Published in final edited form as:
Alcohol Clin Exp Res. 2011 April ; 35(4): 584–594. doi:10.1111/j.1530-0277.2010.01379.x.

Implication of the Purinergic System in Alcohol Use Disorders

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Abstract

In the central nervous system, adenosine and ATP play an important role in regulating neuronal activity as well as controlling other neurotransmitter systems such as GABA, glutamate, and dopamine. Ethanol increases extracellular adenosine levels that regulate the ataxic and hypnotic/sedative effects of ethanol. Interestingly, ethanol is known to increase adenosine levels by inhibiting an ethanol-sensitive adenosine transporter, ENT1 (equilibrative nucleoside transporter type 1). Ethanol is also known to inhibit ATP-specific P2X receptors, which might result in such similar effects as those caused by an increase in adenosine. Adenosine and ATP exert their functions through P1 (metabotropic) and P2 (P2X-ionotropic and P2Y-metabotropic) receptors, respectively. Purinergic signaling in cortex-striatum-VTA has been implicated in regulating cortical glutamate signaling as well as VTA dopaminergic signaling, which regulates the motivational effect of ethanol. Moreover, several nucleoside transporters and receptors have been identified in astrocytes, which regulate not only adenosine-ATP neurotransmission, but also homeostasis of major inhibitory-excitatory neurotransmission (*i.e.* GABA or glutamate) through neuron-glial interactions. This review will present novel findings on the implications of adenosine and ATP neurotransmission in alcohol use disorders.

Keywords

Adenosine; ATP; Alcoholism; Purinergic; Signaling; Neurotransmission

Introduction

In 1972, a purine nucleotide, ATP (adenosine 5'-triphosphate), began to be recognized as a neurotransmitter. As shown in Fig. 1, adenosine is synthesized from ATP and has been known as a neurotransmitter or neuromodulator since it alters neuronal activity (Burnstock, 1972;Burnstock, 2008). In the late 1970's, adenosine receptors have been identified as P1 receptors, which are selectively antagonized by low concentrations of methylxanthines such as caffeine and theophylline (Burnstock, 2008). Currently, four well-characterized G-protein coupled adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3 in the brain are known to mediate the physiological function of adenosine (Fredholm et al., 2005). Adenosine signaling has been implicated in the pathophysiology of many central nervous system (CNS) disorders including sleep disorders, anxiety, and alcoholism (Burnstock, 2008;Dunwiddie and Masino,

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2001;Fredholm, 2010;Fredholm et al., 2005). Furthermore, interaction between adenosine receptors and other G-protein coupled receptors in the striatum regulates motor and motivational behaviors (Ferre et al., 2008;Ferre et al., 1997;Ferre et al., 2002;Ferre et al., 1996).

Ataxia is the earliest and most conspicuous physical manifestation of acute ethanol consumption. The striking similarity between the pharmacology of adenosine and ethanol provided strong circumstantial evidence for an adenosinergic modulation of CNS effects of ethanol. Dar and his colleagues first reported a functional relationship between adenosine and ethanol because dipyridamole, an ENT1 inhibitor, promoted ethanol-induced ataxia and sleep (Dar et al., 1983). Extracellular or synaptic adenosine levels are mainly regulated by nucleoside transporters (Baldwin et al., 1999; Sawynok and Liu, 2003). Among several nucleoside transporters, ENT1 (equilibrative nucleoside transporter type 1) regulates extracellular adenosine levels in response to ethanol treatment in cultured cells (Chen et al., 2010; Choi et al., 2004; Nagy et al., 1990).

ATP-gated P2X receptors (P2XRs) are a superfamily of ligand-gated ion-channels (Bantel et al., 2002; Khakh et al., 2001) that are becoming a focus of investigation in alcohol studies. Currently, seven subunits of the P2XRs have been identified (P2X1–P2X7) that form homo- or heteromeric channels (e.g., P2X1/4, P2X1/5, P2X2/3, P2X4/6) when expressed in *Xenopus* oocytes or mammalian cell lines (Lalo et al., 2008; Le et al., 1998; Lewis et al., 1995; Nicke et al., 2005). Each P2XR subunit consists of two alpha-helical transmembrane (TM) segments, a large extracellular domain (ectodomain), and an intracellular location of amino and carboxy terminals (North, 2002). Recent crystallographic investigations on zebra fish P2X4Rs (Kawate et al., 2009) confirmed previous predictions that functional P2XRs result from the assembly of three subunits (Aschrafi et al., 2004; Jiang et al., 2003).

BRAIN ADENOSINERGIC A_1 MODULATION OF ETHANOL-INDUCED ATAXIA

Using long-sleep (LS) and short-sleep (SS) mice as genetic models with differential sensitivity to the soporific effects of ethanol, Proctor and Dunwiddie (1984) reported that LS mice were sensitive not only to ethanol's soporific effects, but also to the sedative effects of an adenosine agonist, further supporting that adenosine signaling is involved in ethanol intoxication. Other investigators confirmed and elegantly extended these studies that demonstrated a role of adenosine in alcoholism (Gordon et al., 1986; Nagy et al., 1990). A key mechanism in the behavioral effects of ethanol involves inhibition of alcohol-sensitive ENT1 (Nagy et al., 1990). This increases the extraneuronal adenosine levels that activate adenosine receptors and is known to mediate acute and chronic effects of ethanol (Diamond and Gordon, 1994; Dunwiddie and Masino, 2001). Interestingly, mice lacking ENT1 stayed on the rotarod significantly longer compared to wild-type mice when mice were given 1.0 or 1.5 g/kg ethanol (*i.p.*) (Choi et al., 2004). Since ENT1 null mice showed reduced A_1 receptor function (Choi et al., 2004), diminished A_1 receptor may mediate resistance to ethanol-induced ataxia.

Dar and his colleagues have confirmed the A_1 adenosinergic modulation of ethanol-induced ataxia in male CD-1 mice and Sprague-Dawley rats, using intraperitoneal (ip), intracerebroventricular (icv), intracerebellar (ICB), intrastriatal (IST), and intramotorcortical (IMC) administration of adenosine agonists/antagonists. The sensitivity of the cerebellum to ethanol has been associated with cAMP-response-element-binding protein (CREB) transcription activity and cerebellar activation that plays a role in alcoholism (Acquaah-Mensah et al., 2006). It is the significance of cerebellum in alcoholism that directed our focus to the study of ethanol-induced ataxia, although striatum (Meng et al., 1997) and motor cortex (Barwick and Dar, 1998) also mediate ethanol-induced ataxia.

Direct ICB infusion of adenosine A_1-, A_1/A_{2A}- and A_{2A}-selective agonists/antagonists, including N^6–cyclohexyl-adenosine (CHA; an adenosine A_1 agonist) and $^{5'}$-N-ethylcarboxamido-adenosine (NECA; an agonist with A_1/A_{2A} affinity), markedly accentuated ethanol-induced ataxia that was blocked by adenosine A_1 antagonist, 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) (Dar, 1990; Dar, 1998). The role of A_1 receptors in the modulation of ethanol-induced ataxia was further supported indirectly by the results of experiments involving microinfusion of 2-p-(2-carboxyethyl) phenethylamino-5'-N-carboxaminoadenosine (CGS-21680; an adenosine A_{2A}-selective agonist) into the rat motor cortex (Barwick and Dar, 1998) and mouse striatum (Dar, 2001). In spite of its high A_{2A}-selectivity [K_i= 2600 nM (A_1); K_i = 15nM (A_{2A})], at least a 25-fold higher dose of CGS-21680 was required compared to CHA, to produce comparable accentuation of ethanol-induced ataxia (Dar, 2001). The accentuation by CGS-21680 was totally abolished by pretreatment with DPCPX indicating A_1- and not A_{2A}-receptor modulation (Dar, 2001). Further evidence for the modulation of ethanol-induced ataxia by A_1- and not A_{2A}-receptor was provided when intramotor cortex microinfusion of a high dose (4 nmol) of CGS-21680 accentuated ethanol-induced ataxia that was abolished by DPCPX but not by A_{2A}-selective antagonist, 8-(3-chlorostyryl) caffeine (Barwick and Dar, 1998). Thus, the accentuation by CGS-21680 at such a high dose presents the possibility that it is modulating ethanol-induced ataxia through a non-selective effect at the adenosine A_1 receptor. Finally, pretreatment with adenosine A_1 antisense RNA blocked the CHA-induced accentuation of ethanol-induced ataxia, indicating that an inhibition of A_1 receptor expression is associated with a decreased ethanol response (Dar and Mustafa, 2002).

The ethanol-induced cerebellar ataxia was functionally related to: (i) an increase in the maximum number of adenosine A_1 receptors (Clark et al., 1993); (ii) an inhibition of adenosine uptake (Clark and Dar, 1989b) *via* inhibition of alcohol-sensitive ENT1; (iii) an increase in adenosine release (Clark and Dar, 1989a); and, (iv) a decrease in glutamate release in an adenosine-sensitive manner (Clark and Dar, 1989c). The A_1 adenosine receptors are highly expressed in the cerebellar granule cells, their axons, and axonal terminals (Dar, 1997). Since A_1 receptors are coupled with the G_i/G_o protein, the adenylate cyclase/cAMP/PKA signaling system has been implicated in the behavioral responses of ethanol (Newton and Messing, 2006). This signaling pathway participates in ethanol-induced ataxia because ethanol-induced ataxia is augmented by pretreatment with miconazole, an inhibitor of adenylate cyclase (Dar, 1997), and attenuated by forskolin, a stimulator of adenylate cyclase or by cAMP/cpt-cAMP (Dar, 1997). In addition, ethanol-induced ataxia is positively correlated with increased chloride uptake in the striatal microsacs, which is also regulated by adenosine A_1 receptors (Meng et al., 1997). Since $GABA_A$ agonist (muscimol) activated ethanol-induced ataxia while $GABA_A$ antagonist (bicuculline) has an opposite effect (Dar, 2006), GABA receptor-mediated chloride influx into the cells contributes to ethanol-induced ataxia. Interestingly, ethanol is also known to potentiate GABAergic transmission to cerebellar granule cells *via* an increase in Golgi cell excitability (Carta et al., 2004). The GABA released at the granule cell synapse dampens the firing of granule cells, which are the only excitatory cells within the cerebellar cortex (Voogd and Glickstein, 1998). Consequently, in the deficit of this excitatory signal, the GABAergic Purkinje cells inhibit the deep cerebellar nuclei, thereby resulting in ethanol-induced ataxia.

The inhibition of ENT1 by acute ethanol increases extracellular adenosine levels (Nagy et al., 1990; Nagy et al., 1989), which activates A_1 receptors and decreases glutamate release at parallel fibers to the Purkinje cell synapse. This process leads to the expression of ethanol-induced cerebellar ataxia. Ethanol also inhibits nitric oxide synthase (NOS), causing decreased nitric oxide (NO) levels (Al-Rejaie and Dar, 2006a; Al-Rejaie and Dar, 2006b). Cerebellar NOS is located in the granule and basket cells from which soluble NO diffuses to

Purkinje cells (Fedele et al., 1998). The Purkinje cells exhibit strong immunoreactivity for guanylyl cyclase (Marcoli et al., 2006). Thus, NO would stimulate cGMP production in the Purkinje cells, resulting in decreased Purkinje cell firing. The cerebellar glutamate system appears to regulate NO-cGMP signaling as elucidated in our previous studies (Al-Rejaie and Dar, 2006a; Al-Rejaie and Dar, 2006b). As Purkinje cells represent the only inhibitory output in the cerebellar cortex, any event leading to the depression of Purkinje cell firing would cause a decrease in GABAergic transmission within the Purkinje cells and the consequent attenuation of ethanol-induced ataxia. Thus, the overall effect of ethanol is to suppress excitatory glutamatergic transmission and increase GABAergic inhibitory firing by enhancing inhibitory function of Purkinje cells on the deep cerebellar nuclei, all of which lead to ataxia.

A ROLE OF ADENOSINE AND THE WAKE-PROMOTING BASAL FOREBRAIN IN MEDIATING THE SOMNOGENIC EFFECTS OF ETHANOL

It is known that ethanol intake has significant effects on sleep (Brower, 2001; Roehrs and Roth, 2001). However, the cellular substrates responsible for mediating the effects of ethanol on sleep are unknown. Strong and consistent evidence suggest that adenosine, a somnogen, is a key mediator of many behavioral and neuronal responses to ethanol including ataxia, anxiety, tremors, and seizures (Barwick and Dar, 1998; Batista et al., 2005; Concas et al., 1996; Dar, 2006; Dunwiddie and Masino, 2001; Hack and Christie, 2003; Jarvis and Becker, 1998; Kaplan et al., 1999; Newton and Messing, 2006; Phan et al., 1997; Prediger et al., 2006). Acute exposure to ethanol inhibits adenosine reuptake *via* ENT1 in cell cultures. Chronic ethanol exposure down-regulates ENT1 expression (Krauss et al., 1993; Nagy et al., 1990). ENT1 null mice display decreased adenosinergic tone, resulting in increased ethanol consumption in addition to reduced hypnotic (loss of righting reflex) and ataxic responses to ethanol. In contrast, treatment with an A_1 receptor agonist decreased ethanol consumption in ENT1 null mice (Choi et al., 2004).

Previous studies suggest that adenosine is a homeostatic regulator of sleep (Portas et al., 1997; Radulovacki et al., 1984; Thakkar and Mallick, 1996; Thakkar et al., 2003b). During prolonged wakefulness, adenosine, a byproduct of metabolism, accumulates in the wake-promoting basal forebrain (BF) region that includes the horizontal diagonal band, the substantia innominata and the magnocellularis pre-optic nuclei (HDB/SI/MCPO) (Murillo-Rodriguez et al., 2004; Porkka-Heiskanen et al., 1997). Increased adenosine in the BF acts *via* A_1 receptors to inhibit the BF cholinergic and non-cholinergic wake-promoting (GABAergic and glutamatergic) neurons (Alam et al., 1999; Arrigoni et al., 2006; Thakkar et al., 2003a; Thakkar et al., 2003b). Inhibition of BF wake-promoting neurons results in the transition from wakefulness to sleep (Basheer et al., 2004; McCarley, 2007). Lesions of the BF cholinergic neurons abolish prolonged wakefulness, induce an increase in adenosine release, and attenuate the homeostatic response to sleep deprivation (Blanco-Centurion et al., 2006; Kalinchuk et al., 2008; Kaur et al., 2008).

Recent studies suggest that acute intragastric administration of ethanol (3 g/kg) in freely behaving Sprague-Dawley rats produces a significant increase in slow wave sleep (non-rapid eye movement sleep) with a concomitant decrease in wakefulness during the first 12 hours after ethanol treatment. Rapid eye movement (REM) sleep remains unaffected. Bilateral microinjections localized in the HDB/SI/MCPO [see Figure 4 in (Thakkar et al., 2010)] of a selective A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), significantly attenuates the sleep-inducing effects of ethanol, suggesting a role of A_1 receptor in ethanol-induced sleep. Furthermore, intragastric administration of ethanol (3 g/kg) causes a significant decrease in the number of BF wake-promoting neurons with c-Fos immunoreactivity, suggesting ethanol-induced inhibition of BF wake-promoting neurons

(Thakkar et al., 2010). While these results suggest that adenosinergic mechanisms may play an essential role in ethanol-induced sleep promotion by inhibiting the wake-promoting neurons of the BF, the confirmatory test demonstrated that local microdialysis perfusion of ethanol induces a significant and dose-dependent increase in extracellular levels of adenosine in the BF (Thakkar et al., 2010). As expected, local administration of ethanol in the BF region is known to increase the duration of sleep (Mendelson, 2001). These recent results suggest that ethanol causes an increase of extracellular adenosine in the BF. Increased extracellular adenosine inhibits the BF wake-promoting neurons *via* A_1 receptor to promote sleep, which is a key mechanism responsible for sleep induction following acute ethanol intake. Since adenosine is a homeostatic regulator of sleep, and since ethanol has no effect on the circadian timing of sleep onset at lights-on [see Figure 2 in Thakkar et. al., 2010], acute ethanol intake may affect the homeostatic, but not the circadian regulation, of sleep.

INTERACTION OF ADENOSINE RECEPTORS WITH OTHER G-PROTEIN COUPLED RECEPTORS AND ETHANOL PREFERENCE

It is evident that adenosine A_{2A} and dopamine D_2 receptors interact with each other on the plasma membranes or in the cytoplasm through signaling molecules (Ferre et al., 1991; Short et al., 2006a). Similarly, adenosine A_1 and dopamine D_1 receptor can also cross talk (Ferre et al., 1996). Especially, dimerization of A_{2A} receptors with D_2 receptors in the striatum allows the A_{2A} to influence reward-related behavior through modulation of adenylate cyclase activity (Ferre et al., 2008; Ferre et al., 1994). Contrary to the antagonistic actions of these receptors under normal circumstances, in the presence of ethanol, the two receptors can have synergistic activity through Gβγ which increases cAMP levels and PKA activity (Mailliard and Diamond, 2004; Yao et al., 2002; Yao et al., 2003). In rats, A_2 antagonist 3,7-dimethyl-1-propargylxanth (DMPX) attenuated ethanol self-administration (Arolfo et al., 2004; Thorsell et al., 2007), indicating that modulation of A_{2A} receptor plays an essential role in ethanol preference. On the other hand, A_{2A} agonists have been shown to reduce ethanol-withdrawal responses (Kaplan et al., 1999). Paradoxically, mice lacking the A_{2A} receptor demonstrate reduced sensitivity to the hypnotic effects of alcohol, increased alcohol consumption, and reduced alcohol-withdrawal seizures (El Yacoubi et al., 2001; Naassila et al., 2002).

Adenosine A_{2A} receptor is involved in the synergistic interaction between mu-opioid and cannabinoid CB1 receptors in heroin-seeking behaviors (Yao et al., 2006). Since mu-opioid receptors regulate alcohol preference (Herz, 1997) and a genetic variant of human mu-opioid receptor (OPRM1) is a predictor of naltrexone efficacy, an FDA approved medication for alcoholism (Anton et al., 2008), dysregulation of A_{2A} receptor might be an genetic factor of alcohol use disorders in humans. In addition, adenosine A_{2A} receptors also synergistically interact with mGluR5 glutamate receptors in the striatum (Ferre et al., 2002). Since mGluR5 glutamate receptors have an essential role in alcohol preference, especially in the nucleus accumbens (Besheer et al., 2009; Hodge et al., 2006; Olive et al., 2005), indirect regulation of mGluR5 by A_{2A} receptors may also contribute to alcohol use disorders. Co-activation of adenosine A_{2A} and mGluR5 receptors in the neostriatal slices from mice is known to synergistically increase phosphorylation of dopamine- and cAMP-regulated phosphoprotein (DARPP-32) on threonine 34 (Nishi et al., 2003), which promotes inhibition of protein phosphatase-1 (Greengard, 2001; Greengard et al., 1999). Interestingly, mice lacking DARPP-32 self-administer less ethanol than wild-type mice (Risinger et al., 2001), suggesting that co-inhibiting adenosine A_{2A} and mGluR5 receptors might inhibit DARPP-32 and reduce alcohol consumption. The effect of mGluR5 receptor agonist (CHPG) on GABA release in the ipsilateral ventral pallidum was strongly potentiated by co-perfusion with the adenosine A_{2A} agonist, CGS21680 (Diaz-Cabiale et al., 2002), also demonstrating that

synergistic interaction of these receptors promote striatal neuronal activity. On the other hand, motor stimulating effect of mGluR5 receptor antagonist (MPEP) is stimulated by adenosine A_{2A} receptor antagonist (KW-6002), which indicates an in vivo functional interdependence of these receptors (Kachroo et al., 2005). Consistently, the co-administration of sub-threshold doses of an adenosine A_{2A} receptor antagonist (SCH 58261) with an mGlu5 receptor antagonist (MTEP) reduced both alcohol self-administration and cue-induced reinstatement of alcohol-seeking in rats (Adams et al., 2008), suggesting that a combinational drug strategy for treating alcoholism could be considered.

A ROLE OF ENT1 IN REGULATING GLUTAMATE NEUROTRANSMISSION IN REWARD CIRCUITRY

Extracellular or synaptic adenosine levels are mainly regulated by nucleoside transporters (Baldwin et al., 1999; Sawynok and Liu, 2003). Two main plasma membrane transporter families have been characterized. Equilibrative nucleoside transporters (ENTs) mediate nucleoside transport bi-directionally depending on the concentration gradient across the plasma membrane, whereas concentrative nucleoside transporters (CNTs) mediate inwardly directed transport driven by the sodium electrochemical gradient (Baldwin et al., 1999). Three ENT subtypes have been cloned and characterized (Hyde et al., 2001). ENT1 is sensitive to nanomolar concentrations of nitrobenzylthioinosine (NBTI), whereas ENT2 is resistant to NBTI up to 1 mM (Crawford et al., 1998; Griffiths et al., 1997a; Griffiths et al., 1997b; Yao et al., 1997). ENT1 and ENT2 are widely expressed in the CNS (Anderson et al., 1999a; Anderson et al., 1999b; Jennings et al., 2001). ENT3 appears to be expressed outside of the nervous system, and its pharmacological properties have not yet been fully characterized (Hyde et al., 2001). ENT1 and ENT2 are about 50% homologous in amino acid sequence and contain 11 putative transmembrane domains.

Nucleoside transport across the plasma membrane is one of several factors that regulate extracellular adenosine concentrations, which in the brain range from 25–250 nM under basal conditions (Dunwiddie and Masino, 2001). These are sufficient to tonically activate a significant fraction of high affinity A_1 and A_{2A} receptors. Among several nucleoside transporters, ENT1 regulates extracellular adenosine levels in response to acute ethanol treatment in cultured cells (Nagy et al., 1990). Acute ethanol treatment increases extracellular adenosine in cultured cells by selectively inhibiting ENT1 while chronic ethanol exposure decreases ENT1 expression and no longer increases levels of extracellular adenosine, which can be viewed as a cellular model of tolerance (Nagy et al., 1990). Since mice lacking ENT1 exhibit reduced ataxic/hypnotic effects to acute ethanol exposure (Choi et al., 2004) and lowered initial sensitivity (Chen et al., 2010), ENT1 null mice appear to mimic a chronic ethanol treated status. Consistently, ENT1 null mice consume more alcohol compared to wild-type littermates (Choi et al., 2004). Interestingly, mice over-expressing human ENT1 are more sensitive to the acute intoxicating effect of ethanol (Parkinson et al., 2009). Consistently, ENT1 expression appears higher in the striatum of CD1 mice compared with both C57BL/6J and C57BL/6J × CD1 mice (Short et al., 2006b). Since C57BL/6J mice display increased ethanol drinking compared to CD mice (Short et al., 2006b), ENT1 expression might be inversely correlated with ethanol consumption.

One of the neural mechanisms underlying increased ethanol preference in ENT1 null mice is attributed to increased glutamate neurotransmission in the nucleus accumbens (NAc) (Choi et al., 2004). Interestingly, inhibition of presynaptic adenosine A_1 receptor is known to promote glutamate-mediated synaptic neurotransmission in the hippocampus (Manzoni et al., 1994). Consistently, in the NAc, inhibition of adenosine A_1 receptor increases glutamate-evoked synaptic activity (Harvey and Lacey, 1997). Previously, we found that activation of adenosine A_1 receptor reduces ethanol intake in ENT1 null mice (Choi et al.,

2004), suggesting that diminished adenosine activity might be related to increased glutamate levels and increased ethanol intake. A recent study indicates that increased resistance to acute ethanol intoxication is possibly related to increased glutamate signaling in ENT1 null mice (Chen et al., 2010). Moreover, we also found that inhibition of ENT1 expression or activity reduces excitatory amino acid transporter 2 (EAAT2) expression and glutamate activity in cultured astrocytes, which might contribute to increased extracellular glutamate levels in ENT1 null mice (Wu et al., 2010).

Interestingly, hetero-dimerization of adenosine A_1 and A_{2A} receptors in striatal glutamatergic nerve terminals is known to finely tune glutamate release depending on adenosine concentrations (Ciruela et al., 2006; Quarta et al., 2004b). At lower adenosine concentration, high-affinity A_1 receptor inhibits glutamate release, whereas a higher adenosine concentration stimulates glutamate release through A_{2A} receptor (Ciruela et al., 2006). Thus, either decreased A_1 receptor experession or increased A_{2A} receptor function might lead to increased glutamate release. In addition, this interaction regulates dopamine release in the NAc as well (Quarta et al., 2004a).

Choi and his colleagues have demonstrated that altered adenosine-glutamate homeostasis in ENT1 null mice is implicated in the resistance to ethanol-induced locomotion and ataxia by NMDA antagonist, CGP37849 (Nam et al., 2010). ENT1 null mice appear less intoxicated following sequential treatment of CGP37849 and ethanol compared to wild-type littermates in a rotarod experiment. These results indicate that glutamate neurotransmission is critical in regulating the response and susceptibility of alcohol related behavior. Interestingly, a microdialysis experiment revealed that the NAc of ENT1 null mice is less sensitive to the glutamate-reducing effect of the NMDA receptor antagonist (Nam et al., 2010). These findings suggest that glutamate neurotransmission in the NAc is essential to regulate ethanol intoxication.

Increased glutamate signaling is implicated in CREB-mediated gene expression, which regulates several addictive behaviors (Kalivas, 2009; Nestler, 2001). Importantly, excessive glutamate neurotransmission is known to be associated with increased ethanol drinking (Spanagel and Kiefer, 2008; Spanagel et al., 2005). However, it seems paradoxical that both increased glutamate signaling and decreased CREB activity (Pandey et al., 2004) are associated with alcohol use disorders since increased glutamate receptor signaling is likely to increase CREB activity via several signaling mechanisms (Lonze and Ginty, 2002).

A ROLE FOR ATP-GATED P2X4 RECEPTORS IN ALCOHOL CONSUMPTION

P2XRs are widely distributed in the CNS on neurons (Surprenant and North, 2009) and on glial cells (Inoue, 2008; Trang et al., 2006). Tests using brain slice preparations, dissociated neuronal cultures, and P2XR knockout (KO) mouse models proposed possible roles for P2XRs (Surprenant and North, 2009). This includes learning and memory (Labrousse et al., 2009; Sim et al., 2006; Wang et al., 2004), depression and anxiety (Basso et al., 2009), pain perception (Honore et al., 2006; Jarvis et al., 2002; Tsuda et al., 2009; Ulmann et al., 2008), and vascular tone (Yamamoto et al., 2006). In addition, P2XRs have been suggested to play a role in hormonal control of temperature regulation, food and water intake, sexual behavior, and emotional responses (Stojilkovic, 2009), which are also implicated in alcohol use disorders.

The mesolimbic dopamine (DA) system plays an important role in ethanol consumption, ethanol addiction, and reinforcement (Gonzales et al., 2004; Spanagel and Weiss, 1999). P2XRs have been identified on both neurons and glia in the mesolimbic DA system (Heine et al., 2007). A recent study indicates a functional role of the P2XRs in the ventral tegmental area (VTA). P2XRs modulate ethanol's effect on GABAergic synaptic transmission in the

ventral tegmental area (VTA) (Xiao et al., 2008). Taken together, these findings suggest that P2XRs may have an important role in modulating the activity of dopaminergic neurons that are important for controlling ethanol intake.

P2X4Rs are the most abundant P2XR subtype expressed in the CNS (Buell et al., 1996; Soto et al., 1996), and building evidence implicates P2X4Rs in alcohol consumption. First, a recent study using a genomic/phenomic approach of ethanol consumption identified *p2rx4* as one of the "candidate genes" that predisposes to varying levels of ethanol intake across the 28 recombinant inbred rat strains (Tabakoff et al., 2009). The authors concluded that interactions of the products of the candidate genes identified in the study were involved in neurobiological pathways affecting GABAergic neuronal activity in the mesolimbic DA system. Second, Davies and colleagues recently found that mice lacking the *p2rx4* gene displayed significantly different alcohol consumption compared to wild type C57BL/6J or heterozygous littermates (manuscript in preparation). Collectively, these findings support the contention that P2XRs play a role in ethanol drinking.

Developing effective treatments for alcohol related disorders faces a number of challenges due to limited knowledge of the sites and mechanisms of ethanol action in the CNS. Over the past several years, Davies and his colleagues have placed a significant amount of effort focusing on sites of ethanol action in P2XRs. Studies using recombinant expression systems have found that P2X2, P2X3, and P2X4Rs expressed in *Xenopus* oocytes are sensitive to ethanol at intoxicating and anesthetic concentrations (Davies et al., 2005; Davies et al., 2002; Xiong et al., 2000). Also, these studies demonstrated that residues contained within the ectodomain-TM interfaces are important for causing or modulating the effects of ethanol in P2X3 and P2X4Rs (Asatryan et al., 2008; Popova et al., 2010). More specifically, in P2X4Rs, mutational analyses identified amino acid residues within the TM segments at the ectodomain-TM interface (W46, D331, M336) that are critical for ethanol action (Popova et al., 2010).

Recent work has demonstrated that the ectodomain-TM interface of P2X4Rs is also an important site for modulation of the receptor activity by ivermectin (IVM) (Jelinkova et al., 2008; Jelinkova et al., 2006; Silberberg et al., 2007). IVM is a semi-synthetic macrocyclic lactone widely employed in both animals and humans as a broad spectrum anthelmintic (Geary, 2005; Omura, 2008; Richard-Lenoble et al., 2003). The current therapeutic potential of IVM is attributed to action on a non-mammalian, glutamate-gated inhibitory chloride channel (Cully et al., 1994; Dent et al., 1997; Le et al., 1998). However, IVM can also potentiate $GABA_A$ and glycine receptors *in vitro* (Dawson et al., 2000; Shan et al., 2001). More recent studies in humans suggest that IVM also affects other LGICs including nicotinic acetylcholine receptor (Krause et al., 1998; Sattelle et al., 2009) and P2X4Rs (Khakh et al., 1999). Regarding P2X4Rs, IVM is used to selectively identify the participation of P2X4Rs from other P2X family members in ATP-mediated processes (Khakh et al., 1999).

Interestingly, some of the sites or regions that IVM is purported to act on have recently been reported as being important for ethanol modulation (Asatryan et al., 2008; Popova et al., 2010). Based in part of this finding, Asatryan and colleagues tested the hypothesis that IVM would reduce the sensitivity of P2X4Rs to ethanol (Asatryan et al., 2010). In agreement with this hypothesis, IVM antagonized ethanol in a concentration-dependent manner (Asatryan et al., 2010). As illustrated in Fig. 2, these findings were used to construct the first molecular model illustrating overlapping sites of action for ethanol and IVM (i.e. M336) in P2X4Rs (Asatryan et al., 2010). Taken together, the findings suggest that the ectodomain-TM interface of P2X4Rs is a site of action and/or modulation for both ethanol and IVM. In

addition, the newly identified alcohol pocket may represent a potential target for the development of medication for prevention and treatment of alcohol use disorders.

FUTURE DIRECTIONS

It is evident that adenosine and ATP signaling are implicated in several aspects of alcohol use disorders (Fig. 1). In addition to significant recent advances in molecular and neurobiological basis of purinergic metabolism and signaling, further experiments will be required to determine the causal relationship between adenosine and ATP signaling in neuron-glial interactions, and how the interaction finely regulates other major neurotransmitters such as dopamine, GABA, and glutamate signaling in circuitry levels. Considering the apparent function of adenosine signaling as a main contributor for initial ethanol-induced intoxication or tolerance, the molecular basis of ethanol dependence in the context of tolerance to ethanol intoxication still needs to be determined. Reduced ethanol intoxication is a key determinant of increased ethanol consumption (Offenhauser et al., 2006); therefore, unraveling precise adenosine signaling would be essential to identify addictive properties of ethanol. Furthermore, detailed molecular mechanisms underlying involvement of adenosine signaling in ethanol-induced sleep processes or associated disorders will be critical to understand a hangover or negative reinforcing effects of ethanol. Finally, recent studies on P2X4 and its direct interaction with ethanol open a new possibility. Additional preclinical experiments with ivermectin (IVM) in animal models will be required to lead on to clinical trials.

Acknowledgments

We thank D. Frederixon, S. Johng for editing the manuscript. This project was funded by the Samuel Johnson Foundation for Genomics of Addiction Program at Mayo Clinic to D.S.C., by the Harry S. Truman Memorial Veterans Hospital to M.M.T. and by grants from the National Institutes of Health (NIH) to D.S.C. (AA015164, AA018779, AA017830-Project 1), to M.S.D. (AA0701), to M.M.T. (NS059831 and RAA017472A), to L.A. (AA017243, Project 4), to D.L.D. (AA013922 and Integrative Neurosciences Initiative on Alcoholism AA013517, Project-4).

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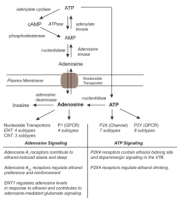

Fig. 1.

Metabolic pathways and receptors of purinergic signaling involved in alcohol use disorders. In the cytosol, adenosine is synthesized from AMP by nucleotidase activity and transported to extracellular region via nucleoside transporter. Among several nucleoside transporters, ENT1 is known to regulate adenosine levels in response to ethanol. Adenosine is also converted from ATP extracellularly by ecto-nucleotidase activity. Extracellular adenosine binds to 4 different G-protein coupled adenosine receptors and is known to mediate ethanol-induced ataxia and sleep. ATP interacts with both ion channel named P2X receptors and G-protein coupled P2Y receptors. Among these, P2X4 receptors contain an ethanol-binding site (see Figure 2) and regulate ethanol drinking. cAMP, cyclic adenosine monophosphate; ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; VTA, ventral tegmental area.

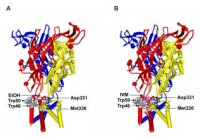

Fig. 2.

Molecular model of the rat P2X4R reveals a putative ethanol and IVM pocket. The model was built by threading the edited primary sequence onto the X-ray crystal structure of zebra fish P2X4R (Kawate et al., 2009). (**A**) A side view of the rat P2X4R showing the ectodomain and the six alpha helices of TM1 and TM2 segments of 3 different P2X4R subunits. Residues W46, W50 in the first alpha helix of one subunit as well as D331 and M336 in the final alpha helix of the adjacent subunit form a pocket that demonstrates a good fit for a molecule of ethanol (in pink) at the same scale. (**B**) A similar view of the rat P2X4R, but with a model of IVM (rendered in balls and sticks) inserted into a putative binding site in a position between the alpha helices like that described in nicotinic acetylcholine receptors (Sattelle et al., 2009). Figure taken from (Asatryan et al., 2010).