Published in final edited form as:

Curr Drug Abuse Rev. 2010 September ; 3(3): 163–174.

An Essential Role for Adenosine Signaling in Alcohol Abuse

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Abstract

In the central nervous system (CNS), adenosine plays an important role in regulating neuronal activity and modulates signaling by other neurotransmitters, including GABA, glutamate, and dopamine. Adenosine suppresses neurotransmitter release, reduces neuronal excitability, and regulates ion channel function through activation of four classes of G protein-coupled receptors, A₁, A_{2A}, A_{2B}, and A₃. Central adenosine levels are largely controlled by nucleoside transporters, which regulate adenosine levels across the plasma membrane. Adenosine has been shown to modulate cortical glutamate signaling and ventral-tegmental dopaminergic signaling, which are involved in several aspects of alcohol use disorders. Acute ethanol elevates extracellular adenosine levels by selectively inhibiting the type 1 equilibrative nucleoside transporter, ENT1. Raised adenosine levels mediate the ataxic and sedative/hypnotic effects of ethanol through activation of A₁ receptors in the cerebellum, striatum, and cerebral cortex. Recently, we have shown that pharmacological inhibition or genetic deletion of ENT1 reduces the expression of excitatory amino acid transporter 2 (EAAT2), the primary regulator of extracellular glutamate, in astrocytes. These lines of evidence support a central role for adenosine-mediated glutamate signaling and the involvement of astrocytes in regulating ethanol intoxication and preference. In this paper, we discuss recent findings on the implication of adenosine signaling in alcohol use disorders.

Keywords

Adenosine; Alcoholism; Signaling; Ataxia; Glutamate; Neuron-Glial Interaction; Pharmacology; Genetics

INTRODUCTION

Adenosine is a ubiquitous nucleoside which has multiple, interrelated functions in the CNS that are crucial to proper brain function [1-3]. As in all living cells, central adenosine is the building block for adenine nucleotides used in nucleic acid synthesis, and can be phosphorylated to produce the ATP used as metabolic currency. Conversely, it is the product of ATP hydrolysis, and thus serves as an indicator of metabolic activity [4]. The physiological effects of adenosine are directly related to its metabolic function. For example,

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adenosine dilates blood vessels in the brain and periphery, thereby directing nutrients present in circulation to metabolically active cells. In addition to coupling the energy demands of neurons with blood flow necessary to provide precursors and maintain their activity, adenosine takes on yet another, related role in the CNS as a modulator of neurotransmission. This role appears particularly important in the fine-tuning of excitatory glutamatergic signaling in the striatum, a brain region that integrates information pertaining to many aspects of reward-related behavior. Dysregulation of adenosine signaling and its impact on striatal glutamatergic transmission has been strongly implicated in mediating the effects and abuse potential for alcohol [5-7].

Astrocytes appear to be significant sources of extracellular adenosine [8]. Moreover, nucleoside transporters, which determine in large part extracellular adenosine concentrations, are predominantly expressed in astrocytes [9]. Mounting evidence suggests that many of the known roles of adenosine in the CNS are a result of neuro-glial interactions [10-12]. In this review, we discuss the regulation of adenosine levels in the brain, mechanisms of adenosine signaling including an extensive summary of the current pharmacological and molecular tools available, and the modulatory role of adenosine on glutamate signaling in alcohol use disorders. Special emphasis is given to summarizing the evidence that 1) elevated levels of adenosine caused by ENT1 blockade mediates several of the behavioral effects of ethanol, particularly sedation and ataxia, and that 2) neuro-glia interactions play an essential role in adenosine-mediated glutamate signaling in alcohol abuse.

REGULATION OF ADENOSINE LEVELS IN THE CENTRAL NERVOUS SYSTEM

Consistent with the interrelated functions of adenosine in the CNS, one of its main known physiological actions is to inhibit the release of other neurotransmitters by reducing neuronal excitability [2,13,14]. Thus, regulation of adenosine levels is essential for the proper function of major transmitter systems in the brain, particularly glutamate [15,16]. Similar to classical chemical messengers such as serotonin, dopamine, and GABA, adenosine is regulated positively by production and release, and negatively by metabolism and uptake, the balance of which determines its concentration in extracellular space. Unlike classical neurotransmitters however, which are synthesized, stored, and released into the synapse in response to electrochemical stimulation, adenosine levels are regulated to a much greater extent by production and transport [1,2]. The result of this atypical regulation pattern is that adenosine levels can change rapidly, an essential feature of its ability to fine-tune the activity of neighboring neurons. Dysregulation of adenosine levels is associated with a number of neurological disease states, and recent evidence indicates that adenosine signaling may be central to the development of alcoholism [4].

Adenosine Production

Adenosine is produced in the CNS both intracellularly and extracellularly from AMP, which depends upon ATP breakdown. Dephosphorylation of adenine nucleotides ATP, ADP, and AMP outside of cells accounts for much of the adenosine found in extracellular space, which ranges from 25 to 250 nM, depending on the brain region of interest [4]. Cyclic AMP can also be converted extracellularly to adenosine, although the slow kinetics indicate that large, prolonged elevations in cAMP are required to substantially affect adenosinergic tone [17]. ATP can be co-released from neurons along with the transmitters acetylcholine, dopamine, serotonin, or norepinephrine [18,19], where it supplies an energy-related message to post-synaptic neurons, and generally results in excitation. Recent evidence also indicates that neurons are capable of releasing adenosine *per se*, whereas astrocytes almost exclusively

release ATP that is metabolized into adenosine by ecto-nucleotidases [20,21], releasing adenosine itself only under pathological conditions, such as hypoxia [22]. The location of adenosine production thus appears largely dependent upon cell type, with astrocytes and neurons showing differential expression of purinergic enzymes and transporters [23]. Both neurons and astrocytes ultimately function as major sources of extracellular adenosine [10-12,24].

Adenosine Metabolism

As is true for other transmitters and messengers in the CNS, metabolism of adenosine represents an important way to terminate its actions. Because adenosine is a precursor to adenine nucleotides used in genetic material and energy production, it is more efficient to conserve adenosine rather than break it down. However, both metabolic pathways exist, and the degree to which each contributes to adenosine levels depends upon 1) the intracellular vs. extracellular location, 2) the cell type, and 3) the physiological conditions at the time. Under normal conditions, adenosine is phosphorylated intracellularly to AMP by adenosine kinase, the high activity of this enzyme ensuring that adenosine concentrations inside of cells are low [4]. Transport of adenosine into cells (discussed below) thus represents a critical factor in determining the fate of the purine. The second mechanism used to terminate adenosine action in the synapse is via its enzymatic conversion to inosine by adenosine deaminase and ultimately to hypoxanthine or xanthine by xanthine oxidase [25,26]. Under normal conditions, adenosine breakdown plays only a marginal role in controlling its levels, but becomes important under conditions of high extracellular adenosine, such as hypoxia and ischemia [27]. A recent study showed that under such conditions, adenosine produced by neurons is not only broken down extracellularly, but is also metabolized to hypoxanthine by astrocytes [28]. Thus, astrocytes appear to play diverse roles in regulating adenosine levels under both normal and pathophysiological conditions [10].

Adenosine Transport

Among the different levels of regulatory control exerted by the CNS on extracellular adenosine concentrations, nucleoside transport across the plasma membrane is arguably the most important. Two main nucleoside transporter families have been characterized, sodium-independent equilibrative nucleoside transporters (ENTs), which mediate bi-directional nucleoside transport determined by the concentration gradient, and sodium-dependent concentrative nucleoside transporters (CNTs), which mediate an inwardly directed transport driven by the sodium electrochemical gradient [29]. Both ENTs and CNTs are expressed in highly metabolically active tissues, including the heart, spleen, lung, liver, testis, kidney and brain. As their names imply, these transporter families serve two very distinct functions, differing in fundamental ways, which are reflected in their localization in cells and specific kinetic profile [29].

Four ENT subtypes (ENT1-4) have been cloned [30], differing mainly in substrate specificity and tissue distribution, but in some cases also in kinetic parameters, such as sensitivity to pharmacological inhibition. ENT1 and ENT2 differ in sensitivity to the inhibitor nitrobenzylthioinosine (NBTI; also known as nitrobenzylmercaptapurine riboside or NBMPR), with ENT1 responding to nM concentrations, whereas ENT2 is resistant up to 1 mM [31-33], suggesting that they are responsible for adenosine uptake (or release) under very different conditions. Other antagonists such as dilazep and dipyrindamole are available for human ENT1 [34], but rat ENT1 is largely resistant to these compounds, precluding the use of rats as an animal model and limiting to some extent characterization of this transporter [35]. However, genetic deletion has been instrumental in characterizing ENT1 function, underscoring this approach as useful to examine other receptor and transporter subtypes, especially in the absence of selective pharmacological agents. Circadian variation

in the expression of ENT1 has been reported in the cortex and basal forebrain of rats [36], which may contribute significantly to the circadian rhythm in adenosine levels [37], and adenosine's role in sleep regulation [38-40]. ENT1 and ENT2 are highly expressed in the striatum [41,42], and are especially abundant in astrocytes [9]. ENT3 appears to be expressed outside the nervous system [43] and mainly intracellularly, where it colocalizes with lysosomal markers in cultured human cells [44]. ENT4 expression and pharmacology has yet to be fully characterized [30], though it appears to be highly selective for adenosine. Expression of ENTs also varies by cell type and can be modulated by physiological conditions. Under basal conditions, ENT inhibition results in enhanced extracellular adenosine concentrations, whereas enhanced ENT activity (e.g. by activation of A_{2A} autoreceptors in the CA3 region of the hippocampus) facilitates adenosine uptake into neurons [45]. Under conditions where intracellular adenosine concentrations are high, such as hypoxia or ischemia, ENTs can be driven in reverse, releasing adenosine into the synapse.

As their name implies, concentrative nucleoside transporters (CNTs) are involved in scavenging nucleosides for use inside cells. Three types of CNTs have been identified in humans, with analogs in rodents, differing mainly in substrate specificity and tissue distribution. CNT1 is expressed in metabolic and excretory tissues, and appears to be inhibited by adenosine [46]. A recent study showed that pyrimidine, but not purine, nucleosides displayed sodium- and concentration-dependent uptake in mCNT1-transfected Cos-7 cells, transport characteristics that are conserved in the human isoform [47]. CNT2, the best characterized of the three transporters, and CNT3 are expressed in a wide variety of tissues, including the brain. However, few studies have mapped the distribution of either CNT2 or CNT3 with regard to certain brain regions. In rats, CNT2 was shown to be expressed in the hippocampus, cerebellum, neocortical areas, and brain nuclei involved in drug and alcohol addiction, including the nucleus accumbens and amygdala [48]. However, a role for CNTs in addictive behavior has not yet been investigated, largely due to the lack of potent inhibitors of CNT family members [35]. This study showed that sleep-deprivation is associated with reduced amounts of cortical CNT2, suggesting that CNT2 is a critical factor underlying the extracellular accumulation of adenosine during periods of prolonged wakefulness. In the rat, CNT2 cloned from vascular tissue of the blood-brain barrier also appears to function in nucleoside transport into the brain from peripheral circulation [49].

ADENOSINE RECEPTORS AND SIGNALING IN THE CENTRAL NERVOUS SYSTEM

Four different adenosine receptor subtypes have been identified, along with several types of nucleoside transporters that regulate the extracellular levels of adenosine and thus contribute significantly to the modulation of adenosine signaling in the CNS and periphery [13]. The information gleaned from the use of various pharmacological tools, cell culture methods, and *in vivo* animal models has allowed for considerable progress toward understanding the role of adenosine signaling in the healthy brain as well as in neurological disorders. Alcoholism and substance abuse are among the most prominent of the CNS-based diseases in which dysregulation of adenosine signaling has been implicated, and a combination of pharmacological and molecular tools has been instrumental in identifying potential therapeutic applications of manipulating this system [13].

Interestingly, several non-selective ligands for adenosine receptors have been used to elucidate some physiological functions of adenosine signaling. Such nonspecific adenosine receptor ligands include the non-specific adenosine agonist, NECA (N-ethylcarboxamidoadenosine), and adenosine receptor antagonists, XAC (xanthine amine congener), theophylline, and caffeine [2]. However, in an effort to characterize the role of individual receptor subtypes in adenosine signaling, several compounds have been

developed to be subtype selective. Additionally, radiolabeling has been used to quantify the binding of particular ligands to adenosine receptors. Despite the significant contribution of pharmacological tools in the characterization of adenosine receptor signaling, this approach has been complicated by factors including interspecies variability in the specificity of receptor ligands for particular subtypes [50] and cross-reactivity of pharmacological agents with multiple receptor subtypes [51]. As a result, cell line and animal model approaches have been used to clarify the results of pharmacological studies. Human recombinant adenosine receptors have been expressed in CHO (Chinese Hamster Ovary) and HEK (Human Embryonic Kidney) cell lines [52]. These model systems are complementary to the pharmacological and *in vivo* studies but results should be considered with the caveat that the density of receptors and signaling molecules may not represent physiological levels [51]. Therefore, mice that have been genetically engineered to either have a specific receptor knockout or overexpression allow for *in vivo* clarification of the role of different adenosine receptors [53].

A₁ Receptors

Several A₁ receptor-specific agonists have been synthesized through modification of adenosine [2]. Partial agonists at the A₁ receptor [54] and indirect A₁ agonists such as adenosine kinase inhibitors [55] or allosteric enhancers of adenosine binding [56] are being developed. Additionally, there are several classes of A₁-specific antagonists derived from xanthine or the parent molecule, adenine [2,51].

Studies utilizing radioligands, antibodies, and analysis of mRNA expression levels have determined that the A₁ receptor is highly expressed throughout the rat cortex, hippocampus, cerebellum, thalamus, and brain stem [57-59] and the striatum [60,61], and is located on both pre- and post-synaptic neuronal membranes [62]. These receptors signal mainly through coupling with G_i/G_o proteins, causing a decrease in adenylyl cyclase activation [63-65]. They have also been shown to activate ATP-sensitive potassium channels, thereby reducing action potential duration [66]. Release of $\beta\gamma$ dimers from G proteins (G $\beta\gamma$) following stimulation of any of the adenosine receptor subtypes, including A₁, leads to the phosphorylation of extracellular signal-related kinase (ERK), albeit via various mechanisms in different cells or receptor systems [67]. The G $\beta\gamma$ subunits also activate protein kinase (PKC) and G-protein-coupled inwardly rectifying potassium channels (GIRKs) [68] to reduce neuronal excitability.

Combining pharmacological approaches with *in vivo* and *ex vivo* studies using A₁ receptor knockout mice has also contributed to the current understanding of the role of the A₁ receptor in the CNS. These studies have been particularly useful in elucidating the A₁ receptor-mediated modulatory actions of adenosine on glutamatergic neurotransmission (discussed below). Briefly, both non-selective adenosine receptor antagonism and A₁-selective antagonism have been shown to block dopaminergic inhibition of glutamate-generated EPSCs, thereby increasing glutamatergic signaling [15]. However, this effect is absent in the hippocampus of mice lacking A₁ receptors [53,69], indicating that adenosine normally inhibits glutamate release from presynaptic membranes via A₁ receptor activation. Behavioral changes observed in A₁ receptor knockout mice, including increased sensitivity to pain, anxiety, and hypoxic damage [69], provide clues as to other systems in which A₁ receptor-mediated signaling is likely to play an important modulatory role. The A₁ receptor has also been shown to mediate many of the effects of ethanol. A₁ receptor antagonists attenuate ethanol-induced motor incoordination, indicating that A₁-mediated signaling is involved in the ataxic effects of alcohol [70]. A₁ agonists have been shown to decrease anxiety-like behavior, tremor, and seizures during acute ethanol withdrawal in mice [71],

raising the possibility that A₁ agonists may be useful in the management of alcohol withdrawal.

A_{2A} Receptors

In contrast to the wide distribution of A₁ receptors in the central nervous system, A_{2A} receptors are expressed primarily in dorsal striatum, nucleus accumbens, and olfactory tubercle of the rat [72]. A_{2A} receptors have a lower affinity for adenosine, and therefore, are activated when extracellular adenosine is more abundant, such as during periods of high activity. Although A_{2A} receptors generally couple with G_s proteins, A_{2A} receptors in the striatum stimulate adenylyl cyclase activity primarily through G_{oif} [72]. Elevated cAMP levels within the cell causes activation of PKA which has numerous consequences, notably the phosphorylation of CREB and an increase in expression of cAMP-dependent genes, such as *c-fos*.

Complex adenosine receptor-receptor interactions involving the A_{2A} receptor in both neurons and astrocytes are thought to provide a means by which adenosine can fine-tune synapses (for review, see [73]). Presynaptic A₁-A_{2A} heterodimer formation inhibits the usual A₁-mediated decrease in glutamate release in the striatum [74], a mechanism thought to explain the paradoxical observation that high levels of adenosine increase glutamate release in this brain region. In addition, dimerization of postsynaptic A_{2A} receptors with D2 receptors in the striatum influences reward-related behavior through modulation of adenylyl cyclase activity [75,76]. The inhibitory action of A_{2A} on D2 receptor-mediated intracellular signaling cascades can also influence glutamate levels in the striatum, a presynaptic effect that is synergistically enhanced by simultaneous mGluR5 activation.

Mailliard and Diamond (2004) state that acute ethanol administration leads to A_{2A}-receptor dependent translocation of PKA to the nucleus, where it can affect gene transcription via CREB activation [77]. A_{2A} agonists have been shown to reduce ethanol-withdrawal responses [71], yet paradoxically, mice lacking the A_{2A} receptor demonstrate reduced sensitivity to the hypnotic effects of alcohol, increased alcohol consumption, and reduced alcohol-withdrawal seizures [53,78,79]. Interestingly, the A₂ antagonist, DMPX (N-ethylcarboxamidoadenosine), was associated with increases in ethanol consumption in rats when administered at a low dose (1 mg/kg), while higher doses (10 and 20 mg/kg) decreased drinking [80]. This bimodal effect may be due to the non-selective nature of DMPX antagonist action on A₂ receptors. Adenosine-mediated fine-tuning of synapses in the striatum and its implication in addiction merits further investigation.

A_{2B} Receptors

Less is known about the role of signaling through the A_{2B} receptor in CNS physiology and pathophysiology, due largely to the low affinity and selectivity of A_{2B} for most agonists [51], such as enprophylline. However, more selective antagonists, such as CVT-6883 have been developed [81]. Despite obstacles to the study of A_{2B} receptors, mRNA analysis has revealed that this receptor can be found throughout the brain, although it is not expressed at high levels [59,82]. Some studies have addressed the challenge of characterizing A_{2B}-mediated signaling in the absence of specific agonists by utilizing non-selective adenosine agonists in combination with specific antagonists targeted to inhibit signaling from other adenosine receptors [83]. Both G_s-mediated activation of adenylyl cyclase and G_q-mediated stimulation of the IP₃/DAG pathway have been demonstrated in a human mast cell line [83,84]. The A_{2B} receptor has also been shown to activate p38 and JNK in addition to the ERK family of mitogen-activated protein kinases (MAPKs) [67]. The development of specific pharmacological agonists of the A_{2B} receptor would greatly enhance understanding of its functional relevance to adenosine signaling.

THE ROLE OF ADENOSINE SIGNALING IN NEURO-GLIAL INTERACTIONS

Adenosine-mediated neuro-glial interactions are essential for the development and maintenance of neuronal networks, and thus have the ability to dramatically influence behavior under both normal and pathological conditions. Astrocytes are situated between neurons and blood vessels, which allows them to act as an intermediary tying neuronal activity with delivery of nutrients. Since one astrocyte contacts tens of thousands of neurons, astrocytes are also perfect candidates for the modulation of neuronal networks. Along with presynaptic and postsynaptic neurons, astrocytes form the tripartite synapse [10], essential to the fine-tuning of synaptic plasticity.

In addition to their important role in neurotransmitter recycling (e.g. the glutamate-glutamine cycle), it is now widely accepted that astrocytes participate in intercellular signaling. Like neurons, astrocytes have been shown to respond to chemical messengers released into the synapse with the induction of various intracellular signaling mechanisms. Also like neurons, astrocytes can signal to other CNS cells through the release of chemical messengers, termed gliotransmitters. Among these are glutamate [85,86], ATP [20,87], D-serine (NMDA receptor co-agonist [88]), and adenosine [20,22]. Although still somewhat contentious, it appears astrocytes release most gliotransmitters via exocytosis (with the likely exception of adenosine itself), a process which depends upon increases in intracellular calcium, but that differs from neuronal exocytosis in that it does not require depolarization-induced action potentials, which astrocytes appear incapable of producing [89].

Through neuro-glial interactions, adenosine is involved in both small-scale processes at the synapse level (Figure 1), as well as large-scale brain processes at the network level. Low levels of adenosine present in extracellular space mediate tonic inhibition of neurotransmitter release at excitatory synapses via activation of presynaptic A₁ receptors in the striatum [15] and several other brain regions. As astrocytes also release ATP in a phasic manner in response to excitation by neurotransmitters (e.g. glutamate), they contribute substantially to activity-dependent accumulation of adenosine in the synapse and thus enhance adenosine-mediated neuromodulation. High levels of adenosine resulting from astrocytic ATP release and reversal of adenosine transport in conditions such as hypoxia and ischemia are essential in neuroprotection (reviewed in [90]).

Using transgenic mice expressing inducible dominant negative SNARE (dnSNARE; a protein essential for exocytosis of transmitters) selectively in astrocytes [12], Haydon's group demonstrated that adenosine accumulated from the hydrolysis of ATP has a depressive effect on adjacent pathways [89,91,92]. In this manner, adenosine is important for coordinating the relative strength of neuronal networks. Recent evidence from our lab has shown that genetic deletion [6] or pharmacological inhibition [93] of ENT1 reduces expression of glutamate transporter EAAT2 and glutamate uptake capacity in astrocytes. Because neuroadaptive processes in the striatum and other components of the reward network, especially in glutamate neurotransmission, are a salient feature of addictive behavior, our results imply that astrocytic adenosine signaling may be central to the pathophysiology of addiction [94,95].

Adenosine derived from astrocytes also appears to have a central role in homeostatic and compensatory sleep. It has been well-established that adenosine receptor antagonists like caffeine promote wakefulness. Likewise, it is no coincidence that both the accumulation of adenosine in extracellular space and the parallel increase in homeostatic sleep pressure are activity-dependent [96]. Another study using transgenic mice expressing dnSNARE selectively in astrocytes shows attenuated baseline slow wave activity (SWA; EEG indicator for sleep pressure), and deficits in SWA accumulation after 6 hour sleep deprivation, the

latter of which was borne out behaviorally in a lack of recovery sleep in these mice [97]. The cognitive consequences of sleep loss were also dependent on astrocytic ATP release and its extracellular conversion to adenosine. Conditional knockout of the A₁ receptor gene in mice results in identical changes in SWA, recovery sleep, and memory function following sleep deprivation as those seen in dnSNARE mice, and confirms previous investigations using A₁ receptor antagonists [98]. Evidence for a role of astrocytic adenosine signaling in complex behaviors such as sleep and addiction represent critical steps in the study of glial cell function, and open the door to new strategies to treat neurological disorders. This may prove particularly meaningful in light of the fact that sleep disorders and alcoholism often show co-morbidity with each other and other psychiatric disorders.

ADENOSINE SIGNALING IN ETHANOL-INDUCED ATAXIA

Ataxia is one of the most apparent behavioral manifestations of ethanol intoxication, which appears to be mediated by an abnormal balance between excitatory and inhibitory neurotransmitters in the brain. Adenosine has been shown to modulate both glutamatergic and GABAergic transmission, and therefore represents a potential target of ethanol in the disruption of excitatory-inhibitory balance [4,99,100].

The adenosine A₁ receptor has been a main focus of ethanol-related investigations because it mediates many of adenosine's modulatory actions on other neurotransmitter systems, and is located in important areas for motor control, such as the motor cortex, striatum, and cerebellum [60]. Not surprisingly, as summarized in Table 1, studies have shown that adenosine mediates ethanol-induced ataxia primarily through A₁ receptors [101-107]. The selective A₁ receptor agonist N⁶-cyclohexyladenosine (CHA) reduced the attenuation by glutamate on ethanol-induced motor incoordination, while the A₁ receptor antagonist DPCPX opposed the facilitatory actions of the NMDA antagonists on ethanol's ataxic effects [108]. CHA also enhanced the accentuation of ethanol's ataxic effect by the GABA_A agonist muscimol, while DPCPX inhibited the effect. This is consistent with evidence that an inhibitory action of A₁ receptor activation on GABA release is not often observed [4]. Moreover, A₁ activation is associated with GABA_A downregulation, which implies that it could have a stimulatory effect on GABA release [109]. However, it is not clear if A₁ receptor activation can directly accentuate ethanol-induced potentiation of GABA transmission.

The A₁ receptor subtype is negatively linked to the adenylyl cyclase-cAMP signaling pathway via G_i/G_o proteins [63,64]. Evidence from studies in *Drosophila* and mice suggest that ethanol-induced motor impairment is negatively correlated with the activation of the adenylyl cyclase-cAMP system [70,110,111]. The *cheapdate* mutation in *Drosophila*, an allele of the *amnesiac* gene believed to encode a neuropeptide that activates adenylyl cyclase to increase cAMP, was found to increase the loss of postural control due to ethanol intoxication. Similarly, forskolin (a stimulator of adenylyl cyclase) and miconazole (an inhibitor of adenylyl cyclase) attenuate and potentiate ethanol-related motor impairment, respectively. In mice, intracerebellar cAMP inhibits ethanol-induced ataxia and the accentuating effect of the A₁-selective adenosine agonist CHA on ethanol-induced ataxia [70].

A₁ receptor activation is also known to stimulate the MAPK pathway in Cos-7 and Chinese hamster ovary cells [112,113] via Gβγ subunits associated with A₁ receptor G_i/G_o proteins and Src non-receptor tyrosine kinases [114]. It is noteworthy that the MAPK pathway has also been linked to alcoholism. The activity of extracellular-signal-regulated kinases 1/2 (ERKs 1/2), a subset of MAPKs, has been shown to be correlated with the duration of ethanol exposure [115]. Acute ethanol exposure increases phosphorylated (activated)

ERK1/2 levels in the central and medial nuclei of amygdala in rats [116]. Further studies on how A₁ receptor activation modulates the activity of the MAPK pathway in the key motor areas will provide more insight into how this pathway may be involved in ethanol-induced ataxia.

In contrast to A₁ receptors, which are located in all brain regions known to regulate motor function, A_{2A} receptors are located mainly in the striatum [61,117]. Significantly higher doses of A_{2A} agonists and antagonists are typically required to exert an observable effect on ethanol-induced motor incoordination in comparison to A₁ selective ligands [102,104]. In these studies, the potentiation of ataxia by A_{2A} agonists was abolished by pretreatment with an A₁ selective antagonist, but not an A_{2A} selective antagonist, indicating that nonspecific A₁ receptor activation was responsible for the potentiation of ataxia. Since heterodimerization between A₁ and A_{2A} receptors in glutamatergic presynaptic terminals in the striatum reduces the affinity of the A₁ receptor for agonists [74], future studies addressing the involvement of receptor-receptor interactions in ataxia and other ethanol-related behaviors is thus an interesting prospect.

DISRUPTION OF ADENOSINE-MEDIATED GLUTAMATE SIGNALING IN ALCOHOLISM

Glutamatergic neuroadaptation in the corticostriatal reward network is a key factor in drug addiction, including alcoholism [118]. In particular, a hyperglutamatergic tone is implicated in drug and alcohol self-administration [119-121]. Importantly, glutamate signaling regulates transcription factors such as CREB, representing a direct link by which alcohol and drugs can affect gene expression, and conversely, a mechanism by which genes can influence responses to alcohol and drugs. Indeed, CREB-mediated gene expression has been shown to regulate several attributes of addictive behaviors [122-124]. Since such changes in glutamate signaling and downstream gene transcription represent both a consequence of, as well as a precursor to, drug abuse, they are considered to play an essential role in dependency and relapse. These findings support a role for pharmacological inhibition of glutamate signaling as a means to attenuate excessive ethanol consumption [125], and to potentially break the cycle of addiction. As glutamatergic neurotransmission is modulated by adenosine, changes in adenosine signaling contribute substantially to the effects of addictive drugs and processes underlying addiction. Ethanol-induced elevation in extracellular adenosine was originally presumed to reflect increased production of adenosine as a by-product of ethanol metabolism into acetate. However, Nagy et al. (1990) found that acute ethanol exposure increases adenosine primarily through selective inhibition of ENT1, while chronic ethanol exposure leads to ENT1 downregulation [126,127]. Although the molecular mechanism of ENT1 inhibition by ethanol remains to be determined, it has been suggested that ethanol alters phosphorylation of the third cytoplasmic loop of ENT1 by protein kinase C (PKC) or casein kinase II (CKII) and thus modifies adenosine transporting activity [128-130]. Alternatively, adenosine levels could be regulated by ENT1 expression. Since the promoter region of human ENT1 contains a transcription factor site for CREB [131], and since deficits in CREB expression and/or activity are associated with increased ethanol preference in mice [132], it is conceivable that downregulation of ENT1 expression results from reduced CREB activity, and may lead to excessive alcohol drinking.

The development of mice lacking ENT1 has become indispensable in the exploration of adenosine-mediated glutamate signaling in alcoholism [133]. The majority of the studies conducted using these mice has focused on the striatum due to its integral functions in regulating locomotor behavior and reinforcement, and because the role of adenosine in modulating glutamate signaling is well-established in this region. ENT1 null mice show reduced A₁ receptor-mediated inhibition of glutamate excitatory postsynaptic currents in the

nucleus accumbens, reduced hypnotic and ataxic responses to ethanol, and greater alcohol intake [6]. Activation of A₁ receptors inhibits this hyperglutamatergic state and reduces alcohol consumption. Recently, our lab has confirmed the role of adenosine-mediated glutamate signaling in the striatum in regulating sensitivity to ethanol intoxication and intake [5]. We have also shown that ENT1 null mice have reduced ataxic responses to NMDA receptor antagonists, supporting a role for this receptor in mediating ethanol's behavioral effects, and indicating that adaptive downregulation of these receptors may result from the constitutively elevated glutamate levels in these animals [7].

Astrocytes play a primary role in regulating extracellular glutamate concentrations, and are now accepted as a main source of extracellular adenosine. As such, neuro-glial interactions are of particular interest in mediating alcohol-related behaviors. Adenosine is generally thought to be produced extracellularly from ATP release by astrocytes. Since ENT1 is primarily expressed by astrocytes, these data suggest that astrocytic release of adenosine itself may significantly contribute to extracellular adenosine levels in the striatum. Moreover, in addition to genetic deletion of ENT1, we have shown that pharmacological inhibition of ENT1 reduces the expression of excitatory amino acid transporter 2 (EAAT2) in striatal astrocytes [93]. This finding is consistent with the observations of [134], who report that acute ethanol decreases glutamate uptake by rat cortical astrocytes, and introduces the possibility that a direct intracellular pathway links the expression and function of ENT1 and EAAT2. A schematic representation of our evidence for disrupted neuro-glial interactions in ENT1 null mice is shown in Figure 2. Several recent studies consistently support an inverse relationship between ENT1 expression and/or function and excessive alcohol consumption [39,135,136]. It is especially noteworthy that a recent clinical study reports that genetic variants of ENT1 are implicated in the development of alcoholism [137].

It is worthy of mention that recent studies have also implicated disrupted adenosine signaling in the effects of addictive opiates. Lu et al. (2010) showed that chronic morphine treatment impairs hippocampal long-term potentiation (LTP) and spatial memory via accumulation of extracellular adenosine acting on A₁ receptors [138]. Levran et al. (2009) found a significant association between heroin addiction and SNPs in *SLC29A1*, the gene encoding ENT1 [139]. While investigation into the role of adenosine in mediating the effects of alcohol and addictive drugs is only in its infancy, it warrants further study, particularly regarding adenosine-mediated neuro-glial interactions.

CONCLUSIONS

The neuromodulatory role of adenosine signaling in the CNS is essential to proper brain function. Pharmacological approaches have been important in characterizing cell signaling pathways involved in adenosine signaling and have been useful in behavioral analyses. Additionally, more recent applications of molecular genetics to the study of adenosinergic neuromodulation have become indispensable in filling in gaps limited by pharmacological lack of specificity. Knockout and overexpression of adenosine receptors and transporters in mice have proven exceptionally useful in establishing key roles for adenosine signaling in behavior, a prime example being the accepted status of the ENT1 null mouse as a valid animal model for adenosinergic regulation of glutamate signaling in alcoholism. Combinations of pharmacological and molecular approaches have shown the relative contributions of different adenosine receptors and downstream signaling pathways in ethanol-related behaviors, including ethanol-induced ataxia, in which adenosine A₁ receptor potentiation is a salient feature. Investigations revealing the multiple levels of adenosinergic regulation of glutamate and dopamine signaling in reward pathways have highlighted

potential therapeutic targets in the treatment of alcoholism, with possible application to advancements in treatments of other addictions.

While the cellular source of adenosine levels in the synapse at a given time remains somewhat contentious, it is becoming increasingly clear that astrocytes determine to a large extent the extracellular concentration of adenosine through a number of mechanisms. Not surprisingly, there is significant overlap between the functions of astrocytes and those ascribed to adenosine, namely the fine-tuning of signaling by neurotransmitters. Advances in the study of astrocytes, particularly molecular techniques used to isolate and characterize astrocyte physiology, have contributed greatly to the understanding of neuro-glial interactions, neuronal network formation and remodeling, and complex behaviors. Future studies of adenosinergic mediation of astrocyte-to-neuron signaling hold promise for the study of certain diseases with high prevalence of comorbidity, such as alcoholism and sleep dysregulation, and the development of treatment strategies that can address several neuropathologies at once.

Acknowledgments

This study was supported by the Samuel Johnson Foundation for Genomics of Addiction Program at Mayo Clinic and by grants from the National Institutes of Health (NIH) to D.-S. C. (R01 AA015164, R01 AA018779, P20 AA017830-Project 1).

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Key Learning Objectives

1. Extracellular adenosine concentrations in the brain are largely controlled at the levels of production and nucleoside transport across the plasma membrane. Both neurons and astrocytes are cellular sources of adenosine, either indirectly (via the release of ATP and its subsequent hydrolysis by ecto-enzymes), or directly through equilibrative nucleoside transporters (ENTs).
2. Four types of ENTs (1-4) mediate passive, bi-directional transport of nucleosides and are better characterized than sodium-dependent concentrative nucleoside transporters (CNTs 1-4), which take up adenosine from extracellular space into cells. This discrepancy is mainly due to a lack of specific pharmacological agonists and antagonists for CNTs.
3. Four adenosine receptors (A_1 , A_{2A} , A_{2B} , and A_3) have been cloned. Pharmacological inhibitors of these receptors are relatively non-selective, but molecular approaches, such as recombinant expression of adenosine receptor subtypes in immortal cell lines and genetic knockout/overexpression in mice have been useful in clarifying their intracellular signaling pathways and physiological roles.
4. Adenosine modulates the activity of neurotransmitters at several levels. Presynaptically, adenosine inhibits release of excitatory neurotransmitters such as glutamate via activation of A_1 receptors. Postsynaptically, adenosine receptor activation reduces neuronal excitability via opening potassium channels. Additionally, subtypes of adenosine receptors form heterodimers with other adenosine receptors and those for other neurotransmitters to fine-tune signaling.
5. Through neuro-glial interactions, astrocyte-derived adenosine modifies neuronal network plasticity. Adenosine-mediated neuro-glial interactions have recently been shown to regulate complex behaviors, such as homeostatic sleep, and adenosinergic fine-tuning of glutamate signaling in reward circuitry is strongly implicated in neuroadaptive processes underlying addiction.
6. Acute ethanol-induced increases in adenosine levels, due to the selective inhibition of ENT1, mediate several of the behavioral effects of ethanol intoxication, the best characterized of which is its role in ethanol-induced ataxia. A_1 receptor activation in several motor associated brain regions, including the motor cortex, cerebellum, and striatum, tips the scales away from glutamate and in favor of inhibitory GABAergic signaling through a variety of presynaptic and postsynaptic intracellular mechanisms. A role for A_{2A} receptors and their interactions with BDNF signaling has been suggested, but difficult to establish.
7. Inhibition or genetic deletion of ENT1 significantly impacts glutamate neurotransmission in the striatum, which is implicated in alcohol and drug reward. Such changes include decreases in EAAT2 expression by astrocytes, increased extracellular glutamate levels presumably reflecting an adaptive response to constitutively elevated glutamate. ENT1 null mice are less sensitive to the aversive aspects of acute ethanol intoxication, and display significantly greater voluntary ethanol intake. As such, the ENT1 null mouse is considered a valid model to study adenosine-mediated glutamate signaling in alcoholism.

Future Research Questions

1. A common issue in pharmacological studies is the specificity of ligands for particular subtypes of receptors and transporters. Synthesis of more specific agonists and antagonists, particularly in the case of adenosine transporters, will greatly enhance our understanding of their functions.
2. Conditional, reversible knockdown and overexpression studies will allow discrimination between potential developmental effects of the permanent absence of a specific gene product and more immediate physiological responses. Moreover, combining this approach with methodologies such as microinjection will determine the contributions of knockdown or overexpression of gene products in specific brain regions to particular behavioral phenotypes.
3. The study of astrocyte physiology and its role in neuro-glial interaction is still in its infancy. Further advances in identification, isolation, and molecular characterization of astrocytes, with emphasis on their role in adenosine signaling, will be of immense benefit to the study of complex CNS processes and disorders. Notably, because astrocytes form networks with one another and appear integral to wiring and remodeling diverse neuronal pathways, future research on these glia shows great promise in the development of treatments for disorders involving dysfunctions in several areas of the brain, such as addiction, depression, and sleep disorders.
4. Adenosine signaling modifies that of glutamate and other major neurotransmitters on several levels, including presynaptic effects on transmitter release, postsynaptic alterations in neuronal membrane potential, and a number of receptor-receptor interactions. Future studies characterizing the conditions during which each plays a role are essential to many fields, particularly addiction research, given the high level of complexity in adenosinergic modulation of reward-related networks.
5. It is apparent that there are many different signaling pathways by which adenosine could modulate ethanol-induced ataxia. Combining pharmacological, molecular genetic, and proteomic approaches to determine the roles of established and novel signaling pathways in specific areas of the brain and in small neuronal populations will greatly advance the field. Because adenosinergic modulation of glutamate signaling may be dysregulated in neuromuscular diseases like Parkinson's [140,141] and Huntington's [142], a better understanding of adenosine-mediated signaling in ethanol-induced ataxia will translate to advances in treating these diseases as well.
6. Clinical applications of ongoing multi-disciplinary research include the identification of polymorphisms in molecular players which may contribute to alcoholism and other diseases related to adenosine signaling, discovery of new diagnostic biomarkers, and most importantly, the development of superior therapeutic strategies to the hit-or-miss pharmacological and social approaches currently in use to treat alcoholics.

Fig. 1.

Adenosine-mediated glutamate signaling in the striatum. In astrocytes, adenosine (Ado) is synthesized from AMP (from ATP breakdown) by endo-nucleotidase (endo-NTase) activity and transported to the extracellular region via equilibrative nucleoside transporters (ENTs). ATP released by neurons or astrocytes can also be converted to adenosine extracellularly by ecto-nucleotidase (ecto-NTase) activity. Adenosine binds to G-protein coupled receptors, including presynaptic A₁ receptors (A₁R), known to inhibit glutamate release. A₁ and A_{2A} adenosine receptors are also located postsynaptically, where they modulate neuronal excitability. Presynaptic neurons synthesize glutamate (Glu) from glutamine (Gln), via glutaminase and then package glutamate into vesicles for release using vesicular glutamate transporters (VGLUTs). Once released into synaptic clefts, glutamate activates ionotropic (iGluR) and metabotropic (mGluR) receptors, and is removed within a few milliseconds by excitatory amino acid transporters (EAATs) located in astrocytes. Astrocytes then convert glutamate into glutamine via glutamine synthetase, and recycle it back to presynaptic neurons.

Fig. 2. Altered glutamate signaling in ENT1 null mice. Deletion or inhibition of ENT1 increases extracellular glutamate levels by inhibiting presynaptic adenosine A1 receptors (A₁R) and/or astrocytic excitatory amino acid transporter type 2 (EAAT2). Our recent behavioral, biochemical, and microdialysis studies suggest that increased resistance to acute ethanol intoxication is related to increased glutamate signaling in ENT1 null mice. Resultant decreases in CREB activity might be associated with increased ethanol drinking in mice lacking ENT1.

Table 1

Adenosine Receptors and Ethanol-Induced Ataxia

Subtype	Drug	Dose	Location	Animal	Ataxia	References
A_1 R agonist	CHA	1, 2, and 4 ng	striatum	CD-1 mice	↑	M.S. Dar (2001)
		4 ng	cerebellum			M.S. Dar (2002)
		0.009, 0.034, and 0.069 nmol	motor cortex	Sprague-Dawley rats		Barwick & Dar (1998)
		9, 12, 15, and 24 ng/200 nl	striatum			Meng & Dar (1994)
A_1/A_{2A} R agonist	R-PIA	15, 30 and 50 ng/5 μ l	i.c.v.	CD-1 mice	↑	M.S. Dar (1990)
	S-PIA	0.525, 1.0, 1.5, and 3.0 μ g/5 μ l				M.S. Dar (2001)
	NECA	1.5, 3, and 6 ng				M.S. Dar (2001)
A_1 R antagonist	DPCPX	10, 25, 50, and 100 ng	striatum	CD-1 mice	↓	M.S. Dar (2001)
		100 ng	cerebellum			M.S. Dar (2002)
		0.164 nmol	motor cortex	Sprague-Dawley rats		Barwick & Dar (1998)
		5 mg/kg	i.p.			L. Connoles <i>et al.</i> (2004)
A_{2A} R agonist	CGS-21680	25, 50, and 100 ng	striatum	CD-1 mice	↑	M.S. Dar (2001)
		4.0, 8.0, and 16.0 nmol	motor cortex	Sprague-Dawley rats		Barwick & Dar (1998)
		536 and 1072 ng/200 nl	striatum			Meng & Dar (1994)
		10, 20, and 30 ng/5 μ l	i.c.v.	CD-1 mice		M.S. Dar (1990)
A_{2A} R antagonist	p-SPT	5 and 10 μ g/5 μ l	i.c.v.	CD-1 mice	↓	M.S. Dar (1990)
	SCH 58261	10 mg/kg	i.p.	Sprague-Dawley rats	No effect	L. Connoles <i>et al.</i> (2004)
A_1/A_{2A} R antagonist	caffeine	2.5, 5, and 20 mg/kg	i.p.	CD-1 mice	↓	M.S. Dar (1988)
		62.5 mg/kg			↑	
		2.5, 5, and 25 μ g/5 μ l			↓	
		75 μ g/5 μ l			↑	
		5 and 20 mg/kg	oral	Sprague-Dawley rats	↓	
		50, 100, and 150 μ g/5 μ l	i.c.v.	CD-1 mice	↓	M.S. Dar (1990)