

## Functional *ADA* Polymorphism Increases Sleep Depth and Reduces Vigilant Attention in Humans

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**Homeostatically regulated slow-wave oscillations in non-rapid eye movement (REM) sleep may reflect synaptic changes across the sleep-wake continuum and the restorative function of sleep. The nonsynonymous c.22G>A polymorphism (rs73598374) of adenosine deaminase (ADA) reduces the conversion of adenosine to inosine and predicts baseline differences in sleep slow-wave oscillations. We hypothesized that this polymorphism affects cognitive functions, and investigated whether it modulates electroencephalogram (EEG), behavioral, subjective, and biochemical responses to sleep deprivation. Attention, learning, memory, and executive functioning were quantified in healthy adults. Right-handed carriers of the variant allele (G/A genotype,  $n = 29$ ) performed worse on the d2 attention task than G/G homozygotes ( $n = 191$ ). To test whether this difference reflects elevated homeostatic sleep pressure, sleep and sleep EEG before and after sleep deprivation were studied in 2 prospectively matched groups of G/A and G/G genotype subjects. Deep sleep and EEG 0.75- to 1.5-Hz oscillations in non-REM sleep were significantly higher in G/A than in G/G genotype. Moreover, attention and vigor were reduced, whereas waking EEG alpha activity (8.5–12 Hz), sleepiness, fatigue, and  $\alpha$ -amylase in saliva were enhanced. These convergent data demonstrate that genetic reduction of ADA activity elevates sleep pressure and plays a key role in sleep and waking quality in humans.**

**Keywords:** adenosine deaminase, cognitive performance, plasticity, slow-wave sleep, synaptic homeostasis

### Introduction

Sleep homeostasis refers to the general principle that elevated sleep need following sleep loss is counteracted by prolonged sleep duration and, especially, by enhanced sleep intensity (Borbély 1980, 1982; Daan et al. 1984). A highly predictable and reliable marker of non-rapid eye movement (REM) sleep intensity is the prevalence of slow-wave oscillations in the electroencephalogram (EEG). Accumulating evidence indicates that EEG slow-wave oscillations in non-REM sleep are causally linked to local synaptic processes, which reflect the duration and quality of prior wakefulness (Rao et al. 2007; Massimini et al. 2009). Homer 1a, brain-derived neurotrophic factor (BDNF), and other molecular markers of brain plasticity were recently suggested to play causal roles in sleep homeostasis (Maret et al. 2007; Faraguna et al. 2008).

Slow (delta)-wave oscillations characterizing deep non-REM sleep consist at the cellular level of rhythmic alternations in the membrane potential of cortical neurons between a hyper-

polarized down-state and a depolarized up-state (Steriade et al. 1993). Given their tight homeostatic regulation (Bersagliere and Achermann 2010), slow waves are thought to be essential for the functions of sleep, which may include synaptic homeostasis, learning, and consolidation of memories (Tononi and Cirelli 2006; Diekelmann and Born 2010). In support of these views, pharmacological and electrical induction of slow-wave oscillations during sleep may potentiate memories and reduce the detrimental consequences of sleep restriction on cognitive performance (Marshall et al. 2006; Walsh et al. 2006, 2010). In contrast, sleep deprivation and experimental slow-wave suppression may impair many cognitive abilities such as attention, perceptual processing and learning, hippocampal activation, and memory encoding (Aeschbach et al. 2008; Landsness et al. 2009; Tomasi et al. 2009; Van Der Werf et al. 2009).

The molecular and neurochemical bases of sleep homeostasis, and the relationships between waking brain activity and sleep slow-wave oscillations are poorly understood. However, adenosine and its receptors play a well-established role in sleep homeostasis (Basheer et al. 2004; Landolt 2008). Moreover, in vitro data show that, for example, the facilitatory action of BDNF on long-term potentiation (LTP) requires endogenous adenosine. More specifically, enhanced LTP by BDNF in hippocampal slices was prevented when adenosine was removed with the adenosine metabolizing enzyme adenosine deaminase (ADA) (Fontinha et al. 2008). Thus, it is possible that BDNF and the adenosine neuromodulator system interact to mediate the consequences of neural activity during wakefulness on the homeostatic regulation of non-REM sleep.

Adenosine kinase (ADK) and ADA contribute to the regulation of extracellular adenosine levels (Fredholm et al. 2005). Not only ADK (Palchykova et al. 2010) but also ADA may be involved in homeostatic sleep-wake regulation. Converging genetic and pharmacological studies in mice and rats indicate an important role for *Ada* in regulating the buildup of non-REM sleep need during prolonged wakefulness, as well as non-REM sleep intensity (Franken et al. 2001; Okada et al. 2003). In humans, a functional G>A transition at nucleotide position 22 of the coding sequence of the *ADA* gene (c.22G>A; rs73598374) is associated with enhanced slow-wave activity in baseline sleep (Rétey et al. 2005). While these data suggest that this genetic variation increases non-REM sleep pressure, the answers to the questions whether it also affects cognitive performance and sleep homeostasis are unknown.

Heterozygous G/A allele carriers of *ADA* show reduced ADA enzymatic activity (Battistuzzi et al. 1981; Riksen et al. 2008)

and may have higher endogenous adenosine levels than homozygous G/G genotype subjects (Hirschhorn et al. 1994). We investigated the functional consequences of the c.22G>A polymorphism of *ADA* in 220 healthy volunteers, and systematically recorded self-reported sleep-wake habits and quantified cognitive abilities including attention, learning, memory, and executive functioning. In a subsequent case-control study in 22 healthy adults, 4-week rest-activity patterns and homeostatic sleep-wake regulation were studied by quantifying neurophysiological, behavioral, subjective, and biochemical responses to a night without sleep. Based on the previous findings, we hypothesized that the G/A genotype subjects would exhibit higher sleep pressure and habitually sleep longer, show more slow-wave sleep and slow-wave activity, and be more strongly affected by sleep deprivation than the G/G homozygotes.

## Materials and Methods

### Subject Recruitment and Genotyping

The Cantonal ethics committee for research on human subjects reviewed and approved the study protocol and all experimental procedures. They were conducted according to the principles of the Declaration of Helsinki. All participants provided written informed consent.

One hundred twenty-seven men and 118 women were genotyped. The prevalence of the G/A genotype was roughly 13% (31/245), whereas 87% had the G/G genotype (214/245) and no individual with A/A genotype was present. These genotype frequencies are in accordance with previous findings in a healthy Italian population (Persico et al. 2000). Self-reported sleep-wake habits, attention, learning, memory, and executive performance were systematically quantified. Because we were also interested in lateralized cognitive functions (see Supplementary Table 1) and right-handedness guarantees a uniform degree of functional lateralization, only the data of right-handed individuals ( $n = 220$ ) were analyzed. Among the G/A genotype subjects, 5 healthy women and 6 healthy men (all Swiss or German) willing to participate in a sleep deprivation study were recruited for the laboratory experiment. They were prospectively matched with 11 G/G homozygotes with regard to sex, age, years of education, habitual alcohol and caffeine intake, body mass index, trait anxiety, diurnal preference, and daytime sleepiness (see Supplementary Table 2). Women were matched with respect to the phase in the menstrual cycle (follicular phase, luteal phase). All participants reported to have no history of neurological and psychiatric disease, not taking any medication, and to be moderate alcohol and caffeine consumers. Two pairs of subjects were matched with respect to cigarette smoking (~10 cigarettes per day); all other participants were nonsmokers.

Genomic DNA was extracted from 3 mL fresh ethylenediaminetetraacetic acid blood with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Genotypes were determined by allele-specific PCR on an MJ Research PTC-225 thermal cycler (MJ Research/Bio-Rad, Reno, NV). HOT FIREPol DNA Polymerase and the following primers were used: forward primer, 5'-gcgacagggccacat-3'; reverse primer, 5'-gaactgcctgsaggagcc-3' (annealing temperature, 67 °C). Sequencing was performed by the Sanger chain-termination method (Sanger et al. 1977) with an ABI PRISM 3100 (16 capillaries) (Applied Biosystems Inc., Foster City, CA) genetic analyzer. All genetic analyses were replicated at least once for independent confirmation of the results.

### Habitual Sleep Duration

Self-reported habitual sleep length on work and leisure days was quantified with the Munich Chronotype Questionnaire (Roenneberg et al. 2003). All participants of the sleep deprivation study wore during 4 weeks a rest-activity monitor (Actiwatch; Cambridge Neurotechnology Ltd, Cambridge, UK) on the wrist of their nondominant arm. Habitual sleep length was estimated from the records of the rest-activity patterns,

together with inspection of sleep-wake diaries. Note that actigraphy-derived "sleep duration" refers to total time in bed, including possible brief intrusions of wakefulness.

### Learning, Memory, Executive Functioning and Attention

The cognitive abilities of all study participants were systematically tested during 2 h.

The validated tests used to assess verbal and nonverbal learning efficiency, working memory, and executive functioning included the Rey Auditory Verbal Learning Test (Strauss et al. 2006), the Rey Verbal Design Learning Test (Foster et al. 2009), the Digit Span Test (Strauss et al. 2006), a Stroop Color-Word interference Task (Stroop 1935; Perret 1974), the Random Number Generation Task (Towse 1998), a go/no-go test (Greenwald et al. 1998), a design fluency test (Regard et al. 1982), and a letter fluency test (Perret 1974).

The d2 attention task (Brickenkamp 1962) is a timed test of selective attention. Fourteen lines of the letters "d" and "p" with 1–4 dashes arranged either individually or in pairs above and below the letters are presented on a sheet of paper. The subjects are allowed 20 s to scan each line and to mark all letters "d" with 2 dashes. Outcome measures include the total number of processed items, a highly reliable measure of processing speed, the sums of errors of omission and commission, and the fluctuation rate across trials (Brickenkamp 1962).

### Laboratory Study to Examine Homeostatic Sleep-Wake Regulation

All participants of the laboratory study were polysomnographically screened in the sleep lab, to exclude poor sleep efficiency and unrecognized preexisting sleep disorders.

The experimental protocol consisted of 4 nights and 2 days in the sleep laboratory. The first and second nights (24:00–08:00) served as adaptation and baseline, respectively. The subsequent 2 days and 1 night, subjects were not allowed to sleep. During the 40-h prolonged wakefulness, they were constantly supervised by members of the research team. Subjects were free to read, study, play games, watch movies, and occasionally take a walk outside the laboratory. They received normal meals 3 times a day, prepared either in the university cafeteria or by themselves in a kitchen adjacent to the laboratory. The last night (24:00–10:00) served as recovery night from sleep deprivation.

### Pre-study Procedures

For 2 weeks prior to the study, volunteers were asked to abstain from all sources of caffeine (coffee, tea, cola drinks, chocolate, and energy drinks), to wear a wrist activity monitor on the nondominant arm, and to keep a sleep-wake diary. For 3 days before and during the study, all subjects had to also abstain from alcohol and to maintain regular 8:16-h sleep-wake cycles. Bedtimes were scheduled from 24:00 to 08:00. When not adhering to the directives, subjects were excluded from the study. The smokers were asked to write down the number of cigarettes per day (not more than ~10 cigarettes per day were allowed). During the study, the 2 pairs of smokers were allowed to smoke at the same predefined times, in order to avoid withdrawal.

### All-Night Polysomnography

Polysomnographic recordings including EEG, bipolar electrooculogram (EOG), mental electromyogram (EMG), and electrocardiogram (ECG) were continuously conducted during all experimental nights, with REMbrandt DataLab (version 8; Embla Systems, Broomfield, CO) and the polygraphic amplifier Artisan (Micromed, Mogliano Veneto, Italy). Analog signals were conditioned by a high-pass filter (EEG: -3 dB at 0.15 Hz; EMG: 10 Hz; ECG: 1 Hz) and an anti-aliasing low-pass filter (-3 dB at 67.2 Hz), digitized, and transmitted via fiber optic cables to a personal computer. Data were sampled with a frequency of 256 Hz. The EEG was recorded from 1 referential (C3A2) and 8 bipolar derivations along the left and right anterior-posterior axes. The data derived from the C3A2 derivation are reported here.

Sleep stages (Rechtschaffen and Kales 1968) were visually scored for 20-s epochs with REMbrandt Analysis Manager (version 8; Embla

Systems). Four-second EEG spectra (fast Fourier transform [FFT] routine, Hanning window, 0.25-Hz resolution) were calculated with MATLAB (MathWorks Inc., Natick, MA), averaged over consecutive 5 epochs, and matched with the sleep scores. Twenty-second epochs with movement- and arousal-related artifacts were visually identified and eliminated. To compute all-night power spectra (0.25-Hz resolution, C3A2 derivation) in non-REM (stages 2–4) and REM sleep, all artifact-free 20-s values were averaged. In the recovery nights, data analysis was restricted to the first 8 h of the 10-h sleep opportunities.

#### **Waking EEG Recordings**

During the 40-h prolonged wakefulness, EEG, EMG, EOG, and ECG signals were recorded in 14 sessions at 3-h intervals in the same way as during the nights, with REMbrandt DataLab and polygraphic amplifier Artisan. The first recording was scheduled 15 min after lights-on from the baseline night. The study participants were instructed to comfortably relax in a chair and to place their chin on an individually adjusted headrest. A 3-min recording period with eyes closed was followed by a 5-min period with eyes open, while subjects fixated a black dot attached to the wall. At least 1 h before each waking EEG recording, subjects had to stay in the laboratory (constant temperature: 19–21 °C, light intensity: <150 lux), and 15 min before each recording, they were left by themselves in their bedroom.

The bioelectric signals were conditioned in the same way as in the polysomnographic recordings. Artifacts in all derivations were visually identified and excluded. The power spectra (FFT, Hanning window) of artifact-free, 50% overlapping, 2-s epochs were computed with MATLAB (MathWorks Inc.). Mean power spectra between 0 and 20 Hz (0.5-Hz resolution, C3A2 derivation) of the 5-min periods with eyes open are reported.

#### **Subjective Sleepiness and Profile of Mood States**

To quantify the evolution of subjective sleepiness, a validated German version of the Stanford Sleepiness Scale (Sturm and Clarenbach 1997) was administered at 3-h intervals throughout extended wakefulness (first assessment at 08:10 after the baseline night). Subjective sleepiness, vigor, depression, and anger were also quantified at 16:45 on days 1 and 2 of prolonged wakefulness with the Profile of Mood States (POMS; McNair et al. 1971).

#### **Psychomotor Vigilance Task**

All participants completed at 3-h intervals during extended wakefulness fourteen 10-min sessions of the psychomotor vigilance task (PVT) (Durmer and Dinges 2005). The task was implemented on a PC, using the software e-Prime (Psychology Software Tools Inc., Pittsburgh, PA). When a digital millisecond counter started to scroll in the center of the computer screen, subjects had to press a button with their right forefinger on a response box connected to the PC. In each session, 100 stimuli were presented (random interstimulus intervals: 2–10 s). Subjects received oral instructions and performed 1 training session on the evening prior to the adaptation night.

#### **Alpha-amylase Activity in Saliva**

Saliva samples (Salivettes, Sarstedt, Nümbrecht, Germany) were collected at 2-h intervals throughout prolonged wakefulness, starting at 08:00 after the baseline night. Salivary  $\alpha$ -amylase activity (sAA), an indirect marker of sympathoadrenal activity (van Stegeren et al. 2006) and a recently proposed biomarker of sleep drive (Seugnet et al. 2006), was determined according to previously reported procedures (Nater et al. 2007).

#### **Data Analyses and Statistics**

Cognitive performance, habitual sleep duration, sleep architecture, sleep and waking EEG, subjective sleepiness, mood states, sustained vigilant attention, and sAA in baseline and during/after sleep deprivation were analyzed in G/A and G/G genotype subjects. All statistical analyses were performed with SAS 9.1.3 software (SAS

Institute, Cary, NC). Variables that were not normally distributed (absolute EEG power values and response lapses on the PVT) were transformed to approximate a normal distribution. Two- and 3-way mixed-model analyses of variance (ANOVAs) with the between-subjects factor “gender” (female, male) and the within-subjects factors “genotype” (G/A, G/G), “condition” (baseline, recovery/deprivation), “non-REM sleep episode” (1–4), “session” (14 assessments during prolonged waking), or “time” (7 time points of sAA determination) were performed. The significance level was set at  $\alpha < 0.05$ . If not stated otherwise, only significant effects of factors and interactions are mentioned. Paired and unpaired, 2-tailed *t*-tests to localize differences within and between groups were only performed when the respective main effects and/or interactions of the ANOVA were significant.

## **Results**

### ***The c.22G>A Polymorphism of ADA Modulates Focused Attention***

Performance on various tasks reflecting learning, memory, and executive functioning was similar in G/A and G/G genotypes of ADA (see Supplementary Table 1). Nevertheless, analysis of the d2 attention task in 29 G/A and 191 G/G genotype subjects with right-hand dominance revealed that the G/A genotype processed roughly 30 items less than the G/G genotype ( $503 \pm 12.6$  vs.  $534 \pm 5.3$ ,  $P < 0.04$ ). This genotype-dependent difference reflects reduced speed in the G/A genotype and was also present in the participants of the laboratory experiment (see below). In contrast, the number of commission and omission errors did not differ between the groups.

### ***The c.22G>A Polymorphism of ADA Does Not Affect Habitual Sleep Duration***

The Munich Chronotype Questionnaire suggested similar sleep length on work days and leisure days in G/A ( $n = 29$ ) and G/G ( $n = 191$ ) genotypes ( $P_{\text{all}} > 0.4$ , data not shown). Four-week rest-activity monitoring in the participants of the laboratory study confirmed this notion. Irrespective of genotype, habitual sleep duration equaled roughly 7.6–7.7 h when averaged over work and leisure days (see Supplementary Table 3).

### ***The c.22G>A Polymorphism of ADA Predicts Individual Differences in Slow-Wave Sleep***

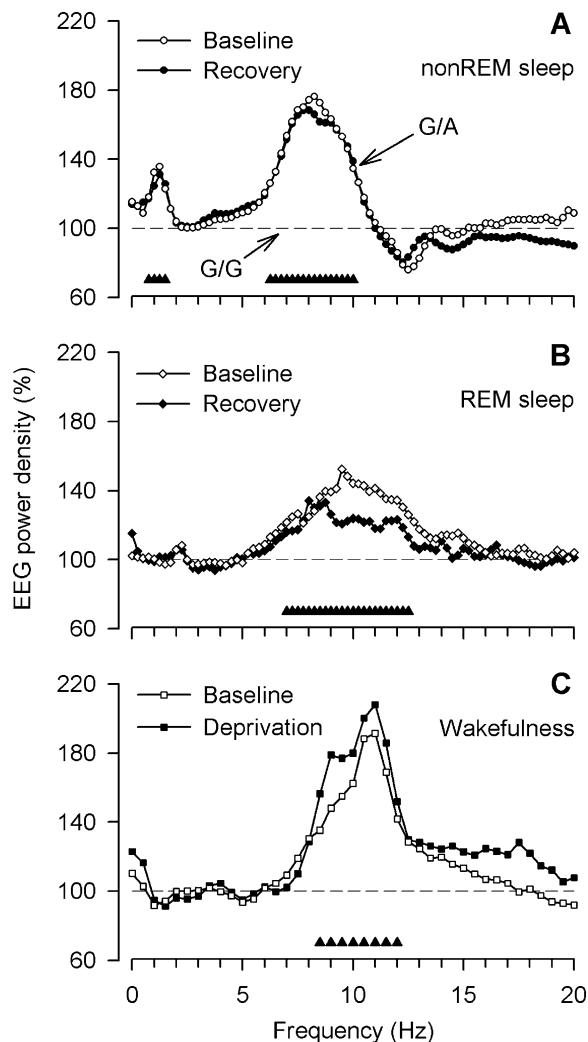
Both genotype groups showed normal sleep architecture, including short sleep latency and high sleep efficiency in the baseline night (see Supplementary Table 4). Nevertheless, corroborating our previous finding (Rétey et al. 2005), the G/A genotype subjects spent more time in slow-wave sleep than the G/G genotype subjects ( $123.9 \pm 7.2$  vs.  $100.3 \pm 6.1$  min,  $P < 0.001$ ).

Sleep episode duration, total sleep time, sleep efficiency, and slow-wave sleep increased in the recovery night after sleep loss when compared with the baseline night. On the contrary, sleep latency, wakefulness after sleep onset, stage 1, stage 2, and REM sleep were reduced. These sleep loss-induced changes in sleep architecture were independent of genotype (see Supplementary Table 4).

### ***The c.22G>A Polymorphism of ADA Predicts Higher EEG Slow-Wave Activity in Non-REM Sleep***

To draw conclusions about possible differences in sleep homeostasis between the genotypes, quantitative EEG analyses in sleep and wakefulness are mandatory. Slow-wave (0.75–1.5Hz)

oscillatory activity in non-REM sleep was higher in G/A genotype than in G/G genotype, in both baseline and recovery nights (Fig. 1A). As a physiological marker of sleep homeostasis, slow-wave activity was highest in the first non-REM sleep episode and declined in the course of the night when sleep pressure dissipated (Fig. 2). This time course and the rebound after sleep loss were similar in both genotypes. The data suggest that the c.22G>A polymorphism of *ADA* does not interfere with the dynamics of sleep homeostasis. Nevertheless, the G/A genotype appears to exhibit higher overt sleep pressure than the G/G genotype. Supporting this hypothesis, the “relative” rebound in the first non-REM sleep episode was significantly smaller in A allele carriers than in G/G homozygotes ( $33.3 \pm 7.7$  vs.  $52.8 \pm 6.9$  %,  $P < 0.05$ ).



**Figure 1.** The functional c.22G>A polymorphism of *ADA* modulates EEG activity in non-REM sleep, REM sleep, and wakefulness. EEG power density (C3A2 derivation) between 0 and 20 Hz in the G/A genotype ( $n = 11$ ) was expressed as a percentage of the corresponding values in the G/G genotype ( $n = 11$ ; horizontal dashed line at 100%). Data in non-REM (A) (stages 2–4) and REM sleep (B) represent all-night values in baseline (white symbols) and recovery nights (black symbols). In the waking EEG (C), averaged power over five 5-min recordings at 8 AM, 11 AM, 2 PM, 5 PM, and 8 PM on day 1 (baseline, white squares) and day 2 (deprivation, black squares) during prolonged wakefulness is represented. Geometric means are plotted for each 0.25-Hz bin in non-REM and REM sleep, and for each 0.5-Hz bin in wakefulness. Black triangles denote a significant effect of “genotype” ( $F_{1,30} \geq 4.2$ ,  $P < 0.05$ ) of a 2-way mixed-model ANOVA with the within-subject factors “genotype” (G/A, G/G) and “condition” (baseline, recovery/deprivation).

### **The c.22G>A Polymorphism of *ADA* Predicts Higher EEG Theta/Alpha Activity in Non-REM Sleep, REM Sleep, and Wakefulness**

The genotype-dependent differences in non-REM sleep were not restricted to the slow-wave range, but also included theta and alpha oscillations. Irrespective of normal (baseline night) or elevated (recovery night) sleep pressure, the G/A genotype subjects exhibited higher activity in the entire 6.25–10 Hz band when compared with the G/G genotype subjects (Fig. 1A). Suggesting that this difference reflects altered EEG generating mechanisms rather than a genotype-specific difference in sleep-wake regulation, similar changes were also present in REM sleep (Fig. 1B, 7–12.5 Hz), as well as in wakefulness (Fig. 1C, 8.5–12 Hz). To examine whether the c.22G>A polymorphism of *ADA* affects homeostatic and circadian influences on EEG alpha oscillations in waking (Cajochen et al. 2002), the time course of activity in the 8.5- to 12-Hz range during extended wakefulness was quantified in G/A and G/G genotypes. Consistent with the conclusion that this genetic variation does not affect the dynamics of sleep-wake regulation, the genotype-dependent difference in alpha activity persisted throughout sleep deprivation and was not modulated by increasing time awake (Fig. 3A).

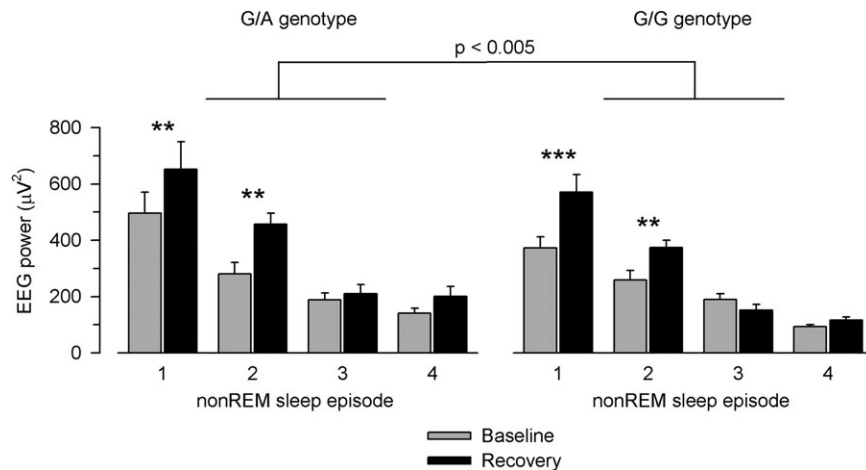
### **The c.22G>A Polymorphism of *ADA* Predicts Higher Sleepiness During Sleep Deprivation**

Previous work suggested that increased alpha activity in waking EEG with eyes open may be associated with higher subjective sleepiness, and reduced alertness and sustained attention (Oken et al. 2006). Investigating the evolution of subjective sleepiness during sleep deprivation showed that sleepiness increased in both groups with prolonged time awake and was also modulated by circadian influences. The G/A genotypes, however, were sleepier than the G/G genotypes, particularly after the night without sleep (Fig. 3B). This conclusion was corroborated by the POMS. While sleep loss reduced subjective state in both groups, fatigue was higher and vigor was lower in the G/A genotype than the G/G genotype (Fig. 4). In contrast, the other POMS subscales were not affected by either sleep deprivation or genotype (data not shown).

### **The c.22G>A Polymorphism of *ADA* Predicts Reduced Sustained Attention During Sleep Deprivation**

Performance on the PVT is a sensitive measure of sustained vigilant attention. Reaction times (RTs) and number of response lapses (RT > 500 ms) on the PVT were impaired by sleep loss in both *ADA* genotypes. However, consistent with increased EEG alpha activity and elevated subjective sleepiness, G/A genotype subjects performed consistently slower and produced more lapses than G/G genotype subjects throughout prolonged wakefulness (Fig. 3C,D). Importantly, the magnitude of the difference between the genotypes was large, comparable with the effects of 1 night without sleep. The data confirm that tonic alertness is impaired in healthy individuals with genetically reduced adenosine metabolism.

To further support this conclusion, performance on the d2 attention task was separately examined in the participants of the laboratory experiment. Corroborating the finding in the entire study sample, the G/A genotype processed significantly fewer items than the G/G genotype (Fig. 5). This difference reflects reduced speed on the d2 task.



**Figure 2.** The functional c.22G>A polymorphism of *ADA* predicts higher low-delta activity (C3A2 derivation, power within 0.75–1.5 Hz) in non-REM sleep (stages 2–4). In contrast, it does not affect the time course and the sleep loss-induced rebound of EEG slow-wave oscillations after sleep deprivation. Mean delta activity in G/A (left panel) and G/G genotypes (right panel) in non-REM sleep episodes 1–4 in baseline (gray bars) and recovery nights (black bars) is plotted. Error bars represent 1 standard error of the mean ( $n = 11$ ). Three-way mixed-model ANOVA with the within-subject factors “genotype” (G/A, G/G), “condition” (baseline, recovery), and “non-REM sleep episode” (1–4) confirmed the significant effect of genotype (genotype:  $F_{1,44.2} = 8.8, P < 0.005$ ). \*\* $P < 0.01$  (recovery vs. baseline; paired 2-tailed  $t$ -test); \*\*\* $P < 0.001$  (recovery vs. baseline; paired 2-tailed  $t$ -test).

### The c.22G>A Polymorphism of *ADA* Predicts Elevated $\alpha$ -Amylase Activity in Saliva

To investigate whether the c.22G>A polymorphism of *ADA* also affects a recently proposed biomarker of sleep drive (Seugnet et al. 2006), we quantified sAA throughout prolonged wakefulness. We found a pronounced diurnal variation, with highest values in the afternoon and lowest values early at night. Interestingly, sAA in the G/A genotype was significantly higher than in the G/G genotype (Fig. 6). These biochemical data are consistent with our neurophysiological, subjective, and behavioral findings, and support the conclusion that the G/A genotype of *ADA* is associated with elevated sleep pressure.

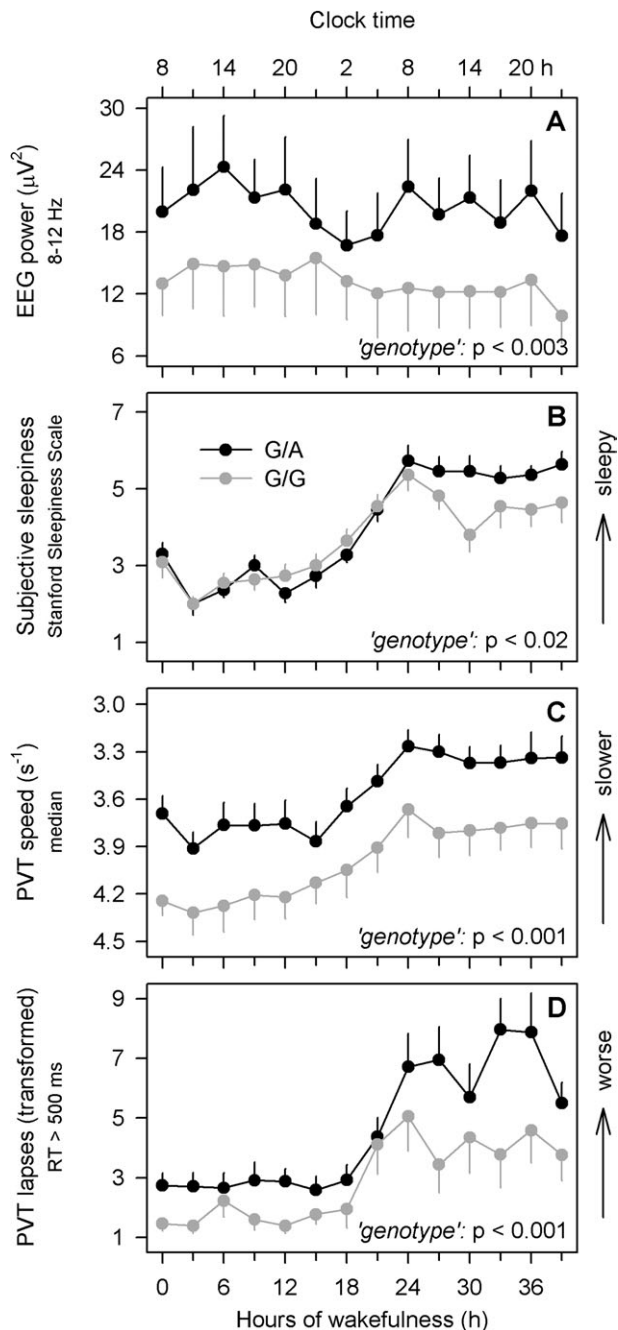
### Discussion

This study demonstrates that healthy adults with genetically reduced ADA activity (G/A genotype) exhibit higher non-REM sleep pressure than individuals with unimpaired ADA activity (G/G genotype). The carriers of the variant allele have more slow-wave sleep, show enhanced brain oscillatory activity within 0.75–1.5 and 6–10 Hz in non-REM sleep (the latter effect is independent of vigilance/sleep state), feel more sleepy and fatigued, are less vigilant (d2 and PVT attention tasks), and present with enhanced  $\alpha$ -amylase activity in saliva when compared with G/G homozygotes. These convergent findings demonstrate that in rested and sleep-deprived state, the functional c.22G>A polymorphism of *ADA* not only modulates sleep structure and intensity but also importantly contributes to interindividual differences in waking quality, including sleepiness and attention. In contrast, habitual sleep length and the dynamics of the homeostatic response to sleep deprivation are unaffected. Taken together, these data suggest that distinct mechanisms underlie the homeostatic regulation of sleep intensity and sleep duration.

It is widely accepted that EEG slow-wave oscillations are the hallmark of deep non-REM sleep (slow-wave sleep) and constitute the primary marker of sleep homeostasis. At the cellular level, slow-wave oscillations consist of depolarized

up-states and hyperpolarized down-states in the membrane potential of cortical neurons (Steriade et al. 1993). The up-states last for 0.4–0.8 s and the down-states for 0.3–0.7 s (Amzica and Steriade 1998). These membrane fluctuations result in EEG slow oscillations with frequencies of 0.65–1.3 Hz (Bersagliere and Achermann 2010), which perfectly coincide with the frequency range that discriminates the EEG in non-REM sleep between G/A and G/G genotypes of *ADA*. Intracellular recordings in non-anaesthetized cats suggest that the long-lasting hyperpolarized down-states represent periods of disfacilitation, a form of inhibition due to reduced activating input from ascending cholinergic and monoaminergic pathways (Steriade et al. 2001; Timofeev et al. 2001). Extracellular adenosine could contribute to cortical disfacilitation by inhibiting basal forebrain and mesopontine cholinergic neurons (Rainnie et al. 1994). Thus, the functional c.22G>A polymorphism of *ADA* may enhance endogenous adenosine levels and amplify cortical disfacilitation in deep non-REM sleep.

Genetic studies in mice and pharmacological experiments in rats and humans also support a role for adenosine in modulating low-frequency slow-wave oscillations in non-REM sleep. Recent insights suggest that integrated brain circuits consisting of neurons and astrocytes regulate extracellular adenosine and adenosine-mediated modulation of neural transmission (Haydon and Carmignoto 2006; Halassa and Haydon 2010). Astrocytes can be activated by neurotransmitters from adjacent synapses and release chemicals by a process called gliotransmission. The primary molecules released by gliotransmission are glutamate, ATP, and D-serine (Oliet and Mothet 2006). The ATP is rapidly hydrolyzed to adenosine and modulates synaptic activity by acting on adenosine receptors. Astrocytic modulation of cortical synapses is relevant for sleep-related EEG rhythms in vivo (Halassa and Haydon 2010). More specifically, genetic inhibition of gliotransmission exclusively in astrocytes decreases the slow oscillation in somatosensory cortex (Fellin et al. 2009). Intriguingly, the dnSNARE mice show reduced slow-wave activity in non-REM sleep in baseline, as well as in recovery sleep after sleep deprivation (Halassa



**Figure 3.** The functional c.22G>A polymorphism of *ADA* predicts higher EEG alpha activity, elevated subjective sleepiness, and impaired sustained attention during prolonged wakefulness. Starting 15 min after waking from the baseline night, 14 test sessions at 3-h intervals consisting of 5-min waking EEG recording, subjective sleepiness rating, and testing of sustained attention were completed in each individual. Ticks on the x-axis are rounded to the nearest hour. Black circles: G/A genotype ( $n = 11$ ); gray circles: G/G genotype ( $n = 11$ ). Data were analyzed with 2-way mixed-model ANOVA with the within-subject factors “genotype” (G/A, G/G) and “session” (14 assessments during prolonged waking). (A) Throughout prolonged wakefulness, EEG activity in the 8.5- to 12-Hz range was consistently higher in G/A genotype than in G/G allele carriers (genotype:  $F_{1,30} = 10.9$ ,  $P < 0.003$ ; session:  $F_{13,239} = 2.3$ ,  $P < 0.007$ ; genotype  $\times$  session interaction:  $F_{13,159} = 0.2$ ,  $P > 0.9$ ). (B) The evolution of subjective sleepiness during sleep deprivation was quantified with the Stanford Sleepiness Scale. ANOVA revealed significantly higher sleepiness in the G/A genotype than in the G/G genotype (genotype:  $F_{1,78} = 6.3$ ,  $P < 0.02$ ; session:  $F_{13,155} = 42.1$ ,  $P < 0.001$ ; genotype  $\times$  session interaction:  $F_{13,162} = 1.7$ ,  $P < 0.08$ ). The difference becomes evident after the night without sleep. (C and D) Sustained attention during prolonged wakefulness was quantified with the PVT. The time courses of speed (1/median RT) and response lapses (RT > 500 ms,

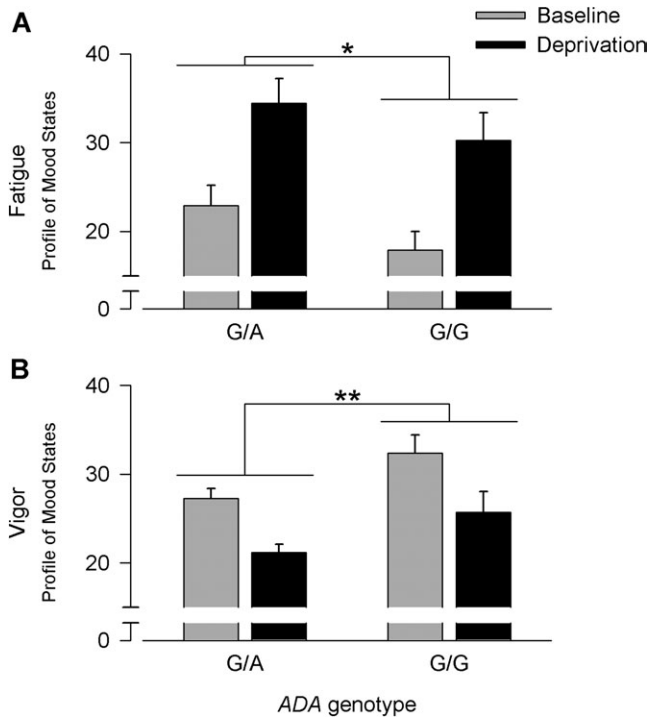
et al. 2009). Reminiscent of the genotype-dependent difference between G/A and G/G allele carriers of *ADA*, the difference to wild-type mice is most pronounced in the 0.5- to 1.5-Hz band in non-REM sleep.

Last but not least, the adenosine  $A_1$  and  $A_{2A}$  receptor antagonist, caffeine, is well-known to primarily reduce EEG low-delta frequencies in non-REM sleep in rested and sleep-deprived states (rats: 0.75–1.5 Hz; humans: 0.75–2.0 Hz) (Landolt et al. 1995, 2004; Schwierin et al. 1996). Genetic studies suggest that  $A_{2A}$  receptors play a primary role for the effects of caffeine on sleep. Mice with  $A_{2A}$  receptor loss of function have reduced sleep and blunted response to sleep deprivation, as well as to the wake-promoting effects of caffeine (Urade et al. 2003; Huang et al. 2005). Consistent with this notion, the human c.1976T>C polymorphism (rs5751876) of the  $A_{2A}$  receptor gene (*ADORA2A*) modulates individual sensitivity to caffeine on sleep (Rétey et al. 2007). This polymorphism also has a striking effect on EEG theta/low-alpha (~6.5–9.5 Hz) oscillations in non-REM sleep, REM sleep, and wakefulness (Rétey et al. 2005). The partial overlap of this frequency range with the findings in the present study suggests that the repercussions of genetically altered *ADA* activity may, at least in part, be mediated by adenosine  $A_{2A}$  receptors.

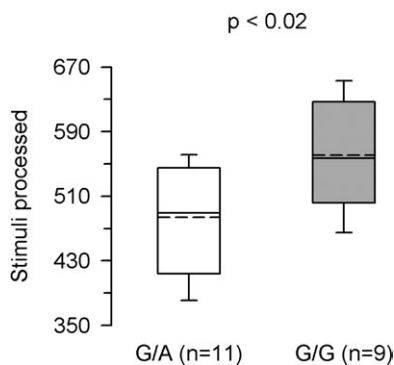
The consequences of genetic abolition of gliotransmission on EEG activity in REM sleep and wakefulness in mice are unknown. Remarkably, however, performance on a novel object recognition task appears virtually unaffected after prolonged waking in dnSNARE mice when compared with wild-type animals (Halassa et al. 2009). This phenotype may be reminiscent of the difference in performance on d2 and PVT attention tasks between human G/A allele carriers and G/G homozygotes of *ADA*. Although the comparison between exploration of novel objects in rodents and attention performance in humans needs to be made with caution, our findings could indicate that the c.22G>A polymorphism of *ADA* increases overt homeostatic sleep pressure by interfering with the astrocyte-dependent regulation of extracellular adenosine. Indeed, *ADA* may be more abundantly expressed in astrocytes than in neurons (Fredholm et al. 2005).

Slow-wave oscillations in non-REM sleep are highest in the beginning of the night and most pronounced in those brain areas that were disproportionately active during preceding wakefulness (Kattler et al. 1994; Ferrara et al. 2002; Huber et al. 2004). Moreover, experimental induction of slow waves may potentiate the beneficial effects of sleep (Marshall et al. 2006; Walsh et al. 2006, 2010), whereas their suppression may impair proper functions of brain and body (Aeschbach et al. 2008; Tasali et al. 2008; Landsness et al. 2009; Van Der Werf et al. 2009). Thus, slow-wave oscillations in non-REM sleep are thought to reflect a restorative function of sleep. Based on this reasoning, the physiological enhancement of sleep pressure and slow-wave oscillations may be a promising strategy to potentiate the positive effects of sleep. Because

transformed by  $\sqrt{x + \sqrt{1 + x}}$  are illustrated. All RT < 100 ms (“errors of commission”) were excluded from analyses. The G/A genotype performed significantly worse than the G/G genotype throughout prolonged waking (speed—genotype:  $F_{1,25} = 15.4$ ,  $P < 0.001$ ; session:  $F_{13,239} = 38.6$ ,  $P < 0.001$ ; genotype  $\times$  session interaction:  $F_{13,146} = 0.3$ ,  $P > 0.9$ ; lapses—genotype:  $F_{1,66} = 24.5$ ,  $P < 0.001$ ; session:  $F_{13,194} = 19.5$ ,  $P < 0.001$ ; genotype  $\times$  session interaction:  $F_{13,144} = 1.1$ ,  $P > 0.3$ ).

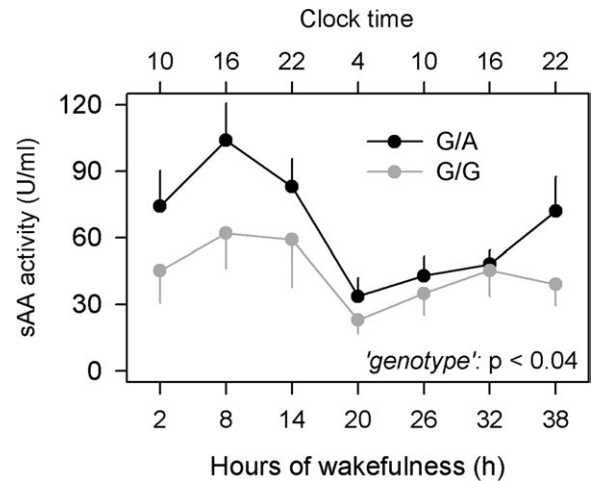


**Figure 4.** Elevated fatigue and reduced vigor in G/A genotype ( $n = 11$ ) compared with G/G genotype ( $n = 11$ ) of *ADA*. The POMS was administered at 16:45 on days 1 (baseline, gray bars) and 2 (deprivation, black bars) of extended wakefulness. Data represent means + standard error of the mean. They were analyzed with 2-way mixed-model ANOVA with the within-subject factors “genotype” (G/A, G/G) and “condition” (baseline, deprivation). In rested and sleep-deprived state, fatigue was higher and vigor was lower in the G/A genotype (fatigue—genotype:  $F_{1,30} = 4.2$ ,  $P < 0.05$  [\*]; condition:  $F_{1,30} = 28.8$ ,  $P < 0.001$ ; genotype  $\times$  condition interaction:  $F_{1,30} = 0.0$ ,  $P > 0.8$ ; vigor—genotype:  $F_{1,30} = 9.2$ ,  $P < 0.005$  [\*\*]; condition:  $F_{1,30} = 16.1$ ,  $P < 0.001$ ; genotype  $\times$  condition interaction:  $F_{1,30} = 0.0$ ,  $P > 0.8$ ).



**Figure 5.** Reduced speed on d2 attention task in the G/A genotype compared with the G/G genotype of *ADA*. The upper and lower lines of the “box and whisker plots” represent the 75th and 25th percentiles of the study sample, whereas the horizontal lines in the middle of the boxes indicate the sample medians (50th percentiles). Mean values of processed items (dashed lines) differed significantly between 11 G/A (white box) and 9 G/G (gray box) genotype subjects ( $484 \pm 19.1$  vs.  $561 \pm 22.6$ ,  $P < 0.02$ ; paired 2-tailed  $t$ -test). In contrast, the number of errors did not differ between the groups.

adenosine and adenosine receptors play a well-established role in sleep homeostasis, this neuromodulatory system could provide a rational target to intensify sleep, for example, in patients with insomnia, shallow sleep, and disturbed vigilance. Apart from possible unrelated unwanted reactions (Landolt



**Figure 6.** Elevated sAA in G/A genotype compared with G/G genotype of *ADA*. Saliva samples were collected at 2-h intervals, starting at 8 AM on day 1 of prolonged wakefulness. The sAA (U/mL) was averaged per 6-h intervals. Error bars represent standard error of the mean ( $n = 11$ ). The data were analyzed with 2-way mixed-model ANOVA with the within-subject factors “genotype” (G/A, G/G) and “time” (7 time points during prolonged waking). ANOVA revealed higher sAA in the G/A genotype (genotype:  $F_{1,21} = 5.2$ ,  $P < 0.04$ ; time:  $F_{6,97} = 12.6$ ,  $P < 0.001$ ; genotype  $\times$  time interaction:  $F_{6,71} = 0.7$ ,  $P > 0.6$ ).

2008), the present genetic study shows that such an approach would likely impair the quality of wakefulness. More specifically, waking functions (e.g., sustained attention) could be reduced to an extent that is similar to the effect of 1 night without sleep. Indeed, with respect to another gene variant possibly involved in sleep-wake regulation, *PER3*<sup>S/S</sup> genotype subjects of the circadian clock gene *PERIOD-3* not only exhibit more slow-wave sleep and EEG low-frequency activity but are also more impaired by sleep deprivation than *PER3*<sup>L/L</sup> homozygotes (Viola et al. 2007). Our findings demonstrate that a similar sleep phenotype can impair waking performance even in well-rested individuals.

## Conclusions

In conclusion, functional polymorphic variation of *ADA* in healthy adults distinctly affects non-REM sleep intensity, EEG theta/alpha frequencies in sleep and wakefulness, attention, subjective sleepiness, and  $\alpha$ -amylase activity in saliva. These differences do not mirror differences in habitual sleep duration and are robust against the effects of sleep deprivation. Thus, they do not reflect a genotype-dependent alteration in the dynamics of sleep homeostasis. This observation is consistent with recent findings in monozygotic and dizygotic twins, showing that the pronounced genetic influences on the sleep EEG are independent of elevated sleep pressure after sleep loss (De Gennaro et al. 2008). Moreover, as shown in rats (Mackiewicz et al. 2003), *Ada* enzymatic activity is not affected by sleep deprivation. The data rather suggest an elevated level in overt non-REM sleep propensity in the G/A genotype compared with G/G homozygotes, which may be due to elevated adenosinergic tone at the synapse because of genetically reduced *ADA* activity. Whether this difference directly underlies the observed phenotypes in sleep and wakefulness, or whether it modulates other molecular systems contributing to the homeostatic regulation of sleep propensity, remains to be elucidated.

## Supplementary Material

Supplementary material can be found at: <http://www.cercor.oxfordjournals.org/>

## Funding

University Research Priority Program “Integrative Human Physiology,” University of Zurich; the Swiss National Science Foundation (grant # 310000-120377).

## Notes

We thank Katharina Hefti and Renate Wehrle for their help with data collection, and Roland Dürr and Peter Achermann for providing us with the dedicated software for EEG analyses. *Conflict of Interest:* None declared.

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