 2017
Unc13/Dunc13/Munc13	*unc-13*	Isoflurane	Resistance to anesthesia and synaptic localization in *C. elegans*	Metz et al., 2007
	Dunc13	Ethanol	Changes in ethanol preference and tolerance in Drosophila	Das et al., 2013; Xu et al., 2018
	munc13–1	Ethanol	Protein expression in mice brain	Ghosh et al., 2017
	munc13–1	Ethanol, butanol, octanol	*In vitro* protein binding	Das et al., 2013; Xu et al., 2018
	munc13–2	Ethanol	Protein expression in monkey brain	Alexander et al., 2018
Complexin 2	*CPLX2*	Ethanol	Protein expression in monkey brain	Alexander et al., 2018
Synaptotagmin 1	*Syt1*	Ethanol	Changes in gene expression in mice	Pignataro et al., 2007
	Syt1	Ethanol	Change in gene and protein expression in mice	Varodayan et al., 2011
Synapsin	*Syn*	Ethanol	Tolerance to ethanol in *Drosophila*	Godenschwege et al., 2004
Dynamin 1	*shibire*	Ethanol	Tolerance to ethanol in *Drosophila*	Krishnan et al., 2012