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ALCOHOLISM: CLINICAL AND EXPERIMENTAL RESEARCH

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Ethanol Actions on Multiple Ion Channels: Which Are Important?

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Background: This review is based on a plenary lecture presented at the 1999 meeting of the Research Society on Alcoholism. It provides an overview of the search for sites of action for ethanol in the brain. Initial studies were directed at interaction of ethanol with membrane lipids, but during the past decade, emphasis has been shifted to protein sites, particularly those on ion channels. Molecular biological techniques have provided the opportunity to study isolated channels in cellular expression systems and also provide the opportunity to manipulate these channels in mutant mice.

Conclusions: There is now compelling evidence that multiple ion channels are affected by ethanol and growing support for the idea that ethanol interacts directly with specific sites on ion channels. The key, and unanswered, question is which of these channels are responsible for alcohol-induced behaviors such as intoxication, tolerance, dependence, or craving. Mutant mice will likely give (some) answers to these questions during the next decade.

Key Words: Acetylcholine, Alcohol, GABA, Glutamate, Ion Channel.

I AM MOST appreciative of being selected for the 1999 Research Society on Alcoholism's Distinguished Research Award, and for this opportunity to present a personal, and biased, view of a 20-year-long search for sites of alcohol action in the brain. With training in chemistry and pharmacology, I was, and continue to be, fascinated by our ignorance of the manner by which molecules as simple as ethanol can produce such complex actions on the brain and behavior. My first studies asked whether either membrane lipids or certain voltage-sensitive calcium or sodium channels might be perturbed by ethanol and other intoxicant-anesthetic drugs. The answer was 'yes', but only with concentrations of ethanol that were either in the severely intoxicating or lethal ranges (Harris and Hood, 1980; Harris and Schroeder, 1981). This lead to an ongoing search for brain proteins that are sensitive to concentrations of ethanol (e.g., 5–30 mM) that produce mild to moderate intoxication in humans.

OVERVIEW

During the past decade, there has been a transition from studies of nonspecific actions of ethanol on membrane lipids to a search for specific sites of action on neuronal proteins. My own conversion from the lipid to protein belief system was completed when Kari Buck, my first doctoral student, showed that A2C (a synthetic compound designed to perturb membrane lipids) did not mimic the effects of ethanol in vivo or in vitro, despite marked changes in membrane physical properties (Buck et al., 1989).

As we focused on brain proteins, the key questions were: Which neuronal proteins (or functions) are sufficiently sensitive to account for the intoxicating action of ethanol? What is the molecular mechanism by which ethanol affects these proteins? and Which neuronal functions determine specific behavioral actions (e.g., activating, sedative, anxiolytic, ataxic) of ethanol? Because of the data implicating low ethanol sensitivity (or "responsiveness") in susceptibility for development of alcoholism (Schuckit, 1980, 1985, 1986, 1994; Schuckit and Smith, 1996), identification of molecular sites for alcohol action in the brain become important as candidate systems for possible therapeutic interventions as well as candidate genes for evaluation in human alcoholism. Molecular techniques make it feasible to pinpoint regions of proteins critical for alcohol action and, more importantly, to construct mutant animals that can tell us if these candidate proteins are indeed responsible for distinct behavioral actions of ethanol in vivo.

MOLECULAR TARGETS OF ALCOHOL

$GABA_A$ Receptors and Ethanol Action

In the early 1980s, a number of laboratories found that drugs (e.g., $GABA_A$ agonists, uptake inhibitors) which augment GABAergic function enhance behavioral actions of

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ethanol and drugs (e.g., $GABA_A$ antagonists, synthesis inhibitors) which inhibit GABAergic function reduce ethanol behaviors (Martz et al., 1983; Deitrich et al., 1989; Koob, 1995). In collaboration with Dick Deitrich, we found that the Long-sleep/Short-sleep (LS/SS) mice, which differ in genetic sensitivity to ethanol, differ in their behavioral sensitivity to GABAergic drugs (Martz et al., 1983). These studies suggested that ethanol may exert some of its effects by enhancing GABA-mediated inhibition. One early electrophysiological study also presented evidence supporting this idea (Davidoff, 1973), but it was not developed further until 1986. At that time, our laboratory and those of Steven Paul and Raj Ticku all demonstrated that intoxicating concentrations (5–50 mM) of ethanol enhance the function of $GABA_A$ receptors (Allan and Harris, 1986; Suzdak et al., 1986; Ticku et al., 1986). These studies used different tissue preparations (mouse cerebellar and cortical microsacs, rat cortical synaptoneurosomes, and cultured mouse spinal neurons, respectively), but all measured the uptake of $^{36}Cl^-$ stimulated by GABA agonists and all obtained similar potentiation of $GABA_A$ receptor function by ethanol. These observations stimulated numerous electrophysiological studies of ethanol action on $GABA_A$ receptor function, and the results were inconsistent. A detailed discussion of this literature is beyond the scope of this review but is covered in other reviews (Deitrich et al., 1989; Mihic and Harris, 1995) and briefly in the next section. At the risk of oversimplification, we conclude that there are ethanol-sensitive and ethanol-resistant $GABA_A$ receptors in the brain, and that this ethanol sensitivity is likely determined both by subunit composition and by posttranslational processing. However, the molecular details that define an ethanol-sensitive $GABA_A$ receptor remain to be determined. It is of interest to note that our first publication in this area (Allan and Harris, 1986) showed that $GABA_A$ receptors of brain membranes from SS mice were resistant to ethanol, whereas those from LS mice were sensitive. Thus, the existence of ethanol-sensitive and -insensitive receptors, as well as their genetic association with ethanol sensitivity in vitro, was anticipated 13 years ago.

The discovery that GABA receptors were sensitive to ethanol fortuitously coincided with the cloning and sequencing of the genes coding for numerous ligand-gated ion channels. This provided a tremendous advance in our understanding of receptors that are critically important for synaptic transmission in the central nervous system. The finding that the subtype of glutamate receptors that is activated by N-methyl-D-aspartate (NMDA) is sensitive to ethanol (Lovinger et al., 1989) provided a major advance in alcohol research. Studies of ethanol actions on ion channels grew rapidly from a very small database in the early 80s to more than 1000 articles by 1998 (Fig. 1).

Ligand-gated ion channels that are activated by neurotransmittters, including GABA, glycine, glutamate, acetylcholine, serotonin, and purines (e.g., ATP), are all affected by ethanol. However, the sensitivity to ethanol often varies

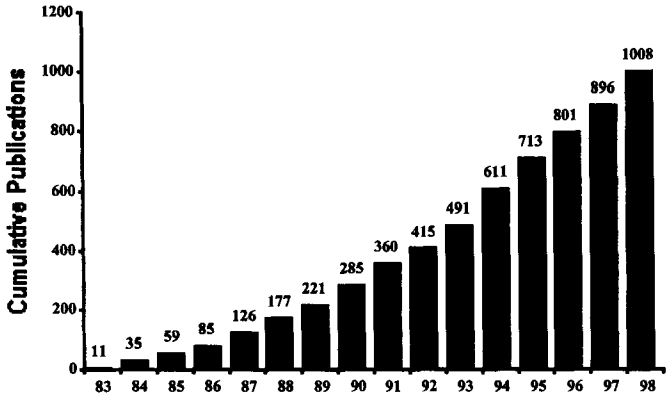

Fig. 1. Cumulative number of publications on alcohol and ion channels. The MedLine database was searched using the terms [(ethanol or alcohol or alcoholism) and (ion channels)].

depending upon the preparation (brain region, cell type) studied. The reasons for this are not clear, but the subunit composition of the receptors as well as posttranslational processing (e.g., phosphorylation) are likely important (Mihic and Harris, 1995). The mechanism of action of ethanol on these channels is not well defined and may well be different for various members of this diverse group of proteins. Possibilities include multiple binding sites on the protein (perhaps at the protein-lipid interface), actions on bulk lipid surrounding the protein (lipid hypotheses are not completely dead!) and action on a discrete binding site or pocket within the protein. In an attempt to disprove some of these possibilities, we focused on GABA and glycine receptors to determine which parts of the receptor/channel complex are critical for alcohol action.

Sites of Alcohol Action on $GABA_A$ and Glycine Receptors

$GABA_A$ and glycine receptors are the primary mediators of inhibitory neurotransmission in the brain and spinal cord, respectively. These receptors form part of a receptor superfamily displaying significant amino acid sequence homology. A number of classes of GABA receptor subunits have been cloned ($\alpha,\beta,\gamma,\delta,\epsilon$, and ρ), with most containing several subtypes; only two classes of glycine receptor (Gly-R) subunits have been identified (α,β). Although many GABA and glycine receptor subunits form functional receptors only when they are heteromerically expressed with other subunits, a few, such as the GABA $\rho 1$ and glycine $\alpha 1$ subunits, do express homomerically. More information on glycine and $GABA_A$ receptor structure and function can be found in reviews by Betz (1990, 1991) and Whiting et al. (1995).

GABA receptor function can be markedly enhanced by pharmacologically relevant concentrations of a number of classes of sedative, hypnotic, and anesthetic agents including: barbiturates, benzodiazepines, alcohols, and the volatile and nonvolatile anesthetics (see Sieghart, 1995; Mihic et al., 1997). Glycine receptors, although insensitive to barbiturates and benzodiazepines, are also affected by eth-

anol, longer chain alcohols, and volatile anesthetics (Celentano et al., 1988; Aguayo and Pancetti, 1994; Engblom et al., 1991; Mascia et al., 1996a,b). The extensive behavioral evidence implicating $GABA_A$ receptors in ethanol action will not be reviewed here but has been presented in detail elsewhere (Koob, 1995; Draski and Deitrich, 1995). A role for the strychnine-sensitive glycine receptor in alcohol action is supported by behavioral evidence that glycine and the glycine precursor serine are able to enhance the depressant effects of ethanol. This action was blocked by strychnine (Williams et al., 1995).

One of the most useful findings from different receptor subunits was the observation that ethanol inhibited the function of homomeric ρ1 GABA subunits and enhanced the function of homomeric α1 glycine receptors. Statistically significant enhancement of glycine receptor function by ethanol was seen at 10 mM, and inhibition of ρ1 receptor function was seen at 25 mM (Mascia et al., 1996b; Mihic and Harris, 1996). These findings formed the basis of a search for the site of alcohol action on chimeric and mutant GABA and glycine receptors (Mihic et al., 1997; Ye et al., 1998; Ueno et al., 1999). In brief, two amino acids were found to be critical for ethanol action on glycine and GABA receptors, and these are located in the transmembrane regions (TM2 and TM3), near the cytoplasmic surface.

Mutation of Ser-267 in TM2 of the Gly-R α1 subunit to Ile produced a receptor that was completely insensitive to the enhancing effects of ethanol. The equivalent amino acid mutation in the GABA-R α1 subunit also abolished ethanol sensitivity in GABA $\alpha1\beta1$ receptors, whereas this mutation in the GABA α1 subunit resulted in a decrease in ethanol enhancement. Furthermore, the mutation of Ala-291 of the GABAα, a subunit (in TM3), also abolished ethanol sensitivity (Mihic et al., 1997). Subsequent work showed that ethanol enhancement of glycine receptor function was only observed when small amino acids (e.g., Ala, Ser) occupied residue 267; substitution with medium-sized amino acids (e.g., Ile, Val) resulted in almost no effect of ethanol, whereas mutation to large amino acids (e.g., Tyr, Phe) produced glycine receptors inhibited by ethanol (Ye et al., 1998). We next addressed the question of whether these amino acids constitute part of the binding site for ethanol, or instead affect the transduction of a conformational change subsequent to the binding of alcohol molecules elsewhere on the receptor. The potencies of n-alcohols in affecting receptor function increase with increasing carbon chain length but not indefinitely: a "cutoff" is reached when there is no further increase in potency. Glycine α1 receptors have an alcohol cutoff at decanol (Mascia et al., 1996b), whereas the cutoff for the related GABA ρ1 receptors is at heptanol (Mihic and Harris, 1996). Because it has been suggested that the alcohol cutoff reflects the size of an alcohol binding site, the shorter cutoff in the GABA ρ1 receptor can be interpreted as it having a smaller alcohol binding site than the glycine α1 receptor. Ser-267 in the Gly-R α1 subunit was mutated to the larger amino acid glutamine, with the resulting receptor possessing a decreased alcohol cutoff (Wick et al., 1999). Conversely, mutation of Ile-307 and/or Trp-328 (equivalent to the TM2 and TM3 residues in Gly-α1) in the GABA ρ1 subunit, to smaller amino acid residues, increased the n-alcohol cutoff. The ability to change the point of alcohol cutoff in these receptors suggests that the amino acid residues we identified in TM2 and TM3 delimit the size of alcohol molecules interacting within a binding cavity or surface.

A critical, and unanswered, question is how binding of ethanol between TM 2 and 3 might alter channel function. It is likely important that TM2 forms the channel pore and controls channel opening and that the neurotransmitter site may be formed in part by the loop between TM2 and 3. Thus, it is reasonable to postulate that the energy of binding of the neurotransmitter is converted into conformational changes that are relayed through TM2 and 3 to the channel gating region of TM2 (Bormann et al., 1993, 1994; Kuhse et al., 1995). In this model, our putative alcohol binding site is strategically located to alter the coupling of neurotransmitter binding with channel opening. We propose that binding of ethanol in this region could either stabilize the open or closed state of the channel and that mutations change this equilibrium. Thus, the function of some receptors is enhanced by ethanol whereas others are inhibited, depending upon the specific amino acids at the positions in TM2 or 3 (Ye et al., 1998; Wick et al., 1999).

An obvious question is how to reconcile this evidence for an alcohol binding site in a protein region near the extracellular surface with other data which suggests that phosphorylation of GABA receptors might be important for ethanol actions. Wafford et al. (1991) and Harris et al. (1997) reported that the eight extra amino acids found in the γ2L, but not γ2S, subunit conferred potentiation of GABA action by low ($\sim$20 mM) concentrations of ethanol. Mutation of the consensus site for phosphorylation by protein kinase C contained in the γ2L insert resulted in the loss of ethanol modulation of GABAergic currents (Wafford and Whiting, 1992). Because anesthetic (>100 mM) concentrations of ethanol produce significant potentiation of GABAergic currents in $\alpha\beta$ receptors and $\alpha\beta\gamma$ receptors (Mihic et al., 1994), it is possible that phosphorylation of the γ2L subunit increases the receptor's sensitivity to low concentrations of ethanol; i.e., the phosphorylation state of the receptor sets the "gain" determining receptor sensitivity to ethanol. It is clear that not all $GABA_A$ receptors are sensitive to subanesthetic (<100 mM) concentrations of ethanol, but the exact determinants of ethanol sensitivity remain to be defined (see Mihic and Harris, 1996a). There is increasing support for the idea that activation of protein kinase C (PKC) is important for ethanol actions on GABAA receptors (Weiner et al., 1997b). There are recent reports that in hippocampus, ethanol sensitivity depends on the population of $GABA_A$ receptors studied (Weiner et al.,

1997a), the activity of PKC (Weiner et al., 1997b), and the degree of activation of $GABA_B$ receptors (Wan et al., 1996). We used null mutant mice lacking PKCγ to link the behavioral and neurochemical observations by demonstrating that this mutation reduces sensitivity to ethanol in vivo and abolishes the action of ethanol on the function of cerebellar $GABA_A$ receptors (Harris et al., 1995). However, null mutant mice that lack the gamma2L subunit have normal responses to ethanol, so even though there is evidence for importance of phosphorylation in ethanol sensitivity, there is no in vivo evidence for a role of the gamma2L subunit (Homanics et al., 1999; Zhai et al., 1998).

Two other emerging targets of ethanol action in the brain are nicotinic acetylcholine receptors and specific potassium channels. This recent literature is reviewed below.

Actions on Neuronal Nicotinic Acetylcholine Receptors

These receptors are possible targets for acute actions of clinically relevant concentrations of ethanol. There is considerable evidence for behavioral interactions between alcohol and nicotine, as well as biochemical evidence, which suggests direct effects of ethanol on nicotinic receptors (Collins, 1995). For example, ethanol enhancement of dopamine release in the nucleus accumbens seems to require activation of nicotinic receptors in the ventral tegmental area (Blomqvist et al., 1997). These results imply that nicotinic receptors may be important for ethanol reinforcement and voluntary intake, and this is supported by the finding that a nicotinic antagonist (mecamylamine) reduces ethanol consumption by rats and that a low dose of nicotine increases voluntary ethanol intake (Blomqvist et al., 1996). These findings raise the question of whether ethanol produces those actions by enhancing the function of neuronal nicotinic receptors. Recent reports support this idea by showing that ethanol enhances specific (bugarotoxin-insensitive) nicotinic responses on cultured cortical neurons (Aistrup et al., 1999). In addition, these investigators found that other nicotinic responses were weakly inhibited by ethanol, which suggests that distinct subtypes of neuronal nicotinic receptors respond differently to ethanol. These results lead us to study the ethanol sensitivity of recombinant human neuronal nicotinic receptors expressed in *Xenopus* oocytes.

Fortunately, we began our studies before we saw a recent, discouraging, study of ethanol on neuronal nicotinic receptors expressed in oocytes. These investigators found that only high concentrations (100–300 mM) potentiated ACh responses on most receptors, and the combination of $\alpha3\beta4$ gave highly variable results, showing both potentiation and inhibition, whereas homomeric $\alpha7$ receptors showed no effect of ethanol (Covernton and Connolly, 1997). We independently obtained exactly the same results from our initial studies of nAChR expressed in oocytes. However, we have since found that the variable and weak effects of ethanol reported by Covernton and Connolly (1997) are due to a lack of pre-exposure to ethanol. Preincubation with ethanol for at least 1 min results in reproducible potentiation of several receptor subunit combinations (Cardoso et al., 1999). We found that ethanol (75 mM) potentiated ACh-induced currents in $\alpha2\beta4$, $\alpha4\beta4$, $\alpha2\beta2$, and $\alpha4\beta2$ receptors. This effect was due to an increase in Emax, without a change in the EC50 or Hill coefficient. The $\alpha3\beta2$ and $\alpha3\beta4$ combinations were insensitive to ethanol. Low concentrations of ethanol (25 and 50 mM) significantly inhibited homomeric $\alpha7$ receptor function, but these receptors showed highly variable responses to ethanol (Cardoso et al., 1999).

Yu et al. (1996) also reported that ethanol inhibited the function of recombinant $\alpha7$ neuronal-type nAChRs and enhanced the function of $5\text{-}HT_3$ receptors expressed in *Xenopus* oocytes. The authors tested a chimeric receptor that contained the amino-terminal domain from the $\alpha7$ nAChR and the transmembrane and carboxy-terminal domain of the $5\text{-}HT_3$ receptors. They found that the function of this chimeric receptor was inhibited by ethanol, which suggested that its site of action on $\alpha7$ nAChRs is on the amino-terminal domain. This is a surprising result in view of the lack of importance of this domain in the actions of alcohols on $GABA_A$ and glycine receptors which share considerable homology with nicotinic receptors. For muscle nAChR, there is evidence that long-chain alcohols bind to the TM2 region within the channel pore (Forman, 1997). However, studies from this group also suggest that ethanol does not bind to the same site as the long chain alcohols (Wood et al., 1991). In contrast to the findings of Yu et al. (1996) (but in agreement with Covernton and Connolly, 1997), we found that ethanol weakly and inconsistently inhibits the function of $\alpha7$ receptors expressed in oocytes (Cardoso et al., 1999). Additional studies are clearly required to determine how ethanol and long chain alcohols affect the function of neuronal nicotinic receptors and whether the sites of action are shared among homologous ligand-gated ion channels.

Actions on Potassium Channels

Ethanol actions on potassium channels have not received as much attention as $GABA_A$ or glutamate receptors, but several papers on the effects of ethanol on voltage-gated potassium channels were recently published. The *Drosophila* Shaw2 channel is inhibited by ethanol with an IC_{50} of 179 mM and this appears to be due to a single, saturable hydrophobic site on the channel protein (Covarrubias et al., 1995). Dopico et al. (1998) reported that ethanol (10–100 mM) increased the activity of large conductance, Ca^+-activated K^+ channels that contribute to the control of hormone release in neurohypophysial terminals. The authors postulate that the actions of ethanol on these K^+ channels could in part explain the reduced release of vasopressin and oxytocin that occurs after ethanol ingestion. A novel twin pore, open rectifying, voltage-independent po-

tassium channel was recently cloned from rat cerebellum and found to be inhibited by ethanol (41% inhibition by 170 mM) (Leonoudakis et al., 1998). Thus, there is evidence that relatively high concentrations of ethanol inhibit the function of two different K channels and that a Ca-dependent K channel is enhanced by lower concentrations of ethanol.

Inwardly rectifying potassium channels are a family of channels activated by neurotransmitters and hormones that are thought to contribute to neuronal excitability and cell signaling in the central nervous system (Jan and Jan, 1997). Ethanol enhances the firing rate of dopaminergic neurons in the ventral tegmental area, and this is accompanied by enhancement of function of an inward rectifying channel (Brodie and Appel, 1998). This is of interest because the increased firing of the VTA neurons is thought to be important for ethanol reinforcement, and it will be important to determine which K channels are affected by ethanol and how they influence the firing rate of the VTA. We are particularly interested in G-protein coupled inwardly rectifying potassium channels (GIRK channels), which are one of the six classes of inward rectifiers. To date, five subunits of the GIRK channel have been identified and designated GIRK 1–5 (Dascal, 1997). These subunits are differentially distributed in the brain and are, therefore, thought to have distinct functions in different neuronal populations (Spauschus et al., 1996; Ponce et al., 1996).

In the central nervous system, GIRK channels may be activated by muscarinic m2, α2 adrenergic, D2 dopaminergic, histamine, $5HT_{1A}$, adenosine A1, $GABA_B$, μ, δ, and κ opioid and somatostatin receptors, all of which are coupled to G-proteins (Spauschus et al., 1996; Dascal, 1997). Activation of the receptor by its agonist leads to the liberation of $G\beta\gamma$, which binds directly to the GIRK channel. When the GIRK channel is open, potassium ions flow out of the neuron resulting in decreased neuronal excitability, and, hence, GIRKs play a major role in regulating inhibitory responses in the nervous system (Neer, 1995; Slesinger et al., 1995; Huang et al., 1997). Results from mutant mice lacking the GIRK2 subunit showed that the postsynaptic actions of $GABA_B$, $5\text{-}HT_{1A}$ and adenosine A1 receptors were absent in these mice, but presynaptic actions of these receptors were not affected (Luscher et al., 1997).

The diversity and complexity of neuronal potassium channels makes it very difficult to determine effects of ethanol on individual potassium channels in neuronal preparations, and there is little information about effects of ethanol on GIRKs in the brain. Ethanol (11–44 mM) enhances muscarinic cholinergic responses in hippocampus (Madamba et al., 1995), which could be due to muscarinic activation of GIRKs, but there is no direct evidence that this is the case. One study of postsynaptic $GABA_B$ responses in hippocampal neurons found no effect of acute application of 10–100 mM ethanol (Frye and Fincher, 1996).

We recently expressed a variety of potassium channels in oocytes and compared their sensitivity to ethanol. All GIRK channels studied were enhanced by ethanol in a dose-dependent manner with GIRK2 channels showing the greatest enhancement (Lewohl et al., 1999). The sensitivity of GIRK2 channels are compared with several ligand-gated ion channels in Fig. 2. A statistically significant (but very small) enhancement of GIRK2 function was found with ethanol concentrations as low as 10 mM. Ethanol did not change the reversal potential or shape of the I/V curves, which indicates that it did not activate additional conductances, but enhanced the GIRK potassium conductance. In contrast, other inwardly rectifying potassium channels (IRK1, ROMK1, ROMK2, and ROMK3) showed no significant effect of ethanol at concentrations up to 200 mM. As a first step to determine the role of a GIRK in ethanol action in vivo, we have obtained GIRK2 null mutant mice (Signorini et al., 1997) and are testing them for behavioral actions of ethanol.

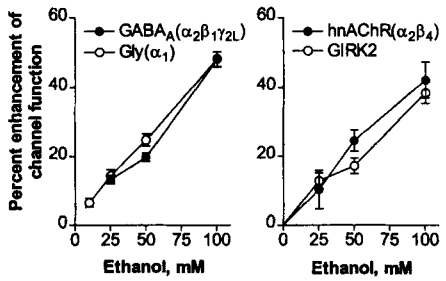

Fig. 2. Ethanol sensitivity of ion channels expressed in *Xenopus* oocytes. Left panel shows ethanol enhancement of function of a heteromeric GABA-A and a homomeric glycine receptor; right panel shows a heteromeric neuronal nicotinic receptor and a G-protein activated inwardly potassium channel. An EC2–5 concentration of GABA or glycine was used, an EC30 concentration of acetylcholine was used; ethanol was preapplied for 2 min. Values are mean ± SEM, n = 5–35, in some cases the error bars are smaller than the symbols. Data are adapted from Cardoso et al., 1999; Lewohl et al., 1999; Mascia et al., 1996b; Ueno et al., 1999.

DETERMINING THE IMPORTANCE OF ETHANOL TARGETS

Transgenic and Mutant Mice

The material presented above shows that many ion channels show some effects of ethanol. None of these consistently show marked effects of low (e.g., 5–20 mM) concentrations, but all have sufficient ethanol sensitivity, at least under some conditions, that they must be considered candidate targets for alcohol action. An immense problem is how to determine if any or all of these proteins are indeed important for any of the diverse behavioral actions of ethanol. The most promising approach currently available is use of transgenic and mutant (knock-out or knock-in) mice.

Genetic manipulation in vivo has contributed markedly to our knowledge of the role of single genes and proteins (especially receptors) in behavior and drug sensitivity. This was first accomplished by natural mutants. For example, the oscillator (*osc*) mutant mouse, lacks a functional glycine α1 subunit, does not develop adult glycine receptors (the

embryonic receptor is formed from α2 and the *osc* mice are normal at birth) and die of seizures within a week or two after birth (Rajendra and Schofield, 1995). In addition, the *weaver* mutant mouse lacks functional GIRK2 channels (Slesinger et al., 1997). More recently, null mutant ("knock-out") mice have been made by homologous recombination in embryonic stem cells where the normal gene is replaced by an inactive gene (Wehner et al., 1996; Chen and Tonegawa, 1997). Possible problems created by this technique are loss of the gene in all tissues (and brain regions) and loss of the gene during development which may produce behavioral effects due to abnormal brain development (Gerlai, 1996). New techniques that overcome these problems include the use of brain region-specific knock outs and inducible knock outs (Furth et al., 1994; Schockett et al., 1995). A potentially more elegant approach is to replace the normal gene with a mutated gene ("knock-in"). For example, mutation of a single amino acid (T286A) in CAM kinase II prevents its activation by autophosphorylation and replacement of the normal gene with this mutation was used to show that the autophosphorylated species of the enzyme is critical for long-term potentiation, learning, and memory (Giese et al., 1998). Another approach to expression of a mutated gene is transgenic mice. These animals express a transgene (which may contain a mutation) in addition to the normal genes (Wei, 1997). Technically, transgenic mice are much easier and cheaper to produce than knock-out or knock-in mice. Overexpression of transgenes may overwhelm the endogenous protein creating new phenotypes (Shyamala et al., 1998; Winder et al., 1998). In addition, the transgene can be placed on a null mutant background, thereby creating the equivalent of a knock-in mouse without the difficulties and expense of homologous recombination. This is also an important approach to assure that a phenotype is caused solely by deletion of one gene by determining if the transgene will "rescue" the animal. This strategy has been used successfully with mice lacking the β3 subunit of the $GABA_A$ receptor and those with a defective β subunit of the glycine receptor (Culiat et al., 1995; Hartenstein et al., 1996). These mutations are quite deleterious to the homozygous mice, but the transgenes were able to completely rescue these mutants, which resulted in healthy transgenic offspring. It should be noted that the feasibility of markedly affecting complex behavioral actions of drugs by mutation of a single receptor has been convincingly shown in a recent study where the α2a adrenergic receptor was inactivated by mutation, and the sedative and analgesic actions of dexmedetomidine were completely eliminated (Lakhlani et al., 1997).

An important issue for the use of transgenic or null mutant mice is the role of the genetic background (Gerlai, 1996; Crawley, 1996). One problem has been the use of mixed genetic background (e.g., 129Sv × C57BL/6 crosses) for mutant animals. This raises the problem of obtaining control animals with the same genotype (except for the transgene) as the experimental animals. Most researchers are now aware of this problem, and mutations are often kept (or placed) onto a homogeneous background. Another potential problem in detecting subtle differences of a mutant mouse is the lack of reliability of some commonly used behavioral tests (Crabbe et al., 1999). However, a discussion of these issues is beyond this review.

SUMMARY

Studies of ion channels, particularly ligand-gated channels, have provided considerable insight regarding alcohol action on brain proteins. However, it is a bit daunting that some of the most interesting questions remain to be answered. We do not know if specific behavioral actions of ethanol are determined by one or few protein targets. For example, are self-administration, tolerance, or dependence strongly determined by alcohol actions on single proteins or does each phenotype require alcohol actions on dozens of proteins? It is perplexing that we do not know how ethanol concentrations of 5–15 mM produce intoxication. Are the small effects seen with multiple channels sufficient? I suspect this is the case, but it is also tempting to propose that a very sensitive site of action is lurking in the cellular soup. Are there structural or functional commonalities among the ethanol-sensitive proteins? Up to now, we have described and cataloged these proteins, but the next phase is a 'periodic table' of ethanol targets that show the functional interrelationship among these proteins. These sorts of questions suggest (to me at least) that we are moving toward the concept of an alcohol receptor—a construct that violates the current dogma of the alcohol field. Like all of science, our field needs less dogma and more imagination!

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