Novel Role for Adenosine in Circadian Rhythms and Alcohol Use Disorders

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Abstract - In the central nervous system, the nucleoside adenosine regulates neuronal activity by modulating the actions of other neurotransmitter systems, thereby influencing many different physiological processes and behaviors. Adenosinergic mechanisms are especially important in fine-tuning glutamatergic neurotransmission. The astrocytic release of adenosine triphosphate and its subsequent extracellular breakdown provides adenosine to drive homeostatic sleep. Acute ethanol (alcohol) exposure increases extracellular adenosine, which mediates the ataxic and hypnotic/sedative effects of alcohol, while chronic ethanol exposure leads to downregulated adenosine signaling that underlies insomnia, a major predictor of relapse. Adenosine gates glutamatergic input to the circadian clock located in the suprachiasmatic nucleus of the hypothalamus, modulating both photic (light-induced) and nonphotic (behaviorally-induced) synchronization of circadian activity rhythms. A recent study using mice lacking the equilibrative nucleoside transporter 1, a well-established animal model of alcohol addiction, suggests an expanded role for adenosine in cellular and behavioral circadian timing and alcohol intake, particularly during chronodisruption.

Keywords - adenosine; glutamate; circadian rhythms; sleep; alcohol; astrocytes; neuroglial interactions

INTRODUCTION

Adenosine is an ubiquitous nucleoside thathas various interrelated functions in the central nervous system (CNS) that are crucial to proper brain function (Burnstock, 2006, 2008; Latini & Pedata, 2001). As in all cells, central adenosine can be phosphorylated to produce the adenosine 5'-triphosphate (ATP) used as metabolic currency. Conversely, it is the product of ATP hydrolysis, and thus serves as an indicator of metabolic activity (Dunwiddie & Masino, 2001). The physiological effects of adenosine are directly related to its metabolic function.

For example, 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 maintain their activity, adenosine takes on a related role in the CNS as a modulator of neurotransmission. This role appears particularly important in the fine-tuning of excitatory glutamatergic signaling (**Fig. 1**; Ruby, Adams, Knight, Nam, & Choi, 2010).

The extracellular adenosine concentration, normally ranging from 25 to 250 nM, is regulated to a great extent by production and transport (Burnstock, 2006, 2008; Parkinson, Ferguson, Zamzow, & Xiong, 2006). This pattern of control allows adenosine levels to change rapidly, which is essential for fine-tuning the activity of neighboring neurons. Adenosine is thought to reach extracellular space primarily through two mechanisms: 1) it is produced extracellularly from ATP released by neurons and astrocytes, and 2) it is released *per se* by neurons (Lovatt et al., 2012; Wall & Dale, 2013) and astrocytes (Parkinson et al., 2006; Parkinson, Xiong, & Zamzow, 2005) via equilibrative nucleoside transporters (ENTs). As such, both neurons and astrocytes appear to be significant sources of extracellular adenosine (Hamilton & Attwell, 2010) and mounting evidence suggests that many of the known roles of adenosine in the CNS are a result of neuroglial interactions (Araque, Parpura, Sanzgiri, & Haydon, 1999; Halassa, Fellin, & Haydon, 2009; Pascual et al., 2005).

Adenosine reduces neuronal excitability and regulates ion channel function through 4 subtypes of G-protein-coupled receptors, A1, A2A, A2B, and A3, each of which has a distinct affinity for adenosine (Fredholm, 2010). Whereas the latter two subtypes have relatively low affinity for adenosine, A1 and A2A

receptors have 10 to 100 nM binding affinity, making these the main receptors activated at physiological levels of adenosine. Adenosine A1 receptors are G_{i}-coupled and expressed ubiquitously in the CNS, where they mediate tonic inhibition of neuronal activity. Presynaptic A1 receptors are especially important in inhibiting the release of glutamate (Halassa, Fellin, et al., 2009). On the other hand, adenosine A2A receptors are G_{s}-coupled, and exerting excitatory influences on neurons by increasing the level of cyclic adenosine 3',5' monophosphate (cAMP) production by adenylate cyclase. Although A2A receptors are excitatory at the cellular level, they are primarily expressed in the indirect (striatopallidal) circuit, resulting in the inhibition of motor activity (Aoyama, Kase, & Borrelli, 2000). A2A receptors are also known to associate physically with other neurotransmitter receptors, including the adenosine A1, dopamine D2, and glutamate mGluR5 receptors (Ciruela et al., 2006; Ferre et al., 2010).

Adenosine has been shown to regulate several complex behaviors in both health and disease, with well-established roles in homeostatic sleep and alcohol use disorders (Asatryan et al., 2011; Cunha, Ferre, Vaugeois, & Chen, 2008; Ruby et al., 2010; Ruby, O'Connor, Ayers-Ringler, & Choi, 2014). While adenosine itself may mediate sleep, adenosinergic fine-tuning of glutamate signaling appears responsible for its modulation of circadian timing and to a large extent, alcohol-related behavior and consumption. Dysregulated modulation of glutamatergic transmission by adenosine in the striatum has been strongly implicated in mediating the effects of, and abuse potential for, alcohol (Chen et al., 2010; Choi et al., 2004; Nam, Lee, Hinton, & Choi, 2010). Recent research implicating a role for adenosine signaling in circadian activity, striatal clock gene expression, and alcohol

intake during chronodisruption may lead to new therapies for the often treatment-refractory circadian and sleep problems arising due to long-term alcohol overconsumption.

1. Mediation of Homeostatic Sleep by Adenosine

Adenosine has been identified as the factor that accumulates with wakefulness and promotes sleep (Porkka-Heiskanen et al., 1997). Specifically, the accumulation of adenosine activates A1 receptors to inhibit the wake-promoting neurons of the basal forebrain (BF; Basheer, Strecker, Thakkar, & McCarley, 2004; Porkka-Heiskanen et al., 1997; Radulovacki, Virus, Djuricicnedelson, & Green, 1984; Thakkar, Delgiacco, Strecker, & McCarley, 2003), driving both sleep pressure and intensity as a function of prior time awake (Brown, Basheer, McKenna, Strecker, & McCarley, 2012; Schmitt, Sims, Dale, & Haydon, 2012). The induction of sleepiness and decreased alertness is mimicked by systemic or central administration of adenosine or A1 receptor agonists (Basheer et al., 2004; Blutstein & Haydon, 2013; Frank, 2013). Studies using mice in which soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated transmitter release (gliotransmission) is attenuated specifically in astrocytes (dnSNARE mice; Pascual et al., 2005) have revealed that astrocytic-release of ATP is a major source of adenosine. dnSNARE mice exhibit a markedly attenuated response to sleep deprivation (Halassa, Florian, et al., 2009). During sleep deprivation, both extracellular adenosine and A1 receptor expression increase (Basheer et al., 2004; Brown et al., 2012) to further promote drowsiness and initiate compensatory rebound sleep. dnSNARE mice show reductions in the power of low frequency slow-wave sleep (0.5–1.5 Hz), a well-established index of

sleep pressure directly related to the enhancement of adenosine tone that accompanies sleep deprivation, as well as total recovery sleep time. Moreover, these effects are mimicked in wild-type mice with systemic administration of the A1 receptor antagonist cyclopentyltheophylline (Halassa, Florian, et al., 2009), suggesting that purinergic gliotransmission regulates sleep homeostasis. Interestingly, as dnSNARE mice do not exhibit an abnormal baseline sleep phenotype, it is possible that release of adenosine via ENTs, particularly ENT1, may be important for basal sleep pressure, while the role of gliotransmission is primarily in recovery sleep.

Astrocytes also play a role in the promotion of wakefulness by clearing adenosine during sleep. Adenosine clearance is mainly mediated by the combined actions of ENTs facilitating the inward flow of adenosine and astrocytic adenosine kinase (AK), which phosphorylates intracellular adenosine to AMP. Reduction of AK within astrocytes causes an increase in inhibitory transmission through A1 receptor activation, while overexpression of AK results in the opposite effect (Diogenes et al., 2014), accelerating adenosine clearance and diminishing recovery sleep after sleep deprivation (Dias, Rombo, Ribeiro, Henley, & Sebastiao, 2013). The second pathway for adenosine clearance is extracellular breakdown to inosine by adenosine deaminase (AD). Bachmann et al. (2012) found that a polymorphism of AD that reduced the conversion of adenosine to inosine leads to significant increases in sleep pressure, waking alpha wave activity, and fatigue, while decreasing attention and vigor (Bachmann et al., 2012). Although much remains to be discovered regarding the roles of various adenosine signaling molecules, it is a clear that neuron-glial mechanisms in astrocytes are indispensable in maintaining

sleep homeostasis. As ATP release by astrocytes appears to be regulated by cellular circadian timing, it is reasonable to hypothesize that astrocytes may serve as an interface between the circadian and homeostatic sleep systems.

2. Role of Adenosine in Alcohol Withdrawal-Induced Insomnia

Alcohol and alcohol withdrawal have profound impacts on sleep, which has been extensively documented in the clinical literature (Colrain, Crowley, Nicholas, Padilla, & Baker, 2009). The estimated cost of alcohol-related sleep disorders in the United States exceeds $18 billion per year (Brower, 2001). Acute ethanol intake in non-alcoholics decreases sleep latency (the amount of time to fall asleep) and increases non-rapid eye movement (NREM) sleep quantity and quality. In contrast, REM sleep is suppressed during the first half of nocturnal sleep time and is followed by a "REM rebound" (increased REM sleep) during the second half. During alcohol withdrawal, recovering alcohol dependent patients commonly experience severe and protracted sleep disruptions manifested as insomnia, reduced slow-wave sleep and increased REM sleep along with excessive daytime drowsiness (Allen, Wagman, Faillace, & McIntosh, 1971; Brower, Aldrich, & Hall, 1998; Colrain et al., 2009; Ehlers & Slawecki, 2000; Kubota, De, Brown, Simasko, & Krueger, 2002; Mukherjee, Kazerooni, & Simasko, 2008; Mukherjee & Simasko, 2009; Roehrs & Roth, 2001; Veatch, 2006). These sleep impairments are severe enough that they serve as a primary indicator of relapse (Brower & Perron, 2010). Consistent with clinical studies, basic research also suggests that ethanol withdrawal is accompanied by insomnia-like symptoms, including increased wakefulness, reductions in total sleep time, and delta activity (Ehlers & Slawecki, 2000; Kubota et al., 2002;

Mendelson et al., 1978; Mukherjee et al., 2008; Mukherjee & Simasko, 2009; Veatch, 2006).

Recent studies indicate that the sleep-inducing effects of acute ethanol exposure may be mediated by adenosinergic mechanisms in the wake-promoting basal forebrain (Thakkar, Engemann, Sharma, & Sahota, 2010). Acute ethanol inhibits adenosine transporter ENT1 in astrocytes, leading to increased extracellular adenosine (Nagy, Diamond, Casso, Franklin, & Gordon, 1990), which may mediate ethanol-induced sedation. Likewise, alterations in adenosine signaling contribute to sleep disruptions during early withdrawal (Sharma, Engemann, Sahota, & Thakkar, 2010). Ethanol-dependent rats display profound insomnia-like symptoms, manifested as markedly increased wakefulness coupled with significant reductions in both NREM and REM sleep. This sleep disruption is accompanied by greater numbers of c-Fos immunoreactive, wake-promoting cholinergic neurons in the BF (Thakkar et al., 2003). Ethanol-dependent rats also lack the normal rise in extracellular adenosine in the BF during sleep deprivation, and have lower BF expression of A1 receptors and ENT1 (Sharma et al., 2010). Consistent with these results, mice lacking ENT1 display a reduced central adenosine concentration (Nam et al., 2011), lower sensitivity to acute ethanol, and higher alcohol consumption compared to wild-types (Choi et al., 2004). Together, these studies support the idea that diminished adenosine tone in the BF resulting from decreased expression of ENT1 and A1 receptors may underlie insomnia during acute withdrawal from alcohol. Given that astrocytes appear to be the main source of adenosine in recovery sleep, it is of great interest to determine whether gliotransmission may be compromised in alcohol-induced sleep

disruption. It will also be important to characterize the impact of ethanol-induced dysregulation of circadian timekeeping processes on sleep homeostasis.

3. Adenosinergic Modulation of Circadian Timing

Despite the well-established role for adenosine in sleep, surprisingly little is known about how adenosine interacts with the circadian system. Mammalian circadian rhythms in physiology and behavior are regulated primarily by the suprachiasmatic nucleus (SCN) of the hypothalamus. At the cellular level, circadian timing is mediated by expression of clock genes. Clock genes are expressed in nearly every cell in the body and exhibit circadian oscillation in many brain regions, including those implicated in addiction (Dibner, Schibler, & Albrecht, 2010), though most require the SCN to maintain synchrony. Circadian rhythms are entrained to the external environment by two main inputs to the SCN: photic (light) input, and nonphotic input (typically alerting stimuli, such as novel environments, sleep deprivation, and exercise). Depending on what time of day or night they are presented, these inputs can advance or delay clock timing, which is termed phase-resetting.

Photic stimulation of the SCN arrives via a direct, glutamatergic projection from the retina (Moore, 1983; Pickard, 1982). Activation of adenosine A1 receptors located presynaptically on retinohypothalamic axon terminals attenuates photic phase-resetting of the SCN clock by inhibiting glutamate release (**Fig. 2**; Hallworth, Cato, Colbert, & Rea, 2002; Sigworth & Rea, 2003). Ethanol, which acutely increases extracellular adenosine level by blocking adenosine transporter ENT1 (Nagy et al., 1990), attenuates photic phase-resetting (Ruby, Brager, DePaul, Prosser, & Glass, 2009; Ruby, Prosser,

DePaul, Roberts, & Glass, 2009; Seggio, Logan, & Rosenwasser, 2007) and shortens free-running circadian period (Seggio, Fixaris, Reed, Logan, & Rosenwasser, 2009). Whether or not adenosine plays a role in these effects has yet to be determined, as ethanol is also well known to inhibit the glutamate N-methyl-d-aspartate (NMDA) receptor, activation of which is responsible for photic phase-shifts (Abe, Rusak, & Robertson, 1991; Colwell, Foster, & Menaker, 1991; Ding et al., 1994; Mintz & Albers, 1997; Mintz, Marvel, Gillespie, Price, & Albers, 1999). Recently, the adenosine A1/A2A receptor antagonist caffeine has been shown to potentiate photic phase-delays and postpone the active phase in mice relative to the photocycle (Verbanes et al., 2014).

Adenosine has also been shown to modulate nonphotic phase-resetting of circadian behavioral rhythms. Antle et al. (2001) demonstrated that A1 receptor agonist N-CHA induced dose-dependent phase-shifts when administered during midday, mimicking the effect of a three-hour sleep deprivation procedure. In contrast, caffeine, while not producing phase-shifts on its own, dose-dependently reduced sleep deprivation-induced phase-advances in hamsters (Antle, Steen, & Mistlberger, 2001). Caffeine consumption also lengthens free-running circadian period in mice (Oike, Kobori, Suzuki, & Ishida, 2011). Although it is tempting to speculate that caffeine lengthens period by reducing drowsiness and thus delaying sleep phase, the evidence that caffeine potentiates photic phase-delays (Verbanes et al., 2014) and inhibits nonphotic phase-advances (Antle et al., 2001) may indicate that caffeine-induced period lengthening is due to its interference with the normal balance of inputs to the SCN.

Interestingly, the relationship between circadian timing and adenosine

signaling may be bidirectional. Adenosine has long been known to contribute to homeostatic sleep regulation (discussed later in this section), so it is not entirely surprising that levels of extracellular adenosine vary in a circadian manner (Murillo-Rodriguez, Blanco-Centurion, Gerashchenko, Salin-Pascual, & Shiromani, 2004). Adenosine accumulates in perisynaptic space in an activity-dependent manner, secondary to ATP hydrolysis (Pascual et al., 2005). Thus, a daily rise in the central adenosine concentration is thought to reflect the homeostatic need for sleep. However, evidence for circadian variation in the expression of A1 receptors and ENT1 in wake-promoting areas of the brain (Alanko, Stenberg, & Porkka-Heiskanen, 2003; Murillo-Rodriguez et al., 2004; Virus, Baglajewski, & Radulovacki, 1984) may indicate that they are targets of cellular circadian timekeeping processes. Likewise, astrocytic release of ATP in the SCN, and presumably adenosine produced from its metabolism, follows a circadian pattern (Marpegan et al., 2011). The notion that a feedback loop may exist between the circadian and adenosinergic systems merits future exploration.

4. Implication of Adenosine in the Chronodisruptive Effects of Alcohol

Similar to its role in other brain regions, one of the main functions of adenosine in the nucleus accumbens (NAc) is to inhibit glutamate release via activation of presynaptic A1 receptors (Harvey & Lacey, 1997). As such, increased inhibition of glutamate release via the rise in adenosine during acute ethanol exposure partially accounts for the intoxicating effects of ethanol (Dunwiddie & Masino, 2001). Mice lacking ENT1 exhibit reduced ataxia and hypnosis in response to acute ethanol exposure and consume more ethanol than their wildtype littermates (Choi et al., 2004). Conversely, ENT1 overexpression in

neurons increases ethanol intoxication in mice (Parkinson et al., 2009). Other recent studies lend further support to the inverse correlation of ENT1 gene expression and ethanol drinking (Sharma et al., 2010; Short, Drago, & Lawrence, 2006). Several genetic variants of ENT1 (SLC29A1) are included among a 130 candidate gene-based array for clinical genomic studies of addiction (Hodgkinson et al., 2008). In addition, variants of ENT1 are associated with alcohol abuse and poor sleep in women (Gass et al., 2010) as well as alcohol dependency with a history of withdrawal seizures (Kim et al., 2011).

The primary neurochemical change induced by the ENT1 deletion in mice appears to be a deficit in extracellular adenosine levels, as measured in the striatum (Nam et al., 2011). In a recent study, Ruby et al. (2014) took advantage of this mouse model to examine a possible role for endogenous adenosine in cellular and behavioral circadian timing. ENT1 null mice showed notable changes in entrainment to a standard 12 hour light-12 hour dark photocycle, becoming active ~40 minutes before wildtype littermates, ~20 minutes prior to the dark-phase (active phase for nocturnal rodents). They also displayed a longer active phase, longer duration of nightly activity, and greater activity intensity than wildtypes (Ruby, Vadnie, et al., 2014). This hyperactivity is consistent with low adenosine tone and the hyperlocomotion produced by caffeine, which is mediated by A2A inhibition in the NAc (El Yacoubi et al., 2000; Lazarus et al., 2011). The unusual circadian phenotype of ENT1 null mice was ameliorated by 5-day treatment with A2A receptor agonist CGS-21680 (Ruby, Vadnie, et al., 2014). This result is intriguing in light of the fact that A2A receptors are not known to be expressed in the SCN, suggesting that another brain region (namely, the A2A receptor-rich striatum) may influence

circadian activity. Given the role of A2A receptors in locomotor activity level, it is reasonable to speculate that they contribute to circadian amplitude. In addition to their baseline heavy alcohol consumption, ENT1 null mice showed further escalation in ethanol consumption during chronodisruption by exposure to a constant light photocycle (Ruby, Vadnie, et al., 2014), reinforcing the notion that circadian misalignment may play a role in their drinking behavior.

Clock gene expression in the NAc and caudate-putamen (CPu) was also altered by ENT1 deletion (**Fig. 3**). The most striking change was a dramatic (60%) reduction in peak levels of Period 2 (Per2) mRNA and protein expression in these regions. It is noteworthy that Per2 deficiency is linked with alcoholism in humans, and that Per2 mutation in mice leads to high alcohol consumption (Spanagel et al., 2005) and a similar (although much more severe) circadian phenotype (Albrecht, Zheng, Larkin, Sun, & Lee, 2001). Both ENT1 null (Nam et al., 2011) and Per2 mutant (Spanagel et al., 2005) mice also show higher brain glutamate levels than their respective wildtype littermates, in line with the importance of adenosine in controlling glutamatergic neurotransmission. Consistent with the behavioral data implicating reduced A2A receptor-mediated signaling in the ENT1 null phenotype, pharmacological A2A receptor inhibition decreased Per2 mRNA in the wildtype striatum, whereas A1 receptor inhibition decreased levels of both *Per1* and *Per2* (Ruby, Vadnie, et al., 2014). A2A receptor agonist CGS-21680 increased *Per2* expression in both ENT1 null and wildtype mice (Ruby, Vadnie, et al., 2014). Interestingly, this agent also increased *Per1* expression in the ENT1 null (but not wildtype) striatum (Ruby, Vadnie, et al., 2014). Importantly, like Per2, there is a

negative correlation between A2A receptor activation/expression and alcohol intake (Arolfo, Yao, Gordon, Diamond, & Janak, 2004; Houchi, Persyn, Legastelois, & Naassila, 2013; Kaplan, Bharmal, Leite-Morris, & Adams, 1999; Naassila, Ledent, & Daoust, 2002). Given the relationship between glutamate and Per2 (Spanagel et al., 2005), and the key modulatory role of adenosine in glutamate transmission, it is plausible that the observations of Ruby et al. involve the well-documented dysregulation of glutamate signaling in the striatum of ENT1 null mice (Ruby, O'Connor, et al., 2014). Future work elucidating adenosinergic mechanisms of clock gene regulation and their impact on behavior is warranted.

CONCLUSIONS

It is clear that adenosine signaling is essential to the regulation of circadian rhythms, sleep, and alcohol intake. While the actions of adenosine on glutamate signaling culminate in neuronal regulation, astrocytes also play a fundamental role through the release of the gliotransmitter ATP and its by-product, adenosine, and via uptake of glutamate. As an endogenous sleep-promoting agent that may be regulated in a circadian as well as activity-dependent manner, adenosine may represent a bridge between the circadian and homeostatic sleep systems. Deficient adenosine signaling appears to underlie the persistent insomnia seen in acute and protracted withdrawal from alcohol, as well as the cellular and behavioral circadian dysregulation observed in mice lacking adenosine transporter ENT1, a well-established model of alcohol dependence. Together, this evidence attests to the utility of targeting adenosine signaling for the development of therapies for alcohol use disorders, particularly those involving treatment-refractory circadian rhythm and sleep disruptions.

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FIGURE LEGENDS

Figure 1. Schematic representation of neuroglial adenosine-mediated fine-tuning of glutamatergic neurotransmission. 1) SNARE-mediated ATP release by astrocytes provides a major source of adenosine (Ade) in extracellular space. 2) Adenosine levels are also highly regulated by ethanol-sensitive equilibrative nucleoside transporter 1 (ENT1), a bidirectional nucleoside transporter expressed in neurons and astrocytes. 3) Activation of presynaptic A1 receptors (A1R) inhibits glutamate release. 4) Activation of postsynaptic A1 receptors inhibits adenylate cyclase (AC) to reduce production of cyclic AMP (cAMP). 5) Activation of G_s-coupled A2A receptors (A2AR) stimulates the production of cAMP by AC. 6) A1 receptor activation on astrocytes regulates the expression of excitatory amino acid transporter 2 (EAAT2), which is responsible for the majority of glutamate (Glu) uptake in the central nervous system. Gln, glutamine. Adapted from Ruby, O'Connor, et al., 2014.

Figure 2. Adenosine-mediated inhibition of circadian photic phase-resetting. Activation of presynaptic A1 receptors (A1R) located on retinohypothalamic tract (RHT) neurons inhibits light-induced glutamate (Glu) release in the suprachiasmatic nucleus (SCN), preventing activation of postsynaptic N-methyl-D-aspartate receptors (NMDAR) and thereby attenuating photic phase-resetting of circadian behavioral rhythms.

Figure 3. Adenosinergic regulation of *Period* gene expression in the striatum. Deletion of equilibrative nucleoside transporter type 1 (ENT1) in mice leads to reduced extracellular adenosine (Ade) and

greatly decreased peak expression of clock gene *Period 2* (*Per2*) mRNA and protein in the striatum. Pharmacological inhibition of A1 receptors (A1R) reduces both *Period 1* (*Per1*) and *Per2* mRNA expression, while inhibition of A2A receptors (A2AR) reduces only *Per2* mRNA expression in the murine striatum. This dysregulation in cellular clock timing may contribute to the baseline heavy-drinking in ENT1 null mice, as well as their further escalation in alcohol consumption during constant light exposure.

