Presynaptic targets for acute ethanol sensitivity

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Abstract

Acute exposure to ethanol is known to modulate signalling within the nervous system. Physiologically these effects are both presynaptic and postsynaptic in origin; however, considerably more research has focused primarily on postsynaptic targets. Recent research using the model organism *Caenorhabditis elegans* has determined a role for specific proteins (Munc18-1 and Rab3) and processes (synaptic vesicle recruitment and fusion) in transducing the presynaptic effects of ethanol. In the present paper, we review these results, identifying the proteins and protein interactions involved in ethanol sensitivity and discuss their links with mammalian studies of alcohol abuse.

Introduction

Alcohol is the most used and abused substance in the world and drunkenness is correlated with a host of social problems. Despite the prevalence in its use, the exact physiological and molecular mechanisms underlying intoxication and alcohol abuse remain poorly understood. Numerous studies offer solid evidence that there are significant genetic components for susceptibility to alcoholism [1,2]. One particularly important genetic determinant is an individual's initial sensitivity to alcohol which contributes ~60% of the variability due to genetic makeup [1,3]. Acute exposure to alcohol has concentration-dependent effects on the nervous system where low concentrations evoke hyperactivity, whereas high concentrations can elicit motor unco-ordination, sedation and eventually death. Increasing our understanding of the molecular mechanisms for intoxication could prove indispensable to our understanding of addiction. As sensitivity to ethanol affects the behaviour of mammals and invertebrates in a similar dose-dependent fashion [4,5], this then has permitted the use of genetically driven investigations into the cellular/molecular targets of alcohol using model organisms.

The synapse as a substrate for the effects of ethanol has been reviewed in detail [6–8]. Original electrophysiological research indicated effects of exogenous ethanol on neuromuscular transmission [9–11], without necessarily specifying a pre- or postsynaptic site of action. Neural effects of ethanol are not simply due to an increase or decrease in synaptic efficacy, as ethanol has been shown to affect synaptic transmission in either direction depending on the experimental model (for example [12,13]). As such, mutations that increase or decrease efficacy do not automatically alter ethanol sensitivity. This then indicates a more specific effect of ethanol on an individual protein's function, protein–protein interactions or physiological processes. Postsynaptically, ethanol modulates the function of a number of neurotransmitter receptors, principally GABA (γ-aminobutyric acid), but also glutamate and serotonin [2]. In addition, other effectors have been implicated in ethanol phenotypes including the postsynaptic actin regulator Eps8 [14] and a number of signalling molecules such as the calcium activated potassium channel *slo-1* [4], protein kinase A [5] and protein kinase C [15]. In contrast, little is known about any effectors for ethanol at the presynaptic terminal, outside of $GABA_B$ (type B GABA) receptor signalling [16]. Recent work using the model organism *Caenorhabditis elegans*, however, has positively identified specific presynaptic targets for ethanol and begun to tease apart genetically the action of ethanol in the modulation of exocytosis.

Key words: alcohol, *Caenorhabditis elegans*, exocytosis, Munc18-1, Rab3, UNC-18.
Abbreviations used: GABA, γ-aminobutyric acid; SM, Sec1/Munc18; SNARE, soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor; VA, volatile anaesthetic.
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Regulated exocytosis

Exocytosis is the process of membrane–membrane fusion that occurs between vesicles and the plasma membrane and the subsequent release of vesicle cargo outside of the cell. In a presynaptic neuron, fusion of neurotransmitter-filled vesicles with the active zone membrane following a stimulated elevation in intracellular calcium underlies the process of synaptic transmission. Considerable information has accumulated describing the sequential steps and molecular machinery involved in regulated exocytosis [17,18] and will not be discussed here in detail. Briefly, vesicles are first formed either by endocytosis from the plasma membrane or by *de novo* synthesis and are then packaged with their secretory contents. At the synapse, these vesicles are then held in reserve pools away from the synaptic release site before being recruited and correctly trafficked to the active zone. Once at the plasma membrane, vesicles are first loosely tethered and then tightly docked with the membrane, before a series of biochemical reactions occur that 'prime' the vesicles to be in a releasable state. Finally, on an increase in intracellular calcium, the two membranes fuse allowing the controlled release of neurotransmitter into the synaptic cleft. As ethanol can have variable results on synaptic efficacy, it has been unclear

which proteins or processes within the presynaptic nerve terminal are affected by ethanol and how this modulation is achieved.

Molecular targets for ethanol: vesicle trafficking proteins

Rab proteins are a large evolutionarily conserved family of GTPases associated with vesicles at all steps during intracellular membrane trafficking [19]. Rab3 is the major Rab isoform involved in regulated presynaptic exocytosis and is implicated in the recruitment of synaptic vesicles into the releasable pool and the subsequent loose tethering with the plasma membrane [20]. Rabs act as molecular switches; when bound to GTP, Rab proteins are associated with the vesicle membrane, producing vesicles that are physiologically competent for docking/fusion. GTP hydrolysis then acts to switch off docking/fusion competence. *Rab3*-null mice display increased synaptic depression [21] illustrating the positive role of Rab3 in vesicle recruitment. In *C. elegans*, null alleles of *rab-3* exhibit only mild phenotypic deficiencies [22]. Mutants exhibit a slower loopy locomotion, indicating an effect on central nervous system signalling, and resistance to aldicarb, demonstrating a reduction in cholinergic release at motor neuron terminals. During a recent screen for altered response to ethanol of existing *C. elegans* mutants with synaptic defects, *rab-3*-null mutants were identified as having decreased ethanol sensitivity [23]. Mutant worms dispersed to a greater extent in the presence of a depressive concentration of ethanol in comparison with wild-type controls. Direct comparison of locomotion rates demonstrated that multiple *rab-3* loss-of-function mutants moved significantly faster in depressive concentrations of ethanol than wild-types, a phenotype that was replicated by a loss-of-function mutant of its GTP exchange factor, *aex-3* [23]. These results indicate that the reduction in ethanol sensitivity was specific to the biochemical GTP-bound state of the Rab protein and not simply due to the loss of Rab3. Therefore, resistance to high levels of ethanol appears to involve a reduction in the recruitment of vesicles from the reserve to the releasable pool. A link between loss of Rab3 and ethanol resistance was subsequently confirmed in knockout mice and, interestingly, these mice were shown to have an increase in voluntary ethanol consumption [23].

Rab proteins are thought to achieve their function via interactions with various effector proteins [18–20]. The best characterized effectors include those proteins involved with initial tethering [HOPS (homotypic fusion and vacuole protein sorting) complex, exocyst] and motor proteins involved in organelle movement within the cell. A newly identified Rab3 effector is the synaptic protein synapsin. Synapsins are neuronal-specific phosphoproteins associated with synaptic vesicles via multiple interactions with both the phospholipid and protein components of the vesicle [24]. As the proposed role for synapsins, to preserve the reserve pool of vesicles within the presynaptic terminal, is intimately associated with vesicle recruitment, it is perhaps not surprising that Rab3 is an interacting partner. Rab3 and synapsin directly interact [25] and the presence of synapsin I stimulates both GTP binding and GTPase activity of Rab3 [26]. Consistent with the observed increase in GTPase activity is a reduction in synaptic vesicle associated Rab3 in synapsin I-null mice [26]. A recent study using a *Drosophila* knockout for all synapsin isoforms also implicated this protein in developed tolerance to ethanol [27]. Here, adult flies were exposed to 50% ethanol vapour until the loss of postural control in a vertical tube occurred. In comparison of the time with postural loss for wild-type and synapsin-nulls, initial sensitivity to ethanol vapour was unaffected. Upon a second exposure, however, there was a significant increase in the time for ethanol to take effect for the synapsin-null flies. Therefore studies using two separate model organisms point to recruitment of vesicles to the releasable pool being a major process affected by ethanol presynaptically.

Molecular targets for ethanol: Munc18-1 and SNAREs (soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptors)

SM (nSec1/Munc18-1) proteins are evolutionarily conserved cytosolic proteins essential to all vesicle fusion steps within cells, including synaptic vesicle fusion [18,28]. This is indeed true for *C. elegans* where *unc-18*-null worms are mostly paralysed with limited, unco-ordinated locomotion [29] and have a large reduction in both their evoked and spontaneous neuromuscular transmission [30]. Although the exact function of Munc18-1 in vesicle fusion remains controversial, it is presumed to involve its strong interaction with the SNARE protein syntaxin-1. Munc18-1 is known to interact biochemically with syntaxin in three distinct, yet interrelated, forms of binding [31]. The first interaction occurs when syntaxin adopts a closed conformation and sits within the pocket of the arched structure of Munc18-1 (here designated Mode 1 binding). This interaction precludes syntaxin from interacting with the other SNAREs and presumably acts as an inhibitor to vesicle fusion. Mutations inhibiting Mode 1 binding have impaired neuromuscular transmission in *Drosophila* [32], altered synaptic vesicle fusion in mice [33] and altered individual fusion kinetics in adrenal chromaffin cells [34,35]. Munc18-1 also interacts in a 1:1 binding reaction with open conformation syntaxin (Mode 2 binding) via syntaxin's N-terminal domain [36] as seen for some yeast SM proteins. This interaction, at least in *C. elegans*, has been shown to be essential for the protein's function as transgenic mutants with inhibited N-terminal binding are phenotypically indistinguishable from the null mutant [37]. In contrast, *C. elegans* transgenic mutants with inhibited closed conformation binding are behaviourally identical to wild-type worms [37]. The final interaction between Munc18-1 and syntaxin occurs while syntaxin is within the assembled SNARE complex (Mode 3 binding). *In vitro* lipid fusion assays show that this interaction acts to accelerate membrane fusion similar to

that seen with yeast Sec1 [38]. A recent report alternatively suggests that the interaction affects synaptic vesicle priming upstream of the membrane fusion step itself [39].

We showed recently that a D216N point mutation in Munc18-1 specifically reduced Mode 3 binding to the SNARE complex [40]. Using a variety of binding assays, we demonstrated that this specific point mutation did not alter the binding affinity of Munc18-1 with either closed conformation syntaxin or its N-terminal domain. The mutation did inhibit binding to the assembled SNARE complex as shown by two distinct assays. Furthermore, immunoprecipitation studies showed that this inhibition resulted in a reduction in the amount of syntaxin bound to Munc18-1 expressed in PC12 cells. This D216N mutation in mammalian Munc18-1 was first isolated as a natural polymorphism between two mice strains with differing phenotypes in response to ethanol [41]. The mutation was then statistically correlated with a number of behavioural differences evident between the strains, including withdrawal effects following removal from ethanol, g/kg consumption of ethanol and preference for ethanol in a two-bottle choice paradigm. Obviously, statistical correlations of phenotypic effects between two inbred mice strains could potentially be the result of other genetic differences. Indeed, changes in brain mRNA levels between the two strains were assessed by microarray analysis and the expression of a large number of other transcripts were found to be altered [41]. Transcripts with changes in expression included both potassium channel modulators and calcium activated proteins, such as NCS-1. As the calcium-activated potassium channel *slo-1* is activated by ethanol in *C. elegans in vivo* [4] and shows alcohol-induced post-transcriptional reorganization of its splice variants in mammals [42], it is certainly possible that the differences in ethanol phenotypes of the two mice strains could be due to something other than the Munc18-1 polymorphism.

We therefore verified a specific role for the D216N mutation in ethanol sensitivity *in vivo* by expressing the mutation in an otherwise isogenic background. *unc-18*-null *C. elegans* were genetically rescued either with the wild-type protein or the orthologous mutation (D214N) and direct phenotypic comparisons were made between multiple independently derived rescue strains. To avoid gross overexpression or mislocalization, the rescuing transgene utilized the endogenous *unc-18* promoter. Transgenic rescues with either the wild-type or D214N mutant created phenotypically normal worms, indicating a lack of severe effect on vesicle fusion [40]. Quantification showed that the mutant worms moved slightly faster than wild-type rescues; however, this was due to alterations in the co-ordinated motor activity governing locomotion rather than a gross change in the end-point neuromuscular junction signalling as sensitivity to aldicarb was identical for mutant and wild-type worms. Analysis of ethanol sensitivity revealed that the D214N point mutation in UNC-18 greatly reduced the phenotypic effects of ethanol on co-ordinated locomotion [40]. At low concentrations, ethanol acts as a stimulant inducing hyperactivity and this increase was completely absent in D214N expressing worms. At high concentrations ethanol acts as a sedative and D214N mutants had a reduced sensitivity to the effects of ethanol to depress co-ordinated locomotion. Another independently derived *unc-18* mutant (I133V) was also found to have inhibited SNARE complex binding and reduced sensitivity to ethanol, but lacked the hyperactive locomotor phenotype [40].

The phenotypic effects of the D214N UNC-18 mutation then may be a direct result of its altered interaction with proteins of the assembled SNARE complex. There have been no studies looking at mutations in SNARE proteins and ethanol sensitivity; however, loss-of-function mutants in *C. elegans* syntaxin (UNC-64) are resistant to the depressive effects of VAs (volatile anaesthetics) such as halothane or isoflurane on co-ordinated locomotion [43]. Addition of VAs reduces the EC_{50} of animals using a radial dispersion assay. By screening existing *C. elegans* mutants, some UNC-64 mutations dominantly conferred VA resistance, whereas other mutations in UNC-64 and the other SNARE proteins VAMP (vesicle-associated membrane protein; SNB-1) and SNAP-25 (25 kDa synaptosome-associated protein; RIC-4) were VA hypersensitive [43]. Although it could be hypothesized that anaesthetics and ethanol may affect the same molecular targets, it is important to note that neither *rab-3* nor *aex-3* loss-of-function altered VA sensitivity [43]. This could indicate that VAs and ethanol affect, at least partially, distinct molecular targets or simply that the different assays have distinct sensitivities. In support of this hypothesis, a loss-of-function mutation in *slo-1* was originally isolated as a suppressor to the UNC-64 locomotion phenotype [44] and these mutants have a reduced sensitivity to both ethanol [45] and VAs [4], indicating at least partial overlap of molecular targets.

Conclusions and future directions

In recent years, research using the nematode worm *C. elegans* has provided evidence for the involvement of specific presynaptic proteins in ethanol sensitivity. Evidence from *Rab3*-null and Munc18-1 transgenic mutants with altered ethanol sensitivity points strongly to vesicle recruitment and the downstream vesicle priming/fusion reaction as potential sites for the neural action of ethanol [23,40]; however, a number of questions remain. The relative contributions of various proteins and processes, both pre- and post-synaptic, need to be identified. It is more than likely, for example, that significant cross-talk between proteins could occur. Indeed, Munc18-1 and Rab3 are direct binding partners [46] and have been implicated in each others function (i.e. Munc18-1 in recruitment and docking [30,46] and Rab3 in fusion [47]). From a clinical standpoint, the relationship between altered sensitivity and complex alcohol phenotypes needs to be explored in more detail. It is promising to note that correlations exist between both the Rab3-knockout and Munc18-1 polymorphism and phenotypes such as preference for alcohol [23,41]. Finally, the molecular similarities and differences between ethanol-induced sedation and hyperactivity need to be delineated. The described Munc18-1 polymorphism alone is reported as a presynaptic effector

of hyperactivity [40], although low ethanol concentrations can affect $GABA_A$ (type A GABA) receptor activity postsynaptically [48]. Given the potential complexities in the genetic and cellular/molecular interactions involved in transducing ethanol phenotypes, the continued use of a tractable model system such as *C. elegans* is essential.

Funding

Research in our laboratories was funded by grants from the Biotechnology and Biological Sciences Research Council (to A.M. and R.D.B.) and the Royal Society (to J.W.B.) and a Research Councils UK Academic Fellowship (to J.W.B.).

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Received 3 April 2009
doi:10.1042/BST0380172