

Astrocytic Modulation of Sleep Homeostasis and Cognitive Consequences of Sleep Loss

Michael M. Halassa,^{1,3} Cedrick Florian,² Tommaso Fellin,^{1,4} James R. Munoz,¹ So-Young Lee,^{1,3} Ted Abel,² Philip G. Haydon,^{1,3,5,*} and Marcos G. Frank^{1,5}

¹Department of Neuroscience

²Department of Biology

University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

³Department of Neuroscience, Tufts University Schools of Medicine, Boston, MA 02111, USA

⁴Present address: Italian Institute of Technology, via Morego 30, 16163 Genova, Italy

⁵These authors contributed equally to this work

*Correspondence: philip.haydon@tufts.edu

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SUMMARY

Astrocytes modulate neuronal activity by releasing chemical transmitters via a process termed gliotransmission. The role of this process in the control of behavior is unknown. Since one outcome of SNARE-dependent gliotransmission is the regulation of extracellular adenosine and because adenosine promotes sleep, we genetically inhibited the release of gliotransmitters and asked if astrocytes play an unsuspected role in sleep regulation. Inhibiting gliotransmission attenuated the accumulation of sleep pressure, assessed by measuring the slow wave activity of the EEG during NREM sleep, and prevented cognitive deficits associated with sleep loss. Since the sleep-suppressing effects of the A1 receptor antagonist CPT were prevented following inhibition of gliotransmission and because intracerebroventricular delivery of CPT to wild-type mice mimicked the transgenic phenotype, we conclude that astrocytes modulate the accumulation of sleep pressure and its cognitive consequences through a pathway involving A1 receptors.

INTRODUCTION

Although astrocytes are not electrically excitable, they exhibit Ca^{2+} elevations activated by metabotropic receptors. Natural stimuli such as whisker movement and visual gratings evoke astrocytic Ca^{2+} signals in the barrel (Wang et al., 2006) and visual cortices (Schummers et al., 2008). Synaptic activation of astrocytes leads to Ca^{2+} signals and in turn to the release of a number of chemical transmitters from these glia. This process of gliotransmission modulates synaptic activity (Jourdain et al., 2007; Pascual et al., 2005).

Using molecular genetics we have shown that by releasing ATP, astrocytes regulate extracellular adenosine acting on synaptic A1 receptors. Astrocytes release gliotransmitters via many pathways including exocytosis (Jourdain et al., 2007).

The exocytotic release of chemical transmitters depends on the formation of a SNARE complex between vesicles and the target membrane (Scales et al., 2000). Conditional astrocyte-selective expression of the SNARE domain of the protein synaptobrevin II (dnSNARE) prevents both tonic and activity-dependent extracellular accumulation of adenosine that acts on A1 receptors in situ (Pascual et al., 2005). Adenosine is a transmitter involved in the homeostatic drive for sleep following prolonged wakefulness (Porkka-Heiskanen et al., 1997). However, the cellular source and mechanism of action of adenosine in the context of sleep are not well understood (Heller, 2006). Since astrocyte-dependent adenosine accumulation tonically regulates synaptic transmission and can be enhanced in an activity-dependent manner, this glial pathway of neuronal modulation is a prime candidate for mediating the progressive changes of the homeostatic drive for sleep. We therefore used astrocyte-specific transgenic mice to determine whether astrocytes contribute to this fundamental behavior. Because astrocytes release a number of transmitters, we additionally tested whether observed astrocyte-dependent transgenic phenotypes result from an adenosine deficit by using adenosinergic pharmacological agents in vivo to perform occlusion and mimicry experiments.

RESULTS

Study Design and Rationale

To inhibit the accumulation of astrocyte-derived adenosine, we used the tet-off system (Morozov et al., 2003) to allow conditional expression of a dnSNARE transgene selectively in astrocytes (Pascual et al., 2005). Astrocyte specificity of transgene expression is achieved by using the astrocyte-specific Glial Fibrillary Acidic Protein (GFAP) promoter to drive the expression of tetracycline transactivator (tTA) only in this subset of glia. GFAP.tTA mice were crossed with tetO.dnSNARE mice. The tet-operator (tet.O) drives the expression of dnSNARE and the EGFP reporter. Thus, in bigenic offspring of this mating, transgenes are only expressed in GFAP-positive astrocytes (Figure 1A). Conditional suppression of transgene expression is achieved by including doxycycline (Dox) in the diet. Dox binds to tTA and prevents it from activating the tet.O promoter.

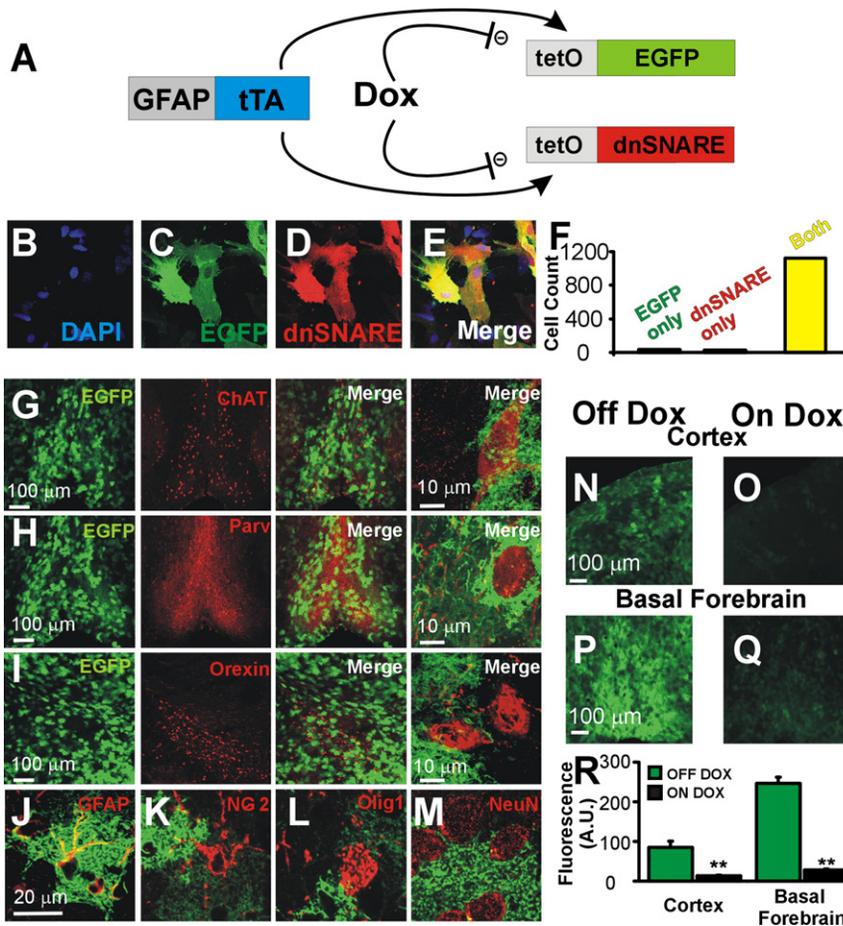


Figure 1. Conditional, Astrocyte-Specific Expression of dnSNARE in Brain Regions Involved in Sleep Regulation

(A) Cartoon depicting GFAP promoter driving the expression of dnSNARE and EGFP (reporter) in astrocytes. Dox suppresses expression of both transgenes. (B–F) EGFP is a reliable marker for dnSNARE, as 97.3% of cultured astrocytes expressing the soluble SNARE domain of synaptobrevin II (dnSNARE) are EGFP-positive. In brain sections, transgene-expressing astrocytes (EGFP; green) are in proximity to cholinergic, choline acetyltransferase (ChAT)-positive neurons (red, [G]) and noncholinergic, parvalbumin- (Parv) positive neurons (red, [H]) in the basal forebrain and surround orexinergic neurons (red, [I]) in the lateral hypothalamus. (J–M) Single optical sections showing that EGFP colocalizes with the astrocytic marker GFAP (J) but not with markers of other glia (NG2, [K]; Olig1, [L]) or neurons (NeuN, [M]). Calibrations for (K)–(M) correspond to (J). Placing dnSNARE mice on a Dox-containing diet suppresses transgene expression in the cortex (N and O) and the basal forebrain (P and Q). (R) Quantification of (N)–(Q) ($n = 3$ animals in each group, $**p < 0.001$, unpaired t test). Differences in EGFP expression between cortex and basal forebrain correlate with relative levels of GFAP expression in these brain regions. Error bars = SEM.

To prevent developmental expression of transgenes, we established and maintained all matings in the presence of Dox until weaning. We confirmed that transgenes are not expressed developmentally by monitoring the expression of EGFP during embryonic development and in early postnatal life (Figure S1 available online).

The EGFP reporter was visually detectable in 97.3% of dnSNARE-transgene-expressing cultured astrocytes ($n = 1182$ cells, 6 coverslips; Figures 1B–1F), establishing it as a reliable marker for dnSNARE transgene expression. Transgene-positive astrocytes were observed in cortex (Halassa et al., 2007), basal forebrain (Figures 1G and 1H), and lateral hypothalamus (Figure 1I), brain regions thought to be important for sleep regulation. EGFP-positive cells colocalize specifically with the astrocytic marker GFAP, but not with neuronal, NG2 glial, or oligodendroglial markers, confirming the astrocytic identity of transgene-expressing cells (Figures 1J–1M). To allow for reversible transgene expression, Dox was removed from the diet at weaning for a period of 2 to 8 weeks (average of 4 weeks without Dox) to ensure that Dox dissipated from the body (Nakashiba et al., 2008), and comparisons were performed between wild-type and dnSNARE mice, assuring that the only variable between these animals was the transgene (Nakashiba et al., 2008). Subsequent addition of Dox suppresses transgene expression within 2 to 3 weeks (Figures 1N–1R).

Gliotransmission Modulates Sleep Pressure Accumulation

The attenuation of gliotransmission did not significantly impact baseline sleep time or architecture. Both dnSNARE and wild-type mice spent similar periods of time in non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep, and wakefulness (Figure S2) and had similar mean 24 hr state-specific EEG spectral profiles (Figure S3). Nevertheless, we found that sleep pressure was significantly reduced when dnSNARE was expressed in astrocytes. Sleep pressure positively correlates with slow wave activity (SWA); the 0.5–4.0 Hz frequency range of the EEG during NREM sleep (Franken et al., 2001). dnSNARE mice exhibited reduced SWA over the course of the light phase (the normal sleep phase) compared with that of their wild-type littermates (Figure 2A).

Net synaptic potentiation during wakefulness is thought to be a cellular correlate of sleep pressure accumulation, and to be related to the role of sleep in synaptic downscaling (Tononi and Cirelli, 2006). Manipulations targeting synaptic potentiation preferentially affect the slower bands of SWA (0.5–1.5 Hz; low-frequency SWA) (Cirelli et al., 2005). We thus examined changes in the NREM EEG spectrum during the light phase at a finer spectral resolution (Figure S4) and found a more pronounced difference between the dnSNARE and wild-type mice in low-frequency SWA (Figures 2B and S4), consistent with an earlier report of

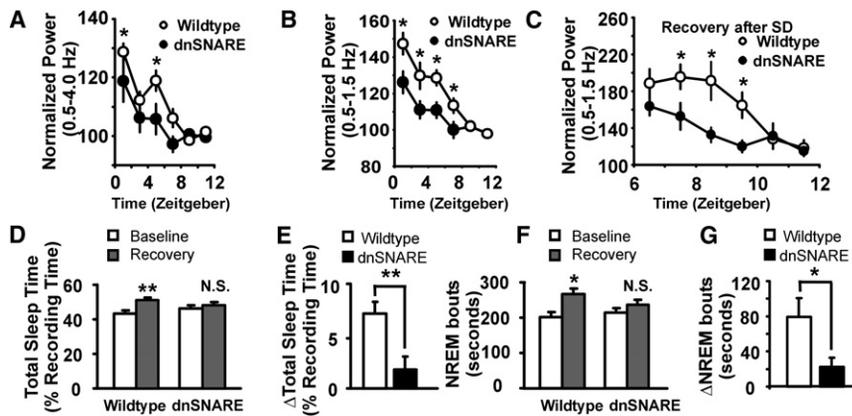


Figure 2. Gliotransmission Is Essential for Sleep Pressure Accumulation

(A) SWA (0.5–4.0 Hz) during NREM sleep across the light phase is decreased in the dnSNARE animals ($n = 7$ animals) compared with that of their wild-type littermates ($n = 8$ animals) (ANOVA, $p < 0.002$, $F = 10.413$, post hoc test, $*p < 0.05$). (B) Low-frequency SWA (0.5–1.5 Hz) is reduced across the light phase in the dnSNARE when compared with wild-type animals (ANOVA, $p < 0.001$, $F = 21.247$, post hoc test, $*p < 0.05$). (C) Following sleep deprivation (SD), low frequency SWA is decreased in the dnSNARE animals (ANOVA, $p < 0.001$, $F = 7.911$, post hoc test, $*p < 0.05$). (D) SD increases TST in wild-type ($n = 9$), but not dnSNARE, animals ($n = 8$) during an 18 hr recovery period compared with a baseline period (** $p < 0.001$). (E) The increase in TST after SD over the 18 hr of recovery in the dnSNARE

mouse is blunted when directly compared with that of wild-type animals (unpaired t test, ** $p < 0.01$; N.S., nonsignificant). (F) SD causes an increase in NREM bout durations in the subsequent 18 hr of recovery in the wild-type animals ($*p < 0.05$). The increase is not statistically significant in the dnSNARE animals. (G) When compared directly, the increase in NREM bout duration is blunted in dnSNARE compared with wild-type animals ($*p < 0.05$). Error bars = SEM.

reduced synaptic potentiation in these transgenic animals (Pascual et al., 2005). This effect was independent of the method of data normalization and was specific to NREM sleep (Figure S5). The difference between wild-type and dnSNARE was limited to changes in SWA, as there were no significant differences in the power of theta and spindles (Figure S6). Following sleep deprivation (Figure S7), wild-type animals exhibited a larger and more prolonged increase in SWA compared with that of dnSNARE mice (Figure S8), which was mainly pronounced in the low-frequency range (Figure 2C).

Increased sleep pressure following sleep deprivation produces a compensatory increase in sleep time (Franken et al., 1991). Previous studies have demonstrated that behavioral recovery following sleep deprivation mainly occurs in the dark phase in C57Bl6/J mice (Kapfhamer et al., 2002). We determined the impact of astrocytic dnSNARE expression on sleep compensation in the 18th hour of recovery. Astrocytic dnSNARE expression reduced the increase in total sleep time (TST) following sleep deprivation (wild-type = $7.53\% \pm 1.13\%$, $n = 9$ mice; dnSNARE = $1.89\% \pm 1.30\%$, $n = 8$ mice; unpaired t test, $p < 0.01$) (Figures 2D, 2E, and S9). Furthermore, astrocytic dnSNARE expression significantly reduced the increase in NREM bout duration following sleep deprivation, another behavioral measure of the homeostatic response (Figures 2F and 2G).

Because astrocytic dnSNARE expression decreases adenosine accumulation, and since adenosine has neuroprotective roles in the nervous system, we wanted to rule out the possibility that the phenotypes observed in transgenic animals were due to inadvertent neurodegeneration that might have occurred when the source of A1 receptor activation was lost. Thus, we added Dox back to the diet for 2 to 3 weeks to inhibit transgene expression (Figures 1N–1R) and asked whether dnSNARE animals can revert to a wild-type phenotype. This is a difficult control to perform since antibiotics have been reported to affect some sleep parameters such as SWA (Moulin-Sallanon et al., 2005). Therefore, we closely examined the effect of Dox on wild-type animals and determined that it had no significant effect on three sleep parameters that we used as markers for reversibility in the

dnSNARE animals. Addition of Dox to transgenic animals restores baseline SWA, baseline low-frequency SWA (Figure S10), and the compensatory increase in sleep time following sleep deprivation to wild-type values (Figures S9 and S10). Reversal of transgenic phenotypes provides compelling evidence that the sleep phenotype we observe in transgene-expressing animals results from the reversible gliotransmission-dependent control of sleep pressure.

Purinergic Gliotransmission Stimulates the A1 Receptor to Modulate Sleep Homeostasis

Though additional gliotransmitters may be affected by our transgenic manipulation, adenosine is a candidate gliotransmitter that may largely explain the sleep homeostasis phenotype observed in these animals. A number of studies have implicated adenosine in the control of sleep homeostasis. For example, individuals with an adenosine deaminase polymorphism, which is expected to result in elevated adenosine, report deeper sleep and exhibit higher SWA (Retey et al., 2005). Thus, a decrease in adenosine in sleep-related brain regions of dnSNARE mice may explain their reduced SWA. We therefore asked whether astrocytic adenosine contributes to the dnSNARE sleep phenotype.

Adenosine levels are decreased at hippocampal synapses by astrocytic dnSNARE expression (Pascual et al., 2005). Because our EEG recordings were sampling synaptic potentials from the cortex, an area of the brain thought to be involved in the homeostatic response to sleep deprivation, we investigated whether A1-receptor-dependent inhibition of intracortical synaptic transmission was reduced in the dnSNARE mice. 8-cyclopentyl-1,3-dimethylxanthine (CPT), an adenosine A1 receptor antagonist, led to an enhancement of synaptic transmission in wild-type slices. However, this enhancement was significantly reduced when slices from the dnSNARE animals were examined (Figures 3A–3C). This was not due to a downregulation of the A1 receptor, as the response to nonsaturating levels of the adenosine receptor agonist 2-chloro-*N*-6-cyclopentyladenosine (CCPA) was similar in slices derived from the two groups of animals (Figures 3D–3F).

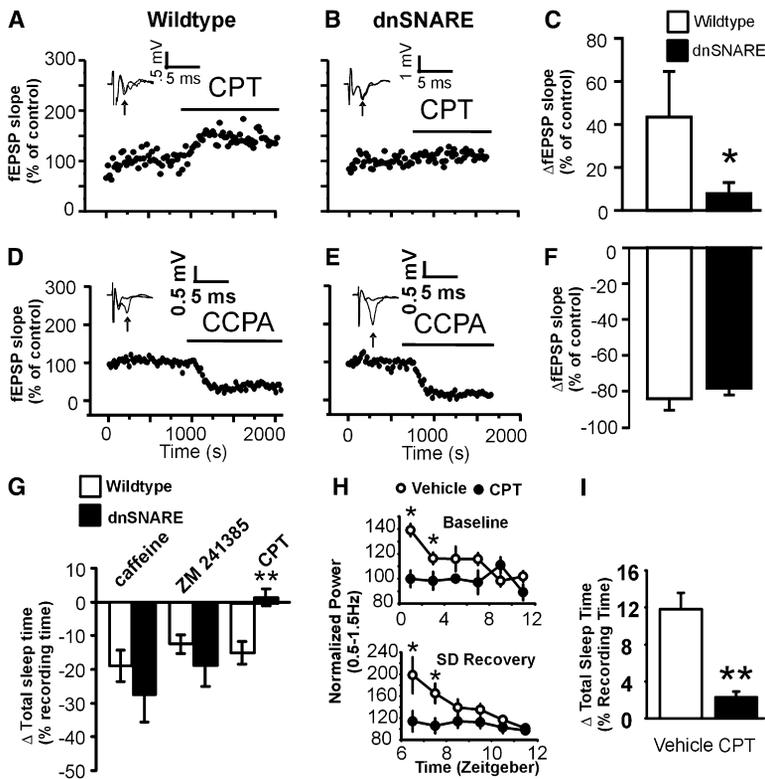


Figure 3. Purinergic Gliotransmission Stimulates the A1 Receptor to Modulate Sleep Homeostasis

(A) The A1 receptor antagonist CPT (100–200 nM) causes an increase in fEPSP slope in slices from wild-type, but not from dnSNARE, mice (B). Inset: average of ≥ 5 fEPSP traces. (C) Average increase in fEPSP slope after CPT application (13 slices, six wild-type mice; 18 slices from six dnSNARE mice; * $p < 0.05$, Mann-Whitney test.) (D–F). The A1 agonist CCPA (500 nM) reduces fEPSP in wild-type mice (12 slices, five animals) and dnSNARE mice (12 slices, four animals; $p = 0.44$, Student's *t* test). (G) Caffeine and ZM 241385 cause equivalent suppression of TST following i.p. injection in wild-type mice (open bars) and dnSNARE mice (closed bars) [caffeine: $t(8) = 0.925$, $p = 0.38$; ZM 241385: $t(8) = 0.925$, $p = 0.38$]. I.p. injection of CPT suppresses sleep only in wild-type animals ($n = 5$ animals per group, unpaired Student's *t* test, ** $p < 0.01$). I.c.v. infusion of CPT reduces low-frequency SWA under baseline conditions (top) (ANOVA, $p < 0.001$, $F = 18$, post hoc test, * $p < 0.05$) and following SD (bottom) (ANOVA, $p < 0.001$, $F = 16$, post hoc test, * $p < 0.05$) (data are normalized to the last 4 hr of the light phase, similar to Figure 2) (H), and sleep compensation following SD was attenuated (unpaired *t* test, ** $p < 0.01$). (I), recapitulating the dnSNARE phenotype. Data in (I) and (H) are from five vehicle-treated animals and six CPT-treated animals. Error bars = SEM.

To determine whether the reduction of synaptic A1 activity translated into sleep-related adenosine deficits *in vivo*, we investigated whether the known sleep-suppressing effects of adenosine receptor antagonists (Huang et al., 2005) were occluded in dnSNARE mice. Astrocytic dnSNARE expression prevented the sleep-suppressing effects of CPT (Figures 3G and S11) but did not affect the sleep-suppressing effects of the A2A antagonist ZM 241385 (Figures 3G and S11), or of caffeine (Figures 3G and S11), which is known to act via A2A receptors in this behavior (Huang et al., 2005).

To determine if the absence of A1 receptor activation contributes to the dnSNARE sleep homeostasis phenotype, we antagonized the A1 receptor in wild-type mice *in vivo* via osmotic minipump intracerebroventricular (i.c.v.) infusion of CPT while recording EEG/EMGs. *In vivo* CPT delivery to wild-type mice recaptured the dnSNARE phenotype. CPT did not affect baseline vigilance states or mean spectral EEG properties (Figure S12). However, the accumulation of sleep pressure as measured by low-frequency SWA under baseline conditions and following sleep deprivation was blunted in CPT-infused animals as compared with vehicle controls (Figure 3H). Further support for the notion that astrocytic adenosine acts through A1 receptors to control sleep homeostasis is provided by the observation that CPT infusion reduced the increase in TST following sleep deprivation (Figure 3I).

Purinergic Gliotransmission Contributes to Memory Impairment Following Sleep Loss

Accumulated sleep pressure caused by prolonged wakefulness can impair cognitive function (Yoo et al., 2007). Because

dnSNARE mice accumulated less sleep pressure, we investigated whether their learning exhibited an altered response to prolonged wakefulness as compared with that of wild-type mice. We chose a memory task that is known to be sensitive to the effects of sleep pressure (Palchykova et al., 2006). Novel object recognition (NOR) is a task that uses the spontaneous preference of rodents for novelty to measure recognition memory (Figure 4A). Both wild-type and dnSNARE mice were able to learn the task equally well when they were trained at the beginning of the light phase and their subsequent sleep was undisturbed (wild-type, $n = 16$, dnSNARE, $n = 16$). However, sleep deprivation following training impaired NOR memory in wild-type animals ($n = 11$; Figure 4B). Recognition memory in the dnSNARE mice, on the other hand, was unimpaired by sleep deprivation ($n = 10$, Figure 4C). This difference is unlikely to be due to sleep-deprivation-induced alteration in the motivation or attention during the test period because mice were left undisturbed for ~18 hr following sleep deprivation, a period sufficient for behavioral and electrophysiological recovery (Figure 2C). Furthermore, this difference cannot be attributed to an alteration in task acquisition because dnSNARE and wild-type mice did not differ in exploration time during task acquisition (Figure S13). dnSNARE mice exhibited neither anxiety-related behaviors nor motor impairment (Figure S14), which could otherwise prohibit performing the NOR task, and did not exhibit a generalized circuit dysfunction as their contextual fear conditioning memory was similar to that of wild-type littermates (Figure S15). Chronic infusion of the A1 receptor antagonist CPT in wild-type mice protected against the memory-degrading effects of sleep deprivation, mimicking the dnSNARE phenotype (Figures 4D and 4E).

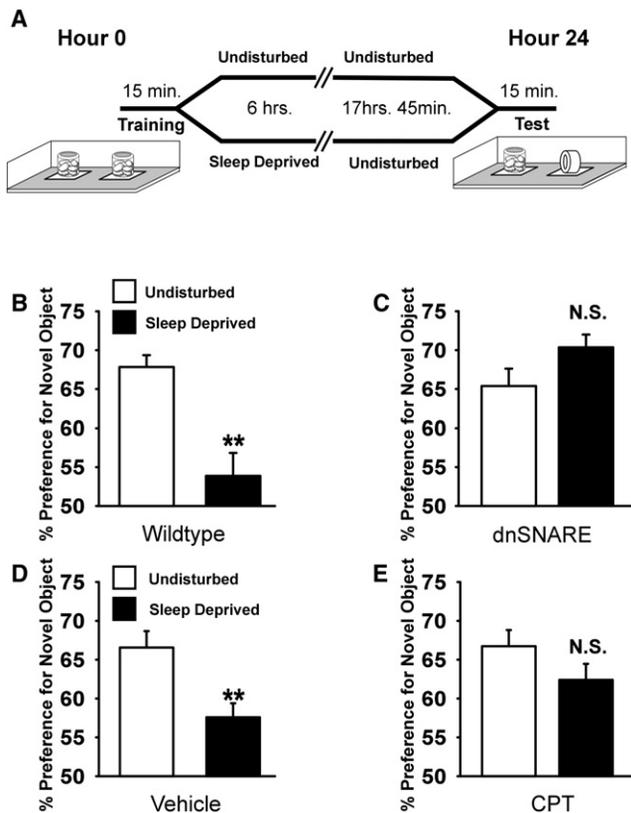


Figure 4. Purinergic Gliotransmission Contributes to Memory Impairment Following Sleep Loss

(A) Novel object recognition (NOR) paradigm: mice are trained to recognize two identical objects and are either left undisturbed or sleep deprived for 6 hr following training. At hour 24, mice are tested for the ability to recognize a novel object replacing one of the familiar objects. (B) SD impairs NOR in wild-type mice [$t(27) = -4.636$; $**p < 0.001$]. (C) dnSNARE mice are unaffected by the effects of SD on NOR memory [$t(25) = 1.56$, $p = 0.132$]. (D and E) i.c.v. delivery of CPT [$t(15) = -1.430$; $p = 0.173$], but not control vehicle [$t(15) = -3.251$; $p < 0.005$], into wild-type mice protects against the sleep-deprivation-induced memory deficit. Error bars = SEM.

To distinguish between the importance of sleep pressure and sleep per se in regulating recognition memory, we performed NOR training at the end of the light phase, when sleep pressure has subsided (Figures 2A and 2B), and then sleep deprived the animals for the subsequent 6 hr during the dark phase. Training wild-type mice at the end of the light phase results in a NOR memory that is insensitive to 6 hours of sleep deprivation following training (Figure S16), indicating that sleep immediately following NOR training is not required for the memory per se. These results lead us to conclude that the acute accumulation of sleep pressure can impair memory consolidation.

DISCUSSION

By genetically inhibiting SNARE-dependent release of transmitters from astrocytes, we demonstrate that gliotransmission contributes to sleep homeostasis. Inhibition of gliotransmission reduces SWA, particularly that in the low-frequency range,

during the course of the light phase (Figures 2A and 2B) and blunts the additional accumulation of low-frequency SWA following sleep deprivation (Figure 2C). Moreover, this reduction in sleep pressure leads to a reversible reduction in TST following sleep deprivation (Figures 2D, 2E, S9, and S10). Because astrocytes release several transmitters, our subsequent pharmacological experiments were critical in determining that the observed transgenic phenotypes involve a decrease in purinergic gliotransmission. In support of previous *in situ* studies, *in vivo* behavioral analyses show that astrocytic expression of dnSNARE prevents the A1 antagonist CPT from exerting its normal actions on wakefulness (Figure 3G). Additionally, i.c.v. administration of CPT mimics the dnSNARE phenotype by preventing the accumulation of sleep pressure (Figure 3H) and changes in TST following sleep deprivation (Figure 3I). Although we cannot discount the possibility that additional gliotransmitters also contribute to the sleep phenotypes we observe, our data indicate that astrocytic adenosine acting through A1 receptors contributes to the modulation of sleep pressure, the regulation of sleep homeostasis (Figures 2 and 3), and cognitive deficits following sleep deprivation (Figure 4). It is important to note that the role of gliotransmission in sleep regulation is likely to be underestimated by our transgenic approach because less than 50% of cortical astrocytes express the dnSNARE transgene (T.F., M.M.H., Miho Terunuma, Francesca Succol, Hajime Takano, Stephen J. Moss, and P.G.H., unpublished data).

There are two potential concerns about the use of the transgenic system we employed in this study. First, widespread transgene expression might lead to generalized dysfunction of the nervous system. However, we have found that astrocytic dnSNARE expression is highly selective for specific sleep phenotypes. Baseline sleep behavior is unperturbed in the dnSNARE animal (Figure S2), and the effect on sleep is limited to that of sleep homeostasis. Brain oscillations under baseline conditions are not affected (Figure S3), arguing strongly against a general circuit deficit in the dnSNARE mouse. The dynamic changes in the NREM sleep spectra are limited to SWA, particularly those in the low-frequency range (Figures S4 and S5). Theta and spindle frequencies are unaffected, and the relative waking and REM sleep spectra are also not affected (Figure S6). Open field behavior (a measure of anxiety), motor learning and memory assessed by the rotarod, contextual fear conditioning, and NOR learning and memory are intact in the dnSNARE animals, arguing against a general circuit dysfunction in these mice (Figures 4, S14, and S15). Second, dnSNARE expression has the potential to nonspecifically interfere with all membrane trafficking pathways in these glial cells. However, we find no perturbation in afferent-induced Ca^{2+} signals (Figure S17), and in a separate study we demonstrate that dnSNARE expression does not affect astrocytic resting membrane potential, input resistance, or current response to afferent activity (T.F., M.M.H., Miho Terunuma, Francesca Succol, Hajime Takano, Stephen J. Moss, and P.G.H., unpublished data) indicating that trafficking of receptors, ion channels, and transporters is not perturbed in these transgenic animals. Thus, in dnSNARE mice astrocytes retain functional integrity when the transgene is expressed.

A number of studies aimed at understanding the role of A1 receptors in the regulation of sleep homeostasis have provided

conflicting results (Porkka-Heiskanen et al., 1997; Stenberg et al., 2003). However, it is worth noting that in cases where manipulations were performed on animals that were allowed to develop normally, the experimental results agree with our current study by demonstrating that the A1 receptor plays a role in the homeostatic response to sleep deprivation. When A1 receptor antisense oligonucleotides were perfused into the basal forebrain, a transient reduction of the A1 receptor protein resulted, and a transient attenuation of the homeostatic response to sleep deprivation was observed at the level of EEG and behavior (Thakkar et al., 2003). More recently, a conditional knockout of the A1 receptor in mice has been shown to have a phenotype of attenuated sleep pressure accumulation assessed by SWA, not dissimilar to the dnSNARE mouse (R. Greene and T. Bjorness, 2008, Purinergic Signaling; abstract). In contrast constitutive A1 receptor knockout mice show a modest sleep phenotype (Stenberg et al., 2003). Because the A1 receptor is involved in a number of physiological processes during development (Kimura et al., 2003), it is conceivable that its permanent absence would lead to compensatory responses. Therefore, in agreement with other studies in which developmental compensation was not a concern, our study demonstrates an essential role for the A1 receptor in mammalian sleep homeostasis. Importantly, we provide the cellular origin for adenosine, and point to its critical role in mediating the cognitive consequences of prolonged wakefulness.

Previous studies have concluded that sleep can play a role in memory consolidation (Stickgold et al., 2001). By demonstrating that NOR memory consolidation does not require sleep immediately following learning, we do not mean to minimize the possible role of sleep in NOR memory consolidation in general. It should be noted that when mice were sleep deprived, they were allowed to sleep afterwards during the 18 hr prior to the test. Thus, in plastic brain processes that require sleep for consolidation (Frank et al., 2001), it will be important to investigate whether gliotransmission plays a role.

Conclusion

Taken together these studies provide the first demonstration that a nonneuronal cell type of the brain, the astrocyte, modulates behavior and provides strong evidence of the important role of A1 receptors in the regulation of sleep homeostasis and the cognitive decline associated with sleep loss. Given that astrocytes express novel receptors (Barres, 2008), these glial cells offer a novel target for the development of therapeutics for sleep and cognitive disorders.

EXPERIMENTAL PROCEDURES

Sleep Recording, Analysis, and Comparisons

EEG/EMG implantation surgery was performed as described before (Frank et al., 2002). EEG/EMG signals were conveyed by a light-weight cable, low- and high-pass filtered at 0.3 and 30 Hz and 10 and 100 Hz, respectively (15 LT Bipolar amplifier system, Astro-Med, West Warwick, RI), amplified, and sampled at 200 Hz.

Stage scoring of computerized EEG/EMG epochs as REM, NREM, and wake was done in 4 s epochs by a trained experimenter blind to genotype using SleepSign software (Kissei Comtec America, INC). Epochs containing movement artifacts were included in the state totals but excluded from subsequent spectral analysis. Following assignment of state scores, data were

analyzed as a percentage of the total recording time (1, 2, 12, or 24 hr bins). Spectral analysis was performed using a Fast Fourier Transform (FFT; Hanning Window). EEG SWA (0.5–4 Hz; or low-frequency SWA: 0.5–1.5 Hz) was used as a quantitative measure of sleep pressure (Cirelli et al., 2005; Franken et al., 2001). NREM SWA for both recording days was normalized to the last 4 hr of the baseline light phase as described (Franken et al., 2001). These latter analyses were restricted to the light phase, because in agreement with a previous mouse study (Franken et al., 2001), there was too little NREM sleep in most mice during the active (dark) phase for accurate SWA assessments.

In Vivo Pharmacology: Acute

Mice prepared for EEG recordings were allowed to recover after surgery and were acclimated as described (Frank et al., 2002). Vehicle or drugs—caffeine (10 mg/kg in 0.9% saline), CPT (20 mg/kg in DMSO), or ZM 241385 (30 mg/kg in DMSO)—were i.p. injected at the beginning of the light phase on separate days. At least 48 hr was allowed between vehicle and drug injection in both groups, and at least 2 weeks was allowed between the injection of CPT and ZM 241385. Vigilance states were scored and quantified in the 3 hr following injection as described previously (Huang et al., 2005). Effects of each drug were expressed as the difference in TST after drug injection versus vehicle injection for each mouse (Figure 3), and the raw data are presented in Figure S11.

In Vivo Pharmacology: Continuous

Micro-osmotic minipumps (model 1002; flow rate 0.25 μ l/day) were filled with either CPT or vehicle (50% DMSO in 0.9% saline), secured by the flow moderator, connected to the brain cannula (The Alzet Brain Infusion Kit 3 [DURECT Corporation, Cupertino, CA]) by flexible tubing, and allowed to equilibrate in 0.9% saline for 24 hr at 37°C. Subsequently one minipump was subcutaneously implanted per animal, and the cannula was stereotaxically placed into the left lateral ventricle at the following coordinates: A/P, -0.8 mm; M/L, -1.0 mm; D/V, -2.0 mm. Animals used in NOR studies were allowed to recover for 1 week before subsequent training and testing.

NOR Task

This task uses differential levels of exploration between familiar and unfamiliar (or novel) objects as a behavioral measure for recognition memory based on the animal's preference for novelty. One week prior to training, mice were individually housed and handled for one minute a day for three days. Mice were habituated to the experimental arena (a rectangular open field [60 cm x 50 cm x 26 cm]) by being permitted to explore it for 15 min in the absence of objects. During training, mice were placed in the experimental arena in the presence of two identical objects and allowed to explore for 15 min. After a retention interval of 24 hr, mice were placed again in the arena in which one of the objects had been replaced by a novel one and were allowed to explore for 15 min. Training and testing was videotaped and analyzed by an experimenter blind to the genotype or drug treatment. Preference for the novel object was expressed as the percent time spent exploring the novel object relative to the total time spent exploring both objects. Statistical analysis was performed using Student's *t* test.

Additional methodology concerning transgenic animal production and maintenance, histological analyses, sleep data analysis, slice electrophysiology, and behavioral tests are described in detail in the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

The supplemental data for this article include Supplemental Experimental Procedures and 17 Figures and can be found at [http://www.neuron.org/supplemental/S0896-6273\(08\)01017-9](http://www.neuron.org/supplemental/S0896-6273(08)01017-9).

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