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Effects of Ethanol on Adenosine 5′-Triphosphate–Gated Purinergic and 5-Hydroxytryptamine$_3$ Receptors

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Abstract

This report of the proceedings of a symposium presented at the 2005 annual meeting of the Research Society on Alcoholism highlights the actions of ethanol on purinergic (P2XRs) and 5-hydroxytryptamine$_3$ (5-HT$_3$Rs) receptors. Both P2XRs and 5-HT$_3$Rs, are modulated by pharmacologically relevant concentrations of ethanol, with inhibition or stimulation of P2XR subtypes and stimulation of 5-HT$_3$Rs, respectively. With regard to ethanol-modulatory actions, these 2 distinctly different receptor classes have been studied to a much lesser extent than other LGICs. The organizers and chairs were Daryl L. Davies and Tina K. Machu. John J. Woodward discusses the molecular pharmacology and physiology of P2XRs and 5-HT$_3$Rs and sets the stage for a detailed investigation into the ethanol sensitivity of these channels by the invited speakers. Daryl L. Davies discusses the results from recent electrophysiological studies conducted in his and Dr. Woodward's laboratories, highlighting the actions of ethanol on P2XR subtypes. Jiang-Hong Ye discusses results from recent studies using loose-patch and whole-cell recordings on purinergic receptors expressed on neurons from the ventral tegmental area (VTA) in rats. Tina K. Machu discusses electrophysiological studies conducted in her and Dr. David Lovinger's laboratories on nonpore lining residues of the second transmembrane domain (TM2) of the 5-HT$_{3A}$ receptor. Li Zhang presents data demonstrating that F-actin cytoskeletons play a critical role in 5-HT$_3$ receptor clustering in hippocampal neurons. Collectively, the presentations provided strong evidence that P2X and 5-HT$_3$ receptors are important targets for ethanol action.

Keywords

5-HT$_3$ Receptor; P2X receptor; Alcohol; Ethanol

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IT WAS NOT that long ago that research involving ethanol was restricted to using it as a solvent to dissolve more interesting and more lipophilic compounds. In fact, ethanol's ability to readily partition into lipid-rich membranes and to disrupt membrane structure and function was thought to be the key to its biological actions. However, a number of investigators, encouraged by Nick Franks and Bill Lieb's findings on novel mechanisms of action for volatile anesthetics, pursued the idea that ethanol may act on protein targets that regulate neuronal activity (Franks and Lieb, 1998). Thus began a successful and ongoing investigation into the role of neuronal ion channels as important targets of ethanol in the brain.

To date, the effects of ethanol on a large number of voltage-sensitive and ligand-gated ion channels (LGICs) have been carefully and systematically studied. Results from these studies demonstrate a remarkable degree of selectivity and specificity of ethanol action among various ion channel subtypes. In particular, a great deal of effort has focused on defining the ethanol sensitivity of γ-aminobutyric acid type A ($GABA_A$) and *N*-methyl-D-aspartate (NMDA) ionotropic receptors that are activated by GABA and glutamate, the major inhibitory and excitatory neurotransmitters in the brain, respectively. These studies reveal that modulation of $GABA_A$ and NMDA receptor function is complex and is affected by subunit makeup of the receptor, interaction with cytoskeletal and scaffolding proteins, and possibly phosphorylation (Lovinger, 1997; Woodward, 2000).

Despite the wealth of knowledge gained from studies of $GABA_A$ and NMDA receptors, there are still gaps in our knowledge of how ethanol affects neuronal signaling. Presentations in this symposium will highlight recent progress toward understanding the effects of ethanol on 2 other important neurotransmitter-gated ion channel families. These are the P2X receptors activated by adenosine 5′-triphosphate (ATP) and the 5-hydroxytryptamine$_3$ ($5\text{-}HT_3$) receptor that is gated by serotonin. These receptors appear to play critical roles in modulating the activity of neurons and both receptor families are sensitive to perturbation by ethanol. Understanding the sites and mechanisms of action of ethanol on these important ion channel proteins is crucial to advancing our understanding of the complex and pervasive effects of ethanol on brain function.

P2X and $5\text{-}HT_3$ receptors are structurally unrelated LGICs that are coexpressed in some nervous tissues. P2X receptors constitute the most recently cloned family of LGICs and have become a focus of investigation in neuroscience. P2XRs are unrelated to either the Cys-loop superfamily or the glutamate superfamily of LGICs (Khakh, 2001; Khakh et al., 2001; North, 2002). 5-Hydroxytryptamine$_3$ receptors are members of the Cys-loop superfamily (Maricq et al., 1991). P2X and $5\text{-}HT_3$ receptors are both cation-selective LGICs that are activated, respectively, by synaptically released ATP and 5-HT. Coapplication of 5-HT and ATP produces current responses that are non-additive, suggesting that these receptors are functionally coupled (Boué-Grabot et al., 2003). Both P2X and $5\text{-}HT_3$ receptors are modulated by pharmacologically relevant concentrations of ethanol, with inhibition or stimulation of P2X receptor subtypes (Davies et al., 2002, 2005; Xiong et al., 2000 and stimulation of $5\text{-}HT_3$ receptors (Lovinger and White, 1991), respectively. In ethanol-modulatory actions, these 2 distinctly different receptor classes have been studied to a lesser extent than other LGICs. Both receptor classes have been implicated either directly or indirectly through the modulation of receptor systems in mediating some of the behavioral actions of ethanol (reviewed in Davies et al., 2005; McBride et al., 2004).

P2X receptors are fast-acting, cation-permeable ion channels that are gated by synaptically released extracellular ATP (Chizh and Illes, 2000; Khakh, 2001; North, 2002). In the CNS, ATP directly mediates fast excitatory synaptic transmission by acting on P2XRs located on postsynaptic membranes. In addition, ATP acts presynaptically on P2XRs to modulate other neurotransmitters such as GABA, glycine, and glutamate.

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Currently, 7 subtypes of the P2X family of LGICs have been identified (P2X1–P2X7), and the respective mRNAs or subunit proteins have all been found in the CNS (Chizh and Illes, 2000; Deuchars et al., 2002; Khakh, 2001; Khakh et al., 2001; North, 2002). To date, P2X1–P2X5 and P2X7 subtypes can form functional ATP-activated homomeric channels (e.g., P2X2, P2X4) and many subtypes can also form functional recombinant heteromeric receptors (e.g., P2X2/3, P2X4/6) when expressed in *Xenopus* oocytes or mammalian cell lines (Chizh and Illes, 2000; Khakh, 2001; Khakh et al., 2001; North and Surprenant, 2000). P2X receptors are multimeric proteins. Although the stoichiometry of the native receptor has yet to be fully elucidated, it is believed that a functional P2X receptor results from the assembly of 3 subunits (Jiang et al., 2003; Khakh, 2001; Khakh et al., 2001; Stoop et al., 1999). To date, 11 different P2XR subtypes have been identified from the 7 known subtypes, but many other combinations are possible (Khakh, 2001; North, 2002).

Several lines of evidence suggest that 5-HT_3 receptors regulate mesocoritcolimbic dopaminergic (DA) neurons and their sensitivity to ethanol. Administration of 5-HT_3 receptor antagonists reduced the spontaneous activity of DA neurons in the ventral tegmental area (VTA) (Rasmussen et al., 1991), whereas local application of 5-HT_3 receptor agonists in the nucleus accumbens enhanced DA release (Campbell and McBride, 1995). Dopamine release evoked by ethanol in the nucleus accumbens and striatum was blocked by 5-HT_3 receptor antagonists (Carboni et al., 1989; Wozniak et al., 1990). In addition, 5-HT_3 receptor antagonists can reduce alcohol intake in alcoholic humans (Johnson et al., 1993) and laboratory animals (Knapp and Pohorecky, 1992). However, ethanol consumption is reduced in animals given free access (Fadda et al., 1991), but not limited access to alcohol (Knapp and Pohorecky, 1992). Furthermore, the presence of the 5-HT_{3A} receptor is required for 5-HT_3 receptor antagonist–dependent reduction of alcohol intake (Hodge et al., 2004). Thus, the 5-HT_3 receptor has a complex role in altering alcohol intake.

The goals of the symposium presenters were to highlight recent findings on ethanol effects on P2X and 5-HT_3 receptors. The differential actions of ethanol at P2X receptor subtypes were presented and will be used in subsequent chimeric receptor studies to identify the molecular sites and mechanisms of alcohol action. The roles of ethanol-sensitive P2X receptors in regulating VTA neuron function by both pre- and postsynaptic mechanisms were demonstrated. The investigation of putative alcohol binding domains in non–pore-facing residues of second trans-membrane domain (TM2) of the 5-HT_{3A} receptor was presented. The interaction of the light chain of microtubule-associated protein 1B with the 5-HT_{3A} receptor and its influence on macroscopic kinetics of whole-cell current were reported; future studies will focus on the possibility that intracellular signaling pathways are involved in alcohol actions at 5-HT_3 receptors.

ETHANOL DIFFERENTIALLY AFFECTS ATP-GATED $P2X_3$ AND $P2X_4$ RECEPTOR SUBTYPES EXPRESSED IN XENOPUS OOCYTES

Daryl L. Davies, Liana Asatryan, Sacha F. Kuo, Brian F. King, and Ronald L. Alkana

Our laboratory and others have shown that ethanol reversibly inhibits ATP-activated function of both P2X2Rs and P2X4Rs (Davies et al., 2002; Xiong et al., 2000) expressed in *Xenopus* oocytes in a concentration-dependent manner, P2X2Rs being less sensitive than P2X4Rs (Davies et al., 2002). The effects of ethanol on other P2XR sub-types have yet to be extensively studied.

The first objective of the present investigation was to systematically test the effects of ethanol on recombinant $P2X_3$Rs expressed in *Xenopus* oocytes using a 2-electrode voltage clamp. We also conducted simultaneous studies of the effects of ethanol on recombinant $P2X_4$Rs

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expressed in oocytes from the same batches of oocytes used for the $P2X_3Rs$ experiments to directly compare the effects of ethanol on $P2X_3$ and $P2X_4Rs$. The second objective was to investigate the effects of zinc ions (Zn^{2+}) and ethanol on $P2X_3$ and $P2X_4$ receptor function. Previous work found that Zn^{2+} potentiates ATP-gated currents in $P2X_3$ and $P2X_4$ receptors (Wildman et al., 1999a; Xiong et al., 1999). We predicted that the ethanol/Zn^{2+} combination studies could provide insight into the mechanism of ethanol and Zn^{2+} action on the P2XRs.

Ethanol Potentiates ATP-Gated Currents in $P2X_3Rs$

Ethanol (5–200 mM) induced a significant, reversible concentration-dependent potentiation of the EC_{10} ATP-gated currents (Davies et al., 2005). The potentiating effects of ethanol appeared to plateau at 100 mM ethanol (Table 1). To begin to investigate the mechanism of ethanol action in $P2X_3Rs$, we performed ATP (0.5–100 μM) concentration–response (*C/R*) studies in the absence and presence of 100 mM ethanol. Ethanol increased ATP potency, in an allosteric manner, as evidenced by a left shift in the ATP *C/R* curve and a 3-fold decrease in the EC_{50} for ATP (2.35±0.10 μM in the absence of ethanol, which decreased to 0.97±0.12 μM). Ethanol did not significantly alter the Hill coefficient or the maximal effect of ATP (E_{max}).

Coapplication of Ethanol and Zinc (Zn^{2+}) Induces a Greater Than Additive ATP Response in $P2X_3Rs$

We first conducted a zinc *C/R* study in the presence of EC_{10} ATP. We found that Zn^{2+} (1–300 μM) induced a reversible, concentration-dependent potentiation of the EC_{10} ATP-gated currents that appeared to plateau at 100 μM. We next performed ATP *C/R* curves in the absence and presence of 100 μM Zn^{2+}. We found that Zn^{2+} increased ATP potency (ATP EC_{50} was 4.59±0.26 μM in the absence of Zn^{2+}, which decreased to 0.87±0.01 μM in the presence of Zn^{2+}) as evidenced by a left shift in the ATP *C/R* curve and a 5-fold decrease in the EC_{50} for ATP. Zn^{2+} did not significantly alter the Hill coefficient or the maximal effect of ATP (E_{max}). We next tested the effects of coapplication of ethanol and Zn^{2+} on ATP-gated currents in $P2X_3$ receptors. Coapplication of ethanol and Zn^{2+} resulted in a synergistic interaction, potentiating the ATP response in a greater than additive manner (Table 2). We also tested the effects of ethanol on a maximally potentiating effect of Zn^{2+} and found that coapplication of 100 mM ethanol plus 100 μM Zn^{2+} increased ATP-gated currents by 31.3±8.75% above the maximal currents achieved when testing 100 μM Zn^{2+} alone (tested in same oocyte).

Ethanol Inhibits ATP-Gated Currents in $P2X_4Rs$

The P2X4R investigation included oocytes from the same batches studied in the $P2X_3R$ investigations reported above and used the same techniques. Ethanol (5–200 mM) induced a concentration-dependent inhibition of the EC_{10} ATP-gated currents in a reversible manner. This finding is consistent with previous work reported by others (Xiong et al., 2000), and extends our initial findings that tested the effects of 50–200 mM ethanol in P2X4Rs (Davies et al., 2002).

Coapplication of Ethanol and Zinc (Zn^{2+}) Induces an Additive ATP Response in $P2X_4Rs$

In contrast to ethanol, Zn^{2+} potentiates ATP-gated currents in $P2X_4Rs$ (Wildman et al., 1999a; Xiong et al., 1999). We extended our ethanol/$P2X_4$ receptor studies by testing the effects of coapplication of ethanol and Zn^{2+} on ATP-gated currents in $P2X_4Rs$. Interestingly, when ethanol and Zn^{2+} were co-applied, the experimentally determined ATP response in P2X4R appeared to be additive. This latter finding is in contrast to the synergistic effects of ethanol and Zn^{2+} found in P2X3Rs.

The factors contributing to the opposite effects of ethanol between $P2X_3$ and $P2X_4$ receptors are unknown, but the present and previous results provide some insight. The differences cannot

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be attributed to the expression system or measurement systems since similar testing protocols were used for both receptors. The different desensitization rates of $P2X_3$ and $P2X_4$ receptors are probably not a contributing factor as the action of ethanol was inversely related to the concentration of ATP in both receptor subtypes. Moreover, at maximal ATP concentrations, there was no significant effect of ethanol in either receptor subtype. Previous work has shown that pH and metal ion composition can influence P2X receptor function (Stoop et al., 1997; Wildman et al., 1999a, 1999b; Xiong et al., 1999) but these variables were controlled in the present study. Previous work has shown that the concentration of ATP can influence the sensitivity of P2X receptors to ethanol (Davies et al., 2002; Xiong et al., 2000). The present study controlled this factor by testing ethanol inhibition of ATP-activated currents using an equivalent EC_{10} concentration for ATP in both studies. Although further investigations are necessary, the current findings indicate that physiological variables cannot explain the differences in ethanol sensitivity between $P2X_3$ and $P2X_4$ receptors.

In conclusion, we found that ethanol potentiated ATP-gated $P2X_3R$ currents but inhibited ATP-gated $P2X_4R$ currents (Davies et al., 2005). The respective potentiation and inhibition of ATP-gated currents were directly related to ethanol concentration in both receptor subtypes. Importantly, the $P2X_4$/ethanol studies used experimental parameters similar to those used in the $P2X_3R$ studies. Thus, the opposite action of ethanol on ATP responses measured in $P2X_3$ (potentiation) versus $P2X_4$ (inhibition) receptors cannot be attributed to differences in experimental paradigms. We also found that ethanol could increase the maximal effect of Zn^{2+} in $P2X_3Rs$. This finding is consistent with the notion that ethanol and Zn^{2+} act on different sites on P2XRs (Davies et al., 2005).

EFFECTS OF ETHANOL ON PRESYNAPTIC P2 RECEPTOR–MEDIATED INHIBITION OF GABA RELEASE IN RAT VTA

C. Xiao, Daryl L. Davies, and Jiang-Hong Ye

The mesolimbic DA system, originating in the VTA, mediates the rewarding effects of drugs of abuse, including ethanol (Gatto et al., 1994; Rodd et al., 2004; Wise, 1996). The discharge of VTA DA neurons is modulated by GABAergic synapses (Johnson and North, 1992; Xi and Stein, 1998; Ye et al., 2004), which seem to regulate ethanol consumption (Melis et al., 2002; Nowak et al., 1998). However, little is known about other factors that may govern this GABAergic inhibition of DA neurons.

Ethanol inhibition of P2X receptors has been shown in a variety of preparations, including bullfrog dorsal root ganglion neuron, hippocampal neuron, and *Xenopus* and HEK293 expression systems (Davies et al., 2002, 2005; Fischer et al., 2003; Li et al., 2000; Xiong et al., 2000). However, the effect of ethanol on presynaptic purinergic receptors (P2Rs) in VTA has not been explored. Considering the critical role that VTA DA neurons play in mechanisms underlying drug abuse coupled with the aforementioned findings, we speculated that activation of P2X receptors could play a role in the rewarding effects of drugs of abuse, including ethanol. To begin to test this notion, the current study tests the hypothesis that presynaptic P2Rs in VTA modulate GABA release and regulate neuronal activity and that these receptors are subject to regulation by ethanol. To accomplish this goal, we examined the effects of ethanol on the function of ATP-activated P2Rs in DA neurons of the VTA of young rats. The neurons examined were dissociated mechanically and preserved some functional synaptic boutons after isolation.

Electrophysiological Properties of VTA DA and GABAergic Neurons

We first tested the effects of quinpirole (D2 agonist) on the activity of VTA neurons using loose-patch cell-attached configuration. We found that spontaneous ongoing discharges of

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VTA DA neurons were reversibly depressed by 100 nM quinpirole. In addition, "voltage sag" was recorded from current-clamped DA neurons. This voltage sag, which was induced by hyperpolarizing currents, is one of the distinctive physiological membrane properties of DA neurons. In contrast, spontaneous firing of GABAergic neurons was not altered by quinpirole, and voltage sag was not observed in GABA neurons.

ATP Increased Spontaneous Firing of VTA DA Neurons

Several studies have shown that a P2X agonist (MeS-ATP) increased dopamine release in both VTA and nucleus accumbens (Krügel et al., 1999, 2003), suggesting an enhancement of the activity of VTA DA neurons. In support of this notion, we found that ATP reversibly enhanced spontaneous firing in 67% (14/21) of the DA neurons in VTA. Moreover, ATP-induced enhancement was concentration dependent (1 μM ATP increased the firing rate of DA neurons by 19±19%; 10 μM ATP by 33±14%; and 100 μM ATP by 70±27%). In contrast, 1 to 10 μM ATP did not alter the mean amplitude of the action. A significant reduction in the amplitude of spontaneous action potentials was observed only when a high concentration (100 μM) of ATP was applied.

ATP Presynaptically Attenuates $GABA_A$ Receptor–Mediated Spontaneous Inhibitory Postsynaptic Currents

Previous studies on rat midbrain synaptosomes have suggested the existence of presynaptic P2Rs (Díaz-Hernández et al., 2001; Gómez- Villafuertes et al., 2001) and that P2Rs modulated GABA release (Gómez-Villafuertes et al., 2001). In support of this notion, we found that 10 μM ATP inhibited spontaneous inhibitory postsynaptic current (sIPSC) frequency and caused a significant rightward shift of the cumulative probability plot of sIPSC interevent interval. Taken together, these results are consistent with a presynaptic action of ATP to inhibit GABAergic transmission.

Ethanol Modulates GABAergic Transmission

Previous studies of ethanol effects on GABAergic synaptic transmission in brain slices yielded controversial results, including potentiation, inhibition, and no effect. However, ethanol effects on GABA release onto DA neurons in VTA have not been well explored. We next examined the effects of ethanol on GABAergic sIPSCs in DA neurons in VTA and found that ethanol (applied alone) has biphasic effects on sIPSC frequency. That is, at low concentrations (10–20 mM), ethanol significantly enhanced the frequency of sIPSCs, whereas ethanol prominently suppressed sIPSC frequency at higher concentrations (40–80 mM). These observations were further supported by the significant shift of the cumulative plots showing that 10 mM ethanol induced a leftward shift of cumulative probability plots, of sIPSC interevent intervals. In contrast, 40 mM ethanol induced a rightward shift of cumulative plots of sIPSC interevent intervals. The effects of ethanol were reversible. The frequency of sIPSCs returned to control levels after washout of ethanol-containing solutions. Ethanol, in all concentrations tested (10–80 mM), did not significantly change sIPSC amplitude. Taken together, the findings suggest that ethanol modulates GABAergic transmission at a presynaptic site.

Effects of Ethanol on ATP-Induced Inhibition of GABAergic sIPSCs

The aforementioned results suggest that the effect of ethanol on sIPSC frequency varies with concentration. However, the effect of a given concentration of ethanol is stable during its application. When a new baseline was established in the presence of ethanol, we examined the effect of ATP. Interestingly, we found that 10 mM ethanol alone induced a significant increase in sIPSC frequency and that the addition of 10 μM ATP induced a prominent inhibition of sIPSC frequency. The frequency of sIPSCs recovered after washout with ACSF. Adenosine 5′-triphosphate (10 μM) also induced a significant rightward shift of cumulative probability of

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interevent interval of sIPSCs. In addition, 10 μM ATP produced a greater inhibition in the presence of 10 mM ethanol compared to the absence of 10 mM ethanol. Adenosine 5′-triphosphate-induced inhibition was also greater in the presence of 40 and 80 mM ethanol.

Taken together, the data are consistent with the notion that functional P2Rs on GABAergic terminals of the VTA DA neurons regulate GABA release and VTA neuronal excitability, and that presynaptic P2Rs in VTA are sensitive to ethanol.

INVESTIGATION OF PUTATIVE ALCOHOL BINDING DOMAINS IN THE 5-HT_{3A} RECEPTOR

Tina K. Machu, Xiang-Qun Hu, Volodya Hayrapetyan, and David M. Lovinger

The 5-HT_3 receptor has been implicated in alcohol intoxication and alcohol-drinking behavior (reviewed in McBride et al., 2004). 5-Hydroxytryptamine$_3$ receptor antagonists, such as ondansetron and zacopride, reduce voluntary alcohol intake in both laboratory animals and humans (Johnson et al., 1993; Knapp and Pohorecky, 1992). Ethanol and other alcohols enhance 5-HT_3 receptor function (Lovinger and White, 1991; Machu and Harris, 1994). The mechanisms through which alcohols enhance the function and the identification of putative alcohol binding sites in the receptor have been a major focus of investigation in our laboratories. This presentation highlights our current findings and relates them to ongoing work in other laboratories investigating alcohol binding “pockets” in related LGICs.

The 5-HT_3 receptor is a member of the Cys-loop family of LGICs, which includes the nicotinic acetylcholine (nACh), $GABA_A$, GABAρ, and glycine receptors (Maricq et al., 1991). These receptors are pentameric, with each subunit containing an extracellular N-terminus, 4 trans-membrane (TM) domains, a large intracellular loop between TM3 and TM4, and an extracellular C-terminus. The TM2 domain lines the pore of the channel. Five subunits of the 5-HT_3 receptor have been cloned (A–E) (Davies et al., 1999; Karnovsky et al., 2003; Maricq et al., 1991), but the C, D, and E subunits are largely restricted to peripheral tissues. The function of only A and B subunit-containing receptors has been assessed. Homopentameric receptors containing only A subunits are functional, but homopentameric B-containing receptors are not. Heteromeric 5-$HT_{3A/B}$ receptors differ from 5-HT_{3A} receptors in their biophysical properties. The B subunit appears to be largely restricted to the periphery in rodents, which suggests that the predominant form of the 5-HT_3 receptor in the brain is the A homomer (Morales and Wang, 2002).

Multiple alcohol binding sites have been proposed in the Cys-loop family of LGICs. Putative binding sites for alcohols mediating allosteric modulation of function include the N-terminus (Yu et al., 1996) and a water-filled crevice near the extracellular face of the receptor that is composed of a non–pore-facing residue in TM2, as well as residues in TM1 and TM3. The proposed binding pocket in the water-filled crevice is of particular interest, because mutation of key residues in GABA and glycine receptors determines the degree and direction of allosteric modulation by alcohols (Mihic et al., 1997). Furthermore, cryoelectron microscopy studies at 4 angstrom resolution also support the existence of this water-filled crevice, as well as confirm the largely α helical structure of TM2 in the muscle-type nACh receptor (Miyazawa et al., 2003). In addition, experiments with the substituted cysteine accessibility method (SCAM) in the 5-HT_{3A} receptor revealed the positions of pore-facing residues in TM2 (Reeves et al., 2001). Thus, we tested the hypothesis that a nonpore residue(s) in TM2 is part of an alcohol binding pocket. We focused on non–pore-facing residues, because pore-lining residues would be more consistent with a channel-blocking effect of alcohols.

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Mechanisms of Alcohol Potentiation of 5-HT_3 Receptor Function

Alcohols (C2–C6) and volatile anesthetics enhance the function of 5-HT_{3A} receptors in a concentration-dependent manner. Recently, the actions of alcohols and anesthetics have been assessed in heteromeric 5-$HT_{3A/B}$ receptors. In general, the heteromeric receptor is less sensitive to volatile anesthetics and higher n-chain alcohols (Stevens et al., 2005) and is insensitive to ethanol (Hayrapetyan et al., 2005). However, the findings presented in this symposium were restricted to the 5-HT_{3A} receptor. Thus, this summary will focus only on alcohol actions at the 5-HT_{3A} receptor.

Alcohols enhance 5-HT_{3A} receptor function in cells that endogenously express receptors (Lovinger and White, 1991), as well as in cells that heterologously express recombinant receptors (Machu and Harris, 1994; Sessoms-Sikes et al., 2003). The effect is rapidly reversible and dependent on the 5-HT concentration used. That is, the greater the concentration, the less the enhancement observed. These results suggest that as the probability of ion channel opening increases, the ability of alcohols to allosterically enhance receptor function is reduced. Furthermore, rapid drug superfusion studies demonstrated that alcohols increase the activation rate and reduce the desensitization rate of whole-cell currents, which favors and stabilizes the open channel state (Zhou et al., 1998). Alcohols enhance currents generated by a saturating concentration of the partial agonist, dopamine, suggesting that increased probability of opening occurs in the absence of any effect on agonist affinity (Lovinger et al., 2000).

Transmembrane Two Mutations, Alcohol Actions, and Putative Alcohol Binding Pockets

Studies in the $GABA_A$ receptors with the SCAM technique demonstrated the accessibility of sulfhydryl reagents to amino acids in the water-filled crevice, formed in part by TM2 and TM3, as well as to TM3 residues located closer to the cytoplasmic aspect of the receptor (Williams and Akabas, 1999). These results raise the interesting possibility that there may be a more cytoplasmic water-filled crevice accessible to small molecules. Therefore, we considered all the non–pore-facing TM2 residues as possible alcohol binding domains. We performed alanine scanning mutagenesis of these residues, as well as serine, cysteine, and tryptophan mutagenesis in some cases. These amino acids were selected because they have a wide range of molecular volumes, polarities, and hydropathies. We measured 5-HT potency, ethanol sensitivity, and n-chain alcohol cutoff in wild-type and mutant 5-HT_{3A} receptors expressed in *Xenopus* ooctyes with 2-electrode voltage-clamp electrophysiological recordings. For some TM2 mutants, we also examined the kinetic properties of whole-cell currents in HEK293 cells expressing receptors, with rapid drug superfusion. We hypothesized that shifts in 5-HT potency might explain loss or gain of ethanol sensitivity. That is, mutants with a low 5-HT EC_{50} would be weakly sensitive or insensitive to ethanol. Mutations in TM2 of the Cys-loop family have been previously shown to alter both agonist potency (Mihic et al., 1997; Sessoms-Sikes et al., 2003) and channel kinetic properties (Scheller and Forman, 2002). Therefore, we anticipated that kinetics properties would be altered in some mutant 5-HT_{3A} receptors and therefore might alter allosteric modulation by alcohols independent of affecting an alcohol binding pocket. The ideal candidate for an alcohol binding domain, when mutated, would have the following characteristics: (1) loss of alcohol sensitivity due to a change in the property of amino acid substituted; (2) a change in alcohol cutoff; (3) minimal effect on 5-HT potency; (4) minimal differences from wild-type receptor on kinetics of whole-cell current in the absence of alcohol; and (5) loss of ethanol enhancement of 5-HT-mediated currents accompanied by no ethanol-induced increases in activation rate.

In the 2-electrode voltage-clamp configuration, we assessed 15 mutant 5-HT_{3A} receptors expressed in *Xenopus* oocytes. These mutations were generated at non–pore-facing TM2 residues at positions 1′,3′,4′,5′,8′,10′,11′,14′, 15′, 18′, and 19′, with position 1′ being at the most cytoplasmic aspect of TM2. Receptors mutagenized at positions 11′ and 14′ were nonfunctional

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and had low current densities, respectively, and thus were not examined further. Shifts in 5-HT potency were observed in mutant 5-HT_{3A} receptors at 1′, 4′, 5′, 10′, and 15′. 5-Hydroxytryptamine EC_{50}s were significantly reduced in 2 mutants and significantly increased in 4 mutants. Increases in ethanol sensitivity were observed in receptors with mutations at 3′ and 10′. Conversely, decreases in ethanol sensitivity were observed in receptors with mutations at 5′, 15′, and 19′. The correlation between 5-HT EC_{50} and ethanol sensitivity was poor (r^2 = 0.44). Changes in n-chain alcohol cutoff were observed with receptors containing mutations at 3′, 5′, 18′, and 19′. Receptors containing mutations at 5′, 15′, and 19′, which were insensitive to ethanol, were sensitive to hexanol. It is unclear why a putative alcohol binding pocket would not be able to accommodate ethanol, but would be able to bind hexanol, a larger molecule. It is unlikely that binding pockets exist at multiple sites in the protein-facing aspect of TM2. Given that TM2 mutations can cause changes in the pharmacological and biophysical properties of the Cys-loop family of receptors, it is possible that some of the changes in allosteric modulation by alcohols that we observed in mutant 5-HT_{3A} receptors were secondary to changes in channel gating instead of an alteration of an alcohol binding domain.

We next tested the hypothesis that some of the mutant 5-HT_{3A} receptors with altered alcohol-modulatory actions might be eliminated as false-positive alcohol binding domains. We measured macroscopic kinetics of whole-cell currents in wild-type and mutant 5-HT_{3A} receptors expressed in HEK 293 cells. We predicted that a false-positive mutant receptor would have altered kinetics relative to the wild-type receptor that would either occlude or mimic the actions of alcohols. All mutations, including the most conservative, that were generated at the 15′ position of TM2 in the receptor altered activation, deactivation, and desensitization kinetics, as well as 5-HT potency. In the majority of mutants tested, ethanol had no effect on 5-HT-mediated currents. Change in alcohol sensitivity could not be related to alteration of any single kinetic parameter, nor could any change in kinetic parameter or in 5-HT potency be used to predict alcohol sensitivity. Another mutation was examined at the 3′ position of the receptor. Despite the observation that ethanol sensitivity was similar to that in the wild-type receptor, the mechanism through which ethanol produces an increase in peak current amplitude appeared to be altered. In the wild-type receptor, ethanol's primary action is to enhance the rate of channel opening. In contrast, in the 3′ mutant 5-HT_{3A} receptor, ethanol had no effect on activation rate, but significantly reduced desensitization and deactivation rates. Thus, kinetic measurements of whole-cell currents in the mutant receptors revealed the ease with which gating properties can be altered and demonstrated no clear relationship between these altered properties and alcohol sensitivity.

In conclusion, mutations at numerous positions in the TM2 domain of the mouse 5-HT_{3A} receptor produce changes in 5-HT potency, channel kinetics, ethanol sensitivity, and n-chain alcohol cutoff. None of the measurements correlated with changes in ethanol enhancement of 5-HT-mediated currents. Furthermore, we have demonstrated previously that mutations at the 16′ position of TM2, a pore-facing residue, eliminate ethanol sensitivity and produce changes in alcohol cutoff. An alcohol binding domain in the channel pore would be more consistent with a channel-blocking effect of an alcohol. Collectively, these results suggest that none of the electrophysiological approaches used in our studies can effectively distinguish between a mutant receptor with a candidate alcohol binding domain and a false-positive mutant receptor with altered alcohol sensitivity secondary to mutation-induced changes in gating properties.

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INVESTIGATION INTO THE REGULATION OF 5-HT_3 RECEPTOR FUNCTION AND THE RECEPTOR'S SENSITIVITY TO ALCOHOL BY INTRACELLULAR SIGNALING PATHWAYS

Li Zhang, Hui Sun, and Xiang-Qun Hu

The serotonin type 3 (5-HT_3) receptor is a member of a LGIC supergene family including $GABA_A$, glycine, and nicotinic acetylcholine (nACh) receptors (Maricq et al., 1991). The 5-HT_{3A} receptors are differentially distributed in a number of important brain areas including the nucleus accumbens and VTA (van Hooft and Vijverberg, 2000). In these brain regions, 5-HT_3 receptors have been found to modulate the release of neurotransmitters such as dopamine and GABA (van Hooft and Vijverberg, 2000). Emerging evidence has indicated that the 5-HT_3 receptor is an important target for the action of alcohol and other drugs of abuse in the central nervous system (Lovinger, 1999). Several lines of studies suggest that 5-HT_3 receptors may play an important role in alcohol preference and reward mechanisms (Lovinger, 1999). Clinical studies have provided evidence that ondansetron, a selective antagonist of 5-HT_3 receptors, can reduce alcohol intake in early onset alcoholic individuals (Johnson et al., 2000). Although ethanol is found to potentiate 5-HT_3 receptor–mediated currents in various types of neurons and cell lines expressing recombinant 5-HT_{3A} receptors, the molecular mechanisms that underlie alcohol dependence remain elusive.

Recent research interest has turned to the regulation of LGIC function, trafficking, and receptor sensitivity to ethanol by intracellular signaling mechanisms. For instance, activation of protein kinase C (PKC) can modulate glycine, $GABA_A$, and NMDA receptors in various types of neurons and in cell lines expressing these receptors. Protein kinase C has been found to induce internalization of $GABA_A$ receptors (Chapell et al., 1998) and, on the other hand, to promote exocytosis of NMDA receptors at the cell surface (Lan et al., 2001). Recent studies have suggested that long-term alcohol consumption can alter the expression and trafficking of certain types of LGICs such as GABA and NMDA receptors (Ron, 2004; Newton and Messing, 2005). Certain types of PKC ioszymes have been identified as key elements that regulate $GABA_A$ receptor sensitivity to ethanol (Newton and Messing, 2005).

Of all LGICs, modulation of 5-HT_{3A} receptor function by PKC has received relatively little attention. Previous studies reported that the application of phorbol esters, activators of PKC, can potentiate 5-HT-activated current in *Xenopus* oocytes expressing 5-HT_{3A} receptors (Zhang et al., 1995). A previous study has revealed an involvement of a tyrosine kinase in PKC enhancement of 5-HT_{3A} receptor function (Coultrap and Machu, 2002). We have recently shown that activation of PKC potentiated 5-HT_3 receptor–mediated responses and enhanced surface immunolabeling and surface expression of 5-HT_{3A} receptors for both *Xenopus* oocytes and in N1E-115 cells (Sun et al., 2003). The increase in surface receptor expression by PKC activation is likely to occur via an increase in receptor trafficking rather than an increase of receptor protein synthesis as the quantity of receptor protein in the total cell extract remained unchanged after phorbol-12-myristate-13-acetate (PMA) treatment. Phorbol-12-myristate-13-acetate potentiation is unlikely to occur via direct phosphorylation of the 5-HT_{3A} receptor protein as the potentiation was not affected by point mutation of each of all putative sites for PKC phosphorylation. Instead, our study suggests that PKC can modulate 5-HT_{3A} receptor function and trafficking through an F-actin–dependent mechanism. This hypothesis is consistent with an observation that 5-HT_{3A} receptors are colocalized and coclustered with F-actin in various neurons and in cells transiently transfected with cloned 5-HT_{3A} receptors (Emerit et al., 2002).

Most neurotransmitter-gated ion channel proteins are anchored to the cytoskeleton network by bridging proteins at synaptic sites. Such a linkage is thought to be critical for receptor targeting,

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clustering, and trafficking. Regulation of neurotransmitter-gated ion channel proteins by cytoskeleton networks has also been the focus of recent studies. However, the role of cytoskeleton proteins in the regulation of 5-HT_3 receptor gating kinetics has not been reported. To address this question, we conducted experiments involving yeast 2-hybrid screening, coimmunopre cipitation, coimmunostaining, and kinetic analysis by patch-clamp recording using a fast perfusion system. Our results suggest that the light chain of MAP1B strongly interacts with 5-HT3A receptor protein. 5-Hydroxytryptamine$_{3A}$ receptors and LC1 were colocalized all along growing axons, particularly in growth cones of hippocampal neurons. Overexpression of LC1 internalized 5-HT_{3A} receptors expressed in HEK 293 cells and remarkably accelerated receptor gating kinetics. Disruption of the receptor–cytoskeleton interaction slowed the desensitization kinetics of the cloned and native 5-HT_3 receptors. The slow kinetics was fully reversed by overexpression of LC1. Moreover, the microtubule disruption increased the open probability but not conductance of single channel current. Thus, an interaction with LC1 internalizes and stabilizes the channels in a closed and desensitized conformational state, thereby controlling the efficacy of 5-HT_3 receptor–mediated synaptic transmission in the brain.

To address whether long-term alcohol consumption alters the expression and trafficking of 5-HT_3 receptor, we administrated transgenic mice overexpressing 5-HT_{3A} receptors with a nutritionally complete liquid diet that was mixed with or without ethanol for 2 weeks. The concentration of ethanol given to the mice started from 3% (v/v) and increased by 1% every 3 days until it reached 6%. While the extent of food consumption and body weight between the alcohol and control-diet mice was not significantly different, long-term ethanol exposure decreased the mRNA expression of 5-HT_{3A} receptors by 24%, as measured by real-time quantitative PCR. The study of longterm alcohol action on 5-HT_3 receptor trafficking is in progress. Also, we are currently investigating alcohol action on 5-HT_3 receptor–cytoskeleton interaction.

CONCLUSIONS

This symposium provided strong evidence that P2X and 5-HT_3 receptors are important targets for ethanol action. Some of the first studies investigating the ethanol sensitivity of P2X receptor subtypes and P2X receptor actions in regulating VTA neuron activity were presented. The difficulty in assessing the role of putative alcohol binding domains in conferring ethanol sensitivity to the 5-HT_{3A} receptor was reported. Finally, the role of the light chain of microtubule-associated protein 1B in regulating 5-HT3A receptor function was described, and the possibility that this interaction may be involved in ethanol's actions at the 5-HT_{3A} receptor was raised.

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Table 1

The Action of Ethanol Is Subunit Dependent in P2X Receptors

Ethanol (mM)	$P2X_3$ receptors % potentiation	$P2X_4$ receptors % inhibition
5	10.5 ± 2.2	6.9 ± 3.6
10	13.3 ± 3.4	10.5 ± 2.0
25	26.4 ± 5.0	21.7 ± 4.3
50	28.3 ± 8.3	28.5 ± 4.2
100	48.7 ± 18.6	38.7 ± 3.6
200	38.7 ± 13.17	65.8 ± 5.2

Ethanol caused a concentration-dependent potentiation of ATP-activated currents in oocytes expressing P2X3 receptors. Ethanol caused a concentration-dependent inhibition of ATP-activated currents in oocytes expressing P2X4 receptors. The data were taken from the results and represented as average percentage increase or decrease of ATP currents by ethanol.

ATP, adenosine 5′-triphosphate.

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Table 2

Experimental EtOH+Zn^{2+} Results From a Coapplication of Ethanol and Zn^{2+} Significantly Increased ATP-Gated Currents Versus Estimated EtOH+Zn^{2+} Currents

EtOH (100 mM)	Zn^{2+} (5 μM)	Estimated EtOH+Zn^{2+}	Experimental EtOH+Zn^{2+}*
20.9 ± 1.7%	25.9 ± 5.0%	46.7 ± 4.4%	63.7 ± 5.7%

ATP, adenosine 5′-triphosphate.