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BK Channels: mediators and models for alcohol tolerance

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Abstract

Enhanced acute tolerance predicts alcohol abuse. We describe work on the role of the calcium- and voltage-gated BK channel in alcohol tolerance, highlighting the lipid environment, BK protein isoform selection and auxiliary BK channel proteins. We show how ethanol, which had the reputation of a nonspecific membrane perturbant, is now being examined at realistic concentrations with cutting-edge techniques, providing novel molecular targets for therapeutic approaches to alcoholism. Addictive disorders impact our emotional, physical and financial status, and burden our healthcare system. Although alcohol is the focus of this review, it is highly probable, given the common neural and biochemical pathways used by drugs of abuse, that the findings described here will also apply to other drugs.

Behavioral tolerance

Behavioral tolerance is a common occurrence following exposure to drugs of abuse, characterized by reduced drug effect on a behavioral parameter, either via altered metabolism of the drug or via altered functionality, where the effects of the drug decrease in spite of unaltered concentration. Consequently, the user will escalate drug intake, to maintain the desired effect, with devastating results. There are multiple classes of tolerance, defined by the timeframe and pattern (e.g. constant versus intermittent) of exposure. Perhaps surprisingly for many readers, acute and rapid functional tolerance can occur within minutes of the first drink (this is addressed below) [1]. Importantly, the presence of enhanced acute behavioral tolerance to alcohol in humans can serve as a marker for the likelihood of the future development of alcoholism [2]. Thus, the teenager who can “drink his friends under the table” could be at greater risk for alcoholism than his buddies. Chronic tolerance is the decrease in sensitivity that develops as a result of repeated exposures to a drug and is measured in days or weeks rather than minutes [1]. Much remains to be established regarding the functional relationship between different classes of tolerance. What is clear is that to fully understand actions of alcohol on behavior, it will be necessary to identify the adaptations to alcohol within individual molecules. Establishing direct causality between effects of alcohol at the molecular/cellular and behavioral levels has proven elusive. Part of

this difficulty stems from the fact that most behavioral tolerance studies have relied on relatively long-term alcohol exposure, whereas most *ex vivo* physiological preparations exhibit much shorter viability. One recent study benefited from the fact that behavioral tolerance to alcohol was readily observed a few minutes after drug injection, making it suitable for comparison with molecular/cellular data obtained in the same timeframe [3]. Remarkably, only a few minutes separated the onset of behavioral tolerance (e.g. ability to maintain balance) and alcohol effects on BK channels and action potential patterns in striatal neurons [3].

In contrast to drugs that have a complex molecular structure and a limited number of direct sites of action, the ethanol molecule is remarkably simple, and potential molecular targets are many and varied. This poses a special problem for understanding actions of ethanol. Effective concentrations of ethanol (and blood levels for legal intoxication) are measured in millimolar concentrations rather than nanomolar levels which are effective for other drugs of abuse. Neuronal synapses, and especially ligand-gated receptors rather than voltage-gated channels, were the major focus of alcohol research for many years [4,5]. However, even within the synapse, voltage-gated channels play a significant role, both in release on the presynaptic side and in reception on the postsynaptic side. Although they come in many different flavors, voltage-gated channels can roughly be divided on the basis of their main charge carrier (i.e. sodium, calcium, potassium and chloride). Many of these channels were found to be remarkably resistant to alcohol action, even at very high concentrations [6,7]. In contrast, the BK calcium- and voltage-activated potassium channel was found to be very responsive to alcohol, generally (but not exclusively [8]), exhibiting potentiated channel activity, resulting from reduced time spent in the closed state. The increased probability of the BK channel adopting an open conformation is observed in brain [9,10], in reconstituted lipid bilayers [11] and in oocytes and HEK 293 cells transfected with mouse, rat and human channels. Potentiation, as well as BK inhibition in arterial smooth muscle [12], is elicited by alcohol concentrations as low as 10 mM (legal intoxication is ~20 mM).

The BK channel has emerged as an important model to study alcohol because it: (i) is abundantly expressed in the brain [13,14]; (ii) plays an essential role in many aspects of neuronal physiology, including neurotransmitter release [15–17], action potential patterning and dendritic excitability [9,18–24]; (iii) activity is regulated functionally by alcohol [4,10,25,26]; (iv) is a key element of behavioral tolerance to alcohol in both invertebrates [27–30] and mammals [3]; and (v) provides a link between ongoing neuronal activity and modulation via intracellular calcium. Four of the mechanisms at play in modulating the response of BK to alcohol are shown in Figure 1.

BK channel

The large conductance Ca^{2+} - and voltage-activated K^+ (BK) channel represents a functional subtype of a large group of K^+ channels [31–34] that play a dominant role in shaping neuronal activity [18,24,35,36]. It is formed by the association of a core and β auxiliary subunits. The α core subunit possesses many of the common structural features of voltage-gated K^+ channels, including an ion-selective pore formed by transmembrane segments S5 and S6 and a voltage-sensing module formed by transmembrane segments S1–S4 (Box 1).

In addition, BK_{Ca} channels have a long intracellular COOH terminus that forms a Ca²⁺-sensing module important for activation of the channel [31–33]. The BK channel is ubiquitous throughout the body, expressed in diverse tissues beyond the nervous system. In the brain, virtually all regions express this subunit although at very different levels [14]. Of the four β auxiliary subunits identified so far, only $\beta 4$ mRNA is expressed in all brain regions examined [37]. Although mRNA coding for $\beta 2$ or $\beta 3$ subunits have been reported in both the brain and smooth muscle, to date there is no evidence that they are expressed and associated with the α subunit in neurons. Finally, the $\beta 1$ subunit is found in both neurons and smooth muscle cells [9,38].

The BK channel as a model for molecular tolerance

Adaptation of BK to alcohol (molecular tolerance) involves both reduced sensitivity to the drug after exposure, which occurs within minutes, and a slower-developing declustering within groups of channels and subsequent internalization of channels from the plasma membrane (leading to decreased current density), measured in hours [39]. After 24-h exposure, BK channels remaining in the membrane are (i) less potentiated by acute challenge; (ii) less clustered; and (iii) less dense within remaining clusters [10]. Overall, the “tolerant” BK population exhibits a higher proportion of internalized channels [10] (Figure 2). The nature of the alcohol–BK interaction is significantly more direct and specific than would have been predicted from prevalent belief several years ago. Much of the initial work establishing the nature of the alcohol–BK interaction was performed in the hypothalamic–neurohypophyseal system [responsible for release of the neuropeptides vasopressin (AVP) and oxytocin (OT)] and includes:

- Establishment of L-type Ca channels and BK channels in neurohypophyseal terminals as targets of acute ethanol action, associated with the inhibition of peptide hormone release from these terminals.
- Discovery that *gating* is the attribute in both L-type Ca channels and BK channels that is targeted by ethanol. Gating refers to the transitions of the channel protein between the closed and the open state, and is a function of membrane voltage and intracellular calcium concentration. Other parameters, such as the ion selectivity and conductance of the channel, are resistant to alcohol.
- Demonstration of tolerance in the acute inhibition of release of AVP and OT from the neurohypophysis in rats placed on an ethanol-containing diet.

It can be postulated that the molecular tolerance observed in voltage-gated calcium channels and BK channels in the terminal are causally related to tolerance to effects of alcohol on peptide release. This, in turn, would result in tolerance to diuretic effects of alcohol observed in both humans and animal models [40]. Although only tolerance of peptide release is being discussed here, the full story is even more interesting (although too complex to discuss here) in that AVP, itself, appears to play an important role in the mediation of forms of behavioral tolerance [41].

We will describe three separate approaches describing the response and adaptation of BK to alcohol, first exploring molecular tolerance from a very reductionist level, then through

analysis in increasingly complex situations, before finally addressing the role of BK channel auxiliary proteins in tolerance and the modulation of drinking behavior in mice. Importantly, the effects of alcohol on human BK channels transfected into cell lines are remarkably similar to the response of BK channels in rodent neurons [42], suggesting that the findings described in this review will be applicable to the human condition. It should be noted that the first approach to be described represents a potential role of lipids in alcohol drinking and tolerance. Hopefully, future research will address the changes in membrane lipids that occur during drinking and also the consequences of these changes for dependence and addiction. The second approach (epigenetic studies) provides a potent mechanism for tolerance but awaits evidence linking it to altered drinking behavior. The last approach, describing the influence for the BK $\beta 4$ subunit on acute tolerance, shows both the effect at the molecular and cellular levels and the consequences for drinking behavior in mice.

Lipid environment affects the immediate and long-term response of BK to alcohol

Theories of molecular mechanisms of action of alcohol in the central nervous system have evolved from an earlier '*lipid hypothesis*', in which actions of ethanol on neuronal membranes was explained mainly in terms of actions on membrane lipids, secondarily affecting proteins, to the recent '*protein hypothesis*', which is predicated on findings (driven by molecular mutagenesis studies) that alcohol can interact directly with membrane proteins to affect function [39,43,44]. Indeed, this change in thinking represents a major paradigm shift. Moreover, our understanding of the relationship between BK molecular structure and ethanol interaction has become increasingly sophisticated [4,45]. However, it might be wrong to replace our focus on lipids as sole targets with a new view in which proteins are the sole targets. In fact, our understanding of the lipid environment at the level of nanoscale domains has advanced significantly and reveals the importance of this heterogeneous group of organic compounds [46]. We believe that the most reasonable interpretation is that although the drug interacts directly with proteins, the lipid environment modulates this interaction or the effects of this interaction.

A useful technique for understanding the influence of lipid environment on membrane proteins is planar bilayer recording, in which proteins are 'extracted' from biological membranes and incorporated into a highly simplified lipid film painted across an opening in the recording chamber. By using transfected cells, from which BK proteins are extracted, channel identity can be controlled. Single channel activity can then be recorded as a function of desired bilayer composition and presence of alcohol. The bilayer preparation allows some very interesting questions to be posed: e.g. 'how much organized membrane and cell are necessary to observe acute molecular tolerance?' The answer is '*remarkably little*' – i.e. acute tolerance is observable in isolated BK channels transposed into reconstituted bilayers [47]. But bilayer composition has a very large effect. The acute ethanol tolerance observed in stable (20:1) phosphatidylcholine–dioleoylphosphatidylethanolamine (PC–DOPE) bilayers is not observed in sphingomyelin–DOPE bilayers [47]. One suggestion to explain this effect is that acute molecular tolerance to ethanol involves interaction with multiple sites perhaps with opposing (i.e. potentiating versus inhibiting) effects within the BK channel,

and changes in lipid environment selectively alter ethanol access to these sites [47]. The activity of BK channels is influenced by the cholesterol/phospholipid ratio and the composition in the surrounding lipid bilayer [48]. Ethanol activation of BK channels is modulated by the cholesterol content in lipid bilayers [49] and certain structural features of phospholipids (such as molecular shape) influence the ethanol response [11].

An intriguing finding from the standpoint of lipid modulation of the ethanol response and adaptation of BK channels derives from the manipulation of bilayer thickness, which can have particular importance in light of evidence for the presence of lipid rafts. Rafts are membrane domains enriched in cholesterol and sphingomyelin. In biological membranes, they are postulated to be equivalent to the liquid ordered (Lo) phase of model membranes, surrounded by a contiguous 'sea' of membrane with a lipid structure equivalent to the liquid disordered (Ld) phase of model membranes [50]. Cholesterol is crucial to maintain these domains in the Lo phase, which retains a high degree of acyl chain ordering, while keeping the translational disordering necessary for transmembrane proteins to maintain functional structure and conformational change [51]. Lipid rafts provide a platform for channels to assemble with signaling molecules [52–55]. Importantly, BK channels reside, at least some of the time, in lipid rafts [56]. Altering the thickness of the bilayer by adjusting the acyl chain length of the component lipids affects the time course of the acute response to alcohol and can transform potentiation to inhibition. BK channels embedded in a thin bilayer are strongly potentiated by alcohol, whereas channels placed in a thicker bilayer are inhibited [47]. Because the channel protein is exactly the same, this is remarkable confirmation of the important role played by lipids in modulating the actions of alcohol.

Insights into the mechanisms by which bilayer thickness affects BK function and pharmacology are becoming more accessible from our growing understanding of channel protein structure. In BK, the linker that connects the S6 gate to the RCK (regulator of K^+ conductance) domain forms a passive spring with the gating ring and is involved in Ca^{2+} -dependent activation [57]. A simple mechanical model has recently been proposed [58], which describes how combining forces of lateral stress within the lipid bilayer with forces generated by local hydrophobic mismatch between membrane lipids and the BK protein might modulate channel properties and alcohol pharmacology. Cholesterol, a major component of lipid rafts [59], regulates BK channel sensitivity to alcohol. Increased cholesterol in the bilayer (which will increase membrane thickness and rigidity and promote formation of lipid rafts) can diminish the alcohol potentiation of the BK channel [60], potentially contributing to channel tolerance.

Certainly, the extreme simplification of the bilayer preparation is both its strength and its weakness. Biological membranes are much more complex than the artificial bilayer. Insights gained in this highly reduced preparation will require validation and reinterpretation when the much greater complexity of biological membranes is examined.

There is evidence that lipid composition is altered by alcohol exposure in biological membranes. Both acute and chronic alcohol exposures change lipid content, particularly cholesterol, in cells. One hour of alcohol exposure depletes plasma cholesterol [61], presumably also decreasing membrane-bound cholesterol, making membranes more fluid or

disordered [61]. By contrast, after days-long exposure to alcohol, cellular membranes become tolerant to the disordering effects of alcohol (so-called ‘membrane tolerance’), responding with alterations in specific phospholipids, such as cardiolipin and phosphatidylinositol [62]. Thus, experiments with BK provide ample evidence of the powerful effects that lipid environment can have in both the immediate and adaptive response to alcohol. However, it is important to note that this line of investigation provides at this time the *potential* for a major mediator of alcohol tolerance (e.g. that BK might move into and out of lipid rafts, thereby changing both the acute response and adaptation to alcohol). Further research must be performed to better understand whether these mechanisms are, indeed, playing a role in addictive behaviors.

Epigenetics and tolerance: MicroRNA mediates a selective degradation of BK isoforms leading to tolerance

One of the more surprising results from the human genome project was the absence of a significantly greater number of genes in the human genome in comparison with ‘less complex’ species [63]. An explanation for this is emerging from our growing appreciation of epigenetic modulation, in which the gene DNA sequence is unchanged, but the expression of gene products is altered. A particularly important element of epigenetic modulation is the potential for influencing gene expression by environment and experience. The development of drug dependency and addiction is a function of both genetic predisposition and environmental factors [64]. Thus, epigenetic mechanisms provide particularly potent machinery for addictive processes. Indeed, the Nestler laboratory has invoked chromatin remodeling, an epigenetic phenomenon, as a key element of cocaine adaptation [65,66]. Alternative splicing is a powerful mechanism for regulating gene expression, contributing to the functional diversity of proteins [67], and enabling cells to adapt to various environmental cues. Particularly relevant to this review are recent studies on mRNAs encoding N-type calcium channels in PC12 cells [68] and *in vivo* rat glutamate receptor NMDAR1 (N- methyl D-aspartic acid receptor 1) subunit [69] demonstrating that alternative splicing can be modulated by alcohol and that alternatively spliced isoforms can differ in their alcohol sensitivity. BK channel alternative splicing has been extensively characterized [13]. In a novel example of an epigenetic mechanism of molecular tolerance, rat brain miR-9, a representative of the recently discovered class of microRNA molecules, is upregulated by alcohol exposure (Figure 3), leading to the selective destruction of those isoforms of the BK channel exhibiting the highest sensitivity to alcohol, while sparing those isoforms that exhibit lower sensitivity. This occurs within minutes of exposure. Thus, these neurons have evolved an elegant mechanism whereby BK function (although compromised) remains, whereas the dysfunction attributable to effects of alcohol on the channel are minimized. Certainly, the evolutionary pressure leading to this strategy is of great interest. One can envision a range of possibilities, from adaptation as a means of protection from intoxicating levels of ingested alcohol from sources, such as rotted fruit, to the ‘hijacking’ of an adaptive mechanism normally invoked in response to other physiological or environmental ‘stressors’.

This mechanism of subtractive isoform reorganization (i.e. the loss of sensitive isoforms) is possible because only some of the BK α -subunit mRNAs within a neuron contain 3'UTRs with a miR-9 Recognition Element (MRE). It is only this subset of BK message that is downregulated by alcohol exposure (Figure 3b). Moreover, this mechanism results in the regulation of additional miR-9 targets relevant to alcohol abuse. Thus, one can envision a concept of miRNA master switches, activated by alcohol, coordinating an integrated and dynamic response pattern among a number of downstream targets.

Phosphorylation significantly affects actions of alcohol

As with other ion channels, the activity of BK splice variants is controlled in part by kinases and phosphatases [15,70–73]. Recent studies have made clear how post-translational modifications such as phosphorylation/dephosphorylation can control the response to alcohol [74–77], as well as provide a potentially elegant mechanism for tolerance. Thus, a recent study demonstrated that the calcium/calmodulin-dependent protein kinase, CaMKII, is crucial in the effects of alcohol on the BK channel α subunit. Incremental CaMKII-mediated phosphorylation of Thr107 in the BK tetramer progressively increased channel activity and gradually switched the channel alcohol response from robust activation to inhibition. Thus, CaMKII phosphorylation of slo Thr107 works as a 'molecular dimmer switch' that could mediate tolerance to alcohol [26].

BK subunit composition predicts alcohol acute tolerance and consumption

Whereas the previous section describes a mechanism of tolerance related to isoform variability in the α subunit of the BK channel, in this section we will describe the influence of the BK auxiliary β subunits on tolerance. The BK channel, which is mainly understood to act as a 'brake' on neural activity [21,78,79] (although excitatory effects have also been reported [20]), is composed of a primary α protein, containing a pore that acts as a conduit for potassium ions, often combined with a variety of auxiliary proteins (β 1– β 4) that modify its function, including alcohol sensitivity (Box 1). Compartment-specific expression of β subunits of the BK channel has been proposed to underlie differences in BK alcohol sensitivity between the somatic, dendritic and terminal compartments of neurons [9]. One of these auxiliary proteins, known as β 4, plays a key role in acute alcohol tolerance. Importantly, one of the most reliable predictors of future alcohol problems in humans is the presence of an enhanced ability to develop acute tolerance in a cognitive task (i.e. performance recovers in spite of the continued presence of a constant blood-alcohol level).

A critical tool in the understanding of the role of the β subunit in tolerance and consumption has been the use of genetically engineered mice, in which the gene encoding the β 4 subunit is 'knocked out' (KO). The KO mouse allows analysis of tolerance at multiple levels, from single channel recording of the individual BK channel protein through to cellular analysis at the level of the action potential and ultimately to drinking behavior [3]. There is remarkable consistency in the results showing that the β 4 subunit inhibits acute tolerance at all levels of analysis and confirming that, in the mouse model, the presence of acute tolerance is an indicator of alcohol consumption, as has been described in human studies (Figure 4). At the single-channel level, ab4 BK channels from the somata of wild type (WT) striatal medium

spiny neurons (a crucial element of the reward and addiction pathway) are potentiated by alcohol, and this potentiation shows no tolerance throughout the recording session (Figure 4b, $\alpha\beta 4$ BK). By contrast, when the $\beta 4$ auxiliary subunit is absent, alcohol-mediated potentiation of BK channel activity dissipates within 5 min of alcohol exposure (Figure 4b, α BK). In several brain regions, BK channels regulate neuronal excitability by shaping action potential patterns. In the striatum, potentiation of BK channels by alcohol strongly depresses neuronal excitability. This effect persisted for 14 min of recording in $\alpha\beta 4$ -expressing MSNs (Figure 4c, WT). In contrast, BK channel activity quickly returned to baseline levels in $\beta 4$ KO mice (Figure 4c, $\beta 4$ KO), indicating that BK channel-mediated tolerance at the molecular level can also be observed at the cellular level. In mice lacking the $\beta 4$ subunit, locomotor activity returned rapidly (within a few minutes) to control level following an initial depression, indicating the presence of behavioral tolerance. Finally, and most interesting, these mice drank markedly more than their wild type counterparts (Figure 4d, BK $\beta 4$ KO). There was no difference found in baseline fluid (water and sucrose) intake in WT and KO mice. These studies underline the concept that alcohol consumption can be linked to the functional properties of an ion channel protein.

Concluding remarks

Here, we have presented data from several approaches that probe the response of the BK channel to ethanol, indicating that the response is more specific and interpretable than textbook doctrine would have suggested for alcohol–protein interactions just 20 years ago. The data described raise many interesting questions. Why are there so many different mechanisms to produce and modify BK alcohol tolerance? Clearly, the neuron finds it important to minimize the consequences of the influence of alcohol on BK activity. This is understandable by virtue of the very high conductance and ubiquity of the BK channel within and outside the nervous system, such that altered activity could have devastating outcomes on function. Thus, not only is the channel quickly desensitized to ethanol potentiation but then a ‘sledgehammer’ approach is apparent in the subsequent internalization of BK, removing it from the functional influence of alcohol. A testable assumption is that different drinking patterns will elicit different varieties of tolerance. For example, characteristics of tolerance produced by drinking that is ‘massed’ (i.e. long drinking bouts) versus ‘spaced’ (i.e. shorter, intermittent bouts of drinking) might produce different types of tolerance, analogous to memories with different characteristics produced by spaced versus massed learning sessions [80]. Ethanol, via both direct and indirect activation of dopaminergic neurons in the ventral tegmental area [7,81], increases dopamine release in the striatum, which has been linked to both motivational and locomotor properties of most abused drugs. Although ion channels are the essential building blocks underlying neuronal activity and behaviors, it will be difficult to understand a complex behavioral trait such as alcohol addiction by simply focusing on the level of individual molecules. Clearly, ignoring the importance of brain circuitry in determining the behavioral inclination towards destructive drinking would be unwise. Depression, similar to alcohol addiction, represents a complex behavioral state, yet an understanding of the role of serotonin reuptake in this disorder has been helpful in the development of the selective serotonin reuptake inhibitors (SSRIs) effective in treatment. Understanding the actions of alcohol at the level of an

individual BK channel protein is an important piece in the puzzle of addiction but is not sufficient to ‘explain’ the basis of addiction. Indeed, it offers both the potential of examining a molecule that underlies some aspects of behavioral tolerance, as well as providing a model to understand in a more generic sense the mechanisms that are at work to produce adaptive responses in other molecular targets, and to other drugs of abuse and environmental perturbations. Why is now such a propitious time for alcohol and addiction research? In our view, it is because of the convergent maturing of our understanding of both structure–function relationships and the molecular biology of molecules controlling nervous system function, and of our growing insight into the nature of addiction at the behavioral level. Thus, one cannot only associate certain genes and gene products with the propensity for alcoholism but we can now understand the manner in which the environment (including drinking history) can, via epigenetic mechanisms, modulate the individualized consequences resulting from genetic makeup. The goal of our research must increasingly progress from studies focused on *either* the world of molecules or the world of behavior, to studies that more successfully bridge the divide. One of the more contentious issues in the addiction field (and society at large) has been the emergence of a disease model of addiction, in which uncontrolled drug use is viewed as a pathology grounded in physical changes in molecules, comparable to those underlying, for example hypertension or diabetes, rather than a lack of ‘will power’. The identification of physical changes in the nervous system, such as those described for the BK channel, make the disease model more plausible to many. Finally, the alcohol field is struggling with a relatively new dynamic; it is becoming clear that alcohol use can have beneficial as well as damaging influences on health [82] and this makes a proper perspective on alcohol and health more complex than before. However, the vast majority of researchers in this field caution that the potential benefits are generally outweighed by the potential dangers of addiction and abuse which might be present in certain individuals. Current treatments available for alcoholism have shown only limited success and include both behavioral approaches, such as 12-step programs, and pharmacological approaches using drugs, such as naltrexone and acamprosate [83]. The identification of promising gene targets, such as the BK (*slo*) gene, as well as several other genes emerging from genetic screens in animal models and human studies will provide new approaches for treatment of alcoholism, as well as prognostic information potentially helpful in prevention (Box 2).

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Box 1. Schematic representation of the structure and characteristics of BK channels

Of all known ionic channels, they display the largest unitary conductance (~250 pS). They are also known as BK, Slo and MaxiK (Gene ID, KCNMA1) and are found in many members of the animal kingdom including nematodes (*Caenorhabditis* [84]), insects (*Drosophila* [27,85]) and mammals [86], a fact suggestive of their importance in cell regulation. Structurally, the BK α subunit consists of seven transmembrane spanning domains (S0–S6) comprising the β -coupling domain (S0), the voltage-sensor (S1–S4) and the pore-forming loop (between S5 and S6). In addition, the channel displays four hydrophobic segments (S7–S10) within the long intracellular carboxyl (C) terminus (a). The narrowest part of the pore, called the selectivity filter, determines the high flux rate of potassium in comparison to other ions. Although coded by a single gene, the α subunit exhibits great diversity owing to its numerous splice sites whose location in the channel amino acid sequence is shown by green arrowheads in (a). An example of the variants found in the supraoptic nucleus is shown in (b). As described in the main text, the expression of α subunit splice variants is regulated by ethanol through a microRNA mechanism [25]. The α subunit is ubiquitously expressed in neurons of the central nervous system, whereas the β subunit is region specific. Of the four β subunits identified so far, only β 4 mRNA is exclusively expressed in all brain regions examined [37]. Although mRNA coding for β 2 or β 3 subunits have been reported in both the brain and smooth muscle, to date there is no evidence that they are expressed and associated with the α subunit in neurons. Finally, the β 1 subunit is found in both neurons and smooth muscle cells. Each channel exists as an assembly of four α subunits and four β regulatory subunits, with a 1:1 stoichiometry (c). There is no evidence that α subunits are associated with more than one β subunit subtype in neurons. Importantly, each β subtype confers a distinct set of biophysical characteristics, and in the case of β 1 makes the channel insensitive to alcohol (Figure I) [9].

Box 2. Outstanding questions

As our understanding of molecular tolerance grows, it will be possible to gain greater insights. For example:

- Does the influence of cholesterol on the alcohol response and molecular tolerance result from a direct interaction with the BK protein, or from a less direct effect on the lipid matrix or other modulatory molecules?
- Can we identify differences in membrane lipid composition or in microlocalization of channels to lipid rafts after alcohol drinking, consistent with a role in behavioral tolerance and dependency?
- Is the role of the BK $\beta 4$ subunit in tolerance and consumption observed in model preparations reflected in human populations of alcoholics?
- What are the dynamics of miR-9 upregulation and decline, and what is the subsequent time course of BK translation and membrane insertion into the neuronal membrane?
- How do the duration, frequency and concentrations of alcohol exposure influence the character of subsequent tolerance (and possibly dependency) at both the molecular and behavioral levels.

It is a particularly exciting time in the alcohol field. A drug that for a long time had the reputation as a membrane perturbant too non-specific to study is now being examined at realistic concentrations and with cutting-edge techniques.

Figure 1.

Alcohol response and adaptation strategies discussed. This review focuses on the BK channel, a calcium- and voltage-activated potassium channel that plays a dominant role in shaping neuronal activity, and which is strongly affected by alcohol. Shown here are four mechanisms influencing or underlying the response and adaptation of the BK protein to alcohol: **(i)** Membrane lipids affect BK function, alcohol response and acute tolerance. Lipid composition might differ between individuals and populations, or might be altered in response to alcohol exposure. Membrane lipid heterogeneity is illustrated by different colors. **(ii)** Epigenetic mechanisms, influencing the post-transcriptional expression of BK are an important mediator of molecular tolerance. MiR-9 is an example of a microRNA mediator that will be discussed in this review. **(iii)** Post-translational mechanisms, such as phosphorylation–dephosphorylation of the channel protein also influence tolerance. **(iv)** BK channel subunit composition might differ between populations and individuals, or might be modified in response to alcohol exposure, altering the response to alcohol. Why is there such a variety of routes? One possibility is that these different mechanisms produce different types of tolerance, modulating, for example the duration of tolerance, and that they are invoked differentially according to parameters such as the timing or concentration characteristics of drug exposure.

Figure 2.

Modulation of BK channel function and distribution in neuronal terminals by chronic ethanol. After 24 h of exposure, BK channels are (i) not potentiated by acute challenge; (ii) less clustered in the membrane; (iii) less dense within remaining clusters; and (iv) more internalized (adapted from Ref. [9]). The light gray boxes indicate the membrane patch exposure to alcohol.

Figure 3.

BK channel α subunit expression levels and sensitivity to alcohol. **(a)** The ratio of mRNAs coding for three of the most abundant BK channel splice variants [Insertless (IL), ALCOREX and STREX] in neurons is illustrated by the size of the channel icon (i). Thus, IL is by far the most abundant variant in naive cells. After alcohol exposure, the ratio IL/ALCOREX/STREX is profoundly altered (ii). IL mRNA representation decreases dramatically, whereas STREX, and to a lesser extent, ALCOREX, increases. This effect is initiated by an alcohol-mediated increase in microRNA (mir-9) and subsequent destruction of a subset of BK transcripts. Green boxes below each splice variant illustrate their respective sensitivity to acute alcohol, as well as the presence or absence of tolerance. Although IL BK channels are potentiated by shorter alcohol exposure, they lose their sensitivity after longer treatment. By contrast, STREX BKs (which represent a greater proportion of the post-exposure BK population) retain their lack of sensitivity after longer exposure to the drug. The sensitivity of ALCOREX sensitivity after chronic alcohol treatment is undetermined. Thus, the overall BK message is reduced and the representation of splice variant species is reordered, resulting in a more alcohol-resistant BK population. **(b)** Regulation of a BK channel transcripts in supraoptic nucleus neurons by alcohol. Only a subset of transcripts contain the miR-9 Recognition Element (MRE; pink boxes) in their

untranslated regions (UTRs). After exposure to alcohol, miR-9 (light gray bands) is upregulated and binds to the MRE region, leading to a downregulation of splice variants [faded bands, (iii)] containing this MRE. The result is an altered isoform landscape in which alcohol-resistant BK isoforms predominate. X4 (green box), STREX (yellow box) and P27 represent three abundant inserts found in this brain region. A similar phenomenon was observed in the striatum [25].

Figure 4.

Role of the BK channel $\beta4$ peptide in tolerance at the molecular, cellular and behavioral levels. Somatic BK channels (green bars) are widely expressed in somata of wild type striatal medium spiny neurons (**a**). At the single channel level, somatic BK channel open probability is rapidly [within 2 min, (**b**); bottom graphs show the timeline of alcohol effects] enhanced by acute alcohol. This effect is transitory when only the BK core α subunit is expressed [(b), α BK]. By contrast, in the presence of the $\beta4$ auxiliary subunit, alcohol potentiation is sustained [(b), $\alpha\beta4$ BK]. In the same neurons, alcohol drastically decreased neuronal excitability by potentiating the activity of BK channels activity, leading to a shortened action potential width [(a), white line models the alcohol effects on action potentials]. Enhanced excitability shows tolerance when $\beta4$ subunits are absent [(c), $\beta4$ KO] but not when they are present [(c), WT]. At the behavioral level, the alcohol intake of mice not expressing the $\beta4$ subunit is markedly higher than that of WT mice (**d**). These studies provide direct evidence for the role of BK, and in particular the BK β subunit, on acute alcohol tolerance and alcohol drinking behavior. Illustration is adapted from Ref. [3].