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The importance of astrocyte derived purines in the modulation of sleep

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Abstract

Sleep is an evolutionarily conserved phenomenon that is clearly essential for survival but we have limited understanding of how and why it is so important. ATP/adenosine signaling has been known to be important in the regulation of sleep and recent evidence suggests a critical role for gliotransmission in the modulation of sleep homeostasis. Here we review the regulation of ATP/adenosine in the nervous system and provide evidence of a critical role for astrocyte derived adenosine in the regulation of sleep homeostasis and the modulation of synaptic transmission. Further understanding of the role of glial cells in the regulation of sleep may provide new targets for pharmaceutical intervention in the treatment of brain dysfunctions, specifically those that are comorbid with sleep disruptions.

Keywords

Adenosine; A1R; Sleep homeostasis; Memory consolidation

We all sleep; we all know it is essential, yet experts still debate its purpose. However, it is clear that sleep is essential for our survival (Banks and Dinges 2007). Even a modest restriction in total sleep time over the course of several days leads to poor performance and cognitive impairments. Indeed there are numerous examples of disasters in society where sleep deprivation has been determined to be a contributing factor. Because of the importance of sleep for human performance there is intensive investigation into the mechanism controlling this process. During the past three years we have become aware that mammalian glia play crucial roles in the regulation of sleep, and here we review evidence for the role of astrocytes in the regulation of sleep homeostasis (Halassa et al. 2009).

Sleep is conserved across the animal kingdom. For example, *Drosophila*, and zebrafish exhibit rest periods that are characterized as sleep. Sleep can be categorized into two distinct core components: the circadian oscillator and the sleep homeostat (Franken and Dijk 2009). The circadian oscillator sets the timing of wakefulness. For example, when we fly across time zones we wake at the wrong time of day/night because our oscillator takes days to reset its rhythm to the light/dark cycle of the new time zone. The sleep homeostat, in contrast, does not set the timing of wakefulness but instead integrates the amount of time that we are awake and provides the pressure or drive to sleep. If one stays awake late at night, the subsequent drowsiness or pressure to sleep is provided by the sleep homeostat. The mechanisms controlling circadian oscillations and sleep homeostasis are distinct.

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Conflicts of Interest

Philip G. Haydon is co-founder and President of GliaCure Inc.

Though there is now abundant evidence that glial cells can control circadian oscillations (Suh and Jackson 2007), this review will focus on sleep homeostasis, where recent evidence has shown the importance of astrocytes in the control of this important daily process.

Astrocytes release gliotransmitters at the tripartite synapse

Much of neuroscience of the past century was dominated by the study of neurons and these investigations catapulted our understanding of brain function. This focus was largely the result of the availability of techniques to study neurons, specifically electrical recording and stimulation protocols. However, glial cells, which are electrically inexcitable, were effectively mute when these approaches were used. The development of imaging and Ca^{2+} sensitive indicators however, allowed the identification of glial excitability: for example that astrocytes respond to receptor activation with Ca^{2+} mobilization (Cornell-Bell et al. 1990). In 1994 it was accidentally discovered that astrocytes can release glutamate in response to receptor induced Ca^{2+} signals (Parpura et al. 1994). Since that time other signals have been demonstrated to be released and numerous studies have now shown that the astrocyte, which contacts pre and postsynaptic structures at the tripartite synapse, can modulate synaptic plasticity *in vitro*, *in situ* and *in vivo* (Halassa and Haydon 2010).

After discovering that astrocytes can release gliotransmitters we have asked whether this process can modulate behavior and have sought to identify underlying mechanisms. In this review we will focus on one class of gliotransmitter – the adenosine triphosphate (ATP)/adenosine system – but by doing so we do not mean to minimize the importance of gliotransmitters for other regulatory processes. Instead we choose to focus here because of our extensive knowledge in this area and because behavioral phenotypes with potential clinical relevance have been identified.

Before discussing the control of sleep by the astrocyte we first provide a framework on understanding of the regulation of adenosine, a critical gliotransmitter that can modulate sleep.

There are many sources of adenosine that can be recruited under different conditions

Adenosine can be derived from many sources in the nervous system as detailed in Figure 1. One prominent idea is that ATP is released and when in the extracellular space it is hydrolyzed to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and ultimately to adenosine. Evidence has accumulated for this form of release being mediated by neurons and astrocytes. ATP can be potentially released by an exocytotic mechanism or via channel mediated release.

Another pathway of release is the direct release of adenosine. Recent evidence has shown action potential-dependent release of adenosine in the cerebellum mediated by a direct release pathway consistent with exocytosis of adenosine (Klyuch et al. 2012). In these studies $\text{CD73}^{-/-}$ mice were used to eliminate the extracellular 5'hydrolase that is necessary for the hydrolysis of AMP to adenosine while monitoring adenosine using adenosine biosensors. Despite the absence of CD73, activity dependent adenosine accumulation was still detected and this release was sensitive to bafilomycin A1, an inhibitor of the proton pump that is required to fill vesicles with transmitter. It is important to appreciate that there are multiple enzymes that can hydrolyze AMP to adenosine (Figure 1), thus a $\text{CD73}^{-/-}$ alone may be insufficient to conclude that adenosine is released directly. However, in support of their contention, they did show that the hydrolysis of exogenous AMP was reduced by 91% in $\text{CD73}^{-/-}$ mice leading the authors to suggest that adenosine is released

from adenosine filled vesicles. The nature of the transporter responsible for filling the vesicles is still to be defined.

Adenosine is taken up into cells via either concentrative nucleoside transporters (CNTs) or equilibrative transporters (ENTs). The ENTs facilitate the transport of adenosine along concentration gradients (Parkinson et al. 2011). Thus, under conditions of elevated metabolic demand, when intracellular adenosine levels rise, adenosine can be directly released through the ENTs. One condition under which this pathway probably contributes significantly to elevating extracellular adenosine is during hypoxia. It is unlikely that this pathway contributes to adenosine-mediated modulation of sleep because application of ENT inhibitors, *in vivo*, lead to an elevation of extracellular adenosine, a result which is only consistent with an alternate source of adenosine and reuptake via the ENT (Porkka-Heiskanen et al. 1997).

An astrocytic source of adenosine

While it is clear that adenosine can accumulate in the extracellular space through numerous pathways, the cell types which provide this nucleoside have been less clear with many assuming that all release is neural activity dependent and due to ATP being co-released along with classical neurotransmitters from synaptic terminals. While this source of release can contribute to the extracellular purine pool, significant evidence has also accumulated showing that astrocytes can provide a source of ATP and adenosine and that this adenosine pathway contributes to the regulation of sleep homeostasis.

In 2005 we published results of studies in which we generated conditional astrocyte-specific transgenic mice, which modified the magnitude of basal excitatory synaptic transmission (Pascual et al. 2005). In these studies we expressed the soluble N-ethyl maleimide-sensitive fusion protein attachment protein receptor (SNARE) domain of the vesicle-associated membrane protein 2 (VAMP2) in the absence of the vesicle tail. This truncated protein acts as a dominant negative SNARE (dnSNARE) competing with the endogenous proteins and preventing the formation of the VAMP2-SNARE complex that is essential for regulated exocytosis (Figure 2). As a consequence, and as discussed beneath, this manipulation led to a selective reduction in extracellular adenosine. It should be noted that there are numerous VAMP proteins each responsible for different aspects of membrane trafficking and that a SNARE from one VAMP will not substitute for the other (Chen and Scheller 2001). Thus, the SNARE domain of VAMP2 does not compete with VAMP8 mediated membrane trafficking events and visa-versa. Moreover, there is accumulating evidence that in nerve terminals different VAMPs regulate the trafficking of different pools of synaptic vesicles: VAMP4 regulates asynchronous release while VAMP2 regulates Ca^{2+} dependent synchronous release and although both can form SNARE complexes, they form macromolecular complexes with discrete proteins (Raingo et al. 2012). Consequently, the SNARE strategy we developed is highly selective and interferes with a small subset of trafficking events. The specificity of the dnSNARE perturbation in astrocytes has been confirmed in our control studies where we show the normal expression of membrane receptors, transporters, K^{+} channels, resting potential, input resistance and Ca^{2+} despite expressing the SNARE domain of VAMP2 for months (Fellin 2009; Pascual et al. 2005).

It is well known that there is an extracellular tone of adenosine that causes a persistent activation of adenosine receptor 1 (A1R) leading to presynaptic inhibition of excitatory synaptic transmission (Dunwiddie and Masino 2001). In hippocampal slices isolated from mice in which dnSNARE was expressed selectively in astrocytes we found an enhanced magnitude of synaptic transmission at the CA3-CA1 synapse. Using pharmacological approaches we asked whether the enhanced synaptic transmission in dnSNARE mice was a

result of reduced A1R activation. Addition of A1R antagonists (either CPT or DPCPX) enhance the magnitude of excitatory synaptic transmission but in dnSNARE mice the relative enhancement was attenuated compared to littermate controls leading us to infer that astrocytic expression of dnSNARE reduced the extracellular level of adenosine (Figure 3). This may be a widespread property in the brain because studies performed *in vivo* have shown a similar role for astrocytes in the control of cortical adenosine tone (Figure 4). Subsequently, the use of adenosine biosensors in hippocampal slices has supported the idea of an astrocytic control of adenosine by showing that astrocytic dnSNARE expression reduces the concentration of extracellular adenosine. (Schmitt et al. 2012) These results demonstrate the fundamental importance of the astrocyte as one source of extracellular adenosine.

We have now used several strategies to probe the mechanism of astrocyte-mediated extracellular adenosine accumulation. We first asked whether adenosine is released down a concentration gradient from an intracellular source via equilibrative nucleoside transporters. However, since pharmacological inhibition of these transporters increased A1R mediated presynaptic inhibition this result is consistent with the transporter being used to take up adenosine into the cytosol. To determine the potential for a SNARE-sensitive release of ATP being the source of adenosine we imaged extracellular ATP in brain slices. Astrocytic expression of dnSNARE significantly reduced extracellular ATP. If ATP is the source of adenosine then pharmacological inhibition of ectonucleotidases should increase extracellular ATP. In agreement with this possibility the addition of the ectoATPase inhibitor ARL6715 increased ATP in slices obtained from wild type but not dnSNARE mice. Final support for an ATP dependent source of adenosine was provided by the ability of exogenous ATP to reconstitute A1R dependent presynaptic inhibition in dnSNARE mice (Pascual et al. 2005).

Taken together these results lead us to conclude that ‘one source’ of extracellular adenosine is SNARE mediated release of ATP which is hydrolyzed in the extracellular space to adenosine. It goes without saying that there can be other sources of ATP and adenosine: presumably each is recruited under different conditions to control different spatio-temporal aspects of the control of extracellular adenosine.

An intriguing question that emerges from these studies is whether the nucleotides upstream of adenosine are merely precursors of adenosine or whether they play significant signaling roles in their own right. ATP is known to be able to act on the purinergic receptors P2X and P2Y receptors (Burnstock et al. 2011). Because of the presence of ectonucleotidases however released ATP is likely to be hydrolyzed rapidly limiting its spatio-temporal action. ADP activates different receptors from ATP. Finally, AMP has recently been proposed to activate a subset of adenosine receptors: AMP activates A1R but not A2BR (and presumably not A2A) (Rittiner et al. 2012). This selectivity is intriguing since it could allow the ATP dependent adenosine pathway to differentially activate A1R from A2A and A2B receptors.

In addition there may be regionally specific roles for adenosine in both overall circuit function and the regulation of sleep and sleep homeostasis. For example, it is known that adenosine acting through adenosine receptor 2A (A2AR) regulates dopamine receptor 2 (D2R) internalization and in the ventral striatum there is a downregulation of D2/D3 receptors after sleep deprivation that may play a role in decreased wakefulness (Volkow et al. 2012). While in the spinal cord, stimulation of the A1R has anti-nociceptive effects on baseline pain (Gong et al. 2010) and dnSNARE mice have a reduced threshold to mechanical nociception (Foley et al. 2011). Below we discuss the role of astrocyte-derived adenosine in both the cortex and hippocampus.

By regulating adenosine, astrocytes modulate circuit function in the cortex

Because adenosine receptors are known to modulate sleep homeostasis (Benington et al. 1995; Bjorness et al. 2009; Thakkar et al. 2003; Ursin and Bjorvatn 1998) and because of the correlation between wakefulness and elevated adenosine (Porkka-Heiskanen et al. 2000; Portas et al. 1997) a natural next step was to move *in vivo* and ask whether circuit activity and/or sleep homeostasis is modulated by the astrocyte. The locations of the circuits involved in sleep homeostasis are complex and beyond the scope of this review, for an in-depth discussion of these circuits please see (Brown et al. 2012). Additionally, while not discounting the importance of other potential targets of adenosine such as orexinergic neurons and the basal forebrain we have focused on the cortex. We performed two classes of experiments: in the first we studied slow oscillations in anesthetized mice and in the second we used cortical electroencephalogram/ electromyogram (EEG/EMG) recordings in freely behaving mice to study sleep homeostasis *per se*.

During non rapid eye movement sleep (NREM sleep) cortical neurons exhibit slow oscillations (also termed up and down states) at a frequency of less than 1Hz (Steriade et al. 1993a; Steriade et al. 1993b). These events are relatively synchronized amongst cortical pyramidal neurons allowing their detection with local field potential measurements (Steriade 2006). When one compares slow oscillations in wild type and dnSNARE mice it is clear that astrocytic SNARE expression reduces the power of the slow oscillation (Fellin et al. 2009). This, together with EEG studies discussed beneath, provides the first evidence that astrocytes modulate the function of neural circuits.

We already demonstrated that astrocytic dnSNARE expression leads to a reduction in adenosine tone so we asked whether adenosine and A1R could influence the activity of the circuits underlying slow oscillations by acutely applying the adenosine receptor antagonist CPT to the cortical surface. In wild type mice we found that the A1R antagonist augmented the power of slow oscillations and that astrocytic dnSNARE expression blunted this A1R modulation (Figure 4) (Schmitt et al. 2012). If indeed this circuit modulation is involved in the control of sleep related behavior then one would predict that astrocytes would exert differential modulation of slow oscillations at different times of day. Recently we have carefully controlled the timing of these studies and have found that a Zeitgeber 0 (ZT0, the time at which lights are turned on in the animal room which is the equivalent of the onset of subjective nighttime) after animals have been awake, there is a large astrocytic SNARE dependent A1R modulation of synaptic transmission and slow oscillations (Figure 3 and 4). However by ZT6 when mice have been left undisturbed and allowed to sleep, the magnitude of the astrocytic adenosine mediated modulation declines significantly. To discriminate between a time of day effect and ask whether this change in adenosine modulation of slow oscillations is wakefulness dependent we sleep deprived mice between ZT0 and ZT 6. In agreement with the possibility that the astrocytic adenosine pool is regulated by a behavioral state, we found that sleep deprivation maintained the elevated adenosine tone in the cortex. (Schmitt et al. 2012)

Astrocytes play a pivotal role in modulating sleep homeostasis

Sleep homeostasis can be monitored using several measures. Central amongst these is the cortical EEG/EMG that is used to measure the amount of time that an animal is in three different vigilance states – wakefulness, NREM sleep and rapid eye movement (REM) sleep, as well as to measure the power of different frequency bands of the EEG in specific vigilance states. It is well known that in response to sleep deprivation the sleep homeostat increases the pressure or the drive to sleep. We can all relate to this personally when we stay up late at night (acute sleep deprivation) we feel drowsy and an increased need or desire to

sleep. Sleep pressure is measure by increased slow wave activity (SWA), the 0.5–4.0Hz frequency range of the EEG during NREM sleep. SWA is elevated normally at ZT0 and decreases as the animal is allowed to sleep, it is also enhanced following a period of sleep deprivation (Figure 5). Increased sleep pressure also causes a compensatory increase in sleep time. (Fuller et al. 2006; Sinton and McCarley 2004)

We used EEG/EMG recordings in freely behaving mice to ask whether sleep homeostasis involves an astrocytic component. Recordings were made for an initial 24hour period (baseline) followed by a subsequent day in which we sleep deprived mice for six hours between ZT0 and 6 which is equivalent to a sleep deprivation being performed for the first half of the subjective night time. Conditional expression of dnSNARE in astrocytes led to reduced sleep pressure (Figure 6). During the baseline day, the power of SWA during NREM sleep was reduced compared to littermate controls. More striking was that in dnSNARE mice the increase in sleep pressure in response to sleep deprivation was significantly attenuated compared to littermate controls. In addition to these changes in EEG, we found that dnSNARE expression prevented the compensatory increase in sleep time that normally follows sleep deprivation (Figure 6) (Halassa et al. 2009).

One concern with any transgenic or knockout study is whether the response observed is physiological and whether it represents the primary response to the genetic manipulation. We took these concerns into consideration and performed several controls: first after observing this sleep phenotype we added doxycycline back to the food, which will turn off transgene expression, and were able to reverse the phenotype. This controls for potential irreversible effects associated for example with neurodegeneration, as well as for transgene insertional effects. Additionally, we were able to phenocopy the dnSNARE mice by infusion of an A1R antagonist intracerebroventricularly. (Halassa et al. 2009)

Based on these collective studies we conclude that astrocytes play an important modulatory role in the control of sleep homeostasis. This is likely due to the ability of the astrocyte to modulate extracellular adenosine and adenosine's consequent actions on neuronal A1R. In support of this possibility is the observation that conditional knockout of forebrain A1R causes a similar phenotype (Bjorness et al. 2009). Manipulation of the expression of adenosine kinase, the enzyme responsible for the phosphorylation of adenosine, and highly expressed in astrocytes, reduces extracellular adenosine and also causes a sleep homeostasis phenotype (Palchykova et al. 2010).

Studies of humans have shown that there are numerous mutations in the adenosine deaminase gene. Many of these lead to severe immunodeficiency. However, a frequent asymptomatic allele in heterozygous carriers is a G-to-A substitution at nucleotide 22 which leads to a substitution of asparagine for aspartic acid. This substitution leads to lower enzymatic activity of adenosine deaminase. Since adenosine deaminase is one of the enzymes responsible for inactivating adenosine, a predicted consequence is that the level of adenosine will be increased, together with the attendant enhancement of sleep. Sleep characteristics of healthy volunteer subjects were analyzed. Studies consisted of two phases. Initially, the subjects provided self-reporting of sleep history. Subjects with the G-to-A substitution in the adenosine deaminase gene report fewer awakenings at night. Subsequently polysomnographic EEG/EMG recordings were performed to quantify sleep characteristics. Subjects with the G-to-A substitution, which is predicted to lead to enhanced adenosine levels showed longer more intense slow wave sleep. These patients have increased sleep pressure and report feeling sleepier. (Bachmann et al. 2012)

Astrocyte-derived adenosine selective acts on A1R and not on A2AR to modulate sleep

There are at least four different adenosine receptors: A1R, A2AR, A2BR and A3R (Burnstock et al. 2011). Most of the actions of adenosine in the brain are currently attributed to A1R and A2AR, so we asked if astrocyte derived adenosine acts specifically on one or both of these receptors. We provided an A1R or A2AR antagonist during the transition from the dark to light phase and determined the resulting enhancement of wakefulness. It is known that A1R selective antagonists as well as A2AR selective antagonists increase wakefulness. This experiment is akin to our drinking a cup of espresso immediately prior to going to bed – most of us know the resulting difficulties to sleep thereafter. We performed parallel studies in wild-type and dnSNARE mice. To our surprise we found the dnSNARE expression selectively prevented the ability of A1R but not of A2AR antagonists to promote wakefulness. (Halassa et al. 2009) There are many interpretations for this initially surprising result. It is known that the distribution of A1R and A2AR is distinct: A1R are expressed in forebrain and A2A more prominently in striatum (Burnstock et al. 2011). However, we did not find a regional expression of dnSNARE matching this receptor heterogeneity. Therefore it is unlikely that astrocyte-derived adenosine is not simply reduced only in A1R expressing brain regions.

A recent study has raised the possibility that AMP could be a signal to activate A1R in addition to adenosine. In this study the authors tested the ability of AMP and non-hydrolysable analogs of AMP to activate A1R and A2BR. While adenosine activated both receptors, addition of AMP selectively activated A1R. One potential confound with this study is the contamination of AMP with adenosine given that AMP could be hydrolyzed to adenosine which is the natural agonist. Thus trace contamination, or hydrolysis could lead to selective A1R activation based on a threshold effect mediated by the differential affinity of the receptors for adenosine. However, an elegant aspect of this study, which provided strong support for AMP being an agonist for the A1R, was the use of a single amino acid substitution that prevented AMP from activating the A1R without altering the EC₅₀ for adenosine. (Rittiner et al. 2012) Together these results strongly suggest that AMP can selectively act on A1R and not A2B receptors and raises the potential that A2A receptors are similarly insensitive to AMP. If this were indeed the case it may explain why astrocytic dnSNARE expression prevents the ability of A1R antagonist, but not A2A antagonist from promoting wakefulness. Perhaps there is sufficient AMP to activate the A1R.

Recently a study has argued that astrocytes do not activate A1R signaling (Lovatt et al. 2012). The basis of their argument was that there is insufficient CD73 activity to give rise to adenosine. However, their argument is flawed given the recent indication that A1R is sensitive to AMP (Rittiner et al. 2012). Moreover, there are several enzymes that can hydrolyze AMP to adenosine (Street et al. 2011) and given the high affinity (~100nM) of the A1R (Fredholm et al. 2011), incomplete pharmacological inhibition or single CD73^{-/-} are not sufficient proof for such an argument. Finally, molecular genetic manipulations of adenosine kinase in mice (Palchykova et al. 2010), polymorphisms in adenosine deaminase in humans (Bachmann et al. 2012), and CD73^{-/-} mice (Zielinski et al. 2012) all exhibit sleep phenotypes providing strong support to the idea that adenosine is a natural signal in the brain that controls sleep.

Sleep deprivation impairs memory consolidation

It is well known that sleep can be important for the consolidation of certain forms of memory and that sleep deprivation can prevent the consolidation of long term memories (Goel et al. 2009; Kalia 2006; Walker and Stickgold 2006). How does sleep deprivation

prevent memory formation? Given the importance of the astrocyte in homeostatic response to sleep deprivation we asked whether this glial cell type contributes to related phenotypes – memory consolidation and late-long term potentiation (L-LTP). To achieve this goal we performed a period of training for either novel object recognition or spatial object recognition tasks (at ZT=0) and then tested the animals for recognition memory 24 hours later. When mice were left undisturbed following training and allowed to sleep *ad libitum*, recognition memory was intact 24 hours later. However, when mice were subjected to sleep deprivation for 6 hours following training, recognition memory was impaired. Expression of dnSNARE in astrocytes dramatically affected this phenotype. When mice were left undisturbed following training and allowed to sleep, recognition memory exhibited 24h after the training period was equivalent to wild type animals. In dnSNARE mice 6 hours of sleep deprivation did not prevent the consolidation of recognition memory. (Halassa et al. 2009)

We then asked whether A1R is required for the sleep deprivation induced impairment of memory consolidation. Initially we infused the A1R antagonist CPT intracerebroventricularly and found that it phenocopied dnSNARE expression. One concern with this approach is that global application of CPT will affect sleep homeostasis as well as memory related processes. Therefore we locally infused CPT only into the hippocampus to allow sleep homeostasis to be unaffected. Hippocampal CPT infusion permitted spatial memory consolidation even in the face of sleep deprivation, suggesting that during sleep deprivation adenosine acts specifically in the hippocampus to impair memory consolidation. (Halassa et al. 2009)

Hippocampal L-LTP requires protein synthesis and is considered to be a cellular correlate of memory consolidation. We therefore asked whether sleep deprivation impacts L-LTP. Hippocampal slices were cut from wild type and dnSNARE mice that had either been left undisturbed or were sleep deprived. Slices obtained from sleep deprived mice failed to exhibit L-LTP whereas normal L-LTP was observed in undisturbed mice. Similar to our behavioral data astrocytic dnSNARE expression or CPT application both rescued L-LTP following a period of sleep deprivation. (Florian et al. 2011)

How could astrocytes and adenosine regulate L-LTP and memory consolidation? While further studies are required to examine this question, it is known that a postsynaptic elevation of cyclic AMP (cAMP) is required for L-LTP and memory consolidation (Vecsey et al. 2009). Since A1R can couple to G_i which reduces cAMP, perhaps a glial source of adenosine during sleep deprivation prevents the neuronal elevation of cAMP that is required for memory consolidation.

The astrocytic control of A1R regulates the trafficking of NMDA receptors

The ability of the astrocyte to impair memory consolidation during sleep deprivation although exciting does not illuminate the adaptive value provided by such a glial pathway. At first sight it would seem that all that the astrocyte does is impair our ability to become sleep deprived and consolidate memories. Are there other effects that are beneficial? To illuminate how the astrocyte might provide such an advantage to the brain requires the understanding that elevated adenosine can regulate neuronal N-methyl-D-aspartate receptor (NMDAR) trafficking.

In our previous studies we noted an internal inconsistency between two sets of information that we could not account for based solely on the astrocytic control of A1R activation. Astrocytic dnSNARE expression reduced the power of slow oscillations and SWA. However, acute administration of A1R antagonist increased the power. This suggests either that dnSNARE is doing something in parallel to the regulation of A1R or that there is a delayed consequence of reducing A1R on an independent system.

Because of the known importance of NMDA receptors for slow oscillations and because astrocytes can release D-serine a co-agonist for the NMDA receptor, we asked whether the magnitude of synaptic NMDA receptors may be reduced in dnSNARE mice. Using different approaches we indeed found a selective decrease in synaptic NMDA receptors without a corresponding alteration in alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors. We monitored the AMPA/NMDA ratio and found that it increased in dnSNARE mice. Using average miniature excitatory post-synaptic currents (EPSCs) we were able to dissect the relative contribution of AMPA and NMDA receptors to the mEPSC and found that astrocytic dnSNARE expression selectively reduced the synaptic NMDA component. Finally, using surface biotinylation approaches we found that the surface expression of the NR2A and NR2B subunits of the NMDA receptor were reduced in dnSNARE mice. In agreement with the biochemistry, pharmacological studies indicated that this effect could not be accounted for solely by a reduction in co-agonist availability, but rather there was a change in the distribution and trafficking of NMDA receptors. (Deng et al. 2011)

We next asked whether this change in NMDA receptor trafficking was downstream of the change in A1R activation. Our results allowed us to conclude that the activation of neuronal A1R (presumably postsynaptically localized) causes an activation of the src family of tyrosine kinases (src kinase), which in turn regulates the phosphorylation of critical residues on the NMDA NR2 subunits. Src kinase dependent tyrosine phosphorylation of NR2 subunits can modulate both channel gating and the rate of subunit endocytosis (Salter and Pitcher 2012). Pharmacological inhibition of A1R caused reduced activation of src kinase, reduced tyrosine phosphorylation of NMDA receptor subunits, reduced surface expression and reduced NMDAR synaptic currents. In contrast, in dnSNARE mice, where NMDAR currents are already reduced, addition of an A1R agonist led to a src kinase dependent increase in tyrosine phosphorylation of the NMDAR subunits and greater synaptic NMDAR currents (Figure 7). (Deng et al. 2011)

Returning to the initial dilemma about our data - we also found that the change in NMDAR currents was delayed (~1h) from the time of pharmacological activation/inhibition of A1R (Deng et al. 2011). This temporal distinction allows us to explain the apparent experimental disconnect. Acute inhibition of A1R will lead to a local dis-inhibition of the circuits and thus an increase in power of slow oscillations. However, more prolonged inhibition of the A1R will lead to a delayed reduction in NMDAR current potentially explaining why dnSNARE expression led to a reduced power of slow oscillations and SWA. In agreement with this possibility when we acutely antagonized both A1R and NMDAR we found a decline in power of slow oscillations (Fellin 2009).

Conclusions and future directions

There is considerable evidence that astrocytes release a variety of gliotransmitters including glutamate, D-serine and ATP (Halassa and Haydon 2010). Much of the work has focused on how these transmitters affect synaptic transmission on a second to second time scale. At one extreme it is now recognized that astrocytes play an important role in spike-timing dependent plasticity with integrative roles on the time course of tens of milliseconds (Min and Nevian 2012). At the other extreme we now know that astrocytes play modulatory roles on the time course of hours and that by controlling the extracellular levels of purines, the astrocyte modulates sleep homeostasis and memory consolidation (Florian et al. 2011; Halassa et al. 2009). Some key challenges that lay ahead are to understand how the same cell type can integrate information over a range of time scales from milliseconds to hours – a temporal range of more than 5 orders of magnitude. Though we have focused on purines in this review, it will be intriguing to determine if there are similar wakefulness dependent

changes in the release of glutamate and D-serine and whether differential mechanisms exist to control the release of each gliotransmitter. Much of our attention has focused on Ca^{2+} , presumably because we can measure it. Recently, however, evidence has been presented to demonstrate that diacylglycerol can stimulate ATP release from cultured astrocytes (Mungenast 2011). Does this allow selective control of ATP release over other gliotransmitters? In addition to stimuli that can cause the release of ATP it must not be forgotten that changes in the expression or post-translational modifications of either the ENTs responsible for uptake of adenosine, or of adenosine kinase, which phosphorylates adenosine, would lead to altered extracellular adenosine.

Concerning sleep homeostasis and learning and memory there is now clear evidence provided by numerous laboratories about the importance of the regulation of adenosine in sleep homeostasis (Basheer et al. 2000; Bjorness and Greene 2009; Bjorness et al. 2009; Florian et al. 2011; Halassa et al. 2009; Palchykova et al. 2010; Schmitt et al. 2012; Stenberg et al. 2003; Thakkar et al. 2003). This extends to humans where polymorphisms in adenosine deaminase affect sleep (Bachmann et al. 2012; Retey et al. 2005). Beyond the control of adenosine, there is also converging evidence from several labs showing that the astrocyte plays an important role in this process.

Sleep disorders are co-morbid with numerous disorders of brain function: depression, schizophrenia, Parkinsons disease, Alzheimer's disease, alcoholism and epilepsy to name a few (Bhatt et al. 2005). We do not fully understand how much the sleep disorder contributes to the dysfunction as opposed to the pathology of the disorder causing a co-morbid sleep disorder. Since the astrocyte is pivotal in regulating sleep homeostasis it will be intriguing to examine whether the astrocyte could be a target to help ameliorate the disorder of the brain.

In closing, the work of the past twenty years has provided an incredible illumination of how astrocytes can influence brain function. Perhaps in the next decade this information can be used to identify astrocytic targets for treatments of certain brain dysfunctions.

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REFERENCES

- Bachmann V, Klaus F, Bodenmann S, Schafer N, Brugger P, Huber S, Berger W, Landolt HP. Functional ADA polymorphism increases sleep depth and reduces vigilant attention in humans. *Cereb Cortex*. 2012; 22(4):962–970. [PubMed: 21734253]
- Banks S, Dinges DF. Behavioral and physiological consequences of sleep restriction. *J Clin Sleep Med*. 2007; 3(5):519–528. [PubMed: 17803017]
- Basheer R, Porkka-Heiskanen T, Strecker RE, Thakkar MM, McCarley RW. Adenosine as a biological signal mediating sleepiness following prolonged wakefulness. *Biol Signals Recept*. 2000; 9(6):319–327. [PubMed: 11025338]
- Benington JH, Kodali SK, Heller HC. Stimulation of A1 adenosine receptors mimics the electroencephalographic effects of sleep deprivation. *Brain Res*. 1995; 692(1–2):79–85. [PubMed: 8548323]
- Bhatt MH, Podder N, Chokroverty S. Sleep and neurodegenerative diseases. *Semin Neurol*. 2005; 25(1):39–51. [PubMed: 15798936]
- Bjorness TE, Greene RW. Adenosine and sleep. *Curr Neuropharmacol*. 2009; 7(3):238–245. [PubMed: 20190965]

- Bjorness TE, Kelly CL, Gao T, Poffenberger V, Greene RW. Control and function of the homeostatic sleep response by adenosine A1 receptors. *J Neurosci*. 2009; 29(5):1267–1276. [PubMed: 19193874]
- Brown RE, Basheer R, McKenna JT, Strecker RE, McCarley RW. Control of sleep and wakefulness. *Physiol Rev*. 2012; 92(3):1087–1187. [PubMed: 22811426]
- Burnstock G, Fredholm BB, Verkhratsky A. Adenosine and ATP receptors in the brain. *Curr Top Med Chem*. 2011; 11(8):973–1011. [PubMed: 21401499]
- Chen YA, Scheller RH. SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol*. 2001; 2(2):98–106. [PubMed: 11252968]
- Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science*. 1990; 247(4941):470–473. [PubMed: 1967852]
- Deng Q, Terunuma M, Fellin T, Moss SJ, Haydon PG. Astrocytic activation of A1 receptors regulates the surface expression of NMDA receptors through a Src kinase dependent pathway. *Glia*. 2011; 59(7):1084–1093. [PubMed: 21544869]
- Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci*. 2001; 24:31–55. [PubMed: 11283304]
- Fellin T. Communication between neurons and astrocytes: relevance to the modulation of synaptic and network activity. *J Neurochem*. 2009; 108(3):533–544. [PubMed: 19187090]
- Fellin T, Halassa MM, Terunuma M, Succol F, Takano H, Frank M, Moss SJ, Haydon PG. Endogenous nonneuronal modulators of synaptic transmission control cortical slow oscillations in vivo. *Proc Natl Acad Sci U S A*. 2009; 106(35):15037–15042. [PubMed: 19706442]
- Florian C, Vecsey CG, Halassa MM, Haydon PG, Abel T. Astrocyte-derived adenosine and A1 receptor activity contribute to sleep loss-induced deficits in hippocampal synaptic plasticity and memory in mice. *J Neurosci*. 2011; 31(19):6956–6962. [PubMed: 21562257]
- Foley JC, McIver SR, Haydon PG. Gliotransmission modulates baseline mechanical nociception. *Mol Pain*. 2011; 7:93. [PubMed: 22136202]
- Franken P, Dijk DJ. Circadian clock genes and sleep homeostasis. *Eur J Neurosci*. 2009; 29(9):1820–1829. [PubMed: 19473235]
- Fredholm BB, AP IJ, Jacobson KA, Linden J, Muller CE. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. *Pharmacol Rev*. 2011; 63(1):1–34. [PubMed: 21303899]
- Fuller PM, Gooley JJ, Saper CB. Neurobiology of the sleep-wake cycle: sleep architecture, circadian regulation, and regulatory feedback. *J Biol Rhythms*. 2006; 21(6):482–493. [PubMed: 17107938]
- Goel N, Rao H, Durmer JS, Dinges DF. Neurocognitive consequences of sleep deprivation. *Semin Neurol*. 2009; 29(4):320–339. [PubMed: 19742409]
- Gong QJ, Li YY, Xin WJ, Wei XH, Cui Y, Wang J, Liu Y, Liu CC, Li YY, Liu XG. Differential effects of adenosine A1 receptor on pain-related behavior in normal and nerve-injured rats. *Brain Res*. 2010; 1361:23–30. [PubMed: 20850420]
- Halassa MM, Florian C, Fellin T, Munoz JR, Lee SY, Abel T, Haydon PG, Frank MG. Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. *Neuron*. 2009; 61(2):213–219. [PubMed: 19186164]
- Halassa MM, Haydon PG. Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. *Annu Rev Physiol*. 2010; 72:335–355. [PubMed: 20148679]
- Kalia M. Neurobiology of sleep. *Metabolism*. 2006; 55(10 Suppl 2):S2–S6. [PubMed: 16979421]
- Klyuch BP, Dale N, Wall MJ. Deletion of ecto-5'-nucleotidase (CD73) reveals direct action potential-dependent adenosine release. *J Neurosci*. 2012; 32(11):3842–3847. [PubMed: 22423104]
- Lovatt D, Xu Q, Liu W, Takano T, Smith NA, Schnermann J, Tieu K, Nedergaard M. Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity. *Proc Natl Acad Sci U S A*. 2012; 109(16):6265–6270. [PubMed: 22421436]
- Min R, Nevian T. Astrocyte signaling controls spike timing-dependent depression at neocortical synapses. *Nat Neurosci*. 2012; 15(5):746–753. [PubMed: 22446881]
- Mungenast AE. Diacylglycerol signaling underlies astrocytic ATP release. *Neural Plast*. 2011; 2011:537659. [PubMed: 21826278]

- Palchykova S, Winsky-Sommerer R, Shen HY, Boison D, Gerling A, Tobler I. Manipulation of adenosine kinase affects sleep regulation in mice. *J Neurosci*. 2010; 30(39):13157–13165. [PubMed: 20881134]
- Parkinson FE, Damaraju VL, Graham K, Yao SY, Baldwin SA, Cass CE, Young JD. Molecular biology of nucleoside transporters and their distributions and functions in the brain. *Curr Top Med Chem*. 2011; 11(8):948–972. [PubMed: 21401500]
- Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG. Glutamate-mediated astrocyte-neuron signalling. *Nature*. 1994; 369(6483):744–747. [PubMed: 7911978]
- Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG. Astrocytic purinergic signaling coordinates synaptic networks. *Science*. 2005; 310(5745):113–116. [PubMed: 16210541]
- Porkka-Heiskanen T, Strecker RE, McCarley RW. Brain site-specificity of extracellular adenosine concentration changes during sleep deprivation and spontaneous sleep: an in vivo microdialysis study. *Neuroscience*. 2000; 99(3):507–517. [PubMed: 11029542]
- Porkka-Heiskanen T, Strecker RE, Thakkar M, Bjorkum AA, Greene RW, McCarley RW. Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. *Science*. 1997; 276(5316):1265–1268. [PubMed: 9157887]
- Portas CM, Thakkar M, Rainnie DG, Greene RW, McCarley RW. Role of adenosine in behavioral state modulation: a microdialysis study in the freely moving cat. *Neuroscience*. 1997; 79(1):225–235. [PubMed: 9178878]
- Raino J, Khvotchev M, Liu P, Darios F, Li YC, Ramirez DM, Adachi M, Lemieux P, Toth K, Davletov B, et al. VAMP4 directs synaptic vesicles to a pool that selectively maintains asynchronous neurotransmission. *Nat Neurosci*. 2012; 15(5):738–745. [PubMed: 22406549]
- Reitey JV, Adam M, Honegger E, Khatami R, Luhmann UF, Jung HH, Berger W, Landolt HP. A functional genetic variation of adenosine deaminase affects the duration and intensity of deep sleep in humans. *Proc Natl Acad Sci U S A*. 2005; 102(43):15676–15681. [PubMed: 16221767]
- Rittiner JE, Korboukh I, Hull-Ryde EA, Jin J, Janzen WP, Frye SV, Zylka MJ. AMP is an adenosine A1 receptor agonist. *J Biol Chem*. 2012; 287(8):5301–5309. [PubMed: 22215671]
- Salter MW, Pitcher GM. Dysregulated Src upregulation of NMDA receptor activity: a common link in chronic pain and schizophrenia. *FEBS J*. 2012; 279(1):2–11. [PubMed: 21985289]
- Schmitt LI, Sims RE, Dale N, Haydon PG. Wakefulness affects synaptic and network activity by increasing extracellular astrocyte-derived adenosine. *J Neurosci*. 2012; 32(13):4417–4425. [PubMed: 22457491]
- Sinton CM, McCarley RW. Neurophysiological mechanisms of sleep and wakefulness: a question of balance. *Semin Neurol*. 2004; 24(3):211–223. [PubMed: 15449215]
- Stenberg D, Litonius E, Halldner L, Johansson B, Fredholm BB, Porkka-Heiskanen T. Sleep and its homeostatic regulation in mice lacking the adenosine A1 receptor. *J Sleep Res*. 2003; 12(4):283–290. [PubMed: 14633239]
- Steriade M. Grouping of brain rhythms in corticothalamic systems. *Neuroscience*. 2006; 137(4):1087–1106. [PubMed: 16343791]
- Steriade M, Contreras D, Curro Dossi R, Nunez A. The slow (< 1 Hz) oscillation in reticular thalamic and thalamocortical neurons: scenario of sleep rhythm generation in interacting thalamic and neocortical networks. *J Neurosci*. 1993a; 13(8):3284–3299. [PubMed: 8340808]
- Steriade M, Nunez A, Amzica F. Intracellular analysis of relations between the slow (< 1 Hz) neocortical oscillation and other sleep rhythms of the electroencephalogram. *J Neurosci*. 1993b; 13(8):3266–3283. [PubMed: 8340807]
- Street SE, Walsh PL, Sowa NA, Taylor-Blake B, Guillot TS, Vihko P, Wightman RM, Zylka MJ. PAP and NT5E inhibit nociceptive neurotransmission by rapidly hydrolyzing nucleotides to adenosine. *Mol Pain*. 2011; 7:80. [PubMed: 22011440]
- Suh J, Jackson FR. *Drosophila* ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron*. 2007; 55(3):435–447. [PubMed: 17678856]
- Thakkar MM, Winston S, McCarley RW. A1 receptor and adenosinergic homeostatic regulation of sleep-wakefulness: effects of antisense to the A1 receptor in the cholinergic basal forebrain. *J Neurosci*. 2003; 23(10):4278–4287. [PubMed: 12764116]

- Ursin R, Bjorvatn B. Sleep-wake and eeg effects following adenosine a1 agonism and antagonism: similarities and interactions with sleep-wake and eeg effects following a serotonin reuptake inhibitor in rats. *Sleep Res Online*. 1998; 1(3):119–127. [PubMed: 11382868]
- Vecsey CG, Baillie GS, Jaganath D, Havekes R, Daniels A, Wimmer M, Huang T, Brown KM, Li XY, Descalzi G, et al. Sleep deprivation impairs cAMP signalling in the hippocampus. *Nature*. 2009; 461(7267):1122–1125. [PubMed: 19847264]
- Volkow ND, Tomasi D, Wang GJ, Telang F, Fowler JS, Logan J, Benveniste H, Kim R, Thanos PK, Ferre S. Evidence That Sleep Deprivation Downregulates Dopamine D2R in Ventral Striatum in the Human Brain. *J Neurosci*. 2012; 32(19):6711–6717. [PubMed: 22573693]
- Walker MP, Stickgold R. Sleep, memory, and plasticity. *Annu Rev Psychol*. 2006; 57:139–166. [PubMed: 16318592]
- Zielinski MR, Taishi P, Clinton JM, Krueger JM. 5'-Ectonucleotidase-knockout mice lack non-REM sleep responses to sleep deprivation. *Eur J Neurosci*. 2012

Figure 1. Adenosine regulation in the nervous system

ATP is released into the extracellular space via exocytosis or through hemichannels (VAAC, Cx43). Once in the extracellular space the ATP is hydrolyzed by ectonucleotidases to ADP, AMP, and ultimately adenosine. Adenosine can act on adenosine receptors and can also be taken into cells through equilibrative nucleoside transporters (ENTs). Intracellularly, adenosine can be further metabolized to inosine, hypoxanthine, xanthine and uric acid. Adenosine kinase phosphorylates adenosine to make AMP.

Figure 2. dnSNARE expression in the brain

A. The tet-O system was used to attenuate gliotransmission *in vivo*. The GFAP promoter drives the expression of dnSNARE and the EGFP reporter in astrocytes. The addition of doxycycline (Dox) suppresses the expression of both transgenes. *tTA*: tet-off tetracycline transactivator. *tetO*: tet operator B. The EGFP reporter is expressed solely in astrocytes and does not colocalize with NeuN in the brain.

Figure 3. Wakefulness-dependent modulation of A1R mediated presynaptic inhibition requires an astrocytic SNARE-sensitive pathway

A. Four hours of sleep deprivation significantly reduced field excitatory postsynaptic potential (fEPSP) slope in wild type (WT) mice ($p < 0.005$, two-way repeated-measures ANOVA, *post hoc* SNK t-test, $*p < 0.05$, $n = 6$). B. fEPSP slopes in slices from dnSNARE mice were not sensitive to sleep deprivation. Insets, fEPSP traces for increasing intensities of stimulation. Scale bar, 0.4mV, 5ms. C. Half-maximal fEPSP slope was significantly higher in dnSNARE slices taken following normal wakefulness (ZT0) and following 4h of sleep deprivation but not following 4h of undisturbed sleep ($p < 0.005$, two-way ANOVA, *post hoc* SNK t-test; $*p < 0.05$, $n = 6$). D. Tonic inhibition by adenosine A1R was significantly increased by sleep deprivation in slices taken from WT but not dnSNARE mice (Mann-Whitney U test; Bonferroni's correction $*p < 0.05$, $n = 6$). Adapted from Schmitt et al 2012.

Figure 4. The effects of the A1R antagonist CPT on cortical slow oscillations are potentiated by sleep loss through an astrocytic SNARE sensitive pathway

Local field potential (LFP) recordings were made in the somatosensory cortex of either WT or dnSNARE mice. Application of CPT produced an increase in power of slow oscillations (0.4–1Hz) in WT but not dnSNARE mice, and this increase was potentiated by sleep deprivation (FFT of 27s spectral segments, normalized to baseline). Inset, Traces show typical LFP recordings during baseline or following CPT (30s recording at time =22min). Scale bar, 2.5mV, 5s. *Adapted from Schmitt et al 2012.*

Figure 5. Typical EEG parameters measured in mice

A. Examples of EEG/EMG signals during Wake, NREM and REM sleep. B. Representative example of power spectral measurements for Wake, NREM and REM sleep. C. Slow wave activity is enhanced in wild-type animals after 6hrs of sleep deprivation.

Figure 6. Gliotransmission is required for sleep pressure accumulation

A. Low frequency slow wave activity during NREM sleep across the light phase is decreased in dnSNARE animals (n=7) compared to WT littermates (n=8). ($p < 0.001$, ANOVA, $F = 21.247$, post-hoc test, $*p < 0.05$). Following sleep deprivation (SD), the low frequency slow wave activity is decreased in dnSNARE animals ($p < 0.001$, ANOVA, $F = 7.911$, post-hoc test, $*p < 0.05$). B. SD increases the total sleep time in wild-type (n=9), but not dnSNARE animals (n=8) during an 18hr recovery period compared with a baseline period ($**p < 0.001$). C. The increase in total sleep time after SD over the 18hr of recovery in the dnSNARE mouse is blunted when directly compared with that of wild-type animals ($*p < 0.05$). Adapted from Halassa et al 2009.

Figure 7. A1R activation enhances NMDAR currents via tyrosine phosphorylation of Src Kinase in dnSNARE mice

A. Slices from dnSNARE mice were incubated in CCPA (100nM) for 1h. In the presence of TTX, CCPA significantly increased tyrosine phosphorylation of Src and NR2B, as well as the surface expression of NR2B (n=4 *p<0.05). B. In slices from dnSNARE, NMDA components of mixed mEPSC significantly increased after CCPA incubation and the increase was blocked by PP2 (control: 13 cells from 7 mice, CCPA: 11 cells from 7 mice, CCPA/PP2: 11 cells from 6 mice). Adapted from Deng et al 2011.