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14. ABSTRACT
Epigenetic changes, including hypermethylation of DNA, are fundamental to progression and maintenance of epilepsy. Using silk-based brain implants engineered to release adenosine we demonstrated that reversal of epigenetic changes prevents epileptogenesis. We identified a novel mechanism by which adenosine reduces DNA methylation in the brain and translated those findings into a new therapeutic strategy (biodegradable silk-based brain implants) to prevent epileptogenesis long term. These findings constitute a novel scientific advance with direct clinical implications. Specifically, using bioengineered silk-based brain implants we demonstrated that transient delivery of a defined focal dose of adenosine to epileptic rats can reverse pathological DNA hypermethylation. Further, we showed that this treatment can prevent epileptogenesis as assessed by the analysis of two independent outcome parameters (seizures and mossy fiber sprouting). To our knowledge this is the first study where a robust antiepileptogenic effect has been demonstrated *after* the onset of epilepsy. Adenosine and silk are FDA approved; thus our findings have direct translational value. In summary, we demonstrated that DNA methylation changes are integral to initiation and progression of epilepsy; these epigenetic changes are modulated by adenosine, which is dysregulated in epilepsy; focal transient silk-based adenosine augmentation reduces epilepsy associated DNA hypermethylation and halts disease progression.

15. SUBJECT TERMS
adenosine releasing silk; polymeric brain implants; focal adenosine augmentation therapy; prevention of seizures; prevention of epileptogenesis; adenosine-based modulation of DNA methylation; the methylation hypothesis of epileptogenesis

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Introduction

Epilepsy is a progressive neurological disorder; even with optimal treatment, ~35% of patients develop pharmacoresistant seizures, leaving them with limited treatment options and poor quality of life. Current drug treatments are designed for symptom control (i.e. seizure suppression) but do not affect the underlying pathophysiological mechanisms and do not prevent epileptogenesis. Adenosine is an endogenous network regulator of the brain with proven antiepileptic properties. Adenosine deficiency is a pathological hallmark of the epileptic brain and directly implicated in seizure generation and epileptogenesis. Importantly, transgenic animals with increased levels of adenosine in the brain are resistant to the development of epilepsy. The goal of this research is to develop a therapeutic approach to utilize adenosine for the prevention of epileptogenesis, which continues to remain an important goal to prevent epilepsy development in OIF and OEF veterans. Unfortunately, life-long systemic adenosine augmentation is not a therapeutic option due to systemic side effects. We therefore selected to develop a silk-based brain implant to deliver a defined dose of adenosine for a predetermined period of time to a critical site in the brain. Our overarching goal was to use those adenosine-releasing silk-based brain implants to prevent epileptogenesis in a rat model of progressive epilepsy development. We determined effective doses, potential side effects, and suitable time points and time frames of therapeutic intervention. Silk is a biodegradable biopolymer. If a silk-polymer can be used to prevent epileptogenesis through the transient delivery of adenosine it would be an ideal therapeutic application following an epileptogenesis triggering event such as TBI. Once its job to prevent epileptogenesis is done, the silk will be resorbed without leaving any residues. As the major outcome of this grant we demonstrated that a transient dose of adenosine can prevent epileptogenesis. In addition, we defined the underlying mechanisms and determined suitable doses and time points of therapeutic intervention.

Body

Task 1: Efficacy and toxicity studies with adenosine releasing silk

Silk production: We engineered and tested two different silk-based formats for the focal delivery of adenosine to the brain: *(i)* rigid polymers designed for the sustained long-term delivery of lower doses of adenosine; and *(ii)* gel-based injectables for the short-term delivery of high doses of adenosine.

(i) We examined the use of transient focal adenosine delivery using a silk polymer to suppress seizures. The rigid polymers had a biphasic adenosine release profile *in vitro* (Fig. 1), consisting of an acute surge of adenosine (6000 ng/day peak at day 1), followed by a sustained release of 1000 ng per day for 12-14 days.

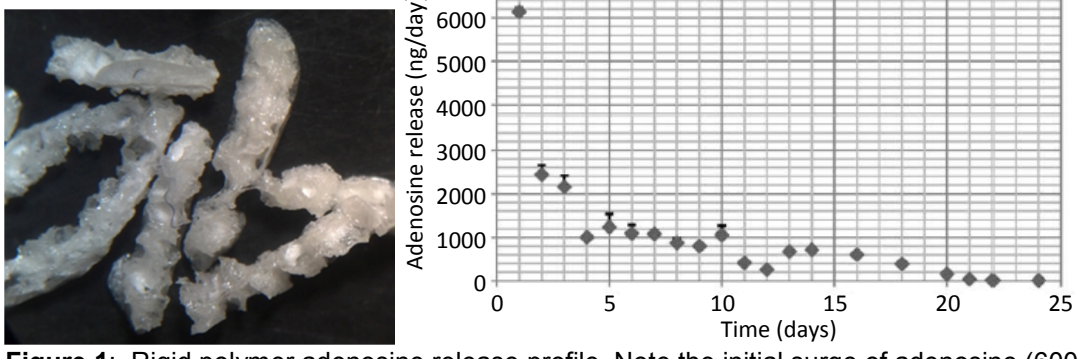

Figure 1: Rigid polymer adenosine release profile. Note the initial surge of adenosine (6000 ng/day) followed by sustained adenosine release (~1000 ng/day) for 2 weeks.

(ii) We further refined our silk polymer design to prepare for a drug delivery format more suitable for future clinical use. A major refinement was to switch from a rigid silk-based brain implant, which requires a major surgical intervention, to a gel-based silk implant that can directly be injected into

the target brain area. This gel-based format allows refinement of the delivery and improved reproducibility of adenosine release. Adenosine delivery can be tailored both by volume of gel and by concentration of adenosine in the gel.

Silk Production Methods

Standard preparation of silk gels start with fibroin that is boiled for 60 minutes in sodium carbonate solution, dissolved in lithium bromide, and dialyzed against water to obtain a pure silk solution. A 4% (w/v) silk solution is sonicated on ice at 30% amplitude for 30 seconds, sterile filtered, and diluted 1:1 with adenosine solution at a concentration of 6mg/mL (final concentration 3mg/mL). This original material has been evaluated for ease of injection, repeatability of injection, and short term release of adenosine- bulk during day 1, with the residual release for two days following.

Modifications of this sonicated silk-adenosine gel were evaluated to determine if the release of adenosine could be extended- drawing out the bulk release, with the residual being delivered over extended time spans. It was of interest to maintain the handling characteristics of the silk gel due to the ease of delivery. Two strategies were explored in parallel: 1.) buffering the solutions used to dissolve adenosine/dilute silk to approach the pKa of silk and 2.) inclusion of silk-adenosine nanoparticles within the gel. The rationale of using buffered solutions was to take advantage of any potential charge interactions of the adenosine and silk by reducing the pH to a target of 4.5, closer to its pKa. Physiological ramifications of changing the pH would be addressed if the buffered system significantly extended release. The inclusion of nanoparticles offers the use of parallel technology developed in our lab, to determine they would accommodate further retention of adenosine over an extended time period.

All gel formulations were loaded into syringes to evaluate repeatability of delivery and the release profile of adenosine in bulk buffered saline solution. Test groups included: a) silk gel, standard pH, control; b) silk gel, standard pH, 3mg/mL adenosine; c) silk gel at buffered pH, control; d) silk gel at buffered pH, 3mg/mL adenosine; e) silk gel at standard pH with silk/adenosine nanoparticles; and, f) silk gel at standard pH, 3mg/mL adenosine, plus silk/adenosine nanoparticles.

Aliquots of 20µL of silk were distributed in 24 well plates, with n=4 for each formulation. Each well received 0.5mL of PBS that was changed in full at each time point of 1, 3, 6, and 24 hours and 4 days. 100µL was sampled for absorbance readings at 258nm.

Adenosine release: Three of the sample groups listed above- b, d, and f—were prepared by diluting silk solution 1:1 with 6mg/mL adenosine solution. The final 20µL aliquots were projected to have a total of 60µg adenosine each, with group f being marginally higher with the adenosine-nanoparticle addition. Adenosine recovery was monitored over 96 hours. The control groups a and c were omitted from Fig. 2 as there was no measurable adenosine or background signal to report. With the starting concentration of 3mg/mL adenosine in the 20µL aliquot, we are not saturating the release solution with adenosine in between each PBS change, suggesting that the silk is delaying the adenosine from immediate release. In between 24h and 96h, only the buffered samples released any measurable adenosine into solution, at the nanogram level. Within each of the groups, we attained consistent release trends at each time point. This was our first evaluation of adenosine-loaded nanoparticles and their potential for extending release by providing an additional diffusion barrier and the potential to enhance the utility of the gels for delivery. This data suggests that nanoparticles, as they were generated for this study, did not delay adenosine release beyond the influence of the silk gel alone.

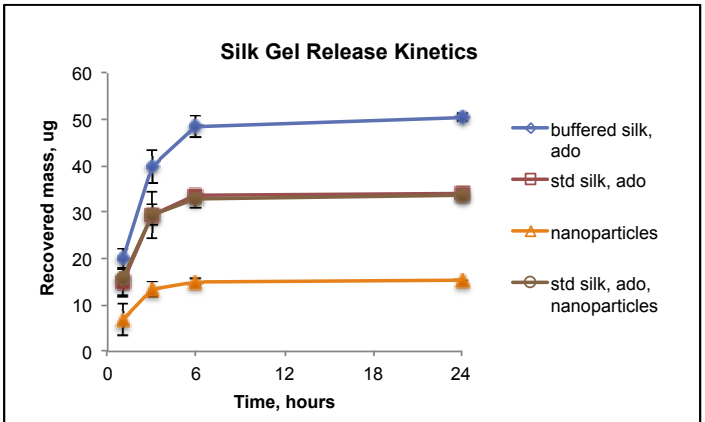

Figure 2: Adenosine release from silk gel.

After evaluation and a repeat trial, our attention is focused on mass balance of our systems. The citrate buffered gels were produced to determine if lowering the pH toward the pKa of silk would enable greater polarity and charge interactions, increasing adenosine retention. While the buffered gels do not extend retention, they seem to enable greater recovery of the loaded adenosine. The two neutral pH groups with and without nanoparticles generated similar release profiles which was an unexpected cumulative result. The influence of pH and the molecular interactions of silk with adenosine are a focus of our continuing work.

Major findings and conclusions

- Development and characterization of two different silk-based adenosine delivery systems.
- Silk-gel can be used as an injectable and is an improved format for silk-based adenosine delivery in light of future clinical applications.
- Silk in a gel format has greater reproducibility for acute delivery of adenosine.
- The non-buffered silk format was selected for further experiments to limit toxicity concerns surrounding pH.

Efficacy studies

Tasks 1a and 1b

120 Rats

We evaluated the efficacy of focal adenosine augmentation in three mechanistically distinct models of evoked seizures: focal electrical stimulation, the chemoconvulsant pentylenetetrazol (PTZ), and maximal electroshock (MES).

In the focal stimulation (kindling) model (Fig. 3A), we utilized high-frequency hippocampal stimulation to evoke an electrical afterdischarge without stereotypical convulsive behavioral activity at the initiation of the experiment. Stimulations were applied at 30-minute intervals (up to 12/day), and the evoked behavior scored according to Racine. Racine stages 1 & 2 indicate limbic seizure activity (chewing, head nodding), and reflect synaptic reorganization. Our results show a clear dose dependence of seizure suppression as reflected by increasing number of stimulations needed to reach equivalent seizure scores (Fig. 3A).

We next examined the dose response of adenosine in blocking seizure generalization evoked using the $GABA_AR$-antagonist picrotoxin (Fig. 3B). This test was performed with different doses of intracerebroventricular chronic adenosine for 5 days, then followed by a standard picrotoxin seizure test. We found a significant reduction in the fraction of generalized seizures with adenosine treatment ($p < 0.01$ by ANOVA). Post hoc tests demonstrate a significant reduction at a dose of 3000 ng adenosine per day (Fig. 3, $p < 0.01$).

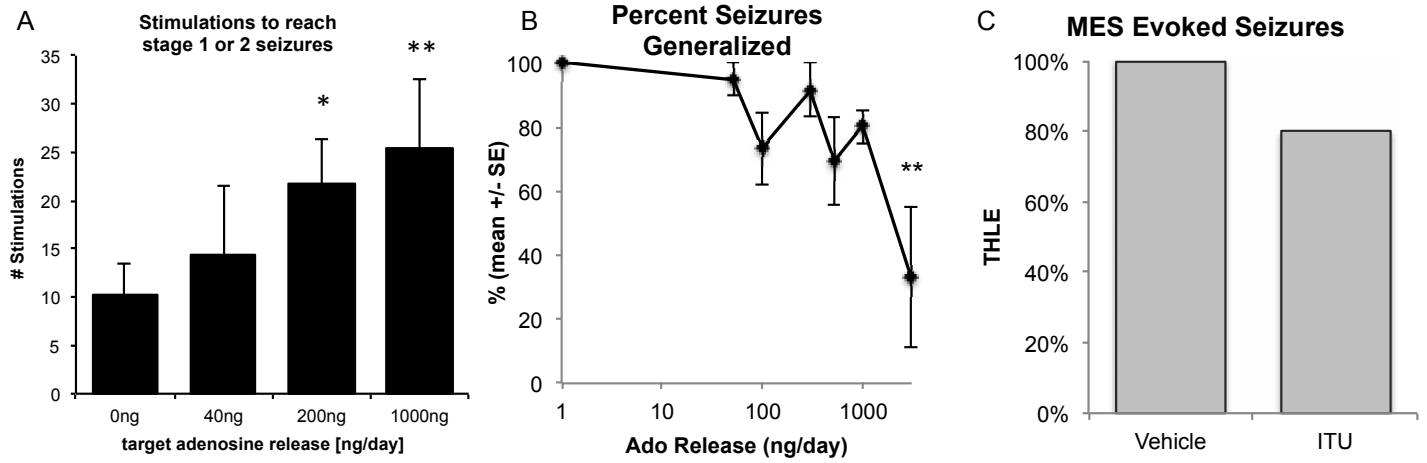

Figure 3: Evaluation of adenosine mediated seizure suppression in three mechanistically distinct models. (A) Focal (intrahippocampal) electrical stimulation. (B) Picrotoxin. (C) Maximal electroshock (transcorneal).

In the maximal electroshock model (MES, Fig. 3C), we were not able to block tonic hind limb extension (THLE) with adenosine augmenting protocols. In rats, we achieved 100% THLE in vehicle treated and naïve rats, and complete protection against THLE using valproic acid, a conventional anticonvulsant with known efficacy in the MES model. However, we were unable to block MES-evoked seizures with focal adenosine. As an alternative to the focal delivery of adenosine we evaluated the outcome after systemic treatment with 5-iodotubercidin (ITU, 3.1 mg/kg), an adenosine kinase inhibitor known to increase brain adenosine levels and inhibit spontaneous convulsive seizures, and still found minimal protection against MES evoked seizures. These data show that adenosine is not effective in the MES model.

Toxicity studies

We evaluated neuroscore and angleboard performance in rats after 5 days of intraventricular adenosine delivery to determine off-target motor effects of adenosine treatment (Fig. 4). Evaluation of the composite neuroscore demonstrated that doses of up to 3000 ng adenosine per day did not affect neurological performance (Fig. 4, not significant by ANOVA). Likewise, evaluation on the angle board demonstrated lack of dose dependent effects, with most doses of adenosine, including the highest 3000 ng/day dose, showing no significant difference from control (not shown, $p > 0.05$ by ANOVA). Overall, rats had no grossly apparent deficits at any doses examined. There was no influence on the weight of the rats at the behavior analysis. In contrast, indirect adenosine supplementation using a systemic ITU administration (at a seizure suppressing dose of 3.1 mg/kg) causes significant sedation.

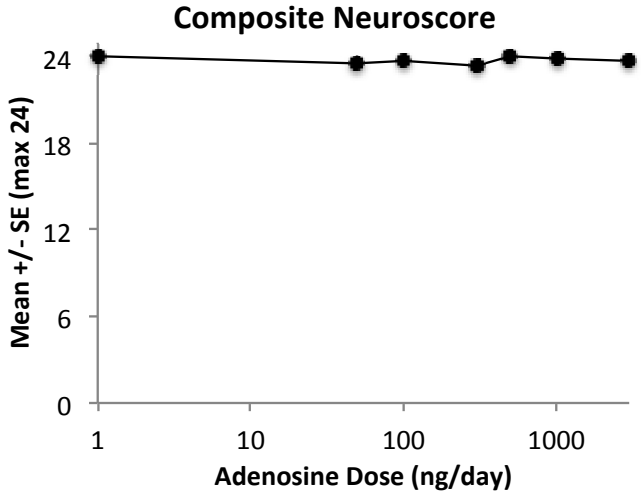

Figure 4: No motor deficits were observed in the composite neuroscore over an adenosine delivery range of 0 – 3000 ng/day. (n= 3 – 9 per dose)

Overall conclusions

- The effective dose range for intraventricular adenosine in a high-frequency stimulation model is between 200 and 1000 ng/day.
- 3000 ng/day adenosine is required to suppress seizure generalization in a GABA-based chemoconvulsant model.
- Intracerebroventricular adenosine has no major side effects over a dose range from 50 to at least 3000 ng per day.
- Adenosine does not inhibit seizures evoked by MES.

Task 2: Assess long-term impact of transient adenosine delivery in clinically relevant model of MTLE

Early stage intervention: treatment with rigid silk-based polymers ***Tasks 2a, 2d***

60 Rats

We first evaluated the antiepileptogenic therapeutic efficacy of rigid silk-based polymers designed to release adenosine with a biphasic kinetic comprised of an acute bolus followed by the sustained 10-day delivery of a dose of 250 ng adenosine per ventricle per day. These studies were performed in the systemic kainic acid (KA) model of epileptogenesis in rats after the onset of epilepsy ('early stage epilepsy'). Spontaneous convulsive seizures were confirmed by video analysis during weeks 4-8 after KA induced status epilepticus (KASE); rats with confirmed spontaneous seizures were assigned to one of three treatment groups: bilateral intrahippocampal silk polymer implants releasing 250 ng adenosine /ventricle /day, or 0 ng adenosine / ventricle / day (silk-only control), or control (sham surgery) (n=8 for each group). Rats were video monitored during weeks 9-13, and 17-21 after implant to evaluate whether the short-term (10 days) delivery of adenosine can prevent the progression of epilepsy (i.e. antiepileptogenic efficacy) long-term (over 3 months) (Fig. 5A). Rats that received the adenosine releasing silk implant had significantly fewer seizures than the untreated rats (Fig. 5B), both during the active adenosine release (weeks 10 and 11), and well after the conclusion of adenosine release (weeks 18-21). There was no effect of the silk implant alone at any time point. These data show that a transient dose of adenosine prevents progression of seizure activity long-term.

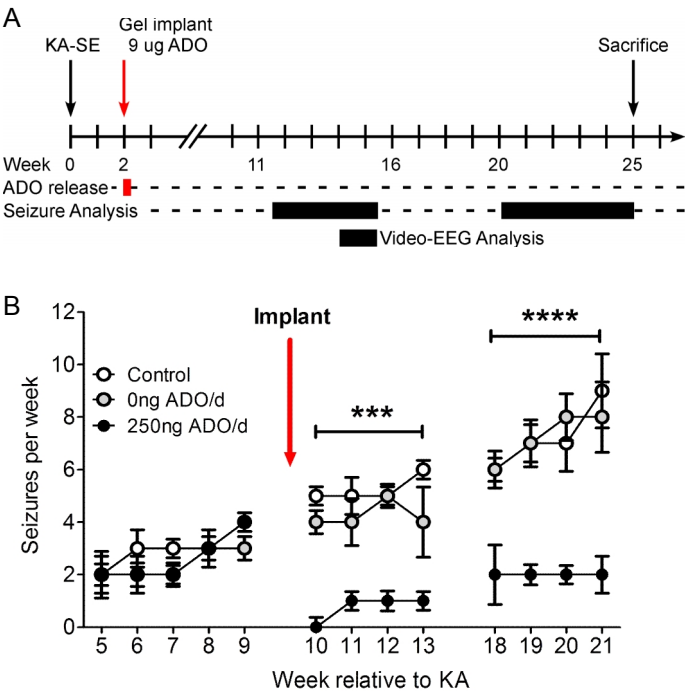

Figure 5: Transient adenosine release prevents epileptogenesis. (A) Study design. (B) Video analysis of convulsive seizure events.

Epileptogenesis is also associated with mossy fiber sprouting in the dentate gyrus of the hippocampus. We examined mossy fiber sprouting using Timm staining of naïve and epileptic rats (9 weeks post KA) and at 21 weeks post KA in surgical sham, 0 ng ("polymer"), and 250 ng adenosine ("ADO") treated rats (Fig. 6). KA-induced mossy fiber sprouting increased in all KA-treated rats compared to the naïve rats. In rats that received a sham surgery or the vehicle polymer, mossy fiber sprouting continued to increase at 21 weeks as compared to the 9 week rats. In rats with adenosine-releasing silk, however, the Timm score did not progress beyond the 9 week measurement, demonstrating that the transient adenosine treatment halted the progression of the epileptogenic remodeling.

The receptor mediated role of adenosine in seizure termination is well established, and our focal adenosine augmentation experiments are based on experiments showing that astrogliosis, adenosine kinase upregulation, and adenosine deficit are hallmarks of temporal lobe epilepsy. While targeting adenosine receptors pharmacologically is effective for seizure suppression, intolerable side effects have limited this approach for epilepsy control. The

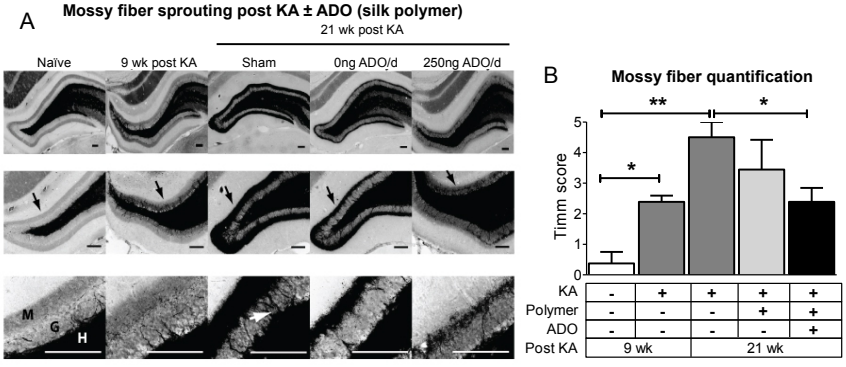

Figure 6: Mossy fiber sprouting in naïve and epileptic rats. (A) Representative histology. (B) Quantification.

decreased mossy fiber sprouting in the adenosine treated rats suggests that the transient adenosine treatment altered the process of epileptogenesis. Since these adenosine-based disease-modifying effects are maintained well beyond activation of the adenosine receptors by adenosine, we hypothesized that the antiepileptogenic effects of adenosine are not based on adenosine receptor activation suggesting the existence of a previously unknown mechanism by which adenosine can affect brain plasticity long-term. Adenosine is an obligatory end product of transmethylation reactions, which also include DNA methylation (Fig. 7A). Increased adenosine kinase (as found in the epileptic brain), by removing adenosine drives the flux of methyl groups through the transmethylation pathway, resulting in increased DNA methylation, a pathological hallmark of the epileptic brain. We suggest that DNA methylation status in the brain critically determines epilepsy progression including mossy fiber sprouting and increased seizure activity.

To test this hypothesis we examined global levels of DNA methylation in epileptic rat hippocampus by ELISA, and found clear hypermethylation in the epileptogenic hippocampus prior to the onset of adenosine treatment (Fig. 7B). Importantly, methylation levels in rats treated with the adenosine releasing polymers was restored to normal levels, an effect which persisted even after the conclusion of the adenosine release (Fig. 7B). To confirm that this effect was specific to adenosine regulation of DNA methylation, we measured DNA methylation levels in naive rats after a single 5 ug dose of adenosine, and found decreased methylation (Fig. 7C). Further, a single dose of homocysteine, a second obligatory end product of the methylation reaction caused the same reduced DNA methylation (Fig. 7C). In contrast, supplementing with S-adenosylmethionine, the methyl source for the reaction, caused increased DNA methylation. These results confirm a regulatory role for adenosine homeostasis in the regulation of DNA methylation, and suggest that the enduring seizure suppressing effects of transient adenosine supplementation may be due to restoration of DNA methylation in the hippocampus.

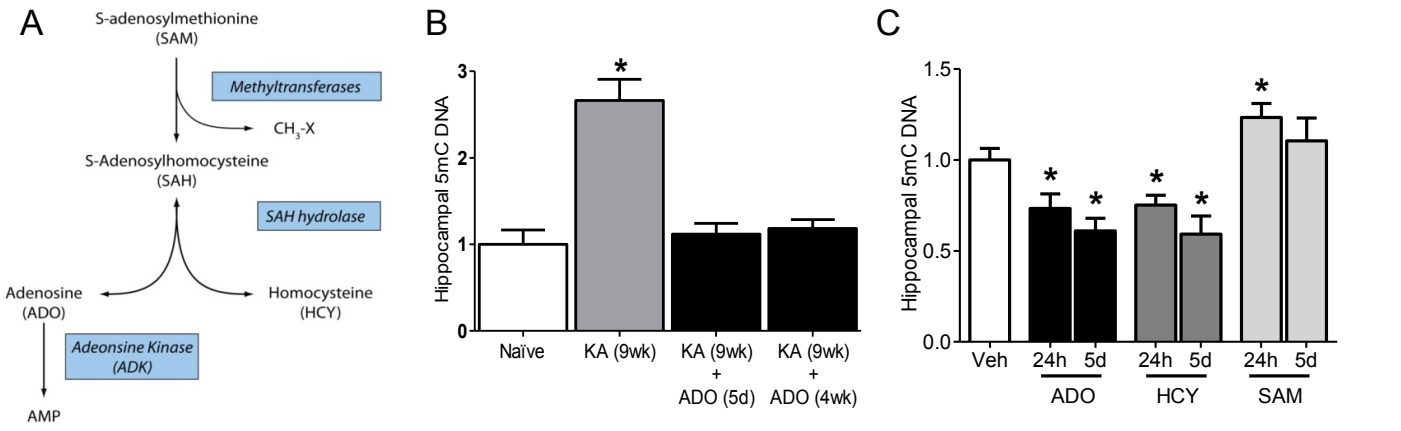

Figure 7: (A) DNA Transmethylation Reaction. (B) Hippocampus from epileptic rats are hypermethylated. (C) Hippocampal DNA methlylation levels is influenced by adenosine and homocysteine, obligatory end products of the transmethylation levels. S-adenosylmethionine, a substrate for the transmethylation reaction, causes hypermethylation.

Major Findings and Conclusions

- A short term dose of adenosine (250 ng/ventricle/d for 10 days) given after the onset of epilepsy in rats completely prevented further disease progression long-term (over 3 months) as evidenced in two independent experimental read-outs: prevention of seizure progression and prevention of mossy fiber sprouting. These findings demonstrate a robust antiepileptogenic effect of adenosine.
- We identified a major underlying mechanism of adenosine-based epileptogenesis: restoration of normal DNA methylation status via a previously unknown adenosine receptor independent effect of adenosine.
- The very positive outcome of these studies motivated us to address the question, whether we could achieve similar antiepileptogenic effect with a less invasive surgical procedure and a shorter duration of active adenosine delivery. In light of future clinical translation we opted for adenosine releasing silk-gel, which we had been developed and tested in parallel. Therefore all subsequent studies were performed with adenosine

releasing silk gel, which permits the focal short-term delivery of a high dose of adenosine with an injectable solution.

Prevention by Focal Adenosine

Task 2c

48 Rats

We next examined the potential for focal adenosine treatment to prevent seizures in a population of rats with a known risk for developing epilepsy (an episode of status epilepticus lasting at least 3 hours), but that had not yet developed spontaneous convulsive seizures. Further, to improve clinical translatability, we used injectable adenosine-releasing silk gel in a less invasive procedure. An additional advantage of adenosine-releasing silk-gel is the opportunity to deliver much higher doses of adenosine over a shorter time frame. This silk gel can be delivered using a 0.04 mm cannula rather than the 1.35 mm cannula required for delivery of the rigid implant. The uniform gel format also improved consistency in the adenosine delivery across implants.

Rats were given two weeks to fully recover from status epilepticus; at that time the animals had been exposed to an epileptogenesis triggering injury, but had not yet developed spontaneous seizures (i.e. 'latent phase of epileptogenesis'). Rats were then divided into two groups: treated with 9 ug adenosine (bilateral gel infusions of 4.5 ug each into the lateral ventricles), and untreated rats. During the same procedure, all rats were fitted with a headset to record cortical EEG. Rats were evaluated during two video-EEG monitoring sessions, during weeks 11-14 after status epilepticus and during weeks 20-24 (Fig. 8A). Seizures were scored by individuals blinded to the experimental conditions. For this initial analysis, seizure quantification was restricted to 8 hours/day. Seizures were first identified using EEG, then scored by video as generalized or partial seizures. Clear evidence of seizure spread beyond the limbic system was required to score a seizure as generalized; absence seizures characterized by a frozen posture and limbic seizures characterized by head nodding and chewing were considered partial. Control rats had an average of 100 – 150 seizures per week for the duration of the recording periods. In contrast, adenosine treated rats had an average of only 5 seizures per week during the first recording period, and only 15 – 65 seizures/week during the later recording period (Fig. 8B) (adenosine treatment significant, $p<0.0001$ by ANOVA), indicating attenuation of the epileptogenic process. Analysis of partial seizures revealed that, during both time windows of analysis, the transient adenosine treatment caused an almost complete

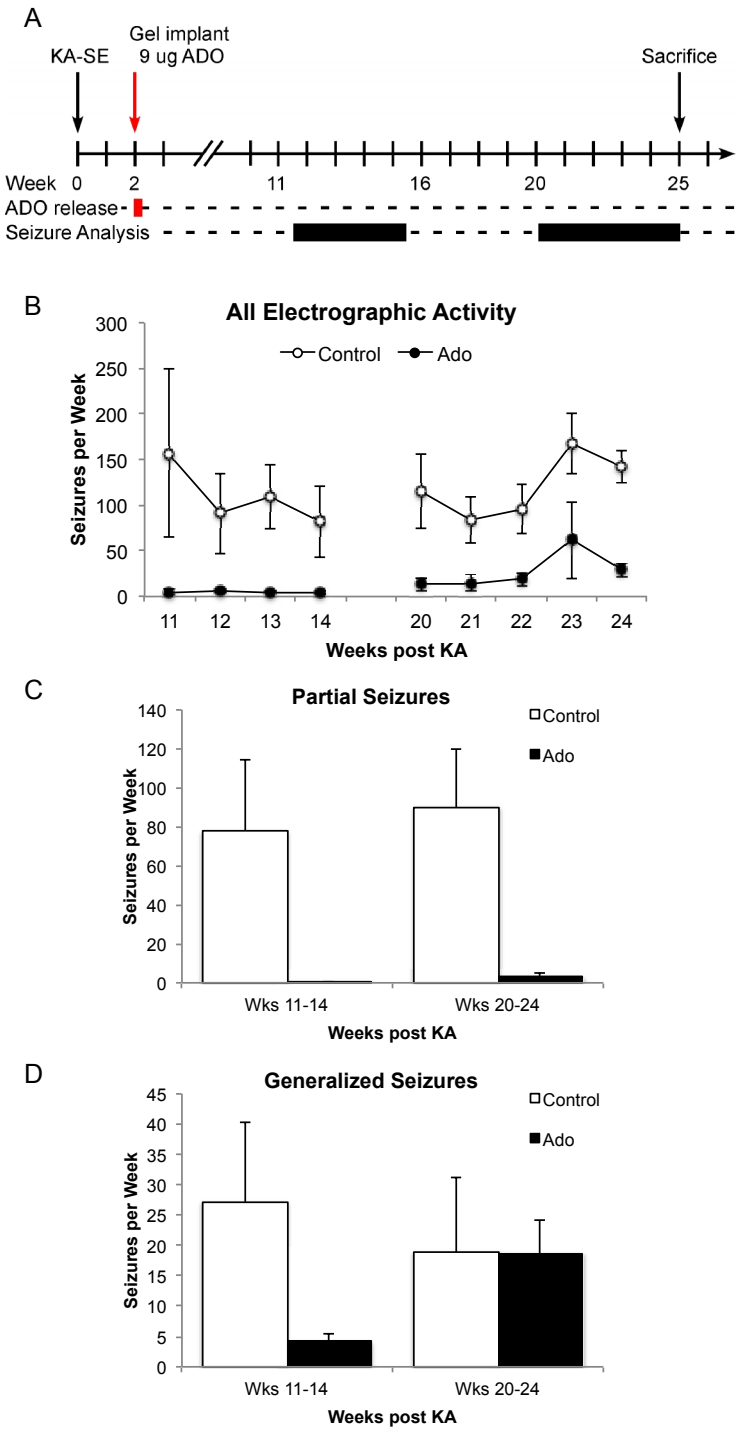

Figure 8: (A) Experimental design. (B) All electrographic activity as assessed by EEG. (C) Partial seizures. (D) Generalized seizures, identified by video analysis.

absence of any partial seizure activity, a major therapeutic outcome that was maintained long-term (treatment significant at $p < 0.005$ by 2-way ANOVA, Fig. 8C). In contrast, convulsive seizure activity was initially reduced, but this effect was not maintained long-term (Fig. 8D).

Major Findings and Conclusions

- An acute bolus of a high dose of adenosine (9 ug) administered during the latent phase of epileptogenesis prevents the development of partial seizures long-term.
- Our data indicate that a transient bolus of adenosine (as opposed to the sustained delivery of adenosine) interferes with a component of epileptogenesis that leads to the development of partial seizure; these findings also suggest that for the long-term prevention of generalized seizures, a prolonged duration of adenosine might be necessary.
- Initial video-EEG analysis was limited to 8 hours/day. However, seizures are known to cluster, and analysis of the full 24 hours/day is ongoing to ensure that the measured effect is not due to missed seizures.

Late Stage Intervention with Adenosine

Task 2b

48 Rats

In the absence of reliable biomarkers it can be difficult to justify an invasive brain procedure based on risk (i.e. 'prophylactic intervention') alone. Therefore, we evaluated the therapeutic efficacy of gel-based adenosine supplementation after the emergence of convulsive seizures (i.e. after the 'diagnosis of epilepsy'). Rats were placed under video monitoring for 8 hours/day starting 2 weeks after status epilepticus. Once a seizure was identified (i.e. 'diagnosis of epilepsy'), rats received a bilateral implant (1.5 ul/ventricle) consisting of either plain silk gel or silk gel with 3 mg/ml adenosine, along with cortical EEG recording electrodes. Rats returned to the colony for 6 weeks, then were placed on video-EEG for seizure analysis (Fig. 9A). Seizures were scored and analyzed according to Task 2C (see above).

In contrast to the rats treated prophylactically with the gel-based adenosine bolus (see Figure 8), we found that treatment with a short-duration adenosine bolus after the emergence of epilepsy did not result in a sustained reduction of generalized (Fig. 9B, $p=0.47$, unpaired t-test) or partial (Fig. 9C, $p=0.49$, unpaired t-test) seizure activities.

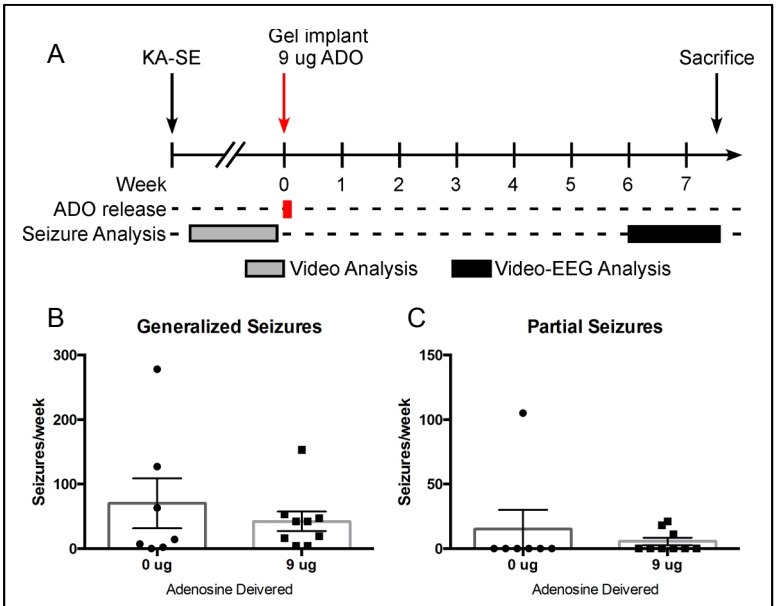

Figure 9: (A) Experimental design. (B) Generalized seizures, identified by video. (C) Partial seizures.

Conclusions and Limitations

- Once generalized seizures occur and chronic epileptic behavior is established, a single short-term bolus of adenosine is not sufficient to alter epileptogenesis; however, as shown in Fig. 5 & 6, the addition of the sustained delivery of a lower dose of adenosine over 10 days is sufficient to prevent epilepsy progression after the onset of epilepsy.
- Our data show a major heterogeneity in seizure outcome in our control group. Data shown are based on video-EEG analysis that was limited to 8 hours/day. However, seizures are known to cluster, and

analysis of the full 24 hours/day is ongoing to ensure that the measured effect is not due to missed seizures.

Key Research Accomplishments

- Development and characterization of two silk-based formulations for the focal delivery of adenosine to the brain.
- Determination that an intraventricular dose range of 200 to 1000 ng adenosine per day effectively suppresses induced epileptic seizures.
- Demonstration that the focal delivery of at least 3000 ng adenosine per day does not trigger any sedative or motor-function related side effects.
- Demonstration that the focal short term delivery of a low dose of adenosine (250 ng/ventricle/day for 10 days) epilepsy progression (after the onset of epilepsy) long-term (for at least 3 months); this robust antiepileptogenic effect became evident by sustained prevention of seizure progression and sustained block of progressive mossy fiber sprouting (a key finding from our histopathological analysis in Task 2d).
- Identification of a novel epigenetic mechanism (reduction of DNA methylation status) by which adenosine prevents epilepsy progression.
- Demonstration that adenosine-releasing silk-gel is a versatile alternative for the prevention of epileptogenesis.
- Demonstration that adenosine therapy is a versatile therapy for the prevention of epilepsy and its progression, when administered either prior to or after the diagnosis of epilepsy.
- Demonstration that a short term bolus of a high dose of adenosine can be used to prevent partial epilepsy.

Reportable Outcomes

Manuscripts

Williams-Karnesky RL, Sandau US, Lusardi TA, Lytle NK, Farrell JM, Pritchard EM, Kaplan DL, Boison D (2013) Epigenetic changes induced by adenosine augmentation therapy prevent epileptogenesis. *Journal of Clinical Investigation* 123(8):3552-63.

Diamond ML, Ritter AC, Jackson EK, Conley YP, Kochanek PM, Boison D, Wagner AK (2015) Genetic Variation in the Adenosine Regulatory Cycle is Associated with Post-traumatic Epilepsy Development. *Epilepsia* 56(8):1198-1206.

Boison D (2013) Adenosine kinase: exploitation for therapeutic gain. *Pharmacological Reviews* 65:906-943.

Invited lectures since August 2013 (D. Boison)

59th Annual Meeting of the Radiation Research Society, New Orleans, LA
Symposium: CNS effects of radiation damage
"Prenatal radiation exposure – a risk factor for the development of epilepsy."

Safar Center for Resuscitation Research, University of Pittsburgh, Pittsburgh, PA
"Translational adenosine research."

Warwick University, School of Life Sciences, Coventry, UK
"Translational adenosine research."

Oregon Health and Science University, Portland, OR
Biology of Neurodegeneration (BOND) interest group
"Comorbidities in Neurology: the search for common mechanisms"

PAME (Partners Against Mortality in Epilepsy) Conference, Minneapolis, MN
Plenary Session: SUDEP Mechanisms: Respiratory
"Adenosine in Brainstem."

Purines 2014 (International Conference on Signaling, Drugs, and Targets), Bonn, Germany
Symposium: Adenosine deaminase and intracellular purine metabolizing enzymes
"Adenosine kinase: exploitation for therapeutic gain."

Twelfth Eilat Conference on New Antiepileptic Drugs (EilatXII), Madrid, Spain
Plenary lecture: *"Adenosine-releasing silk*".

4th Global Symposium on Ketogenic Dietary Therapies, Liverpool, UK
Plenary lecture: *"Adenosine & Epigenetics: Stopping and preventing epilepsy."*

48th Annual Winter Conference on Brain Research, Big Sky, MT
Panel: The science of intractable epilepsy: when small molecules fail.
"Adenosine: seizure suppression and epilepsy prevention."

University of Calgary, Alberta Children's Hospital, Calgary, AB
Developmental Neurosciences Grand Rounds
"EAT: Epigenetics for Antiepileptogenic Therapy".

Oregon Health and Science University, Portland, OR
Biology of Neurodegeneration (BOND) interest group
"EAT: Epigenetics for Antiepileptogenic Therapy".

Antiepileptic Drug and Device Trials XII, Aventura, FL
"Adenosine Augmentation Therapy."

Oregon Health and Science University, Portland, OR
Research Seminar: Department of Anesthesiology and Perioperative Medicine
"Maladaptive changes of adenosine homeostasis after brain trauma."

Spring Hippocampal Research Conference, Taormina, Italy
Session organizer and presenter.
Session: The role of astrocytes in epilepsy
Presentation: *"Astrocyte-driven epigenetic mechanisms of epileptogenesis"*

Neurotrauma 2015; 33rd Annual Symposium of the National Neurotrauma Society, Santa Fe, NM
Symposium: Purines – forgotten mediators in CNS injury
"Role of adenosine in posttraumatic seizures and epilepsy: a potential new target."

UCB Pharma, Braine-l'Alleud, Belgium
Plenary Lecture: *"Therapeutic adenosine augmentation for the prevention of epilepsy."*

Pending Applications

P01 application "EAT – Epigenetics for Antiepileptogenic Therapy" submitted to the NINDS on August 26, 2015; Narrative: *Seizure progression in temporal lobe epilepsy (TLE) can be prevented by a metabolism-based epigenetic mechanism. In our proposed P01 project program **EAT** (Epigenetics for Antiepileptogenic Therapy) leading experts in the areas of metabolism, epigenetics, and epileptogenesis will work together in three coordinated Research Projects and a dedicated Research Core to identify and characterize mechanisms that couple metabolic therapies with substrate-based and epigenetic mechanisms that mediate antiepileptogenic activity in TLE. The expected outcome of this initiative is the translation of metabolism-based antiepileptogenic therapies into novel epigenetic target-based therapeutic approaches for the prevention of epilepsy progression.*

R01 application "Biochemistry of epileptogenesis: therapeutic potential of small molecule drugs" submitted to the NINDS on October 5, 2015; Narrative: "*Clinical as well as experimental evidence demonstrates that*

epilepsy and its progression can be prevented by biochemical manipulations and those that target previously unrecognized epigenetic functions contributing to epilepsy development and maintenance of the epileptic state. Here we will study biochemical interactions between adenosine and glycine that converge on the transmethylation pathway that also controls DNA methylation. Since adenosine and glycine regulating drugs have already been developed and tested in preclinical and clinical settings, research from this application could lead to the fast-paced development of antiepileptogenic treatments that influence the DNA methylome through mass action."

Personnel

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Conclusions and Future Outlook

Acquired epilepsy is a progressive disorder, frequently manifesting many months or years after a precipitating brain injury such as TBI. The progression of epileptogenesis continues even after convulsive seizures begin to manifest, causing increased reliance on antiepileptic drugs and the development of drug resistance for up to 30% of patients. None of the currently used antiepileptic drugs affects the underlying pathogenetic mechanisms of epilepsy and none of those drugs halts or prevents epileptogenesis. Our key finding is the demonstration that a transient dose of adenosine delivered locally via a silk-based brain implant can suppress the development of epilepsy long-term in a rat model of progressive epilepsy. To further improve clinically translatability we developed an injectable silk-based gel formulation to provide a high dose of local adenosine and demonstrated antiepileptogenic efficacy of this new formulation for local adenosine delivery. Clinically, an injectable formulation of adenosine releasing silk will offer a more versatile and less invasive alternative for antiepileptogenic treatment. The translational impact of our studies is high. Focal adenosine augmentation could easily be implemented as a safe treatment option for patients with early signs of epilepsy as well as those at risk of developing epilepsy. For example, therapeutic adenosine augmentation could be used as a preventative measure following severe TBI or following epilepsy surgery, which bears an inherent risk of secondary epileptogenesis. An important aspect is our finding that the transient increase of adenosine provides long-lasting benefit.

Towards the development of small molecule drugs for the prevention of epilepsy At the beginning of this century, adenosine-elevating adenosine kinase (ADK) inhibitors have been the subject of pharmaceutical drug development efforts for the indications seizure suppression in epilepsy, chronic pain, and chronic inflammatory conditions. Although highly effective in rodent models, further clinical development of those agents was limited by toxicity (liver) and sedative side effects associated with the need of chronic sustained drug dosing for those chronic conditions. However, if we determine that the *transient* use of an ADK inhibitor provides long-lasting therapeutic benefits, then ADK inhibitors might have a future for further clinical drug development. To test this possibility, we already initiated new studies to generate preliminary data in support of the short-term use of ADK inhibitors for the prevention of epilepsy.

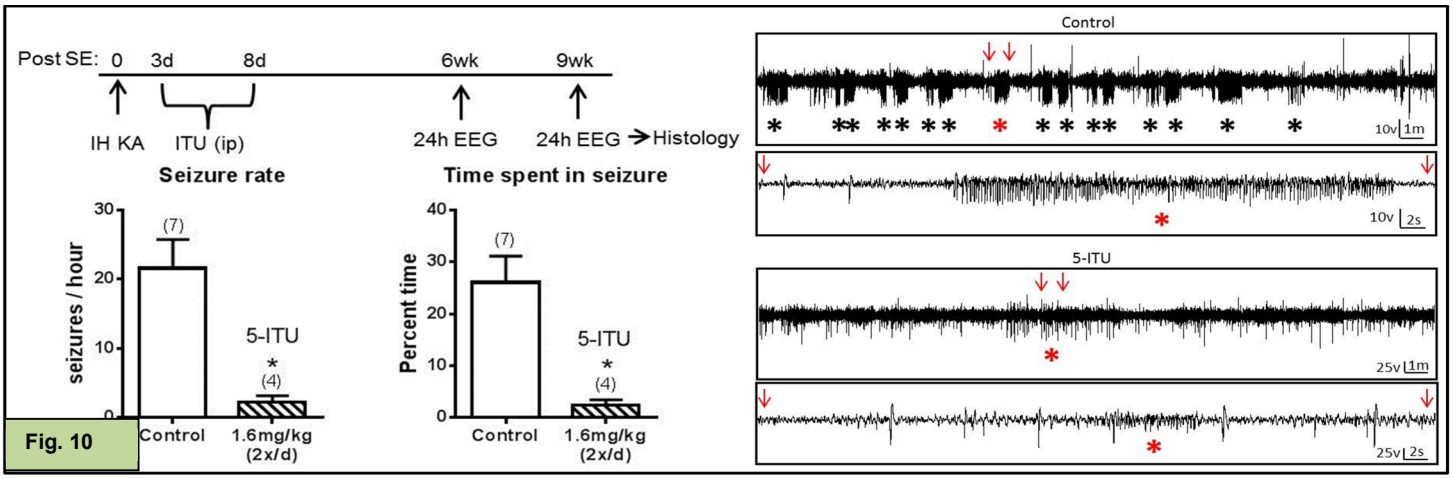

Fig. 10

Prevention of epileptogenesis through transient ADK inhibition Excitingly, our preliminary data **(Fig. 10)** show that the transient and systemic use of a small-molecule ADK inhibitor (5-iodotubercidin; 5-ITU) prevents epileptogenesis in the intrahippocampal KA model of TLE. Three days after intrahippocampal KA-induced SE in mice, 5-ITU was injected i.p. at a dose of 1.6 mg/kg bid for a restricted time span of 5 d. Six and 9 wks after the SE, seizures were quantified by intrahippocampal EEGs. While epileptic control animals (n=7), which received vehicle instead of 5-ITU, had developed a typical seizure rate of >20 seizures per hour, in the animals transiently exposed to an ADK inhibitor (n=4) both the seizure rate as well as the total time spent in seizures were almost completely abolished ($P<0.05$) **(Fig. 10).** Representative 30 min EEG sections (top rows) show 16 and 1 seizures marked by astrisks in the control or 5-ITU-treated animals, respectively. The bottom rows show individual seizures (marked by red asterisks) at higher resolution. *These data show that the transient systemic use of a small molecule ADK inhibitor can prevent epileptogenesis.*

Future directions

Since a systemic short term treatment with an ADK inhibitor effectively prevents epilepsy in a clinically relevant model of temporal lobe epilepsy, our next goal is to focus on the development of the next generation of ADK inhibitors and to perform preclinical efficacy and toxicity studies in order to prepare for future clinical implementation of antiepileptogenic therapies. We are currently seeking out funding opportunities to enable such studies.

Epigenetic changes induced by adenosine augmentation therapy prevent epileptogenesis

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Epigenetic modifications, including changes in DNA methylation, lead to altered gene expression and thus may underlie epileptogenesis via induction of permanent changes in neuronal excitability. Therapies that could inhibit or reverse these changes may be highly effective in halting disease progression. Here we identify an epigenetic function of the brain's endogenous anticonvulsant adenosine, showing that this compound induces hypomethylation of DNA via biochemical interference with the transmethylation pathway. We show that inhibition of DNA methylation inhibited epileptogenesis in multiple seizure models. Using a rat model of temporal lobe epilepsy, we identified an increase in hippocampal DNA methylation, which correlates with increased DNA methyltransferase activity, disruption of adenosine homeostasis, and spontaneous recurrent seizures. Finally, we used bioengineered silk implants to deliver a defined dose of adenosine over 10 days to the brains of epileptic rats. This transient therapeutic intervention reversed the DNA hypermethylation seen in the epileptic brain, inhibited sprouting of mossy fibers in the hippocampus, and prevented the progression of epilepsy for at least 3 months. These data demonstrate that pathological changes in DNA methylation homeostasis may underlie epileptogenesis and reversal of these epigenetic changes with adenosine augmentation therapy may halt disease progression.

Introduction

Epilepsy is the third most common neurological disorder, affecting nearly 50 million people worldwide. Despite decades of research, satisfactory seizure suppression is still only achieved in just over half of affected individuals. Current antiepileptic therapies fail to address the underlying causes of epilepsy and do not halt epileptogenesis (1). Epileptogenesis is characterized by a progressive increase in frequency and severity of spontaneous recurrent seizures (SRS). Several mechanisms are thought to be implicated in the epileptogenic cascade, including neuroinflammatory responses, selective neuronal cell loss, mossy fiber sprouting, aberrant connectivity, and gliosis coupled with adenosine (ADO) dysfunction. One potential unifying factor behind many of the pathological changes in epileptogenesis may be epigenetic modifications, which are likely further potentiated by epileptogenesis itself (2, 3). Epigenetic modifications, which alter gene transcription without modifying the underlying DNA sequence, are highly plastic and can respond rapidly to environmental cues, an important endogenous mechanism for temporally and spatially controlling gene expression. Changes in histone acetylation and methylation as well as changes in DNA methylation, once thought to occur only in dividing cells, have been shown to also occur in mature cells in the CNS (4, 5). Tellingly, these changes occur regularly and rapidly. Even a single episode of neural synchronization exceeding 30 seconds in the hippocampus induces DNA methylation-dependent alterations in transcription of immediate early genes and initiates a cascade of transcription factors, contributing to long-term neuronal and circuit alterations (6).

Methylation of DNA in the CNS has attracted increasing attention recently, with new research showing activity-induced proliferation of neural precursor cells via active DNA demethylation (5). Altered DNA methylation in the brain has also been implicated in psychiatric and neurological conditions, including epilepsy (5, 7). The methylation hypothesis of epileptogenesis suggests that seizures by themselves can induce epigenetic chromatin modifications and thereby aggravate the epileptogenic condition (2). Despite new insights into the role of pathological DNA methylation changes in disease and the fact that 2 DNA methyltransferase (DNMT) inhibitors are currently FDA approved (azacytidine and decitabine), direct manipulation of DNA methylation has not been tested in human epilepsy or in animal models of the disease (8).

DNA methylation requires the donation of a methyl group from S-adenosylmethionine (SAM), a process that is facilitated by DNMT enzymes (Figure 1A). The resulting product, S-adenosylhomocysteine (SAH), is then further converted into ADO and homocysteine (HCY) by SAH hydrolase. Critically, the equilibrium constant of the SAH hydrolase enzyme lies in the direction of SAH formation (9); therefore, the reaction will only proceed when ADO and HCY are constantly removed (9, 10). In the adult brain, removal of ADO occurs largely via the astrocyte-based enzyme ADO kinase (ADK) (11–13). If metabolic clearance of ADO through ADK is impaired, SAH levels rise (10). SAH in turn is known to inhibit DNMTs through product inhibition (14).

ADO is an endogenous anticonvulsant in the brain (15) acting via activation of pre- and postsynaptic ADO A_1 receptors (A_1R) to decrease neuronal excitability (16, 17). The ambient tone of ADO is determined by neuronal ADO release (18) and ADK-driven reuptake through equilibrative nucleoside transporters in astrocytes, which form a "sink" for ADO (19). Since disruption of ADO homeostasis and ADO deficiency has been implicated in epileptogenesis, local therapeutic ADO augmentation is an effective strategy to acutely

Authorship note: Rebecca L. Williams-Karnesky, Ursula S. Sandau, and Theresa A. Lusardi contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

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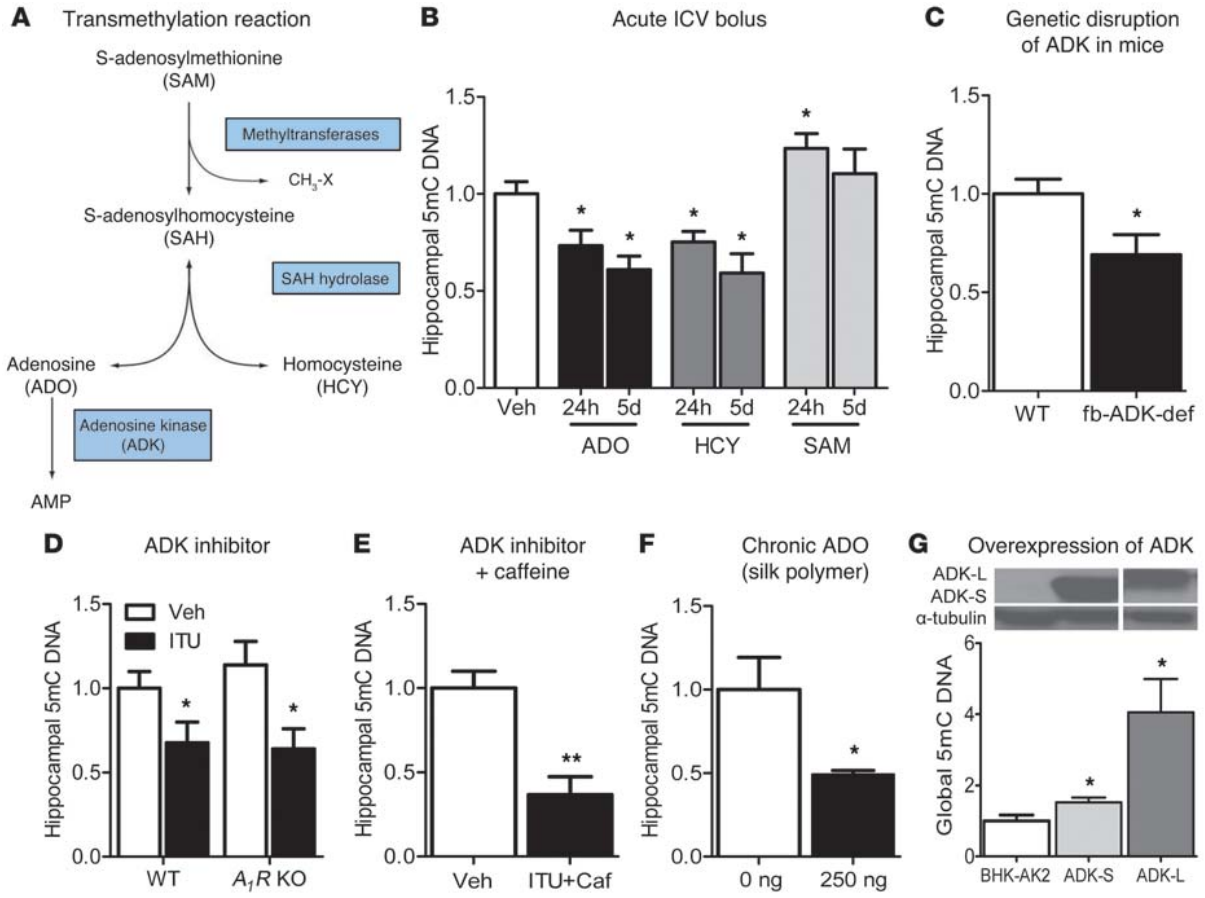

Figure 1

ADO regulates DNA methylation through interference with the transmethylation pathway. (**A**) Biochemistry of the transmethylation reaction. (**B**) A single i.c.v. bolus of ADO (5 μg) or HCY (250 μg) decreases global DNA methylation in the hippocampus at 24 hours and 5 days following the injection, whereas SAM (16 μg) increases global DNA methylation at 24 hours. (**C**) Reduced ADK expression leads to a decrease in hippocampal DNA methylation in vivo as seen in transgenic fb-Adk-def mice with a forebrain-selective reduction of ADK. (**D**) The ADK inhibitor 5-ITU (3.1 mg/kg, i.p. once daily for 5 days) reduces global DNA methylation in hippocampus of WT and homozygous ADO *A1R*-KO mice. (**E**) Coadministration of the nonselective ADO receptor antagonist caffeine (Caf, 25 mg/kg, i.p.) and ITU reduces hippocampal DNA methylation in WT mice. (**F**) Intraventricular ADO-releasing silk (250 ng/d) decreases hippocampal DNA methylation in naive rats 5 days after implantation. (**G**) Overexpression of ADK leads to DNA hypermethylation in ADK-deficient BHK cells (BHK-AK2). Western blot shows protein expression from 3 pooled experimental replicates of BHK-AK2 cells transfected with the cytoplasmic (ADK-S) or nuclear (ADK-L) isoform of ADK and nontransfected control cells. Quantification of DNA methylation was assessed using 3 experimental replicates. ADK-L increases DNA methylation to a greater extent than ADK-S. Data are displayed as average ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 4–9$.

suppress seizures in modeled epilepsy (20). However, possible epigenetic effects of ADO augmentation in the treatment of epilepsy, including the potential to modulate DNA methylation status, have not been studied to date. Based on ADO's role as an obligatory end product of DNA methylation, we hypothesized that an increase in ADK and the resulting decrease in ADO, as seen in chronic epilepsy (21, 22), would lead to an increase in global DNA methylation in the brain. Further, we hypothesized that therapeutic ADO augmentation might be an effective strategy to reverse this pathological DNA hypermethylation and thereby prevent the progression of epilepsy.

Results

Increased ADO and reduced ADK expression induce DNA hypomethylation in the brain via interference with the transmethylation pathway. To provide mechanistic evidence that ADO contributes to the regulation of DNA methylation in the brain, we used a variety of techniques to manipulate ADO. To identify the role metabolic intermediates play in vivo to regulate DNA methylation, we administered a single intracerebroventricular (i.c.v.) bolus of either ADO, HCY, or SAM (Figure 1, A and B). ADO and HCY, both end products in the transmethylation pathway, significantly decreased global DNA methylation in the hippocampus within 24 hours, an effect that was maintained for at least 5 days after infusion (Figure 1B). Conversely, injection of SAM, the primary methyl donor for transmethylation reactions, transiently increased global methylation by 24% at 24 hours (Figure 1B).

We next investigated whether changes in endogenous ADK expression might modulate DNA methylation in the brain. First, we examined transgenic mice (fb-Adk-def mice) with a forebrain-selective reduction of ADK expression (21). We predicted that the resulting 3.3-fold increase in hippocampal ADO concentration (23) would suppress transmethylation and result in decreased DNA methylation. Indeed, a significant 31% decrease in global DNA methylation was seen in hippocampal isolates from fb-Adk-def

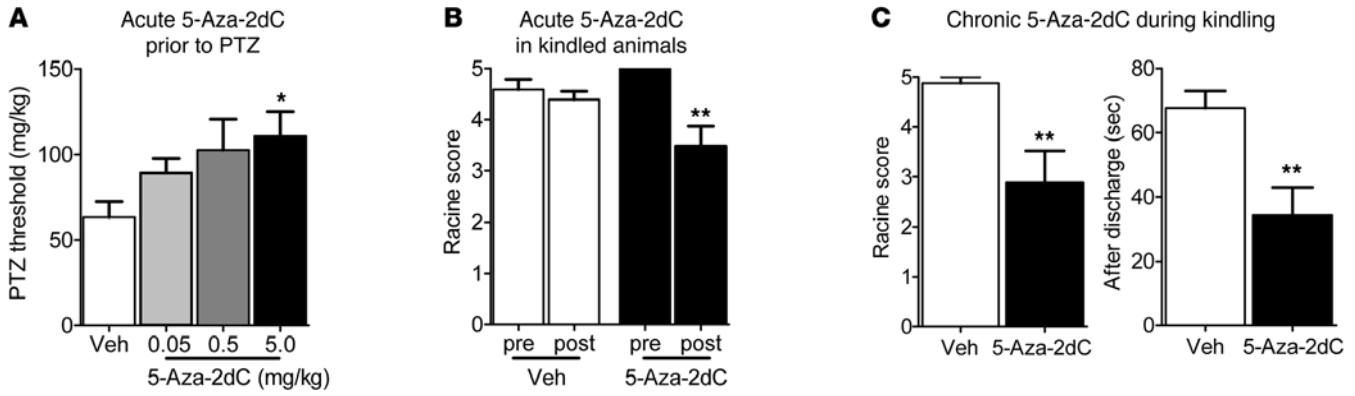

Figure 2

The DNMT inhibitor 5-Aza-2dC attenuates ictogenesis and epileptogenesis. (**A**) Dose response of WT mice pretreated with 5-Aza-2dC (0.05, 0.5, 5.0 mg/kg) 10 minutes prior to continuous PTZ infusion shows a significant increase in seizure threshold with the high-dose 5-Aza-2dC (5.0 mg/kg) compared with saline-treated controls. (**B**) A single injection of 5-Aza-2dC administered to fully kindled rats significantly reduces the average Racine score in subsequent stimulations (post–5-Aza-2dC) compared with the average Racine score prior to 5-Aza-2dC treatment (pre–5-Aza-2dC). There was no difference in the average Racine score in the rats prior to or following vehicle injection. (**C**) Rats kindled while being chronically treated with 5-Aza-2dC have a significant decrease in the average Racine score and after discharge duration compared with control kindled rats in response to a test stimulation administered after an 11-day stimulus- and drug-free period. Data are displayed as the average ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. $n = 6–9$.

mice (Figure 1C). Likewise, chronic administration of the ADK inhibitor 5-iodotubercidin (5-ITU) (3.1 mg/kg i.p. once every day, for 5 days) led to a significant (35%) decrease in global DNA methylation in the hippocampus of WT mice (Figure 1D). Importantly 5-ITU–dependent hypomethylation was maintained in mice with a genetic disruption of the ADO A_1 receptor (*A1R* KO), indicating that activation of the key receptor responsible for the anticonvulsant effects of ADO (24) is not required for the induction of ADO-induced hypomethylation (Figure 1D). To further demonstrate the biochemical basis of methylation interference (Figure 1D) and independence of ADO receptors, we coadministered the nonselective ADO receptor antagonist caffeine with ITU, which likewise resulted in a robust decrease in hippocampal DNA methylation (Figure 1E). Together, these findings show that modulating ADO tone either directly or via modulation of ADK expression can affect DNA methylation status in the hippocampus. In addition, our findings demonstrate what we believe is a novel ADO receptor–independent function of ADO, which acts by direct biochemical interference with the transmethylation pathway.

The nuclear isoform of ADK plays a key role in the induction of DNA hypermethylation. Mammalian ADK exists in 2 alternatively spliced isoforms, ADK long (ADK-L) and ADK short (ADK-S), which reside in the nucleus and cytoplasm, respectively (25). To investigate whether the nuclear isoform of ADK plays a unique role in the regulation of DNA methylation, we transfected cultured *Adk*-deficient BHK-AK2 cells (26) separately with an expression plasmid for either ADK-L or ADK-S and quantified global DNA methylation. Compared with the parental BHK-AK2 cells, recipients of ADK-L showed a robust 400% increase in global DNA methylation, whereas recipients of ADK-S showed only a modest 50% increase in global DNA methylation (Figure 1G). These results demonstrate that, while increases of both isoforms of ADK lead to increases in global DNA methylation, the nuclear isoform appears to be more effective in the regulation of DNA methylation status, suggesting the existence of cell-autonomous (nuclear ADK) and non–cell-autonomous (cytoplasmic ADK) effects of ADK.

Therapeutic delivery of ADO modulates DNA methylation. To investigate the therapeutic potential of ADO, we used ADO-releasing silk-based polymer implants to alter DNA methylation. We previously generated and characterized silk-based biodegradable brain implants able to deliver local doses of 8 to 1000 ng ADO per day (27, 28). These implants successfully suppressed seizures in kindled rats (27) with no adverse effects. Here, we used implants designed to release a controlled dose of 250 ng ADO per implant per day during a restricted time frame of 10 days (27). Five days following bilateral intraventricular implantation of ADO-releasing polymers in naive animals, global DNA methylation was significantly reduced (by 51%) in the hippocampus when compared with that of animals receiving control polymers (Figure 1F). These data suggest that ADO-releasing polymers could be used as a therapeutic delivery device to modulate DNA methylation in vivo.

Inhibition of DNA methylation attenuates seizures and kindling-induced epileptogenesis. We have previously shown that increased ADK expression and the resulting decrease in ADO tone within the cortex and hippocampus are sufficient triggers for spontaneous focal seizures independent of an acute injury (21). Here, we establish that these conditions contribute to increased DNA methylation (Figure 1). Thus, we sought to determine whether changes in DNA methylation contribute to seizure susceptibility and epilepsy development. To address this question, we first performed a dose response study with the DNMT inhibitor 5-Aza-2′deoxycytidine (5-Aza-2dC) in a timed pentylenetetrazol (PTZ) seizure threshold test. WT mice treated with the highest dose of 5-Aza-2dC (5.0 mg/kg, i.v.) 10 minutes prior to continuous PTZ infusion had a significant delay in latency to the extensor phase of seizures (Figure 2A). Similarly, in fully kindled rats, an acute bolus of 5-Aza-2dC significantly attenuated the average Racine score to 3.5 compared with the reproducible Racine stage-5 seizures induced either prior to drug injection (pre–5-Aza-2dC) or in vehicle-treated controls (Figure 2B). Next, we assessed whether inhibiting DNMT activity during kindling acquisition would suppress epileptogenesis. Rats that were treated with 5-Aza-2dC throughout the kindling paradigm

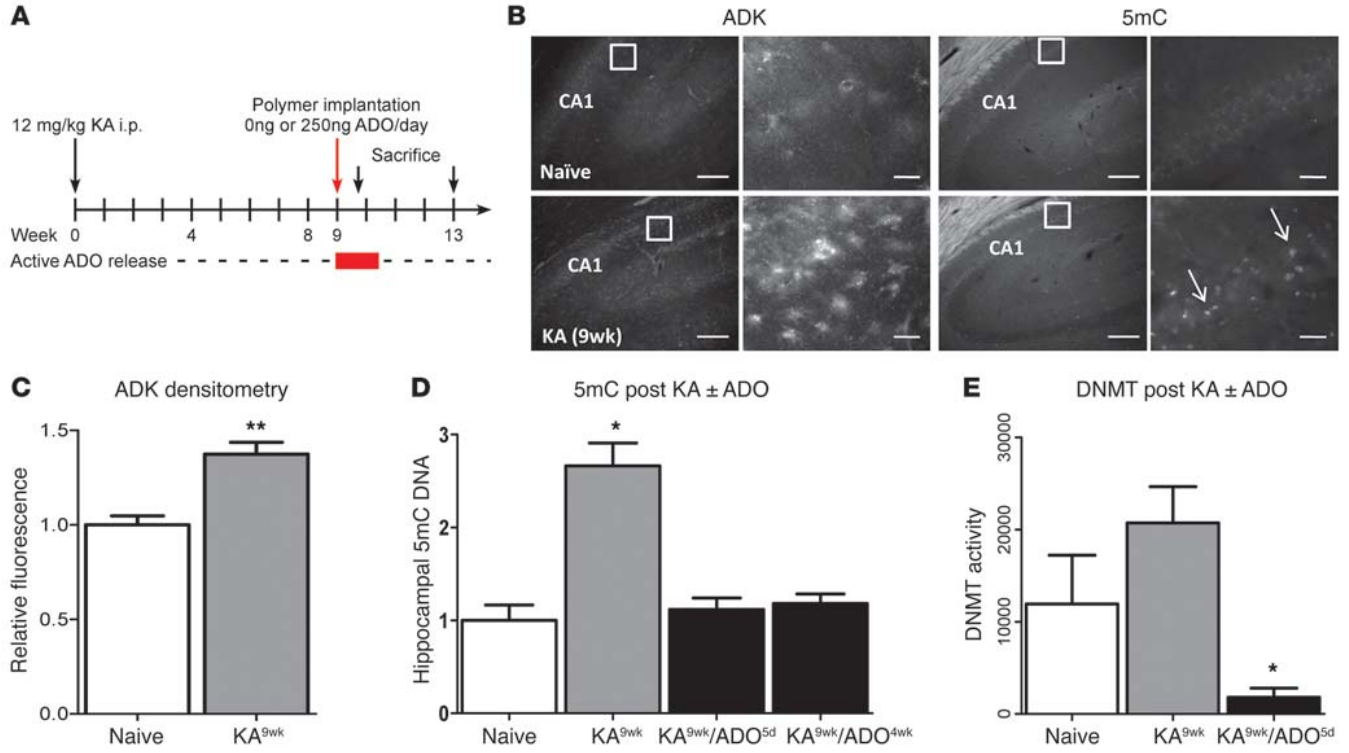

Figure 3

Intraventricular implants of ADO-releasing silk reverse DNA hypermethylation in the epileptic brain. (**A**) Experimental time line. (**B**) Hippocampal immunofluorescence images showing staining of ADK (left), and 5mC immunoreactivity (right) at low and high magnification in a naive rat (upper panels) and an epileptic rat at KA^{9wk} injection (lower panels). Note the regional match (in CA1) of ADK and 5mC (see arrows) immunoreactivity intensities. The white boxes in the low magnification images demarcate the region depicted at high magnification. Scale bars = 150 μm and 15 μm (low and high magnification, respectively). (**C**) Quantification of ADK immunofluorescence indicates a significant upregulation of ADK in the CA1 subregion of the hippocampus at KA^{9wk}, compared with naive controls. (**D**) ADO-releasing silk attenuates the KA-induced increase in hippocampal DNA methylation in epileptic rats. Note that hippocampal DNA methylation, as quantified by ELISA, in ADO polymer–treated KA rats was reduced to naive levels. This reduction in hypermethylation persisted for at least 4 weeks following cessation of ADO release from the polymers. (**E**) DNMT activity increases in epileptic animals 9 weeks after systemic KA, an effect that was strongly and significantly suppressed by ADO released from the polymer. Data are displayed as average ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. $n = 3–8$.

had a significantly reduced average Racine score after receiving a single test stimulation compared with that of saline-treated controls. Furthermore, the average after-discharge duration was reduced by 51% in animals kindled in the presence of 5-Aza-2dC (Figure 2C). Although these experiments are limited to the use of only 1 DNMT inhibitor, which may also exhibit additional off-target effects, these data suggest that inhibition of DNMT activity reduces seizure susceptibility and epilepsy acquisition.

Pathological ADK overexpression in the epileptic hippocampus correlates with DNA hypermethylation. Astrogliosis-associated increases in ADK expression and resulting ADO deficiency have been independently identified as pathological hallmarks of the epileptic brain (21, 22). Based on our findings linking the ADO tone to the global DNA methylation status, we predicted that increased ADK expression in epilepsy would lead to increased DNA methylation. To investigate this hypothesis, we employed a model of temporal lobe epilepsy (TLE) in rats characterized by the development of SRS triggered by systemic kainic acid–induced (KA-induced) status epilepticus (SE) (Figure 3A). Using immunohistochemical methods, we compared ADK and 5-methylcytidine (5mC) expression patterns found in the hippocampus of naive rats and rats sacrificed 9 weeks after the induction of epilepsy (Figure 3B). As predicted (21, 29), astrocytic ADK immunoreactivity was increased throughout the hippocampal formation with highest increases (37%) found near CA1 (Figure 3, B and C). In line with increased ADK and reduced ADO, we also found increased 5mC immunoreactivity in the epileptic hippocampus, most prominently seen in and near CA1 (Figure 3B). The spatial match of ADK overexpression with increased 5mC immunoreactivity suggests a functional interaction between ADK and DNA methylation status. Overexpression of ADK in astrocytes and DNA methylation changes in neurons suggests a non–cell-autonomous effect of ADO, which is also supported by our interference experiments with the transmethylation pathway (Figure 1).

Intraventricular implants of ADO-releasing silk reduce DNA hypermethylation in the epileptic brain. To determine whether transient ADO delivery could reduce DNA methylation in the epileptic brain, we implanted ADO-releasing polymers, which reduce DNA methylation in naive rats (Figure 1F), into the brain ventricles of epileptic animals at 9 weeks after KA (KA^{9wk}) (Figure 3A). Global DNA methylation in whole hippocampal isolates was increased at KA^{9wk} injection compared with that in naive animals (166%; $P = 0.012$) (Figure 3D). In contrast, on day 5 of ADO treatment, DNA methylation levels were restored to the naive state in epileptic rats with ADO polymer ($P = 0.60$) (Figure 3D). Importantly, this change

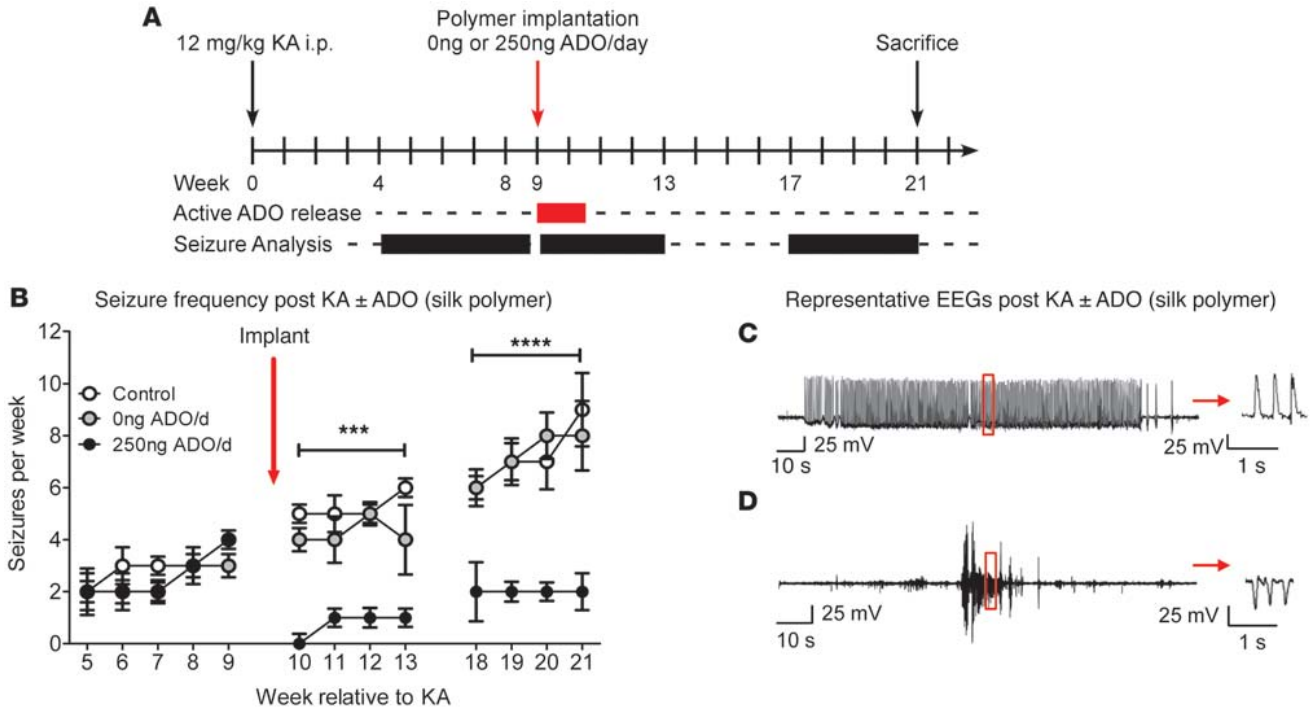

Figure 4

ADO-releasing silk polymers prevent progression of epilepsy development. (**A**) Experimental design. (**B**) Prevention of epileptogenesis by ADO-releasing silk. At KA^{9wk}, epileptic rats received bilateral intraventricular implants of silk polymers releasing either 250 ng (black circles) or 0 ng (gray circles) ADO/d, or underwent sham surgery (white circles). Polymers transiently released a therapeutic dose of ADO for 10 days. Seizures were monitored by video analysis, and weekly seizure numbers are presented. Data are displayed as average ± SEM, $n = 8$ for ADO-implant recipients and 5–8 for control groups. Significant differences are represented between group comparisons (ADO vs. controls) as analyzed by a 2-way ANOVA. ***$P < 0.001$; ****$P < 0.0001$. (**C**) Representative hippocampal EEG trace showing a 2-minute seizure in an animal that received control polymer (0 ng ADO/d) 9 weeks after systemic administration of KA. (**D**) Example of an epileptiform burst in an animal that received ADO-releasing polymer (250 ng/d) at KA^{9wk}. Note, these events were rare in ADO-treated animals and lasted less than 15 seconds. Higher resolution traces (regions demarcated by red boxes in **C** and **D**) are depicted to the right of the red arrows.

persisted for at least 3 weeks after cessation of ADO release from the polymers (4 weeks after implantation) (Figure 3D). These data suggest that a transient dose of ADO delivered locally can have a long-lasting effect on DNA methylation status. To understand the mechanism by which ADO augmentation changes DNA methylation status, we quantified the enzymatic activity of DNMT in epileptic rats. Nine weeks following the systemic injection of KA, DNMT activity in the epileptic animals was elevated almost 2-fold (174%) compared with sham-injected nonepileptic control animals (Figure 3E), consistent with hypermethylation of hippocampal DNA in those animals (Figure 3, B and D). At 5 days of active ADO release, DNMT activity was almost completely blocked (15%; $P < 0.03$) in the epileptic rats (Figure 3E), consistent with restoration of normal DNA methylation status in these animals (Figure 3D).

ADO-releasing silk prevents progression of epilepsy development. Since seizure susceptibility and epilepsy development are partially dependent on changes in DNA methylation (Figure 2), we hypothesized that blocking pathological increases of DNA methylation with ADO therapy could halt long-term epilepsy progression. Because epileptogenesis is a lifelong process that continues after onset of the first SRS and leads to a progression in seizure frequency and severity (30), previous studies aimed at identifying antiepileptogenic drugs were frequently confounded by early initiation of treatment (1). Therefore, to rigorously test the antiepileptogenic potential of transient ADO therapy, we initiated treatment in "early epilepsy" after the onset of SRS using the systemic KA model of TLE (Figure 4A). Epilepsy progression was continuously monitored (24/7) from weeks 5–9 following systemic KA administration. Continuous epileptogenesis was reflected by a progressive increase in the number of seizures after initial SE (control animals, Figure 4B and ref. 31). Epileptic animals (9 weeks after SE; >10 SRS) subsequently received polymer implants that release ADO for a limited duration of 10 days (Figure 4A and ref. 27). Following polymer implantation, epilepsy progression was monitored in two 4-week recording sessions from weeks 10–13 and weeks 18–21 (Figure 4A). As expected, ADO-releasing polymers almost completely prevented any seizures during the first week following implantation (Figure 4B). Remarkably, reduced seizure activity was maintained far beyond the time window of active ADO release (first 10 days) up to at least 12 weeks after polymer implantation (75% reduction of SRS incidence, 250 ng vs. 0 ng ADO/d; $P < 0.001$) (Figure 4B). Importantly, during weeks 18–21 following KA, animals that were transiently exposed to ADO did not show a significant ($P > 0.05$) increase in seizure frequency, while control animals continued to worsen and 3 died due to excessive seizures. Together, these data demonstrate a potent antiepileptogenic role of transient focal ADO delivery. EEG recordings were performed in a separate cohort of animals to avoid potential confounds on DNA methylation analysis and histopathology. Those animals received intrahippocampal and cortical EEG recording electrodes during

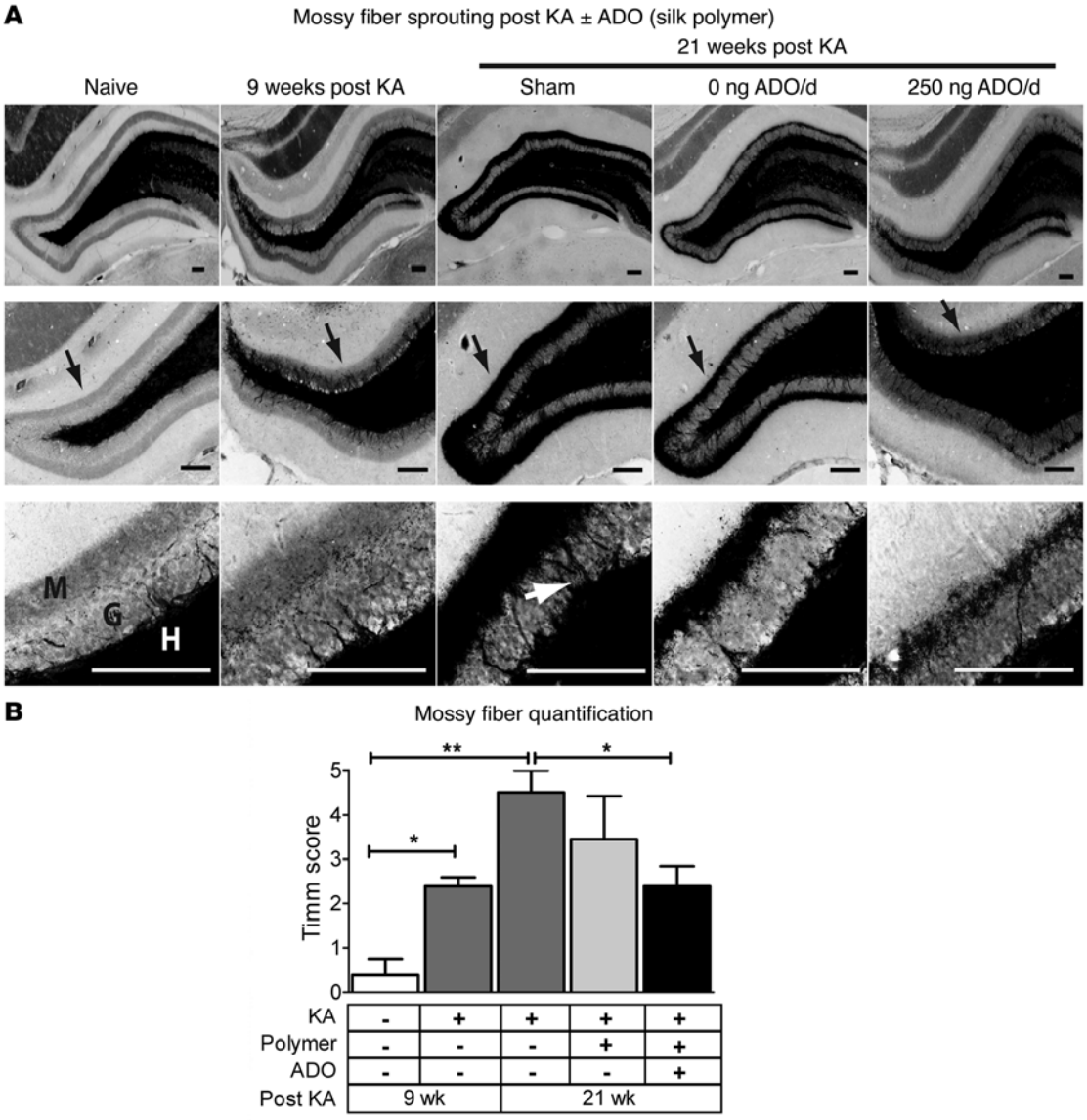

Figure 5

ADO-releasing silk implants prevent mossy fiber sprouting. (**A**) Transient ADO augmentation therapy in epileptic rats prevents mossy fiber sprouting in the dentate gyrus. Top row: representative low magnification images of Timm staining of mossy fibers in hippocampus in naive animals, KA^{9wk} animals, animals 21 weeks after KA (sham), and animals 21 weeks after KA that received polymers releasing either 0 ng (control) or 250 ng ADO/d for a duration of 10 days (implanted at KA^{9wk}). Middle row: higher magnification images that depict Timm granules (black arrows) that correspond to mossy fiber synaptic terminals present in animals 21 weeks after KA (sham and 0 ng ADO/d animals but not in the 250 ng ADO/d animals). Bottom row: high magnification images that illustrate extensive sprouting of mossy fiber axons in animals 21 weeks after KA (white arrow). M, molecular layer; G, granular layer; H, hilus. Original magnification, ×5 (top row); ×10 (middle row); ×40 (bottom row). Scale bars: 500 μm. (**B**) Quantitative analysis of Timm staining shows that transient ADO delivery significantly reduced mossy fiber sprouting compared with that in animals 21 seconds after KA (sham) within the 3-month time span between weeks 9 and 21. Data represent group average Timm score in hippocampus and are displayed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. $n = 3$ for all groups.

the polymer implantation surgery. Electrographic seizures were monitored in these animals from week 10–13 after KA. Whereas sham or control-polymer–receiving animals displayed robust seizures in the EEG (Figure 4C), seizure activity was markedly attenuated in recipients of the ADO-releasing silk polymers (Figure 4D).

ADO-releasing silk implants prevent mossy fiber sprouting. To provide an independent outcome measure for the antiepileptogenic role of silk-based ADO delivery, we assessed the degree of granule cell axon (mossy fiber) sprouting (Figure 5A). Mossy fiber sprouting is thought to be a fundamental epileptogenic mechanism responsible for the formation of new recurrent excitatory circuits in the dentate gyrus (32). Nine weeks after SE, epileptic rats showed a significant increase in mossy fiber sprouting when compared with naive control animals, with visible axons beginning to spread from the hilar layer into the granular cell layer (Figure 5A). In sham-treated animals, mossy fiber sprouting was progressive; at 21 weeks after KA, axon sprouting increased and Timm granules, which correspond to mossy fiber synaptic terminals, presented

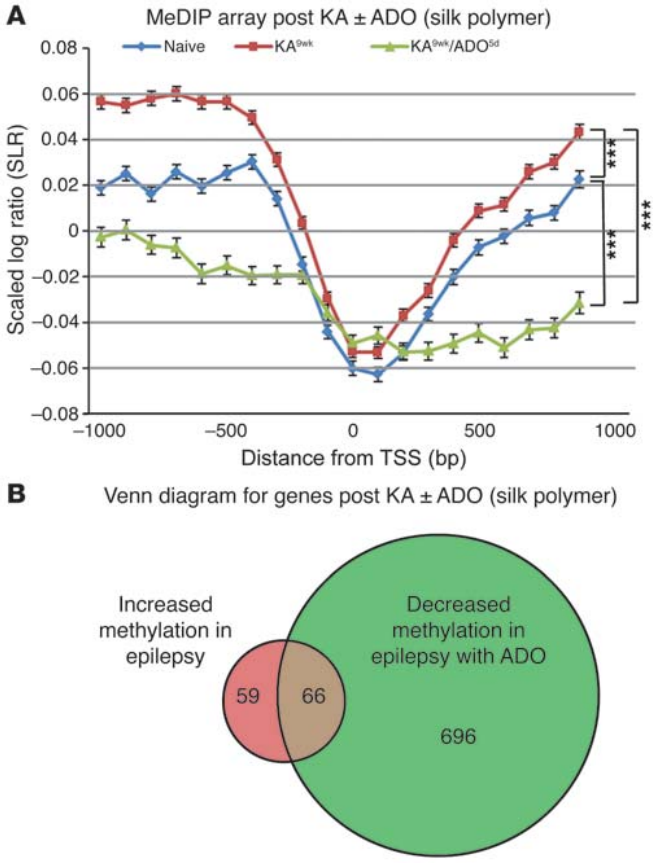

Figure 6

Whole-genome regulation of DNA methylation by ADO. (**A**) Genome-wide analysis of hippocampal DNA extracts shows increased DNA methylation in the epileptic brain (red) and a robust decrease in DNA methylation in response to ADO treatment (green). Pooled (n = 3–4/group) genomic DNA extracts from naive (blue), epileptic (KA^{9wk}) and ADO-treated epileptic (KA^{9wk}/ADO^{5d}) rats were subjected to a MeDIP array followed by a comparison of the average ± SEM SLR of all probes within 1000 bp of the TSS. Note that with exception to the –100 to 100 bp range, active ADO release reduces DNA methylation in the otherwise hypermethylated epileptic hippocampus. Repeated measures ANOVA (Statview) demonstrated a significant effect of the experimental group ($P < 0.0001$) and distance from TSS ($P < 0.0001$) as well as a significant interaction between experimental group and distance from TSS ($P < 0.0001$). Post hoc tests (Sheffe's F) demonstrate that there are significant differences among the 3 experimental groups (*$P < 0.0001$). (**B**) Venn diagram for the number of genes where the DNA methylation is (a) increased in epilepsy (125; red) or decreased with ADO treatment (762, green); and (b) both increased in epilepsy and decreased in ADO treatment (66, overlap). The criterion for genes to be included in the Venn diagram is that 25% of probes within ± 1000 bp of the TSS exceed a dSLR greater than 1.0 for KA^{9wk} vs. naive or less than –1.0 for KA^{9wk}/ADO^{5d} vs. KA^{9wk}.

throughout the molecular layer of the dentate gyrus (Figure 5B). In stark contrast, animals that had transiently been exposed to ADO at 9 weeks after SE had no significant change in mossy fiber sprouting 12 weeks later (Figure 5, A and B). Thus, transient exposure to ADO prevented further mossy fiber sprouting, a major contributor to disease progression in epilepsy.

ADO is a homeostatic regulator of global DNA methylation. Our data demonstrated that ADO homeostasis affects global DNA methylation through interference with the transmethylation pathway (Figure 1) in a non–cell-autonomous (Figure 1, B and F, and Figure 3B) and ADO receptor independent (Figure 1, D and E) manner. Importantly, transient ADO augmentation also reduced global DNA methylation status in epileptic animals (Figure 3D). Our initial ELISA-based data sets demonstrate that ADO exerts homeostatic control over the global DNA methylation status. To validate those findings with an independent experimental strategy, we performed a methylated DNA immunoprecipitation on ChIP assay (MeDIP-on-ChIP) and compared the relative fraction of methylated and unmethylated genomic regions (log ratio) in pooled samples from KA^{9wk}, rats exposed to 5 days of ADO delivery 9 weeks after KA (KA^{9wk}/ADO^{5d}), and naive rats. Array-wide, scaled log ratio (SLR) values were normally distributed and centered at 0. We restricted our array analysis to probes within 1,000 bps of a transcription start site (TSS) according to the Rat RGSC 3.4 assembly, as there was consistent probe representation on the array both up- and downstream of the TSS within this range. Within this range, we found that methylation nearest the TSS was lower in all experimental conditions (Figure 6A). On average, we found that the methylation status in the epileptic hippocampus, KA^{9wk} was higher than in naive hippocampus (Figure 6A), consistent with our ELISA-based predictions (Figure 3D). In the ADO-treated epileptic rats KA^{9wk}/ADO^{5d}, we found a uniform reduction in methylation (Figure 6A), again consistent with predictions from our ELISA-based results with ADO treatment alone (Figure 1B) and in the epileptic brain (Figure 3D). These results support our hypothesis that the epileptic brain is hypermethylated and that ADO treatment reduces methylation. To identify probes with the largest increase in methylation status in epileptic rats, we calculated the δ SLR (dSLR) between KA^{9wk} and naive control rats and the dSLR between KA^{9wk}/ADO^{5d} and KA^{9wk}. We considered the methylation status of a probe to be significantly increased if the dSLR was greater than or equal to ±1, a threshold chosen because it identified the top 2.5% of changed probes in our restricted data set (within 1,000 bps of each TSS). In the Nimblegen array, each TSS was associated with 11 to 20 probes. If at least 25% of the probes associated with a TSS had a KA^{9wk} vs. naive dSLR of 1 or more, we considered the associated gene to be a candidate for significantly increased methylation in the epileptic brain. Using these criteria, we identified 125 genes with substantially increased methylation in the epileptic brain. We demonstrated the phenotypic relevance of these DNA methylation changes in epileptic vs. control rats by comparison of mRNA expression changes from a published mRNA array data set (GEO GSE14763) consisting of pilocarpine-induced epileptic rats compared with controls. From our MeDIP array, we chose the 10 targets with the most positive dSLR values of genes also represented on the rat gene expression array (i.e., the "top 10 list" of targets with the most hypermethylated probes); 70% of these genes (*Unc13c*, *Exoc8*, *Mtmr14*, *Phkg2*, *Umps*, *Spata9*, and *Gsta5*) indeed have decreased mRNA expression in epileptic versus control rats and thus confirm the prediction that increased DNA methylation relates to decreased gene expression. This comparison further validates the MeDIP array results. By similar criteria, comparison of KA^{9wk}/ADO^{5d} with KA^{9wk} rats and a resulting dSLR of ≤ –1 or less, we identified 762 genes that showed reduced methylation status during ADO treatment. Sixty-six genes were identified as common within the 2 groups (see Supplemental Table 1; supplemental material available online with this article;

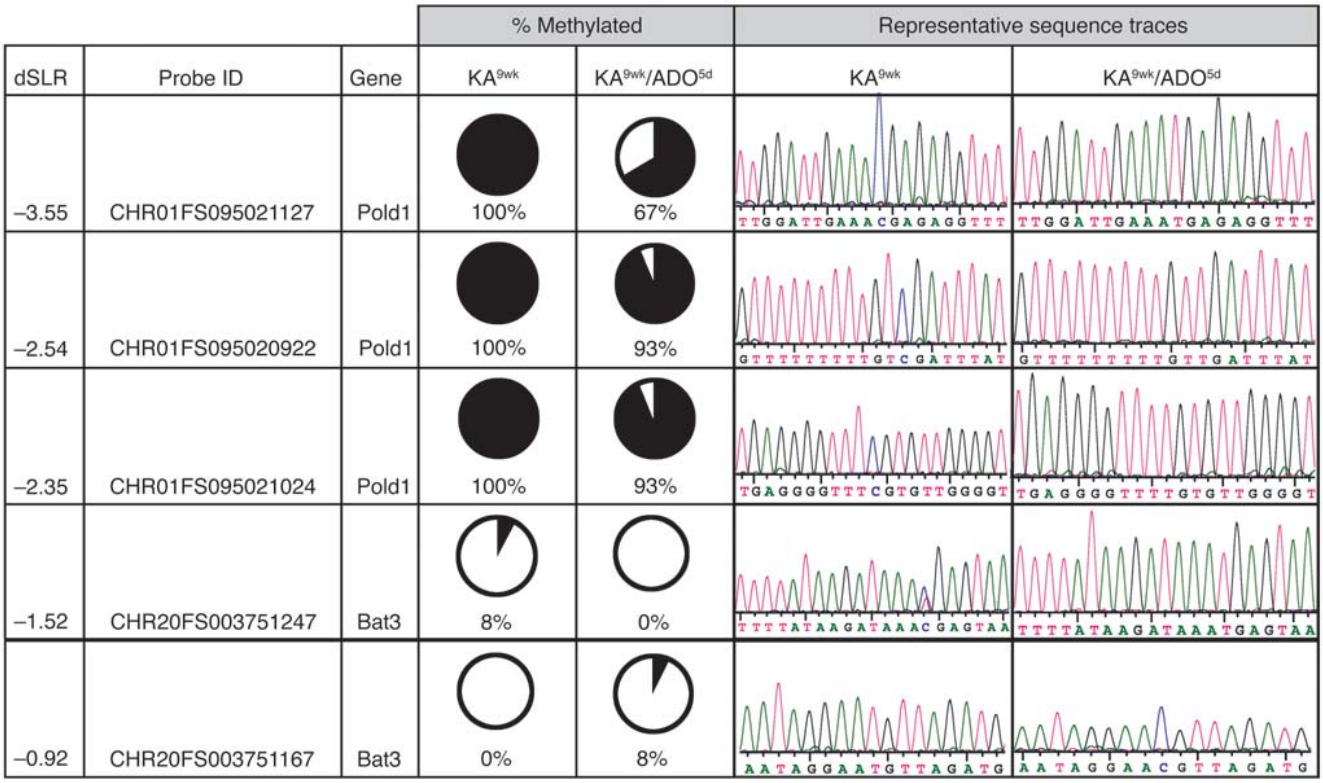

Figure 7

ADO treatment reduces genomic DNA CpG methylation in the epileptic hippocampus. Analysis of bisulfite sequencing of hippocampal DNA from epileptic and ADO-treated epileptic rats confirms that predictions from the MeDIP array are translated to changes in the methylation status of CpGs. Bisulfite sequencing was performed on DNA extracts from KA^{9wk} and KA^{9wk}/ADO^{5d} vs. KA^{9wk} rats (n = 3 animals/treatment and 4–5 clones/animal). The methylation status of individual probes, which contain only 1 CpG and which span a dSLR range from –0.92 to –3.55 KA^{9wk}/ADO^{5d} vs. KA^{9wk} was compared between treatment groups. Changes in methylation are displayed as percentage of methylated (pie chart, black corresponds to the percentage of methylated CpGs; 1 CpG per probe; 12 to 15 clones total) and in the representative sequence traces with methylated cytosines retained (blue peaks) and unmethylated cytosines converted to thymine (pink peaks). Note that at a KA^{9wk}/ADO^{5d} vs. KA^{9wk} dSLR threshold of less than –3.0, there is a robust decrease (33%) in ADO-mediated CpG methylation in 3 out of 3 animals. Probes with a dSLR greater then a –3.0 threshold (–0.92 to –2.54) yielded only slight variations (8%) in the methylation status.

doi:10.1172/JCI65636DS1). In summary, these data demonstrate what we believe to be a novel function of ADO as a homeostatic regulator of global DNA methylation status, which — according to the underlying biochemistry (Figure 1A) — does not directly provide for target specificity. We demonstrate that global hippocampal DNA methylation increased during epileptogenesis and decreased following transient ADO treatment, validating our previous findings (Figure 3) in an independent experimental approach.

ADO treatment reduces genomic DNA CpG methylation in the epileptic hippocampus. The MeDIP array results predict that, within 1,000 bps of each TSS, DNA methylation throughout the genome is consistently increased in epileptic rats and decreased in ADO-treated epileptic rats (Figure 6A). To validate the general robustness of the MeDIP data set, bisulfite sequencing was conducted on genomic regions corresponding to a total of 5 individual probes that each contained only a single CpG site and that covered a wide representative range (–3.5 to –0.92) on the KA^{9wk}/ADO^{5d} vs. KA^{9wk} dSLR (Figure 7). When comparing untreated and ADO-treated epileptic rats, probes with a more negative dSLR (i.e., –3.5) are expected to have a robust difference in the percent methylation. For each probe, the methylation status of its single CpG dinucleotide was compared between bisulfite converted hippocampal DNA from KA^{9wk}- and KA^{9wk}/ADO^{5d}-treated rats (n = 3 animals/group and 4–5 clones/animal). Importantly, the greatest ADO-mediated reduction in percentage of methylation (33%) was associated with the probe that had the largest negative dSLR value (–3.55). This probe was associated with the gene *PolD1*; its CpG was 100% methylated in the KA^{9wk} rats, while we observed a 33% reduction in CpG methylation of this *PolD1* probe in KA^{9wk}/ADO^{5d} rats. ADO dependent reduction of methylation was found in 3 out of 3 animals and in 1 to 3 out of 5 sequenced clones per animal. In contrast, 4 additional probes from 2 different genes (*PolD1* and *Bat3*) that spanned an KA^{9wk}/ADO^{5d} vs. KA^{9wk} dSLR range from –0.92 to –2.54 had CpG methylation changes of 7%–8% (i.e. in only 1 out of 12 to 15 sequenced clones) between KA^{9wk}/ADO^{5d} and KA^{9wk} rats. These data validate that ADO therapy causes decreased methylation in individual CpG sites at dSLR values of –1 or greater and demonstrate that dSLR values of –3 or greater predict robust decreases in DNA methylation across all animals and several clones. Thus, the magnitude of the KA^{9wk}/ADO^{5d} vs. KA^{9wk} dSLR calculated from the MeDIP array positively correlates with a reduction in percentage of methylation in ADO-treated rats as confirmed by bisulfite sequencing.

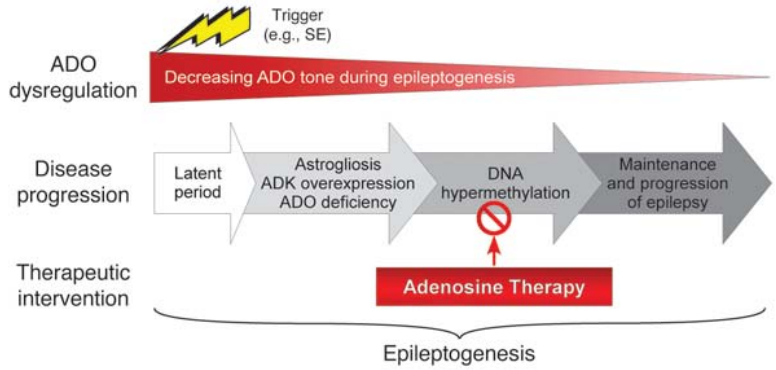

Figure 8
Model for the role of ADO and associated DNA methylation changes in epileptogenesis. As part of ongoing epileptogenesis astrogliosis with associated overexpression of ADK occurs, this results in subsequent ADO deficiency. These alterations lead to DNA hypermethylation and maintenance and progression of the epileptic phenotype. Therapeutic ADO augmentation interferes with DNA methylation and may interrupt this process. For details, see main text.

Discussion

In the present study, we identify what we believe is a novel epigenetic function of the purine ribonucleoside ADO as a homeostatic regulator of global DNA methylation. Our findings demonstrate that there is an increase in DNA methylation in the hippocampi of epileptic animals and that transient ADO therapy effectively reduces this pathological DNA methylation status. Remarkably, this transient ADO therapy also effectively prevents epileptogenesis. Previously, ADO augmentation has been well characterized as an A_1R-dependent anticonvulsant modality (19); however, these receptor-mediated effects are limited to the time period of therapeutic intervention (27). Here, we show that ADO tone can directly modulate DNA methylation in vivo and thereby exert additional epigenetic effects via biochemical interference with the transmethylation pathway. These changes affect the DNA methylome on a homeostatic level, are maintained long after therapy is suspended, are non–cell autonomous, and are ADO receptor independent. By broadly targeting homeostatic functions of multiple intracellular pathways via genome-wide changes in the DNA methylation status, we demonstrate here that ADO-induced changes in the DNA methylome in a global homeostatic sense could be used to attenuate disease progression in epilepsy. Together, these data ascribe a function to the brain's endogenous anticonvulsant ADO as a biochemical regulator of the methylome and directly support the "methylation hypothesis of epileptogenesis" (2).

The studies presented here show that local ADO augmentation via implantable biodegradable polymers can inhibit DNA methylation in the CNS of both healthy and epileptic animals. There is increasing evidence to support the idea that even a brief exposure to an epigenetic modulator may lead to long-lasting changes (33), which can best be explained by homeostatic network effects on the "epigenomic landscape." For example, recent work in cancer biology has shown that exposure to transient low doses of DNA demethylating agents results in long-term anti-tumor effects, modulated by genome-wide promoter methylation, which persist well beyond drug withdrawal (34). In agreement with these studies, our transient biochemical manipulation has long-lasting effects, preventing progression of epileptogenesis for at least 3 months in a model of mesial TLE. The antiepileptogenic effect of transient ADO delivery was documented in 2 independent outcome measures. First, we demonstrated that transient ADO delivery resulted in a sustained reduction of seizures over a time span of at least 3 months, during which all control animals continued to progress in seizure intensity (Figure 4). Second, we demonstrated a suppression of mossy fiber sprouting, a well-recognized pathophysiological phenomenon of TLE (ref. 32 and Figure 5).

To examine the role ADO plays in affecting methylation homeostasis on the network level, we followed 2 independent experimental approaches. Using an ELISA-based assay as well as a rat-specific MeDIP-on-ChIP analysis, we compared the global methylation state of hippocampal DNA derived from experimentally naive rats with that of untreated epileptic rats as well as with that of epileptic rats treated with an ADO-releasing silk polymer for 5 days. In both assays, we found an increase in global DNA methylation status in epileptic rats versus nonepileptic control rats (Figure 3D and Figure 6A). Importantly, 5 days of exposure to ADO reduced the global DNA methylation status in epileptic animals (Figure 3D and Figure 6A). These data independently demonstrate that ADO provides homeostatic regulation of the "DNA methylation landscape." This novel function of ADO is consistent with the underlying biochemistry (Figure 1A), which does not provide any mechanism for target specificity. These homeostatic control functions are also consistent with a non–cell-autonomous effect of ADO (Figure 1B and Figure 3B) and with independence from ADO receptor activation (Figure 1, D and E). Although not the direct focus of our study, the control of methylation homeostasis by ADO does not exclude the possibility for targeting specific changes afforded by ADO therapy. Among the targets that showed reduced DNA methylation during active ADO release (Supplemental Table 1), several specifically interact with DNA or play a role in gene transcription and translation (*PolD1*, *Polr1e*, *Rps6kl1*, *Snrpn*, *Znf524*, *Znf541*, *Znf710*), making them likely candidates as mediators for ADO-dependent changes in major homeostatic functions. Although individual targets need to be validated in additional studies, our conceptual MeDIP predictions have been validated with bisulfite sequencing, which confirms that ADO-mediated changes in global DNA methylation (ELISA and MeDIP) are directly attributable to site-specific changes in genomic DNA CpG methylation. Furthermore, we selected a CpG site from *PolD1*, a gene that encodes a component of the DNA polymerase δ complex, as an illustrative example for a CpG site that displays a robust ADO-mediated site-specific change in the DNA

Table 1
Target and primer information for bisulfite sequencing

Gene	Product size (nt)	Tm	Direction	Primer sequence
PolD1	384	57.7	Left	GGAGGTTAAAAGATATATTTGGGATTT
			Right	TCTCTCCTATAAACCAAAAACAACTAC
Bat3	233	58.1	Left	GGTGAGGATAGGTTAAATAAAATTTGTT
			Right	TAATAAAAACCCTAAACTCTCAAACTC

Tm designates the melting temperature (°C) of the primers.

methylation status. The magnitude of the KA^{9wk}/ADO^{5d} vs. KA^{9wk} dSLR calculated with the MeDIP data set also positively correlated with reduced methylation, adding confidence in the MeDIP predictions (Figure 7). Although not validated by independent replicates on a target basis, the results of our MeDIP-on-ChIP array suggest that a large number of genes associated with DNA structural elements and transcription factors are altered by ADO treatment in the epileptic brain; thus, the protective effect of ADO augmentation might not be mediated via a single gene, but through a network of gene expression changes.

ADO treatment is not expected to decrease methylation at all CpG sites at the same time. Whereas site-specific methylation is regulated by targeting DNMT complexes by a variety of mechanisms (35), a change in the ADO concentration shifts the equilibrium constant of the transmethylation pathway, thereby either permitting or preventing the act of DNA methylation. Within the epileptic brain, high ADK and low ADO will drive SAM methyl group donation to DNA, resulting in a pathological hypermethylation status. Conversely, increasing ADO levels with the silk polymer shifts the equilibrium constant to preventing methylation and restores normal methylation levels in the epileptic brain. At this point, we therefore do not propose the direct involvement of active DNA demethylation. However, since ADK overexpression drives DNA hypermethylation (Figure 1G), we cannot rule out the possibility that the epileptic brain compensates to a certain degree by upregulation of active DNA demethylation. Importantly, dynamic demethylation has been demonstrated in neurons in response to experimentally induced seizures, in which hippocampal *Bdnf* and *Fgf2* were both rapidly demethylated in a GADD45B-specific manner (5). GADD45B expression was shown to rise as an acute response to electrical stimulation, suggesting that seizures trigger a transient increase of GADD45B and thus promote active demethylation (36). Our data show that during active ADO delivery, DNA methylation status is rapidly reduced within 5 days of ADO treatment (Figures 3, 6, and 7). This reduction of the DNA methylation status following ADO treatment is compatible with blockade of DNA methylation through product inhibition of DNMTs (ref. 14; see also suppression of DNMT activity after 4 days of ADO exposure; Figure 3E), likely under conditions of increased compensatory DNA demethylation, an intriguing possibility that warrants further investigation.

We previously demonstrated that dysregulation of ADO homeostasis due to overexpression of the key ADO-metabolizing enzyme ADK leads to exacerbation of epilepsy (21, 37). Similarly, kindling epilepsy was associated with a loss of adenosinergic control mechanisms, in particular with a decrease of ADO A_1R densities in the epileptic brain (38). In light of the epigenetic findings presented herein, we propose a refined model of the ADK hypothesis of epileptogenesis (ref. 39 and Figure 8). Once the epileptic phenotype is established with overt astrogliosis, overexpression of ADK, and ADO deficiency (21, 22, 37), there is a pathological hypermethylation of DNA. Increased methylation in the epileptic brain in turn is thought to perpetuate and to exacerbate epileptogenesis (2). As we have demonstrated here, transient ADO therapy might prevent perpetuation of ongoing epileptogenic processes (Figure 8) by intervening with biochemical mechanisms that maintain the hypermethylated state in epilepsy. We cannot exclude, however, the possibility that ADO may have additional distinct effects on ADO receptor expression, an intriguing possibility that warrants further investigation.

It is important to note that intracellular changes in ADK expression within astrocytes may have both cell-autonomous and non–cell-autonomous ramifications. ADO levels within astrocytes and neurons are regulated by equilibrative and concentrative nucleoside transporters (40, 41). Thus, an increase in ADK in astrocytes, as observed in the epileptic hippocampus, may directly affect DNA methylation within the affected astrocyte (cell-autonomous function). Additionally, a pathological increase of ADK in astrocytes reduces the global ADO tone through the transport and metabolism of extracellular ADO to AMP, thereby indirectly modulating the activity of neighboring cells (i.e., epigenetic changes in neurons). This non–cell-autonomous effect is supported by our findings that increased astroglial ADK expression in epileptic rats leads to increased 5mC immunofluorescence in adjacent neurons (Figure 3).

In order for a new antiepileptogenesis intervention to be clinically relevant, the window of effectiveness is particularly important. Previous reports on "antiepileptogenesis" were based on early intervention within hours, or at most a few days, before or after an epileptogenesis-precipitating injury. Even though partial antiepileptogenic effects were reported in some studies, it is not clear whether epileptogenesis was truly suppressed or whether the precipitating injury was modified (1). Our present study differs because we delayed therapeutic intervention until all animals developed "early epilepsy" (at least 10 SRS); thus, we were able to monitor long-term disease progression (i.e., epileptogenesis) without any confounds related to injury modification.

When considering how to advance ADO-based therapies to clinic applications, safety and feasibility must be taken into consideration. Following surgical resection of an epileptogenic focus, seizures recur in about 50% of patients and secondary epileptogenesis is a major problem (42). Placement of ADO-releasing silk into the resection cavity following epilepsy surgery might be used as preventative treatment. Similarly, transient ADO delivery might be used as prophylaxis in patients at risk for developing epilepsy, e.g., following a severe traumatic brain injury. Finally, since epileptogenesis is a lifelong ongoing process in patients with epilepsy, local treatment with ADO-releasing silk might be envisioned as a feasible therapeutic strategy for preventing disease progression with its sequelae of comorbidities and pharmacoresistance.

Methods

Animal models of epilepsy. For seizure threshold testing, male CD-1 mice (25–35 g) were used. 0.75% (w/v) PTZ was prepared in isotonic saline. 5-Aza-2dC was prepared in isotonic saline and administered via tail vein 10 minutes prior to initiation of PTZ infusion. PTZ was infused via tail vein at a rate of 0.2 ml/min using a Hamilton microsyringe (Harvard Apparatus) in freely moving animals. Times to first twitch, first clonus, and final extensor phase were recorded. Infusion was stopped after a final extension or at a maximum volume of 0.9 ml, whichever came first.

For the kindling model and associated 5-Aza-2dC drug testing, male Sprague Dawley rats (300–350 g) were implanted with bipolar, coated stainless steel electrodes (0.20 mm in diameter; Plastics One) into the right hippocampus (stereotactic coordinates relative to Bregma: AP, –4.2 mm; ML, –4.6; DV, –5.6). For experiment 1, the animals were kindled based on a rapid kindling protocol (43). Briefly, using a Grass S-88 stimulator, rats received 12 stimulations per day (1-ms square wave pulses of 200 μA, 50-Hz frequency, 10 s duration at an interval of 30 minutes between stimulations) every 2–3 days until a stable Racine stage-5 seizure was generated. Following a 2-day stimulus-free period, rats received 2 stimulations, then a single i.p. bolus of either saline or 5-Aza-2dC (0.5 mg/kg) 15 minutes prior to a subsequent series of

9 stimulations. All stimulations were delivered at an interval of 30 minutes, and rats were scored for mean Racine score and after-discharge duration. There was a 5-day drug- and stimulus-free period between the saline and 5-Aza-2dC trials. For experiment 2, animals were kindled as described above while being chronically treated with either saline or 5-Aza-2dC (0.4 mg/kg, i.p.) administered 12 hours prior to the first kindling session and every successive 12 hours until the saline-injected controls achieved a stable Racine stage-5 seizure score. Following an 11 day drug- and stimulus-free period, animals received a stimulus and were scored for seizure stage and duration.

For the TLE model, young male Sprague Dawley rats (130–150 g) received a single acute dose of KA (12 mg/kg, i.p.) to trigger SE. Only rats that exhibited at least 3 hours of convulsive Racine stage-4 seizures were used. Starting 4 weeks after KA administration, animals were continuously video monitored to quantify the number of convulsive stage 4–5 seizures per week. The number of stage 4–5 seizures typically increased to more than 3 seizures per week at KA^{9wk}, and animals experienced at least 10 spontaneous convulsive stage 4–5 seizures (inclusion criterion). All animals were further monitored during weeks 10–13 and 18–21 after KA administration. Behavioral seizures were confirmed by EEG analysis in selected animals, with bipolar recording electrodes implanted into the right hippocampus (AP, –4.2 mm; ML, –3.0; DV, –3.3). Electrical brain activity was amplified (Grass Technologies) and digitized (PowerLab; AD Instruments). EEG files were analyzed manually by an observer blinded to the animals' treatment. EEG seizure activity was defined as high-amplitude rhythmic discharges that clearly represented a new pattern of tracing that lasted at least 5 seconds.

Polymer implant design and implantation. Cylindrical silk-based polymer implants designed to deliver the target dose of 250 ng ADO per day for a limited period of 10 days were designed and fabricated as described (27, 28). Consistency of ADO release and in vivo efficacy have previously been validated (27). ADO-releasing polymers (250 ng ADO/d) or control polymers (0 ng ADO/d) were implanted bilaterally into the lateral brain ventricles using a stereotactic implantation device as described (44).

Pharmacology. Bilateral i.c.v. bolus of saline, ADO (5 μg/ventricle), HCY (250 μg/ventricle), or SAM (16 μg/ventricle) was injected using a transiently placed guide cannula and injection volumes of 5 μl. ITU (3.1 mg/kg in 20% DMSO, i.p.) was administered daily for 5 days to WT and homozygous *A1R*-knockout mice. Caffeine (25 mg/kg in saline, i.p.) was administered 15 minutes prior to ITU.

Immunohistochemistry. Staining for ADK and 5mC was performed using standard protocols (45). For each staining paradigm, high-resolution digital images were acquired under identical conditions and all image processing was applied identically across experimental groups. ADK expression was quantified by densitometry by analyzing fields of 500 μm encompassing the entire CA1. Corresponding fields from 2 sections from each animal (n = 3 animals per group) were analyzed using ImageJ. Levels in each analysis field were measured as arbitrary density units and are represented as averages ± SEM normalized to controls. Timm staining was performed and quantified as described (46).

Cell culture. BHK-WT and BHK-AK2 cells (26) were cultured in DMEM (supplemented with 10% FBS). A pc-DNA3.1 vector with a human CMV promoter was used to drive expression of the ADK-long (provided by R. Gupta, McMasters University, Hamilton, Ontario, Canada) or ADK-short (47) cDNA. Transfection was carried out in parallel experimental replicates using a standard calcium phosphate transfection protocol. Subsequently, cells were harvested for DNA from 3 separate experimental replicates or, to quantify ADK protein expression, 3 transfection replicates were pooled and used for Western blotting.

Western blotting. Cells were harvested for aqueous protein extraction. 25 μg of protein was loaded into a 10% Bis-Tris gel, separated by standard gel electrophoresis, transferred, and incubated overnight using a primary ADK antibody (1:5000) (29). To normalize ADK immunoreactivity with protein loading, a mouse monoclonal anti–α-tubulin antibody (sc-8035, 1:5000; Santa Cruz Biotechnology Inc.) was used to reprobe the same blot and the optical density ratio of ADK to α-tubulin was calculated.

Global DNA methylation assay. Total genomic DNA was isolated from fresh frozen tissues using a DNeasy Blood and Tissue Kit (QIAGEN). Global DNA methylation status was assessed using the MethylFlash Methylated DNA quantification kit (Epigentek) per the manufacturer's instructions.

DNMT activity assay. Nuclear proteins were isolated from fresh frozen tissue using an EpiQuik Nuclear Extraction Kit I per the manufacturer's instruction (Epigentek). DNMT activity was quantified from freshly isolated nuclear proteins using a fluorimetric EpiQuick DNMT Activity/Inhibition Assay Ultra kit per the manufacturer's instruction (Epigentek).

MeDIP array. Hippocampal DNA extracts from naive control rats or those sacrificed at KA^{9wk}/ADO^{5d} (250 ng ADO per day) were pooled (n = 3–5/experimental group) and analyzed by MeDIP array (05924545001, NimbleGen). The Rat DNA Methylation 3x720K CpG Island Plus RefSeq Promoter array is a rat-specific, whole-genome, tiled format containing at least 3 internal replicates for each probe, with each probe containing from 1 to 18 CpG sites. Tissue samples were prepared according to the manufacturer's instructions, then sent to NimbleGen for hybridization and detection. Each tissue sample was split into 2 fractions, the first enriched for methylated DNA using immunoprecipitation with an antibody against 5mC and labeled with Cy5 and the second total DNA fraction labeled with Cy3. The 2 fractions were mixed and then hybridized on the array. Each probe is represented as the SLR, where the ratio indicates the methylated versus the unmethylated signal. Each ratio is $\log_2$ transformed, then scaled using the biweight mean. Gene targets were predicted by associating enriched regions of DNA methylation with their nearest neighbor TSS (upstream or downstream according to the ± strand) according to the rat RGDS 3.4 assembly. Array data have been deposited in GEO (GSE45932).

Bisulfite sequencing. Hippocampal DNA extracts (1 μg) from KA^{9wk} and KA^{9wk}/ADO^{5d} rat (n = 3/group) were bisulfite converted using the Epitect Bisulfite Conversion Kit according to the manufacturer's protocol (QIAGEN). Primers specific to bisulfite-converted DNA (bs-DNA) were designed using MethPrimer (ref. 48 and Table 1). For the analysis of *PolD1*, we chose 3 different probes spanning a dSLR range from –3.55 to –2.35, with each of those probes containing one CpG site. For the analysis of *Bat3*, we chose 2 different probes spanning a dSLR range from –1.52 to –0.92, with each of those probes containing 1 CpG site. Bisulfite-converted DNA was amplified using HotStarTaq DNA polymerase (QIAGEN) and a standard PCR protocol: initial activation (95°C, 15 minutes); 40 cycles of denaturation (94°C, 30 seconds), annealing (Tm in Table 1, 35 seconds), extension (72°C, 1 minute); and final extension (72°C, 10 minutes). Amplified products were gel extracted and ligated into pGEMT Easy Vector and transformed into JM109 competent cells (Promega). Individual clones from each transformation were PCR screened with M13f/r primers for correct insert size. For each DNA target (PolD1 and Bat3), 4–5 clones from each animal were purified and sequenced with M13 primers resulting in 12–15 individual replicates per treatment group (Molecular and Cell Biology Core, Oregon National Primate Research Center). Data were analyzed by aligning sequences with SeqMan Pro Lasergene 10 software (DNAStar) to a theoretical target backbone that is bisulfite converted with methylated CpGs (Bisulfite Sequence Converter, Nephew Lab, Indiana University; http://cancer.informatics.indiana.edu/Nephew_lab/bisulfite_sequencing.htm). Within each sequence, individual CpG sites were scored as either a cytosine or thymine residue. The percentage methylation was calculated for those probes that cover only 1 CpG site and is presented as pie charts.

Statistics. Quantitative data were analyzed using GraphPAD Prism software. In vivo seizure data from the i.p. KA-injected model are based on n = 8

for ADO and $n = 85$ to 8 for controls depending on dropouts (weeks 18–21 after KA administration) due to lethal seizures in the control groups. Stages 4 and 5 seizure counts were averaged by experimental group as seizures per week and analyzed in 1-week bins. All data are presented as mean ± SEM. KA seizure data were analyzed using 2-way ANOVA on ranks using an unweighted means analysis followed by a Bonferroni test. Global methylation, IHC, PTZ, and kindling data were analyzed using nonparametric Mann-Whitney 2-tailed *t* tests assuming non-Gaussian distribution of the data or ANOVAs as appropriate. $P < 0.05$ was considered significant.

Study approval. All animal procedures were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with protocols approved by the Legacy Institutional Animal Care and Use Committee.

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FULL-LENGTH ORIGINAL RESEARCH

Genetic variation in the adenosine regulatory cycle is associated with posttraumatic epilepsy development

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Summary

Objective: Determine if genetic variation in enzymes/transporters influencing extracellular adenosine homeostasis, including adenosine kinase (ADK), [ecto-5′-nucleotidase (NT5E), cluster of differentiation 73 (CD73)], and equilibrative nucleoside transporter type-1 (ENT-1), is significantly associated with epileptogenesis and posttraumatic epilepsy (PTE) risk, as indicated by time to first seizure analyses.

Methods: Nine ADK, three CD73, and two ENT-1 tagging single nucleotide polymorphisms (SNPs) were genotyped in 162 white adults with moderate/severe traumatic brain injury (TBI) and no history of premorbid seizures. Kaplan-Meier models were used to screen for genetic differences in time to first seizure occurring >1 week post-TBI. SNPs remaining significant after correction for multiple comparisons were examined using Cox proportional hazards analyses, adjusting for subdural hematoma, injury severity score, and isolated TBI status. SNPs significant in multivariate models were then entered simultaneously into an adjusted Cox model.

Results: Comparing Kaplan-Meier curves, rs11001109 (ADK) rare allele homozygosity and rs9444348 (NT5E) heterozygosity were significantly associated with shorter time to first seizure and an increased seizure rate 3 years post-TBI. Multivariate Cox proportional hazard models showed that these genotypes remained significantly associated with increased PTE hazard up to 3 years post-TBI after controlling for variables of interest (rs11001109: hazard ratio (HR) 4.47, 95% confidence interval (CI) 1.27–15.77, $p = 0.020$; rs9444348: HR 2.95, 95% CI 1.19–7.31, $p = 0.019$) .

Significance: Genetic variation in ADK and NT5E may help explain variability in time to first seizure and PTE risk, independent of previously identified risk factors, after TBI. Once validated, identifying genetic variation in adenosine regulatory pathways relating to epileptogenesis and PTE may facilitate exploration of therapeutic targets and pharmacotherapy development.

KEY WORDS: Traumatic brain injury, Posttraumatic epilepsy, Epileptogenesis, Genetics, Adenosine, Rehabilomics.

Traumatic brain injury (TBI) is a leading cause of death and disability in the United States, affecting over 1.5 million people annually.[1] Those who survive can develop secondary complications such as posttraumatic epilepsy (PTE). Prevalence and likelihood of developing PTE varies widely in the literature and depends on injury type and severity.[2]

Key Points

- Genetic variation in the adenosine regulatory cycle may accelerate epileptogenesis and increase posttraumatic epilepsy (PTE) risk after TBI.
- rs11001109 rare allele homozygosity and rs9444348 heterozygosity are linked with shorter time to first seizure and higher 3-year seizure rates.
- Confirmatory studies are needed to establish adenosine cycle genetic variation associations with accelerated epileptogenesis and PTE risk.
- If confirmed, adenosine regulatory pathways may be a viable therapeutic target and point of patient stratification for clinical intervention.

Although, PTE accounts for 10–20% of symptomatic epilepsy in the general population and 5% of all epilepsy.[3] It is notable that PTE is associated with less favorable recovery and increases the risk of poor functional outcome following TBI.[4] PTE often requires long-term antiepileptic drug (AED) treatment, increasing the risk of adverse side effects, need for regular monitoring, and limiting cognitive recovery. However, not every patient presenting with clinical risk factors will develop PTE. Recent studies suggest differences in the secondary injury response post-TBI and individual genetic variability may contribute to epileptogenesis.[5,6] Therefore, identifying genetic variation in pathways involved in epileptogenesis may help explain variation in PTE risk and present future points of intervention.

Adenosine is a potent endogenous anticonvulsant, regulated by a complex adenosine regulatory cycle.[7] Within the brain, adenosine homeostasis is largely under astrocyte control, providing a metabolic clearance system for intra- and extracellular adenosine.[8] Briefly, extracellular adenosine triphosphate (ATP) is dephosphorylated into adenosine via a chain of processes involving ecto-nucleotidases (including the 5′-ectonucleotidase or cluster of differentiation 73 [CD-73], encoded by the NT5E gene), equilibrative nucleoside transporters (including the primary type-1 ENT-1 transporter, encoded by the *SLC29A1* gene) for maintenance of intra/extracellular adenosine levels, and intracellular adenosine rephosphorylation to inactive 5′-AMP (via adenosine kinase (ADK), encoded by the ADK gene). Differential regulation of the biochemical processes within this cycle, as well as extracellular adenosine receptors and intracellular epigenetic functions mediated by adenosine, represent a potentially large group of both therapeutic targets and biomarkers for evaluating epileptogenesis.

Reactive astrogliosis is prevalent in both human epilepsy and in TBI,[9,10] potentially contributing to PTE development. Previous research shows intra- and extracellular adenosine homeostasis actively influences epileptogenesis[11] in nontraumatic and posttraumatic seizure models.[12,13] In addition, adenosine activation of the inhibitory adenosine A_1 receptor (A_1R) has a powerful anticonvulsant effect following chemically induced as well as chronic recurrent seizures[12] and on posttraumatic seizure development in a TBI mouse model.[13] Our previous work shows that variation at two single nucleotide polymorphisms (SNPs) within the adenosine A_1R gene (ADORA1) is associated with an increased PTE risk in a population with severe TBI.[5]

Thus, using a Rehabilomics approach,[14] identifying mechanisms and biomarkers of epileptogenesis may have significant prognostic and personalized medical management value in TBI populations for preventing PTE throughout rehabilitation and recovery. SNPs are a potentially useful biomarker for PTE, but few studies have assessed the link between genetic variation and epileptogenesis. We hypothesized that genetic variation within candidate genes encoding components critical for maintenance of adenosine homeostasis, ADK, NT5E, and SLC29A1, would be associated with epileptogenesis and increased PTE risk, as demonstrated by time to event analysis.

Methods

Study design and subjects

This study was approved by the University of Pittsburgh Institutional Review Board. Candidate SNPs were genotyped and subject information regarding PTE was abstracted from medical charts for 208 subjects with severe, nonpenetrating TBI (admission Glasgow Coma Scale [GCS] score ≤8, head region Abbreviated Injury Score [AIS] ≥3) from a single academic medical center as part of a larger study investigating genetic relationships with TBI outcome. Subjects included in this longitudinal historical cohort study were 18–70 years old, had a positive computed topography (CT) scan confirming TBI, and had no history of premorbid seizures. This

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study was restricted to subjects that self-reported race as white due to racial differences in allelic frequency obtained from the database of single nucleotide polymorphisms (dbSNP: http://www.ncbi.nlm.nih.gov/snp). Four subjects were subsequently excluded (n = 204).

Critical care management

All subjects were admitted to the neurotrauma intensive care unit at our level 1 trauma center and received treatment consistent with The Guidelines for the Management of Severe Head Injury.[15] Treatment initially consisted of extraventricular drain (EVD) placement, central venous and arterial catheters, and further surgical intervention when clinically necessary. Intermittent electroencephalography (EEG) recordings were obtained by treating physicians as part of standard of care when suspicion of clinical or nonconvulsive seizures arose. In accordance with guidelines for seizure prophylaxis therapy,[16] the majority of subjects received AEDs for the first week postinjury.

Demographic and injury data

Subject demographic and injury information was abstracted from electronic medical records. Demographic and premorbid variables, including age and sex, were recorded. Injury information including mechanism of injury, GCS score (best in the first 24 h postinjury), subdural hematoma (SDH), depressed skull fracture, and acute care length of stay (LOS) was obtained from ambulance, emergency room, surgical, and radiographic reports. Information regarding AED administration during the acute care period was considered only if used for seizure prophylaxis or treatment. Isolated TBI status and Injury Severity Scores (ISS) were calculated using the AIS scoring system, with data for these measures obtained from the University of Pittsburgh Medical Center (UPMC) trauma registry. The AIS scoring system determines the severity of a specific injury based on survivability of that injury.[17] ISS is defined as the sum of squares of AIS scores from the three most severely affected body regions.[18] Isolated TBI was defined using AIS criteria, where a head region AIS score of ≥3 and a score of <3 in all other body regions was considered an isolated TBI. Nonisolated TBI was defined as a head region AIS score of ≥3 and an AIS score ≥3 in at least one extracranial region.

Seizure assessment

Time to first seizure was the primary variable of interest, and our cohort was further restricted to comply with the most current definition of PTE.[19] PTE was defined as at least one documented seizure occurring after the first week postinjury. Therefore, subjects who had seizures (n = 11) or died (n = 31) during the first week postinjury were excluded. A final cohort of 162 subjects met the inclusion criteria based on our PTE definition.

Information regarding PTE occurrence was collected using all available inpatient and outpatient electronic medical records from our health system. Clinical records were reviewed to determine time to first seizure and included ambulance and emergency room reports, nursing notes, progress reports, EEG reports, patient history and physical reports, and discharge or transfer summaries. PTE follow-up was censored at 3 years postinjury to allow for comparable and complete ascertainment of PTE information for all subjects. Any indications of seizures, convulsions, status epilepticus (SE), or diagnosed seizure disorders were classified as PTE occurrence. Any mention of possible seizure activity that was either ambiguous or nonconclusive was categorized as no-PTE.

DNA extraction

Deoxyribonucleic acid (DNA) was extracted from whole blood samples or cerebrospinal fluid (CSF) collected from each subject. Blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes. Samples were centrifuged and processed to retrieve the buffy coat, and DNA was extracted using a simple salting-out procedure.[20] If no blood sample was available for DNA extraction, DNA was extracted from white blood cells obtained from passively drained CSF using the QIAamp DNA extraction protocol for extraction from body fluids (Qiagen Corporation, Venlo, The Netherlands).

SNP selection and genotyping

SNPs were selected to assess the variability across the entire ADK, NT5E, and *SLC29A1* genes. This required a total of 14 tagging SNPs (tSNPs), including nine ADK (rs10824218, rs11001111, rs10824094, rs946185, rs11001109, rs11000980, rs1908335, rs4746209, and rs7899674), three NT5E (rs9444348, rs4431401, and rs9450282), and two *SCL29A1* (rs324148 and rs760370) tSNPs. These tSNPs were selected via HapMap database build 36, using a minor allele frequency (MAF) ≥20% and CEPH (Utah residents with northern/western European ancestry) data. All 14 tSNPs were genotyped using the i-PLEX Gold SNP Assay (Sequenom Incorporated, San Diego, CA). Hardy-Weinberg equilibrium was verified for all SNPs, indicating genotype distributions were within the expected proportions. Information regarding the degree of linkage disequilibrium (LD) between SNPs in this study was obtained using HapMap database build 36 (http://hapmap.ncbi.nlm.nih.gov). LD and MAF were examined within the specific study population using Haploview[21] software (Broad Institute, Cambridge, MA, U.S.A.; Table S1). We used the most recent Genome Reference Consortium release, GRCh38, to determine SNP location and to examine reported LD from the CEPH population when assessing SNPs with significant associations with time to first seizure and PTE risk.

Statistical analysis

Statistical analyses were performed using SAS version 9.3 (Cary, NC, U.S.A.) and R version 3.03 (http://www.r-project.org/). Summary statistics were calculated for demographic and injury characteristics of interest. Chi-square, using Fisher's exact test when appropriate, and Mann-Whitney statistics were used to assess differences in demographic and injury variables between PTE/no-PTE groups. All mean values were reported as the mean ± standard error of mean (SEM).

There were no a priori hypotheses regarding genetic models of disease for the selected SNPs of interest. Therefore, we initially examined autosomal dominant and autosomal recessive models for ADK and ENT-1 to determine if either genetic model was associated with seizure activity using chi-square analyses. Because ecto-5′-nucleotidase is known to form homodimers,[22] we compared seizure activity between heterozygous and homozygous individuals for the NT5E SNPs, in addition to autosomal dominant and recessive models. For all genes, the most significant genetic model was selected for further examination in primary analysis.

To screen for SNPs potentially associated with time to first seizure, Kaplan-Meier survival curves were generated for each SNP, using the genetic groupings indicated above, and compared using the log-rank statistic. Due to the observed correlation among the SNPs of interest, as evidenced by LD from examination using Haploview (described above and see Fig. 1), it was likely the number of truly independent tests would be less than the number of SNPs analyzed. Therefore, the minimum number of effective tests (M_{eff}) was calculated individually for each gene[23] and summed across genes to generate a total M_{eff} of five. Because NT5E and SLC29A1 both reside on chromosome 6, a M_{eff} was also calculated for both genes simultaneously to determine if there was significant correlation between genes. When examined together, the M_{eff} for NT5E and SLC29A1 did not show correlation between genes, and the total M_{eff} of five was subsequently used as the true number of independent tests. To correct for multiple comparisons during screening procedures, a Sidak correction was applied to the initial screening level of significance ($\alpha = 0.10$), using the M_{eff} as the number of tests. Log-rank statistics were then compared to a corrected level of significance of $\alpha = 0.021$.

SNPs that were significant using the Sidak corrected α, and that met assumptions of Cox proportional hazards modeling, were further examined in multivariate Cox models, adjusted for demographic and injury characteristics found to differ by PTE status in univariate analysis ($p < 0.10$). Following examination of individual SNPs in multivariate Cox models, SNPs were entered simultaneously into the adjusted model.

Additional post hoc descriptive analyses were conducted to assess genetic variant concordance between nominally significant SNPs and SNPs surviving correction for multiple comparisons. Cross tabulations were used to explore concordance among SNPs within genes.

Results

Study population

One hundred sixty-two subjects met inclusion criteria for PTE analysis. The average age was 33.27 ± 1.10 years; 80.2% were male, and 33 subjects were deceased at the conclusion of the 3 year follow-up. The median GCS score was

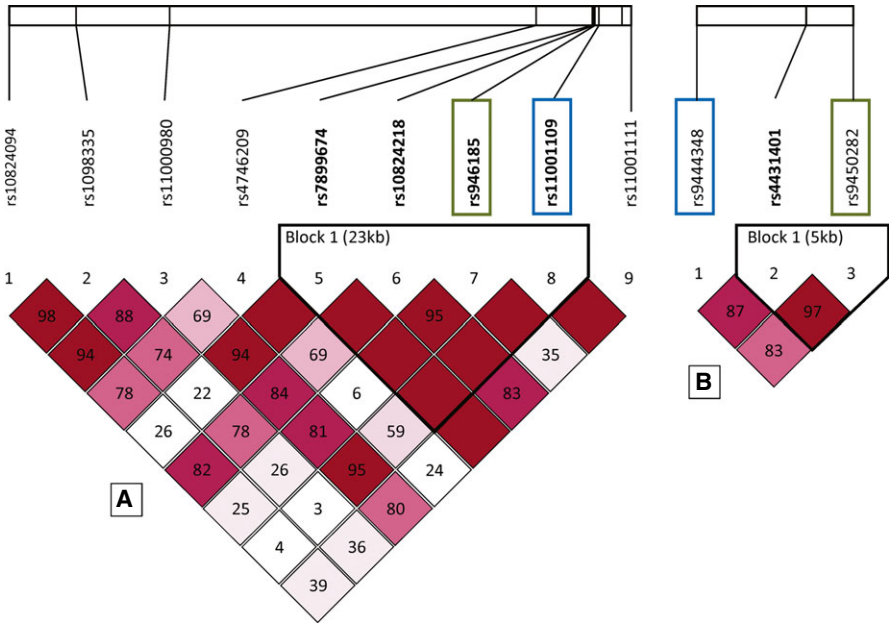

Figure 1.
Haploview LD maps of D′ values (within diamonds) using an LOD heatmap (LOD ≥ 2 in shades of pink/red) for (**A**) ADK and (**B**) NT5E. SNPs significant in Kaplan-Meier curves after correction for multiple comparisons shown in blue boxes; SNPs marginally significant in Kaplan-Meier curves shown in green. LD blocks outlined in black as calculated by 95% confidence bounds on D′.
Epilepsia © ILAE

6, and the mean ISS score was 35.42 ± 0.72. Common mechanisms of injury included motor vehicle accidents (59.3%). The acute care LOS ranged 7–61 days, with a mean of 23.62 ± 0.84 days. During the first week postinjury, 95.7% of subjects received AEDs for seizure prophylaxis including all subjects with PTE. Consistent with previous PTE studies,[3] 14.8% (n = 24) of subjects had documented evidence of PTE upon medical record review. Two additional subjects developed PTE outside of the 3 years follow-up but were included in the "No PTE" group for analyses. Of those with PTE, 30.8% had ≥1 EEG during acute care, and 50% had an EEG at the time of initial seizure presentation. There were significant differences in demographic variables by PTE status (Table 1). Those with isolated TBI more often developed PTE versus those with nonisolated TBI (p = 0.033). Subjects with SDH tended to have higher PTE frequencies (p = 0.089). Mortality was inversely associated with PTE (p = 0.007), with those who died having lower PTE rates. Age was not a significant factor associated with PTE.

Evaluation of tagging SNPs and PTE risk

Tagging SNPs for ADK, NT5E, and SLC29A1 were independently evaluated for their associations with time to seizure using a Kaplan-Meier model approach (Table 2). Within the ADK gene, individuals homozygous for the minor allele at rs11001109 (AA) had significantly shorter times to first seizure and higher seizure rates compared to major allele carriers (p = 0.018) (Fig. 2). Within NT5E, rs9444348 heterozygous subjects had shorter times to first seizure and higher seizure rates compared to homozygous subjects (p = 0.021) (Fig. 2). rs946185 (TT; ADK) minor allele homozygous and rs9450282 (NT5E) heterozygous subjects tended to have shorter time to seizure and higher seizure rates, although these findings were not statistically significant. Remaining SNPs evaluated showed no association with time to first seizure.

Multivariate SNP associations with PTE

After adjusting for ISS, isolated TBI status, and SDH in multivariate Cox models, there were significant differences

Table 1. Comparison of demographic and injury variables between subjects who did and did not develop PTE

	Adenosine Genetics Population (n = 162)		
Variable	No PTE	PTE	p-Value
Sex			
Women	25 (78.1%)	7 (21.9%)	p = 0.210
Men	113 (86.9%)	17 (13.1%)	
AED treatment			
Yes	131 (84.5%)	24 (15.5%)	p = 0.259
No	7 (100%)	0 (0.00%)	
GCS score			
3–4	50 (87.7%)	7 (12.3%)	p = 0.339
5–8	73 (83.0%)	15 (17.0%)	
9–12	13 (92.9%)	1 (7.1%)	
13–15	1 (50.0%)	1 (50.0%)	
Depressed skull fracture			
Yes	18 (75.0%)	6 (25.0%)	p = 0.128
No	120 (87.0%)	18 (13.0%)	
Injury mechanism			
Motor vehicle	83 (86.5%)	13 (13.5%)	p = 0.695
Fall	15 (88.2%)	2 (11.8%)	
Motorcycle	21 (77.8%)	6 (22.2%)	
Other	19 (86.4%)	3 (13.6%)	
Isolated head injury			
Yes	43 (76.8%)	13 (23.2%)	**p = 0.033**
No	93 (89.4%)	11 (10.6%)	
Mortality			
Alive	105 (81.4%)	24 (18.6%)	**p = 0.007**
Dead	33 (100%)	0 (0.00%)	
Age	33.6 ± 1.23	31.3 ± 2.27	p = 0.736
ISS	35.9 ± 0.78	32.4 ± 1.82	**p = 0.047**
Acute Care LOS	23.3 ± 0.91	25.6 ± 2.10	p = 0.288
Subdural hematoma			
Yes	78 (81.3%)	18 (18.7%)	p = 0.089
No	60 (90.9%)	6 (9.1%)	

Characteristics significantly associated with PTE shown in bold

Table 2. Comparison of Kaplan-Meier curves for SNP associations with PTE using log-rank statistics

SNP	Allele	p-Value	3-year seizure rate (%)	Mean days to seizure (SE)
ADK Gene				
rs10824218	TT	0.101	25.3	430.8 (31.6)
(n = 157)	AA + AT		14.3	903.3 (25.4)
rs11001111	GG	0.071	29.5	265.5 (25.7)
(n = 159)	AA + AG		15.3	898.4 (23.9)
rs10824094	TT	0.436	27.3	459.1 (50.5)
(n = 156)	CC + CT		16.9	878.1 (26.0)
rs946185	TT	0.046	31.0	406.0 (48.7)
(n = 158)	CC + CT		15.3	895.5 (24.5)
rs11001109	**AA**	**0.018**	**42.9**	**30.6 (3.3)**
(n = 158)	**GG + AG**		**16.0**	**890.5 (23.4)**
rs11000980	AA	0.355	10.3	578.8 (36.1)
(n = 151)	GG + AG		18.6	860.6 (28.9)
rs1908335	GG	0.264	28.6	446.0 (44.5)
(n = 157)	TT + GT		16.2	883.8 (25.6)
rs4746209	TT	0.841	16.8	805.3 (31.5)
(n = 159)	GG + GT		17.8	868.1 (35.6)
rs7899674	CC	0.228	14.6	905.5 (28.3)
(n = 157)	GG + GC		22.2	540.5 (27.4)
NT5E Gene				
rs9444348	**GG + AA**	**0.021**	**10.2**	**948.5 (21.8)**
(n = 156)	**AG**		**23.8**	**529.8 (25.6)**
rs4431401	CC + TT	0.431	16.0	903.8 (30.7)
(n = 152)	CT		20.4	543.3 (24.6)
rs9450282	GG + AA	0.034	12.9	912.3 (26.9)
(n = 151)	AG		27.4	726.3 (47.4)
SLC29A1 Gene				
rs324148	CC	0.161	20.9	860.9 (36.9)
(n = 158)	TT + CT		11.4	584.6 (19.0)
rs760370	AA	0.410	13.7	814.4 (37.3)
(n = 155)	GG + AG		20.1	859.5 (32.7)

SNPs significant using the adjusted α of 0.021 are shown in bold.

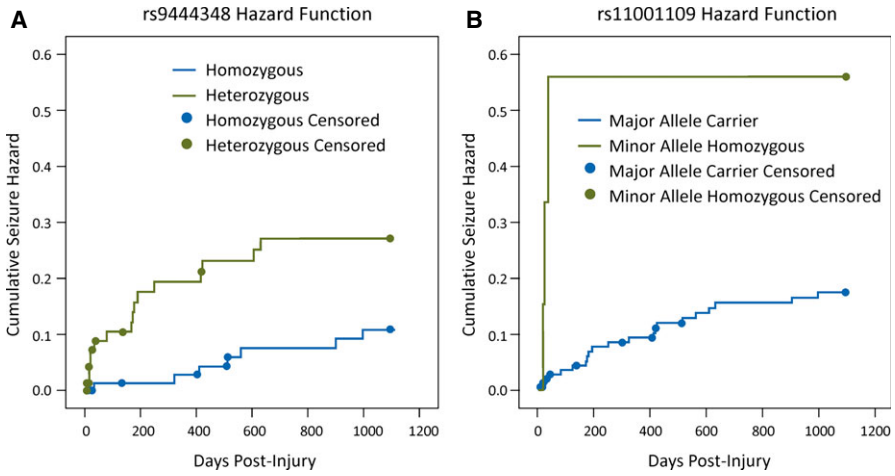

Figure 2.
Kaplan-Meier curves depicting significantly different time to first seizure and 3-year seizure rate by genetic model for (**A**) NT5E: rs9444348, p = 0.021 and (**B**) ADK: rs11001109, p = 0.018.
Epilepsia © ILAE

Table 3. Multivariate cox proportional hazard models

Model	Hazard ratio	Confidence Interval	p-Value
Model 1 (n = 156)			
rs11001109: AA (compared to AG + GG)	4.47	1.27–15.77	**0.020**
ISS	0.99	0.92–1.05	0.674
IHI	2.50	0.80–7.83	0.116
Model 2 (n = 154)			
rs9444348: AG (compared to GG + AA)	2.95	1.19–7.31	**0.019**
ISS	0.98	0.91–1.04	0.492
IHI	1.51	0.48–4.69	0.481
Model 3[a] (n = 151)			
rs11001109: AA (compared to AG + GG)	3.30	0.91–11.90	0.069
rs9444348: AG (compared to GG + AA)	2.52	1.00–6.32	0.050
ISS	0.98	0.91–1.04	0.474
IHI	0.58	0.18–1.87	0.36

All models adjusted for ISS, IHI and SDH.
Due to nonproportionality, models are stratified by SDH status.
[a]SNPs entered simultaneously. SNPs with significantly different HRs shown in bold.

in HRs for rs11001109 (p = 0.020) and rs9444348 (p = 0.019) (Table 3). The risk of developing PTE was ~4.5 times higher for individuals who were minor allele homozygotes for rs11001109 compared to major allele carriers. rs9444348 heterozygous individuals had ~3 times higher risk of developing PTE than homozygous individuals within 3 years post-TBI. When entered simultaneously into a multivariate Cox model, both SNPs remain marginally significant (rs11001109: p = 0.067, rs9444348: p = 0.050) (Table 3).

Post hoc descriptive analyses

Nominally or statistically significant variants in Kaplan-Meier models were explored to examine levels of within gene concordance among these SNPs. Of interest, when SNPs located within the ADK gene were examined, all subjects that were minor allele homozygous at rs11001109 were also minor allele homozygous at rs946185 and rs11001111. These subjects represented a minority of the population (4.5%). In the NT5E gene, 29.3% of subjects were found to be heterozygous for both rs9450282 and rs9444348, whereas 41.5% were homozygous.

Discussion

To determine whether genetic variation within integral components of the adenosine regulatory cycle is associated with epileptogenesis and PTE risk, we investigated a total of 14 SNPs within the ADK, NT5E, and SLC29A1 genes (9, 3, 2 SNPs, respectively). It is notable that SNPs associated with increased HR, rs11001109, and rs9444348 remain significantly associated with PTE risk after adjusting for potential confounders, including SDH, which is consistently cited as a risk factor for posttraumatic seizure.[2,3] To our knowledge, this is the first clinical investigation evaluating how ADK, NT5E, and SCL29A1 genetic variation influences time to first seizure and PTE risk. Based on International League Against Epilepsy (ILAE) definitions[19] and the time to event analytical approach, our data show that there is a genetic risk within ADK and NT5E for PTE over the first 3 years after severe TBI. Both rs11001109 and rs9444348 are tagging SNPs with no known functionality at this time. However, these data do support the hypothesis that genetic variation within adenosine regulatory pathways can accelerate or shorten the latent period between injury and PTE, suggesting a possible genetic role in epileptogenesis following TBI.

The ADK gene, located on chromosome 10q11-q24, is 546-kb long and is one of the largest in the human genome.[7] Despite its size, the coding sequence produced by the ADK gene is only about 1.1 kb long and comprises 11 short exons, each of which range from 36 to 765 nucleotides in length.[7] rs11001109 is located within intron 10, about 13 kb

from the end of exon 10 and 25 kb from the 3′-end of the ADK gene, and it is in LD with multiple other tagging SNPs across a large DNA block of ~80 kb, ranging from intron 9 into the 3′ end of the ADK gene. The region of LD tagged by rs11001109 includes exon 10, which contains the catalytic site of ADK.[24] Variation within rs11001109 may represent functional variation within this exon of the ADK gene. In addition, a missense polymorphism, rs397514452, causing a residue change from alanine to glutamic acid, has been identified in this exonic region within a small case series of individuals with hypermethioninemia and epileptic seizures.[25] These findings further suggest that rs11001109 may reflect functional variation in the represented DNA block. However, future studies are needed to determine what, if any, effects in ADK protein or gene expression result from variation represented by tagging SNPs explored in this current PTE study.

NT5E is located on chromosome 6q14.3, is about 1/10th the size of ADK (55 kb), and consists of 9 exons. rs9444348, is located within intron 1, and as shown from the 1000 Genomes CEPH population, is in LD with a region extending across exon 1 and approximately 22 kb 5′ of NT5E (http://www.broadinstitute.org). Using data obtained from RegulomeDB (www.regulomedb.org), variation represented by rs9444384 may affect binding capacity. The lack of association between ENT-1 genetic variation and PTE in our study is consistent with findings by Wiesner et al.,[26] and further specifies the importance of enzymatic regulation of adenosine in epileptogenesis compared to adenosine transport.

The importance of adenosine regulation and its neuroprotective role has been well established through in vitro and rodent models and extensive reviews of the literature.[27–29] Physiologically, ADK and NT5E are critical components of adenosine regulation as part of a complex adenosine regulatory cycle. In addition, multiple adenosine receptors (i.e., A_1R and $A_{2A}R$), with regional expression patterns and functionality, contribute to this regulatory cycle. As part of the adenosine cycle, two metabolic processes contribute to extracellular adenosine. Bi-directional nucleoside transporters are driven by intracellular adenosine metabolism via ADK, and extracellular ATP dephosphorylation to adenosine occurs via ectonucleotidases, including 5′-ectonucleotidase. Numerous studies consistently document significantly higher levels of extracellular adenosine during pathophysiologic processes, such as those initiated by TBI.[27,28] It is important to note that increased adenosine can contribute to the development of reactive astrogliosis through increased activation of $A_{2A}Rs$.[30] Astrogliosis is a common pathology associated with both TBI and epilepsy[9,10] and associated with ADK upregulation.[31] Genetic variation in NT5E, the activity of which parallels $A_{2A}Rs$ and/or ADK, may increase gliosis, possibly impacting epileptogenesis.

Experimental manipulation of adenosine cycle components and adenosine receptors has shed light on mechanisms of epileptogenesis and the pathophysiology of epilepsy. The extracellular tone of adenosine has a direct inhibitory effect on ictogenesis via A_1R activation that couples to inhibitory G proteins, mediates presynaptic inhibition, and stabilizes the postsynaptic membrane potential.[7,32] The failure of endogenous seizure prevention mechanisms could promote epileptogenesis in the sense that "seizures beget seizures." A_1R agonists have been shown to reduce seizure activity. However, application of various ADK inhibitors can achieve similar results with an improved side effect profile.[26,33] Combined A_1R and $A_{2A}R$ pharmacologic modulation may provide more fine-tuned seizure control.

Rodent studies investigating kainic acid (KA)–induced status epilepticus (SE) report ADK overexpression and increases in ADK enzymatic activity contribute to epileptogenesis.[11,33,34] Experiments in a similar model demonstrate that seizures originate from focal areas of ADK overexpression and generalize to the cortex following disruption of adenosine signaling through A_1R blockade.[34] ADK overexpression, and the resulting adenosine deficiency, is associated with nontraumatic epilepsy development in humans and rodents.[35] Genetically modified mice having reduced ADK expression result in reduced incidence of KA-induced SE, protection against brain injury associated with acute seizures, and resistance to epileptogenesis.[11] Together this work suggests ADK's role in epileptogenesis may be based on the ability of ADK to regulate extra-/intracellular adenosine homeostasis.

Of interest, recent data suggest that intracellular adenosine may facilitate epileptogenesis by regulating the DNA methylome.[36] Adenosine is an obligatory end product of DNA methylation and needs to be metabolically cleared by ADK, presumably via the nucleus-based enzyme isoform[29,36] in order to maintain transmethylation reactions. It is notable that increased ADK activity leads to DNA hypermethylation thought relevant for epileptogenesis.[29,36] Indeed, the methylation hypothesis of epileptogenesis suggests that seizures by themselves can induce epigenetic modifications, thereby aggravating the epileptogenic condition.[37] In addition, the NT5E gene has a CpG island regulated through methylation.[38] It is notable that experimental TBI studies suggest that injury itself reduces epigenetic marks compared to controls,[39] lending greater importance to epigenetics as a mechanism for PTE development. ADK polymorphism associations with epigenetic marking profiles could shed light on the contribution of epigenetic mechanisms and possibly lead to the identification of additional biomarkers to inform PTE risk and pathology.

Although the literature regarding manipulation of 5′-ectonucleotidase is not as expansive as the literature involving ADK, synthesis of current evidence suggests that disruption in 5′-ectonulceotidase function affects adenosine regulation and epileptogenesis. Data suggest that 5′-ectonucleotidase is the enzyme most responsible for the last step of ATP degradation: dephosphorylation of AMP to adenosine and a free

phosphate.[40] Significant reductions in extracellular adenosine have been shown when using 5′-ectonucleotidase inhibitors and NT5E knockout mice.[41] In vitro studies also demonstrate reduced adenosine production following 5′-ectonulceotidase inhibitor administration.[42] It is possible that genetic variation, as observed in our results, may alter 5′-ectonucleotidase function, decrease extracellular adenosine, and increase seizure activity.

There are two functional domains for NT5E, with the N-terminal domain responsible for catalytic activity, and the C-terminal domain for substrate binding.[43] Two binding conformations result in different orientation of these two functional domains,[43] and variation within this gene may affect any of these components required for normal enzymatic function. Outside of its purinergic enzymatic activity, NT5E influences T-cell immunoactivation and inflammatory cell adhesion,[43] potentially implicating this enzyme with inflammatory pathways affecting epileptogenesis after TBI. Our previous work implicates inflammation, specifically genetic variation in the IL-1β gene and IL-1β levels, to PTE risk and accelerated epileptogenesis.[6]

rs11001109 and rs9444348 were marginally significant when presented in the same multivariate model, suggesting that these variants capture some shared variance with regard to PTE risk. This finding is not surprising, given the interrelatedness of these two genes in managing the adenosine regulatory cycle pathway. Multivariate models also linked isolated TBI status and increased PTE occurrence. Several studies have reported differences in inflammatory responses in serum following isolated TBI compared to nonisolated TBI,[44] including IL-1β,[45] suggesting that individuals with isolated TBI may have an inflammatory response more conducive to epileptogenesis compared to those with nonisolated TBI. In addition, SDH was associated with PTE in univariate analysis, supporting previous work reporting an association between SDH and PTE.[2] Some literature suggests age may be a risk factor for PTE.[2,3] However, there were no significant differences between individuals that did/did not seize when we assessed age as a continuous variable (see Results) or when dichotomizing age at previously published cut points (data not shown).

It is important to acknowledge limitations when interpreting the study findings. Because the primary hypothesis centered on genetic variation in epileptogenesis following TBI, time to first seizure was the primary variable of interest and was collected via medical record abstraction. Although the medical system whose comprehensive electronic record system was reviewed is the largest provider in the geographic area, subjects may have presented to physicians outside the system for subsequent seizure development, resulting in misclassification. Nonconvulsive seizures frequently occur early after TBI.[46] Therefore, seizure incidence could also have been misclassified based on the first convulsive seizure. To control for population stratification, we limited analyses to white individuals. Without ancestral data regarding race, it is possible that residual population stratification exists. Conversely, limiting analyses to self-reported white individuals prevents generalization to other racial populations. Although this cohort is one of the largest single center TBI cohorts with genetic data, analyses are limited by small sample size. Future studies are needed to validate the current findings.

In addition, one SNP within the ADK and one SNP within the NT5E genes were marginally associated with time to first seizure using Kaplan-Meier models, but were not statistically significant using the α level appropriately corrected for multiple comparisons (Table 2). With a larger sample size, there could be significant associations with time to first seizure and accelerated epileptogenesis that survives correction for multiple comparisons with these SNPs that are only nominally/marginally significant with our sample size. Future studies can investigate how variation at functional SNPs (e.g., rs201127930, ADK) and the 5′ region of NT5E, located within the regions of LD identified, are important to epileptogenesis to validate our findings. Despite these limitations, this study presents biologically plausible preliminary evidence that genetic variation in the adenosine regulatory cycle is a risk factor for epileptogenesis and PTE in a clinical population with severe TBI. If validated, appropriate therapeutics targeting this pathway can be explored.

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Disclosures

No author has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm this report is consistent with those guidelines.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Minor allele information for study population.

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Adenosine Kinase: Exploitation for Therapeutic Gain

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ABBREVIATIONS: A-134974, *N*7-[(1′*R*,2′*S*,3′*R*,4′*S*)-2′,3′-dihydroxy-4′-aminocyclopentyl]-4-amino-5-iodopyrrolopyrimidine; A-286501, *N*7-((1′*R*,2′*S*,3′*R*,4′*S*)-2′,3′-dihydroxy-4′-amino-cyclopentyl)-4-amino-5-bromo-pyrrolo[2,3-*a*]pyrimidine; AAV, adeno-associated virus; ABT-702, 4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-*d*]pyrimidine; ADA, adenosine deaminase; ADK, adenosine kinase; ADK-L, long isoform of ADK; ADK-S, short isoform of ADK; A_1R, adenosine A_1 receptor; $A_{2A}R$, adenosine A_{2A} receptor; $A_{2B}R$, adenosine A_{2B} receptor; A_3R, adenosine A_3 receptor; AraA, 9-β-D-ribofuranosyladenine; bp, base pair; BHK, baby hamster kidney; CNS, central nervous system; ERK, extracellular signal-regulated kinase; ES, embryonic stem; GP-515, 4-amino-1-(5-amino-5-deoxy-1-β-*d*-ribofuranosyl)-3-bromo-pyrazol[3,4-*d*] pyrimidine; GP-3269, 7-(5-deoxy-β-D-ribofuranosyl)-*N*-(4-fluorophenyl)-5-phenyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine; 5-ITU, 5-iodotubercidin; GP-3966, 4-*N*-(4-fluorophenyl)amino-5-phenyl-7-(β-D-erythrofuranosyl) pyrrolo[2,3-*d*]pyrimidine; HIF, hypoxia inducible factor; IBD, inflammatory bowel disease; 5-ITU, 5-iodotubercidin; kb, kilobase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCAO, middle cerebral artery occlusion; MES, maximal electroshock; MK-801, dizocilpine; mTOR, mammalian target of rapamycin; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; PK, protein kinase; PLC, phospholipase C; PPI, prepulse inhibition; REM, rapid eye movement; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SIDS, sudden infant death syndrome; SUDEP, sudden unexpected death in epilepsy; TBI, traumatic brain injury; TNF-α, tumor necrosis factor α.

***Abstract*——Adenosine kinase (ADK; EC 2.7.1.20) is an evolutionarily conserved phosphotransferase that converts the purine ribonucleoside adenosine into 5′-adenosine-monophosphate. This enzymatic reaction plays a fundamental role in determining the tone of adenosine, which fulfills essential functions as a homeostatic and metabolic regulator in all living systems. Adenosine not only activates specific signaling pathways by activation of four types of adenosine receptors but it is also a primordial metabolite and regulator of biochemical enzyme reactions that couple to bioenergetic and epigenetic functions. By regulating adenosine, ADK can thus be identified as an upstream regulator of complex homeostatic and metabolic networks. Not surprisingly, ADK dysfunction is involved in several pathologies, including diabetes, epilepsy, and cancer. Consequently, ADK emerges as a rational therapeutic target, and adenosine-regulating drugs have been tested extensively. In recent attempts to improve specificity of treatment, localized therapies have been developed to augment adenosine signaling at sites of injury or pathology; those approaches include transplantation of stem cells with deletions of ADK or the use of gene therapy vectors to downregulate ADK expression. More recently, the first human mutations in ADK have been described, and novel findings suggest an unexpected role of ADK in a wider range of pathologies. ADK-regulating strategies thus represent innovative therapeutic opportunities to reconstruct network homeostasis in a multitude of conditions. This review will provide a comprehensive overview of the genetics, biochemistry, and pharmacology of ADK and will then focus on pathologies and therapeutic interventions. Challenges to translate ADK-based therapies into clinical use will be discussed critically.**

I. Introduction

All living systems need efficient self-regulatory mechanisms to adjust metabolic demand to available energy sources. The purine ribonucleoside adenosine is the core partial structure of ATP and has been termed a “retaliatory metabolite” (Newby et al., 1985) in the sense that any drop in energy supplies and ATP lead to increased adenosine, which in turn provides negative feedback inhibition to reduce metabolic demand to save energy. Adenosine is not only part of the energy metabolites AMP, ADP, and ATP of the cell but also an integral component of RNA. In addition, it is part of several adenine-containing coenzymes such as NAD or FAD, part of second messenger systems such as cAMP, and is a central metabolite of biochemical pathways such as the transmethylation pathway. Given its tight link to the energy pool of the cell and to central biochemical reactions and messengers, it is not surprising that adenosine fulfills a key role as a metabolic regulator of energy homeostasis (Fredholm et al., 2011b). Adenosine thus controls important physiologic functions, such as blood supply, glucose homeostasis via interactions with both insulin and glucagon, and lipolysis (Hjemdahl and Fredholm, 1976; Fredholm and Sollevi, 1977). Under conditions of stress or distress adenosine levels rapidly rise, largely by breakdown of adenine nucleotides (Fredholm, 2007). Under those conditions adenosine exerts a multitude of protective functions on many different levels (Linden, 2005; Fredholm, 2007). Those include mechanisms to 1) increase oxygen supply or to decrease oxygen demand by regulation of blood flow, body temperature, and cell work; 2) induce tolerance to hypoxic damage by mechanisms of preconditioning; 3) regulate angiogenesis; and 4) regulate immune responses (Linden, 2005). Most of these physiologic functions of adenosine are mediated by four types of G-protein-coupled adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, A_3R) (Fredholm et al.,

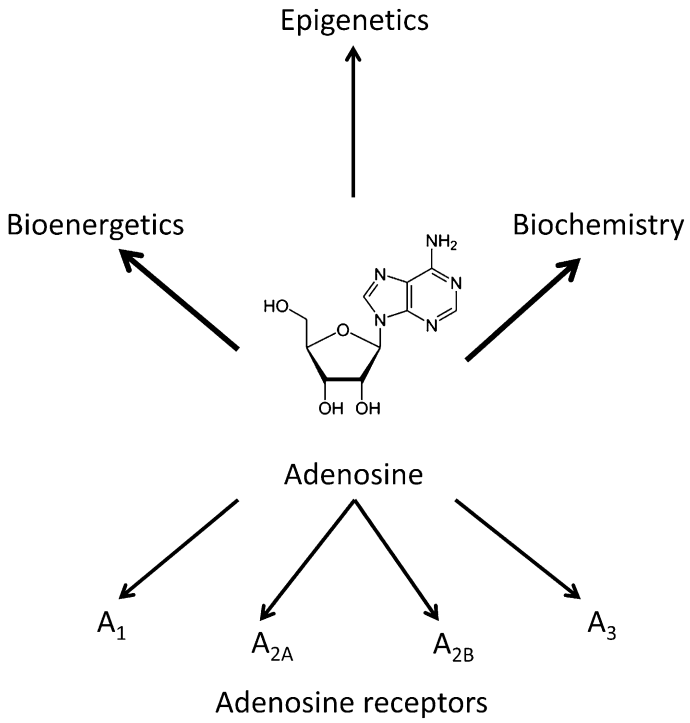

Fig. 1. Adenosine acts as a homeostatic network regulator via multiple adenosine receptor-dependent and -independent pathways.

2000, 2001a, 2011a), although adenosine receptor independent functions of adenosine might also play a role (Fig. 1). In the following sections, I will discuss the existing literature on adenosine kinase (ADK) comprehensively and in detail. The extensive literature on adenosine and its receptors has been reviewed in several comprehensive review articles to which the reader is kindly referred (Camm and Garratt, 1991; Dunwiddie and Masino, 2001; Fredholm et al., 2005b, 2007, 2011a,b; Hasko et al., 2005; Jacobson and Gao, 2006; Fredholm, 2007, 2010; Sawynok, 2007; Cunha, 2008; Headrick and Lasley, 2009; Sebastiao and Ribeiro, 2009a; Stone et al., 2009; Burnstock et al., 2011). Therefore, the discussion of the general literature on adenosine and its receptors has been limited to selected and more recent articles and reviews.

A. Evolutionary Considerations

Adenine, the purine base of adenosine, might have played a role in prebiotic evolution. Importantly, adenine was shown to form nonenzymatically from hydrogen cyanide, a reaction that might have occurred on our primitive Earth (Oro, 1961). Therefore, it is most likely that adenine was already among the primordial compounds that played crucial roles in the origin of life on Earth (Miller and Urey, 1959a,b). Of note, the evolution of life started with self-replicating adenosine-containing RNA ("RNA world") and not with deoxyadenosine-containing DNA, which evolved much later (Joyce, 2002). Three prerequisites for the origin of life have been suggested: energy (ATP), information (RNA), and membranes (Melendez-Hevia et al., 2008). Chemical evolution most likely led to the first proto-cells (Melendez-Hevia et al., 2008). It is tempting to speculate that in those first primitive organisms adenosine assumed a central position between energy and information. To construct a primordial self-regulatory system, a simple, rapid, and efficient method was needed to adjust metabolic demand to available energy supplies. Thus, if energy drops, ATP declines, adenosine increases, and it is this increase in adenosine that exerts a global inhibitory activity. Primordial regulatory systems needed to be simple and based on key biochemical and metabolic pathways. An early evolutionary appearance of adenosine as a key regulator of metabolism and energy homeostasis is supported by its ubiquitous involvement in many physiologic processes (Dunwiddie and Masino, 2001; Fredholm et al., 2011b). It is this primordial function as "master regulator" that is still maintained in all living systems today. More sophisticated regulatory systems as we know them today were added later, on different layers, to tune and fine-tune the system. It is logical that any disruption of this energy homeostasis-based primordial regulatory system has severe consequences for health and disease. Consequently, adenosine homeostasis needs to be kept under tight control.

B. Physiologic Role of Adenosine Kinase

Biochemically, adenosine can be formed by dephosphorylation of AMP via 5′-nucleotidase (EC 3.1.3.5) or by cleavage of *S*-adenosylhomocysteine (SAH) via SAH-hydrolase (EC 3.3.1.1). The major routes of adenosine removal are based on deamination to form inosine via adenosine deaminase (EC 3.5.4.4) or phosphorylation to AMP via adenosine kinase (ADK; EC 2.7.1.20). Importantly, 5′-nucleotidase and ADK are part of a highly active substrate cycle between adenosine and AMP, which enables a cell to rapidly respond to changes in the energy status; it has been shown that minor changes in ADK activity rapidly translate into major changes in the concentration of ambient adenosine (Bontemps et al., 1983, 1993a,b). Since levels of intracellular AMP, ADP, and ATP are high (millimolar range) and levels of adenosine are low (nanomolar range), any changes in the adenosine/AMP substrate cycle flow selectively effect the adenosine concentration without having major impact on the equilibrium of the phosphorylated compounds (Fredholm et al., 2005a; Boison et al., 2010). Several lines of evidence support the notion that ADK, which is a low-capacity and low-K_m enzyme, is the primary enzyme for metabolic adenosine clearance under baseline conditions, with the goal to keep adenosine levels low (Boison et al., 2010). Thus, ADK expression levels are highest in those organs, in particular liver and placenta (Andres and Fox, 1979), which have the highest needs for metabolic adenosine clearance (Finkelstein and Martin, 1986). In contrast, ADA is a high-capacity and high-K_m enzyme, which assists in metabolic

adenosine clearance under conditions in which adenosine levels become excessive (e.g., due to pathologic activity) and the capacity of ADK is exceeded (Boison et al., 2010). Of note, ADK is an evolutionary ancient and highly conserved enzyme, which is directly related to bacterial ribokinases and fructokinases (Spychala et al., 1996; Park and Gupta, 2008). On the basis of these early evolutionary roots, it is not surprising that ADK has been identified in almost all living organisms that have been analyzed genetically, including microorganisms, yeasts, plants and animals, and in every tissue assayed.

II. Gene Structure and Transcription

The *Adk* gene at a remarkable size of 546 kb in humans and 390 kb in the mouse is, together with the human dystrophin gene (Tennyson et al., 1995), one of the largest genes known (Singh et al., 2001; Singh and Gupta, 2004). It has been located on chromosome 10q11-q24 in the human and on chromosome 14 A2-B in the mouse (Klobutcher et al., 1976; Samuelson and Farber, 1985). Although the size of the gene that encodes human ADK is 546-kb long, the coding sequence is only about 1.1 kb. Thereby, the human *Adk* gene has the highest intron/exon ratio of all known mammalian genes (Park and Gupta, 2012). Human *Adk* cDNAs encode proteins with sequence-derived molecular masses of 38.7 and 40.5 kDa, which differ in their N-terminal 21 amino acids (McNally et al., 1997).

A. Gene Structure and Homologies

The *Adk* gene has been characterized, cloned, and expressed from many species, including human (Singh et al., 1996, 2001; Spychala et al., 1996; McNally et al., 1997; Park et al., 2007), mouse (Singh et al., 1996; Boison et al., 2002b), rat (McNally et al., 1997), plants (Moffatt et al., 2000, 2002; Vanderpoorten et al., 2004), and human pathogens (Darling et al., 1999; Long et al., 2003). All known mammalian *Adk* genes have identical structures and comprise 11 relatively short exons (36 to 765 nucleotide range), which yield a coding sequence of ~1100 bp. In contrast, the intervening introns are huge and range from 4.2 to 128.6 kb in humans. The large size of the *Adk* gene seems to be a characteristic feature of amniotes (Park and Gupta, 2012), whereas the *Adk* genes in phylogenetically older eukaryotes, such as in fish or amphibians, are smaller in size (20 to 25 kb) (Singh et al., 2001; Singh and Gupta, 2004). Remarkably, the *Adk* coding sequence is highly conserved in evolution among vertebrate animals. *Adk* cDNA from *Homo sapiens* is 98% identical to *Adk* from *Macaca mulatta,* 88% identical to *Bos taurus*, 84% identical to *Mus musculus,* 83% identical to *Rattus norvegicus,* and even 76% identical to *Xenopus* sp. The *Adk* genes in the invertebrates *Drosophila melanogaster* and *Cenorhabditis elegans* diverge more in sequence similarities and are even smaller in size at 1.5 and 1.3 kb, respectively. The *Adk* gene of the plant *Arabidopsis thaliana* has only 10 small introns located within a 2.4-kb gene (Moffatt et al., 2000, 2002).

The enormous size of the *Adk* gene in amniotes, including human and mouse, is biologically intriguing. Its transcription alone should take about 4 hours (Tennyson et al., 1995). Therefore, it is unlikely that *Adk*-expression undergoes rapid regulatory changes at the transcriptional level. It is therefore more likely that the *Adk* gene drives the expression of a stable long-term product that might be subject to developmental regulation within the context of extended time spans. No additional genes have been identified within intronic *Adk* sequences of human and mouse.

B. Alternative Splice Variants

Two isoforms of ADK (ADK long or ADK-L, and ADK short or ADK-S) are present in mammalian cells (Juranka and Chan, 1985; Sahin et al., 1996, 2004; Sakowicz et al., 2001). Both isoforms are identical except for the amino acids encoded by their first exons (exon 1 and exon 1A) with exon 1A being located in the intron between exon 1 and 2. Differential splicing of the unique first exons with the remaining *Adk* exons gives rise to the two isoforms (Cui et al., 2011).

C. Alternative Promoter Use

Recent findings, based on the analysis of deletion mutants derived from cultured Chinese hamster cells and data mining of the human genome sequence, have identified two independent promoters driving the expression of each of the two isoforms (Singh and Gupta, 2004; Cui et al., 2011). The promoter driving the expression of ADK-L is bidirectional at least in human, hamster, and other mammals, and is linked in head-to-head orientation with the clathrin adaptor mu3A protein (Singh and Gupta, 2004), which is thought to be involved in protein sorting at the Golgi membrane (Drake et al., 2000). Recent blast searches of the human genome with the nucleotide sequence specific for ADK-S and its upstream noncoding region have identified a putative promoter region within the first intron of ADK-L and 350 bp upstream of the initiator codon of ADK-S. This putative promoter is located within a CpG island, and several transcription factor binding sites have been identified in its proximity (Cui et al., 2011). Although the functionality of this promoter region needs to be validated experimentally, this finding offers the intriguing possibility that each of the two isoforms of ADK is regulated independently at the transcriptional level. Independent transcriptional regulation might in turn suggest different physiologic functions of the two isoforms.

III. Biochemistry

First attempts at the biochemical characterization of ADK go back some 45 years and initially focused on mammalian tissue extracts or human tumor cells (Lindberg et al., 1967; Schnebli et al., 1967). The original interest in ADK was its tight link to nucleic acid metabolism as a salvage pathway for adenosine utilization. Despite the long interest in ADK and despite the wealth of biochemical information derived from modern technologies, the regulatory mechanisms that determine ADK activity, and hence adenosine homeostasis, still remain largely enigmatic. In the following, available information has been summarized and gaps of knowledge identified.

A. Catalytic Reaction

ADK is an ATP:adenosine 5′-phosphotransferase catalyzing the following phosphorylation reaction (Kornberg and Pricer, 1951): ATP + adenosine → ADP + AMP. This is an uncommon reaction type in which donor (ATP) and acceptor (adenosine) of the phosphoryl group share the same structural motif (adenine ring). ADK contains two catalytic sites: a high-affinity site, which binds adenosine and AMP selectively, and a site for ATP and ADP (Pelicano et al., 1997). These unique features of the ADK reaction complicated the interpretation of kinetic data and both a two-site ping-pong mechanism (Chang et al., 1983) and an ordered Bi-Bi mechanism (Henderson et al., 1972; Palella et al., 1980; Mimouni et al., 1994) have been proposed. Information obtained from the crystal structures of human (Mathews et al., 1998) and *Toxoplasma gondii* (Schumacher et al., 2000) ADK have confirmed an ordered Bi-Bi mechanism and a more detailed mechanism has recently been proposed (Park and Gupta, 2008). In a first step, inorganic phosphate or an activator compound binds to a conserved NXXE motif. Binding of an activator facilitates the binding of free Mg^{2+} and adenosine to the active site of ADK, causing a conformational change of the enzyme, which in turn increases the affinity for MgATP inducing the formation of an anion hole. This stabilizes the pentacovalent transition state, which is typical for an in-line S_N2 displacement reaction. The magnesium ion plays a catalytic role and enhances the electrophilicity of the γ-phosphate of ATP, whereas the bound inorganic phosphate may increase the electrophilicity of its β-phosphate. This weakens the oxygen bridge between the two phosphate groups. At the same time the 5′-hydroxyl end of adenosine is deprotonated, attacking the positive center of the γ-phosphate. In a final step the γ-phosphate is transferred to adenosine, and products are released in the order of ADP and AMP (Park and Gupta, 2008).

B. Protein Structure

1. Isoforms. Catalytically active ADK exists as a monomer (Sen et al., 2006; Park and Gupta, 2008). Although monomer-stabilizing interaction partners have been identified in ADK from parasites (Sen et al., 2006), it remains to be determined whether similar interaction partners exist for mammalian ADK. Alternative promoter use and splicing (see above) yields two isoforms of mammalian ADK (Juranka and Chan, 1985; Singh et al., 1996; Spychala et al., 1996; McNally et al., 1997). Human *Adk* cDNAs encode proteins with sequence-derived molecular masses of 38.7 and 40.5 kDa, differing only in their N-terminal 21 amino acids (McNally et al., 1997). The long isoform of ADK, ADK-L, contains 21 additional N-terminal amino acids (MAAAEEEPKPKKLKVEAPQAL in human ADK-L), which replace four N-terminal amino acids of the short isoform ADK-S (MTSV in human ADK). Both isoforms are enzymatically functional and show no obvious differences in their kinetic properties (Sakowicz et al., 2001; Sahin et al., 2004).

2. Subcellular Localization. ADK is expressed in most organ systems of the mammalian body with highest expression levels in liver, pancreas, and placenta (Andres and Fox, 1979; Fedele et al., 2005; Cui et al., 2011). Founded on algorithms that predict subcellular localization based on sequence similarities, it was initially speculated that both isoforms of ADK are located in the cytoplasm (Nakai and Horton, 1999; Sakowicz et al., 2001). A recent study however, identified specific subcellular localizations of both isoforms of ADK (Cui et al., 2009). ADK-immunofluorescence analysis of cultured mammalian cells that expressed only ADK-L revealed only nuclear labeling, whereas cells that expressed both isoforms showed labeling in nucleus and cytoplasm (Cui et al., 2009). Transfection of cells with ADK-L or ADK-S carrying a C-terminal fusion with a c-myc epitope or a green fluorescent protein tag confirmed nuclear expression of ADK-L and cytoplasmic expression of ADK-S in vitro. Overexpression of an ADK-S transgene in an *Adk*-null background in the mouse revealed cytoplasmic localization of ADK-S (Fedele et al., 2005), a finding that was replicated by adeno-associated virus (AAV)-based overexpression of ADK-S in mouse brain (Shen et al., 2011; Theofilas et al., 2011).

Thus, independent lines of evidence from in vitro and in vivo studies show that ADK-S is located in the cytoplasm, whereas ADK-L is specific for the nucleus. The N-terminal sequence of ADK-L contains a cluster of conserved amino acids (PKPKKLKVE). When KK in this sequence was replaced by either AA or AD, nuclear localization of ADK was abolished; further fusion of this sequence to other proteins redirected their localization to the nucleus (Cui et al., 2009). These findings suggest that ADK-L contains a novel nuclear localization signal. The nuclear localization of ADK-L suggests a specific function for gene regulation (see below) (Studer et al., 2006). Interestingly, both isoforms of ADK are differentially expressed in a variety of mammalian tissues. Whereas both isoforms of ADK are

prominently expressed in kidney, liver, lung, and pancreas, there is a predominance of ADK-L in brain, and ADK-S expression dominates in adrenal gland, spleen, and thymus; heart and muscle appear to express only ADK-S (Cui et al., 2011). The functional significance of isoform specific expression patterns in different organs has yet to be determined.

3. Crystal Structure. The crystal structure of ADK was first identified for human ADK (Mathews et al., 1998) and subsequently for the parasitic protozoan *T. gondii* (Cook et al., 2000; Schumacher et al., 2000). More recently, the crystal structures of several different eukaryotic and prokaryotic ADKs have been resolved (Reddy et al., 2007; Cassera et al., 2011; Kuettel et al., 2011). Identification of the crystal structure of ADK yielded important insights into the catalytic mechanism and provided information for the design of drugs acting on ADK. As outlined above, the enzymatically active form of ADK is a monomer. Crystallographic studies have identified a larger $\alpha\beta\alpha$ three-layer sandwich domain with a smaller "lid." The larger domain is composed of a central β-sheet with nine strands, which is flanked by 10 α-helices and provides the binding sites for the substrates adenosine and ATP (Mathews et al., 1998). The smaller domain is composed of a five-stranded mixed β-sheet flanked by two α-helices and forms a lid over the active site of the enzyme (Mathews et al., 1998). The two domains are connected by four peptide segments and adenosine binds in the cleft between those domains (Mathews et al., 1998). Studies from *T. gondii,* in which ADK was crystallized both as apo-enzyme and in its substrate-bound forms, revealed a major conformational change of the enzyme upon adenosine binding, reminiscent of opening and closing of the "lid" domain (Cook et al., 2000). In this model, the apo-enzyme is in the open confirmation with the adenosine-binding pocket exposed to the solvent environment. The substrate-bound form, in contrast, is in the closed confirmation, with the lid hiding the substrate binding pocket (Cook et al., 2000). This major conformational change is likely accomplished by a "GG-switch" composed of residues Gly68 and Gly69 (Cook et al., 2000). Although the sequences of *Mycobacterium tuberculosis* and human ADK are less than 20% identical, their overall structures, including the flexible lid, are similar (Mathews et al., 1998; Reddy et al., 2007). Remarkably, this structural similarity extends to bacterial ribokinases, suggesting an early evolutionary origin of ADK (Park et al., 2007; Park and Gupta, 2008, 2012).

4. Catalytic Site. Crystallography studies performed in different species (Mathews et al., 1998; Cook et al., 2000; Schumacher et al., 2000; Reddy et al., 2007; Cassera et al., 2011; Kuettel et al., 2011) uniformly revealed that the large domain of ADK contains the catalytic core, which is located at the domain interfaces, where adenosine binds in a deeply buried cavity and is covered by the smaller lid domain. The ATP binding site is located at an adjacent site in the large domain with the γ-phosphate group pointing near the 5′-end of the ribose moiety of adenosine. Binding of adenosine to the open apo-form of the enzyme induces a 30° rotation of the lid domain relative to the large domain. Thereby adenosine will be sequestered and formation of the ATP binding site in the large domain will be initiated at the same time. Local structural changes are induced by binding of ATP leading to the formation of an anion hole. Once ATP has bound, a closed conformation is achieved in which the small domain of ADK brings an evolutionary conserved catalytic arginine to the active site when adenine is bound (Schumacher et al., 2000). This catalytic arginine forms a hydrogen bond to the γ-phosphate of ATP and orientates the γ-phosphate into the catalytic position for a typical in-line S_N2 displacement reaction (Schumacher et al., 2000).

5. Regulatory Site. The occurrence of substrate inhibition of ADK and a dual regulatory character of some adenosine analogs suggested the existence of an additional regulatory binding site for adenosine with a lower affinity for adenosine (Pelicano et al., 1997; Lin et al., 1988). Based on competition studies, this regulatory site was reported to differ from the catalytic site and might play a role under conditions of high adenosine production, such as during times of ischemia or seizures (Fisher and Newsholme, 1984; Hawkins and Bagnara, 1987; Lin et al., 1988). The existence of a second adenosine-binding site was further supported by chemical modification studies, which demonstrated that a highly active thiol group was essential for activity (Neudecker and Hartmann, 1972, 1978). Adenosine concentrations equivalent to the dissociation constant for the second binding site were shown to effectively protect the reactive thiol group from inactivation by 5,5′-dithio-bis(2-nitrobenzoic acid); consequently, this thiol group has been associated with the second regulatory binding site for adenosine that is different from the ATP binding site (Hawkins and Bagnara, 1987). The presence of two binding sites for adenosine has been confirmed in the crystal structure of human ADK (Mathews et al., 1998). The authors of this study identified a second adenosine-binding site at the ATP-binding site of the enzyme and concluded that substrate inhibition of ADK was due to competitive inhibition of ATP binding (Mathews et al., 1998). This conclusion, however, is not consistent with kinetic data from human placental ADK, which suggest that adenosine is a non-competitive inhibitor of ATP binding (Palella et al., 1980), and with the 5,5′-dithio-bis(2-nitrobenzoic acid) inactivation studies, which suggest that the regulatory adenosine binding site is different from the ATP binding site (Hawkins and Bagnara, 1987).

6. Modeling Studies. Crystallography studies in combination with modeling studies for the binding of

nucleoside and nonnucleoside inhibitors of ADK have revealed more details regarding the conformational changes of ADK. A semi-open conformation intermediate between open and closed, with a small lid-domain rotation of 12° degrees, was first described in *T. gondii* ADK (Zhang et al., 2007). In this model residues Gly143-X-X-Gly146 were suggested to be subject to torsional changes upon substrate binding, which together with a Gly68-Gly69 switch were predicted to induce a hinge bending of the lid domain. The authors of this study concluded that the intermediate conformation suggests that ATP binding is independent of adenosine binding. The possible existence of a semi-open conformation was subsequently confirmed in human ADK by modeling the binding of larger tubercidins, which were thought to stabilize the semi-open conformation (Bhutoria and Ghoshal, 2010). By use of an automated ligand-docking program with a genetic algorithm to explore the full range of ligand conformational flexibility and partial flexibility of the protein (Jones et al., 1997), it was shown that the semi-open conformation, resulting from a smaller degree of ligand-induced movement of the binding site, was sufficient to accommodate aryl compounds (Bhutoria and Ghoshal, 2010). Pharmacophore modeling suggested the existence of three distinct pharmacophoric elements for closed, semi-open, and open-state binders (Bhutoria and Ghoshal, 2010).

C. Kinetic Studies

Early kinetic studies on ADK were based on enzyme purified from ADK-rich tissues such as liver (Miller et al., 1979a,b; Yamada et al., 1981), placenta (Palella et al., 1980), brain (Yamada et al., 1980), or heart (Fisher and Newsholme, 1984). Remarkably, no major differences were observed in the kinetic properties among the different adenosine kinases (Yamada et al., 1982). The key properties of purified ADK can be summarized as follows: maximal enzyme activity is found at pH 6.5–6.8. Under those conditions ADK has an apparent K_m for adenosine of 0.2–0.4 μM and an apparent V_{max} of 2.2 μmol of AMP formed per minute per milligram of protein. In most tissues investigated, the K_m values of ADK for adenosine were between one and two orders of magnitude lower than those of adenosine deaminase (ADA) (Arch and Newsholme, 1978; Phillips and Newsholme, 1979). On the basis of those kinetic data and studies with ADK and ADA inhibitors, it was concluded that under conditions that provide adequate oxygen and glucose, ADK plays a much greater role than ADA in regulating the extracellular concentration of adenosine. Only under conditions of increased energy depletion when adenosine formation is increased, ADA becomes important in regulating extracellular adenosine concentration (Lloyd and Fredholm, 1995). Furthermore, 5′-nucleotidase and ADK are simultaneously active in many tissues including liver or brain, so that a substrate cycle between AMP and adenosine results (Arch and Newsholme, 1978; Bontemps et al., 1983). The difference in K_m values between ADK and ADA indicates that, via this substrate cycle, small changes in the activity of either ADK or 5′-nucleotidase produce rapid changes in adenosine concentration (Arch and Newsholme, 1978; Bontemps et al., 1983).

D. Transcriptional Activation of Adenosine Kinase

Few studies have addressed the transcriptional regulation of the *Adk* gene. ADK expression was found to be reduced in streptozotocin-induced diabetes mellitus in rats (Pawelczyk et al., 2000), whereas insulin was shown to restore *Adk* mRNA to normal levels within the first 7 hours of insulin treatment (Sakowicz and Pawelczyk, 2002). Mechanistic studies in splenocytes isolated from diabetic rats have shown a 3.9-fold increase in *Adk* mRNA 4 and 5 hours after the incubation of the cells with 10 nM insulin (Pawelczyk et al., 2003). Insulin-dependent activation of *Adk* transcription required activation of the mitogen-activated protein kinase (MAPK) pathway, since transcriptional activation of the *Adk* gene was blocked by the MAPK inhibitor PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one]. Insulin exposure also resulted in increased phosphorylation of ERK1/2 and Elk-1 and sustained elevation of c-Jun and c-Fos protein, whereas those changes could be prevented by incubating the cells with PD98059. The authors concluded that insulin activates *Adk* gene transcription via activation of the MAPK cascade and subsequent phosphorylation of Elk-1 and increased expression of c-fos and c-jun (Pawelczyk et al., 2003).

E. Transcriptional Repression of Adenosine Kinase

Hypoxia is known to lead to a rapid rise in adenosine—likely a homeostatic protective response of a tissue (Berne, 1963; Berne et al., 1974; Decking et al., 1997; Frenguelli et al., 2003). At least one transcriptional mechanism might contribute to this phenomenon. It was demonstrated in vitro that hypoxia induced in endothelial cells caused a robust repression (85% reduction) of *Adk* transcript levels. Transcription factor binding assays, hypoxia inducible factor 1-α (HIF-1α) loss- and gain-of-function studies, as well as abrogation of *Adk* transcriptional repression by ambient hypoxia in conditional HIF-1α mutant mice, demonstrated a definitive role of HIF-1α in the transcriptional repression of the *Adk* gene (Morote-Garcia et al., 2008).

F. Regulation by Metabolites

Uniquely, ADK activity is regulated by its own substrates and products, as well as by factors that reflect the energy state and health of a cell. Thus, ADK fulfills the role of a sensor for the energy state and bioenergetic equilibrium of a cell, and at the same time

ADK acts as a switch determining ambient levels of the "retaliatory metabolite" adenosine to adjust metabolic demand to available energy supplies.

1. Adenosine. Surprisingly, ADK from several tissues is inhibited by its own substrate adenosine (Miller et al., 1979a; Fisher and Newsholme, 1984). The magnitude of substrate inhibition increases with rising concentrations of Mg^{2+} (Fisher and Newsholme, 1984). In human placental ADK, substrate inhibition was observed at adenosine concentrations greater than 2.5 μM at pH 7.4, with ATP and Mg^{2+} 0.2 mM, i.e., ~10 times higher than the K_m of the enzyme for adenosine (Palella et al., 1980). Substrate inhibition was likewise found in ADK from human erythrocytes, where the degree of inhibition was found to be pH and Mg^{2+} dependent (Hawkins and Bagnara, 1987). In human liver, substrate inhibition was reported at significantly lower concentrations of adenosine (above 0.5 μM), indicating a rather narrow activity range of ADK in regard to ambient adenosine concentrations (Yamada et al., 1981). Similar adenosine concentrations for substrate inhibition were reported in rodent samples (Yamada et al., 1982; Fisher and Newsholme, 1984). Whereas physiologic adenosine concentrations in the range of 25–300 nM (Lonnroth et al., 1989) are not likely to affect ADK activity, substrate inhibition of ADK by higher concentrations of adenosine might be an important physiologic mechanism to potentiate endogenous adenosine responses under conditions of stress or distress, which can lead to micromolar concentrations of adenosine (Clark et al., 1997; Fredholm, 2007).

2. AMP. The activity of ADK also depends on the concentrations of AMP. It was found that AMP concentrations below 5 mM activated the enzyme, whereas concentrations above 5 mM inhibited the enzyme (Hawkins and Bagnara, 1987). Therefore, under physiologic concentrations of AMP in the range of 0.3 mM (Boesiger et al., 1994), ADK is expected to be activated by AMP, whereas only excessive AMP concentrations are likely to inhibit ADK, e.g., under conditions of severe energy stress, a meaningful physiologic response to augment adenosine signaling in stressful situations. Inhibition of ADK activity by higher concentrations of AMP was found to be competitive with respect to adenosine and noncompetitive with respect to ATP (Palella et al., 1980).

3. ADP. ADP was found to be a noncompetitive inhibitor with regard to adenosine and ATP (Palella et al., 1980; Rotllan and Miras Portugal, 1985; Mimouni et al., 1994). Hyperbolic inhibition was observed during noncompetitive inhibition of adenosine kinase by AMP and ADP (Palella et al., 1980).

4. ATP. ADK activity critically depends on available ATP levels in a Mg^{2+}-dependent manner (Lindberg et al., 1967), whereas free ATP was found to inhibit ADK, the Mg^{2+}-complexed form of ATP activated ADK (Palella et al., 1980; Rotllan and Miras Portugal, 1985). The K_m of ADK for MgATP was determined as 75 μM (Palella et al., 1980). It needs to be mentioned that ATP can also be replaced by GTP as phosphate group donor (Miller et al., 1979b).

5. Magnesium. In most kinase reactions the true phosphate donating substrate is a complex of ATP^{4-} and a divalent metal ion, typically Mg^{2+} forming $MgATP^{2-}$, which then binds the enzyme. In agreement with this concept, a lack of Mg^{2+} in the medium resulted in lack of ADK activity, whereas maximal enzyme activity was achieved in the presence of Mg^{2+} at pH levels where ATP and Mg^{2+} existed primarily in the complexed, chelated form (Palella et al., 1980). The magnesium ion is thought to partly neutralize the negative charges on the phosphate groups of the nucleotide, which otherwise would prevent binding to the enzyme (Mildvan, 1987). After saturation of available ATP, a further increase in Mg^{2+} will result in free Mg^{2+}. ADK activity increases further with increases in free Mg^{2+}; however, once optimal activity levels have been reached, further increases in Mg^{2+} will inhibit the enzyme (Palella et al., 1980; Rotllan and Miras Portugal, 1985; Maj et al., 2002). This free, catalytic Mg^{2+} ion is thought to bind to the active site of the enzyme and induce the transition state of the reaction by increasing the electrophilicity of the μ-phosphorous atom of the nucleotide via its interaction with the oxygen atoms (Parducci et al., 2006). Furthermore, the free Mg^{2+} may optimize the spatial arrangement of the substrate's functional groups (Rivas-Pardo et al., 2011).

6. Inorganic Phosphate. Interestingly, ADK displays a dependency on inorganic phosphate or other pentavalent ions as was first demonstrated in ADK isolated from Chinese hamster cells (Hao and Gupta, 1996). In those studies, the addition of inorganic phosphate, but also of arsenate or vanadate, increased the V_{max} of the reaction and decreased the K_m for adenosine. In contrast, these pentavalent ions did not change the K_m for ATP. Dependency of the enzyme reaction on inorganic phosphate has been confirmed in ADK preparations derived from many different species (Maj et al., 2000, 2002; Park et al., 2006).

7. pH. Maximal activity of ADK derived from human placenta has been observed at pH 6.5 (Palella et al., 1980), whereas ADK from rat brain and human liver displayed a biphasic pH optimum with a sharp pH peak of activity at pH 5.5 and a broad peak of activity at pH 7.5–8.5 (Yamada et al., 1980,1981). A broad pH optimum in the pH 6–8 range was also reported for rat heart ADK (Fisher and Newsholme, 1984). In a more recent study it was shown that under more acidic conditions (pH 6.2) the presence of inorganic phosphate became a necessity for activation (Maj et al., 2000). The pH optimum at close to physiologic conditions implies that a drop in pH as occurs during or after injury is

expected to inactivate ADK, thus contributing to an injury-induced surge of protective adenosine.

8. NO. Several studies have shown that nitric oxide (NO) induces the release of adenosine. Mechanistic studies performed on cultured neurons or hippocampal slices suggest that NO raises adenosine through inhibition of ADK (Rosenberg et al., 2000; Arrigoni and Rosenberg, 2006). However, it was not resolved whether inhibition of ADK was a direct effect of NO or an indirect effect caused by substrate inhibition.

G. Posttranslational Modifications

ADK does not seem to be a target for posttranslational modifications. A screen of a panel of protein kinases for their ability to phosphorylate recombinant mouse ADK yielded negative results (Sahin et al., 2004). Accordingly, ADK is most likely not an efficient substrate for PKA, PKC, PKG, CaMKII, CK1, CK2, MAPK, Cdk1, or Cdk5 (Sahin et al., 2004). Given the early evolutionary origin of ADK it might not seem too surprising that ADK is not regulated by mechanisms that evolved much later.

H. Protein-Protein Interactions

Protein-protein interactions might play important roles in the regulation of ADK activity. Seminal biochemical studies performed on ADK from the parasitic protozoan *Leishmania donovani* suggest a very attractive regulatory model, which is based on aggregation and disaggregation of the enzyme. With increasing concentrations, fully active *L. donovani* ADK formed soluble aggregates, resulting in inactivation of the enzyme. By using the aggregated inactive enzyme as the substrate, it was shown that a cyclophilin from *L. donovani* could induce complete disaggregation, leading to reactivation of the enzyme. It was further shown that the reactivating ability of cyclophilin remained unaffected even in the presence of cyclosporine A and macromolecular crowding agents. The reactivation occurred noncatalytically and was reversible (Chakraborty et al., 2002). The prevention of ADK aggregation by cyclophilin was shown to be mediated by an isomerase-independent chaperone function of cyclophilin (Chakraborty et al., 2004). It was further shown that ADP stabilized the aggregated form of ADK and that cyclophilin was able to disaggregate and activate ADK (Sen et al., 2006). Under conditions of cellular stress a rise in ADP is expected to stabilize the inactive aggregate of ADK and thereby promote a rise in adenosine, which in turn will suppress energy-consuming activities. On the other hand, a cyclophilin-based chaperone function may reactivate ADK any time, even under conditions of energy depletion. Whether mammalian ADK is regulated by a similar chaperone-based mechanism remains to be demonstrated. Intriguingly, it was shown that cyclosporine A and FK506 (tacrolimus) decreased ADK activity in T-lymphocytes (Spychala and Mitchell, 2002). Clinically, cyclosporine A and FK506 treatment led to a rise in plasma adenosine in kidney transplant recipients, suggesting that the resulting increase in plasma adenosine contributes to the immunosuppressive effects of these agents (Guieu et al., 1998).

I. Influence on Downstream Pathways

1. Adenosine Homeostasis. As outlined above, the concentration of adenosine in a tissue is mostly determined by the activities of adenosine-producing nucleotidases, by adenosine-producing transmethylation reactions, and by adenosine-removing ADK and ADA as well as by transmembrane transporters for adenosine (Boison et al., 2010). Extracellular adenosine flows into adenosine-metabolizing cells through equilibrative nucleoside transporters (Baldwin et al., 2004). Because those transport functions depend on the intracellular metabolic clearance rate of adenosine to maintain the inward flux of adenosine, the velocity of intracellular metabolic clearance of adenosine determines the rate of adenosine removal from the extracellular space. Thus, under steady-state conditions of adenosine production, the extracellular adenosine concentration is determined by the rate of intracellular adenosine clearance (Greene, 2011). ADK is the metabolic enzyme with the highest affinity for adenosine. Because of its low capacity, the rate of metabolic adenosine clearance—and therefore its extracellular concentration—seems to be largely dependent on the V_{max} of ADK under physiologic conditions (Arch and Newsholme, 1978). Since ADK needs to bind both ATP and adenosine to transfer a phosphate from ATP to adenosine, resulting in the release of AMP and ADP, and since ADP might inactivate ADK by promoting its aggregated state, the velocity of the enzyme reaction and the resulting adenosine concentration in the extracellular space depend largely on the ATP/ADP ratio and the energy state of the tissue. Under physiologic conditions the homeostasis of adenosine is largely under the control of ADK, whose activity directly depends on the energy state of the cell. Adenosine affects several adenosine receptor dependent and independent pathways simultaneously (Fig. 1), as will be outlined in the following sections.

2. Adenosine Receptors. Adenosine activates four types of known G-protein-coupled adenosine receptors, which are designated as A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R. The pharmacology and physiologic functions of the adenosine receptors have extensively been reviewed (Fredholm et al., 2000, 2001a, 2011a,b; Jacobson and Gao, 2006; Sebastiao and Ribeiro, 2009a; Stone et al., 2009) and only key functions will briefly be outlined below.

a. Adenosine A_1 receptor. Activation of the A_1R, which is coupled to pertussis toxin-sensitive G_i proteins, leads to inhibition of adenylyl cyclase activity

(van Calker et al., 1979; Cooper et al., 1980) and to increased activity of phospholipase C (PLC) (Rogel et al., 2005; Tawfik et al., 2005). In the CNS as well as in the heart, A_1R stimulation leads to activation of K_{ATP} channels and pertussis-toxin-sensitive K^+ channels, whereas it leads to inhibition of Q-, P-, and N-type Ca^{2+} channels (Fredholm et al., 2001a, 2011a). In the heart, coupling to K^+ channels mediates the bradycardia effects of adenosine (Belardinelli et al., 1995); whereas modulation of p44/42 extracellular signal-regulated protein kinase (ERK) signaling through A_1R activation has been implicated mechanistically in the phenomenon of ischemic preconditioning (Reid et al., 2005).

b. Adenosine A_{2A} receptor. In contrast, activation of the $A_{2A}R$ leads to an increase in adenylyl cyclase activity. In peripheral tissues the $A_{2A}R$ couples predominantly to G_S proteins, whereas in striatum, a brain area that is particularly rich in $A_{2A}Rs$, the receptor couples predominantly to G_{olf}, which likewise couples to adenylyl cyclase (Kull et al., 2000). $A_{2A}R$ activation was found to facilitate noradrenaline release and activation of the PLC and adenylyl cyclase pathways in tail arteries of the rat (Fresco et al., 2004). In addition, $A_{2A}R$ activation induced the formation of inositol phosphates, thus raising intracellular calcium and activating protein kinase C in COS-7 cells (Offermanns and Simon, 1995).

c. Adenosine A_{2B} receptor. The $A_{2B}R$ couples positively to both adenylyl cyclase and PLC (Daly et al., 1983; Brackett and Daly, 1994; Peakman and Hill, 1994; Feoktistov and Biaggioni, 1997) and plays a major role in inflammation. $A_{2B}R$ activation was found to evoke interleukin-8 secretion via induction of inositol phosphate formation in a human mast cell line (Feoktistov and Biaggioni, 1995) and to mediate human chorionic vasoconstriction via activation of the arachidonic acid pathway (Donoso et al., 2005).

d. Adenosine A_3 receptor. Activation of the A_3R leads to inhibition of adenylyl cyclase (Zhou et al., 1992), stimulation of PLC (Abbracchio et al., 1995), and mobilization of calcium (Englert et al., 2002; Fossetta et al., 2003; Shneyvays et al., 2004, 2005). A_3Rs can protect cardiomyocytes through activation of K_{ATP} channels (Tracey et al., 1998), and the anti-ischemic effect of A_3R activation was found to be dependent on rhoA-phospholipase D1 signaling (Mozzicato et al., 2004). The A_3R might also play a role in cancer and cell growth, as well as in cell differentiation, survival, and death, since the A_3R couples to MAPK (Schulte and Fredholm, 2002, 2003) and since the WNT signaling pathway was found to contribute to A_3R activation-dependent suppression of melanoma cells (Fishman et al., 2002). Furthermore, proliferation of human melanoma cells was found to be inhibited after A_3R-dependent activation of the phosphatidylinositol 3-kinase-protein kinase B-ERK1/2 pathway (Merighi et al., 2005)

3. Adenosine Receptor-independent Pathways. The examples outlined above illustrate a multitude of AR-dependent pathways in multiple tissues and organ systems that directly depend on adenosine homeostasis. However, given the early evolutionary origin of adenosine and the relative late evolutionary appearance of the adenosine receptors (Burnstock and Verkhratsky, 2009; Fountain and Burnstock, 2009) it becomes plausible that adenosine might have additional, primordial functions that do not require ARs and that rely on biochemical and bioenergetic functions of adenosine.

a. Transmethylation. Adenosine is an obligatory end product of transmethylation reactions, which involve the transfer of a methyl group from *S*-adenosylmethionine (SAM) to a methyl group acceptor (e.g., ethanolamine or DNA) resulting in the formation of SAH, which in turn can be cleaved into adenosine and homocysteine by *S*-adenosylhomocysteine hydrolase when adenosine concentrations are kept low (Hoffman et al., 1979; Boison et al., 2002b; Mato et al., 2008) (Fig. 2). Importantly, transmethylation reactions can only be maintained when adenosine is constantly removed by ADK (Boison et al., 2002b; Mato et al., 2008). Therefore, it is not surprising that liver, the organ in which 80% of all mammalian transmethylation reactions take place, also has the highest expression levels of ADK (Yamada et al., 1981; Cui et al., 2011; Park and Gupta, 2012). Thus, transmethylation is not only a major source for adenosine (Lloyd et al., 1988; Deussen et al., 1989; Kroll et al., 1992) but 95% of the SAH-derived adenosine was found to be salvaged by ADK in isolated guinea pig heart preparations (Lloyd and Schrader, 1993). Conversely, if adenosine is not constantly removed by ADK, the thermodynamic equilibrium of the SAH hydrolase reaction favors the formation of SAH, which is a potent inhibitor of transmethylation reactions (Finkelstein and Martin, 1986; Finkelstein, 1998; Mato et al., 2008). Given the important role of ADK for metabolic clearance of SAH-derived adenosine, ADK is expected to be a regulator of transmethylation reactions. Indeed, the constitutive genetic disruption of the *Adk* gene in mice (Boison et al., 2002b) or in the plant *Arabidopsis* (Moffatt et al., 2002) provided the first direct evidence that ADK expression is a requirement for the maintenance of transmethylation. In mice, the deletion of ADK resulted in increased levels of SAH in the liver and microvesicular hepatic steatosis; all homozygous mutants developed steatotic liver and died within 14 days after birth (Boison et al., 2002b). Likewise, ADK-deficiency in *Arabidopsis* resulted in increased SAH and inhibition of SAM-dependent transmethylation reactions; affected plants were affected by reduced size and failure to elongate the primary shoot (Moffatt

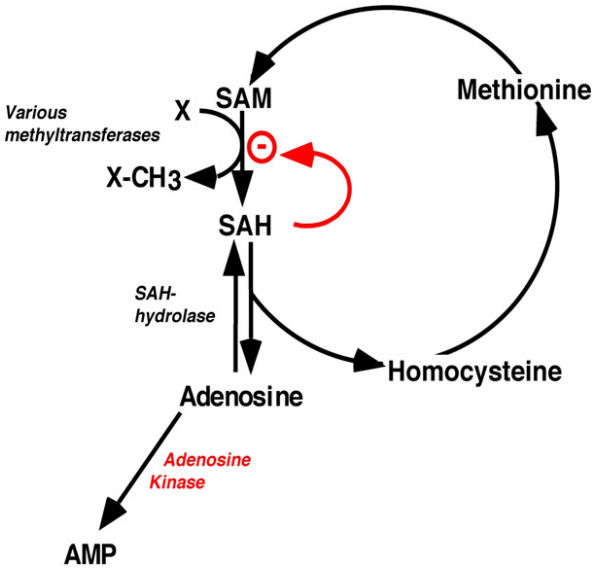

Fig. 2. Transmethylation pathway. Adenosine is an obligatory end product of transmethylation reactions, including those catalyzed by DNA methyltransferases. If adenosine is not constantly removed by adenosine kinase, increased levels of adenosine drive the *S*-adenosylhomocysteine hydrolase reaction toward . SAH synthesis. SAH is a potent inhibitor of methyltransferases, which use SAM as methyl group ($-CH_3$) donor.

et al., 2002). More recently, six human patients with *Adk* mutations resulting in a functional ADK deficiency have been described (Bjursell et al., 2011). These human mutations resulted in disruption of the transmethylation pathway and the development of liver pathology and encephalopathy (Bjursell et al., 2011). SAM-dependent transmethylation reactions also determine the methylation status of CpG islands in promoter regions. Therefore, I hypothesized that the ADK/adenosine system might likewise determine the methylation status of DNA and thereby exert a novel function as epigenetic regulator (Williams-Karnesky et al., submitted manuscript). Infusion of adenosine or homocysteine into the hippocampus of rats induced global DNA hypomethylation, whereas the infusion of SAM induced hypermethylation of the DNA, demonstrating that the methylation status of DNA directly depended on the adenosine-sensitive transmethylation pathway. Importantly, blocking ADK with 5-iodotubercidin or genetic reduction of ADK expression resulted in hypomethylated DNA in the brain, whereas overexpression of either the cytoplasmic or the nuclear isoform of ADK resulted in increased DNA methylation in cultured cells, with the nuclear overexpression of ADK being more efficient in increasing the methylation status of the DNA (Williams-Karnesky et al., submitted manuscript). These data suggest a novel—likely adenosine receptor-independent—role of ADK in regulating the methylation status of DNA and thereby acting as an epigenetic regulator.

b. Mitochondrial bioenergetics. As a "retaliatory metabolite" adenosine is directly linked to mitochondrial bioenergetics and energy homeostasis (Newby et al., 1985; Sommerschild and Kirkeboen, 2000; Peart and Headrick, 2007; Masino et al., 2009). It needs to be stressed that under basal conditions, levels of adenosine (~100 nM in brain) are nearly 10,000-fold lower than ATP (Pazzagli et al., 1995; Delaney and Geiger, 1996). Therefore, even minor decreases in ATP levels can result in dramatic rises in adenosine levels. In line with this notion, adenosine levels increased as brain energy levels decreased following a variety of excitatory stimuli (Shepel et al., 2005). Interestingly, mitochondria are capable to release adenosine (Bukoski et al., 1983, 1986), and a mitochondrial adenosine-producing 5′-nucleotidase has been identified (Raatikainen et al., 1992). Because a concentration-dependent adenosine output from mitochondria by diffusion or facilitated diffusion has been suggested (Raatikainen et al., 1992), it is tempting to speculate that metabolic clearance of mitochondria-derived adenosine by cytoplasmic ADK drives mitochondrial adenosine production. Through this mechanism ADK could directly affect mitochondrial bioenergetics. In support of this notion hepatocyte mitochondria from ADK-knockout mice display a severe mitochondrial pathology (Boison et al., 2002b).

4. Nitric Oxide Metabolism. The interactions between adenosine and nitric oxide metabolism and signaling is a major topic and worth a dedicated review. However, relatively few studies have directly focused on the interactions between NO metabolism and ADK. Several studies have shown that pharmacological inhibition of ADK reduced lipopolysaccharide (LPS)-induced NO production and the induction of inducible NO synthase, most likely via an A_2R-dependent mechanism (Lee et al., 2005; Petrov et al., 2005). On the other hand, as discussed above, NO triggers a rise in adenosine and subsequent inhibition of ADK by substrate inhibition (Rosenberg et al., 2000; Arrigoni and Rosenberg, 2006). This interrelationship between adenosine and NO homeostasis could be a self-limiting mechanism to terminate NO-dependent signaling.

IV. Pharmacology

A. Methods for Drug Development. Adenosine kinase inhibitors have received much attention in pharmaceutical drug development efforts during the late 1990s and early 2000s. Based on the rationale that ADK inhibitors would prevent the metabolic clearance of adenosine and thus potentiate the protective actions of *endogenous* adenosine, they were expected to augment adenosine signaling in a site- and event-specific manner and thus provide all the benefits of A_1R activation, but with reduced potential for widespread systemic side effects (Kowaluk et al., 1998; Kowaluk and Jarvis, 2000). The primary applications for ADK inhibitors were considered to be anti-inflammatory,

antinociceptive, and anticonvulsant therapy (Wiesner et al., 1999; Kowaluk and Jarvis, 2000; McGaraughty et al., 2001b, 2005). ADK inhibitor development was initially based on 5-iodo-7-β-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*] pyrimidin-4-amine (5-iodotubercidin, 5-ITU, Fig. 3) and 5′-amino-5′-deoxyadenosine as lead compounds (Cottam et al., 1993; Kowaluk et al., 1998; Wiesner et al., 1999), which were studied kinetically for inhibition of purified ADK activity (Cottam et al., 1993). A valuable strategy to modify and optimize existing lead molecules to improve their potency, bioavailability, or toxicity profile is based on fragmentation of existing leads and NMR-based screening of those fragments with the goal to identify suitable replacement of the fragments and incorporation of the newly identified fragments into the original scaffold (Hajduk et al., 2000). Structure-activity relationships and computational studies led to the identification of a wide range of ADK inhibitors (Cowart et al., 2001; Zheng et al., 2001; Gfesser et al., 2003; Perner et al., 2003; Ugarkar et al., 2003; Gomtsyan et al., 2004). A virtual screening approach led to the discovery of 2-aryloxazolopyrimidines as ADK inhibitors (Bauser et al., 2004). High throughput derivatization and liquid phase parallel synthesis of the 7-amino and the 2-aryl groups were subsequently used to generate highly potent derivatives (Fig. 3) (Bauser et al., 2004). To optimize ADK activity assays, capillary electrophoresis assays were developed, in which the enzymatic reaction was either performed in a test tube and subsequently

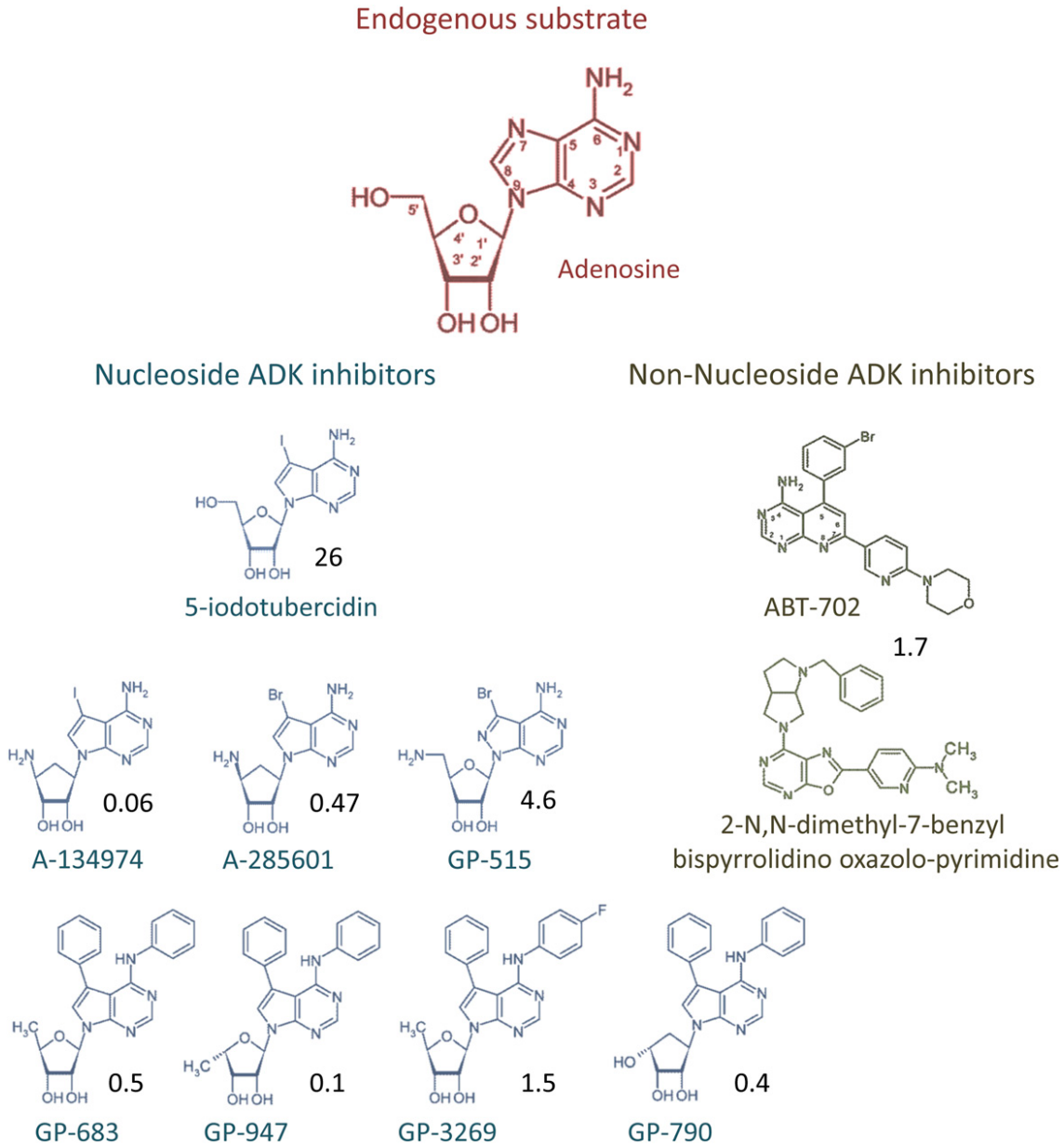

Fig. 3. Chemical structures of ADK's endogenous substrate adenosine and of selected nucleoside and nonnucleoside ADK inhibitors. Numbers in black refer to the IC_{50} of the inhibitor in nanomolars for rat cytosolic ADK. For details and references, please refer to main text.

injected into the capillary or in which the enzymatic reaction was directly performed in the capillary (Iqbal et al., 2006). The latter approach led to further sampling size reductions and increased throughput (Iqbal et al., 2006). To date, several classes of ADK inhibitors have been developed and characterized, which broadly fall into the categories of nucleoside and nonnucleoside ADK inhibitors.

B. Nucleoside Adenosine Kinase Inhibitors. Nucleoside adenosine kinase inhibitors have a hydroxylated ribose or cyclopentane ring and an appended purine or pyrimidine heterocyclic base. The prototype of nucleoside ADK inhibitors is 5-iodotubercidin (Fig. 3), which is a derivative of adenosine in which the 5-aza group of the purine ring has been replaced by a carbon that is linked to an iodine moiety; those compounds compete with adenosine for binding to the enzyme (Kowaluk et al., 1998; Kowaluk and Jarvis, 2000; McGaraughty et al., 2001b, 2005). Development of ADK inhibitors was initially based on the generation of 5-iodotubercidin analogs with modifications of the 5′-group of the ribose moiety to include hydroxyl-, chloro-, azido-, deoxy-, amino-, or fluoro-groups in the 5′-position; however, none of those compounds exceeded the potency of 5-iodotubercidin (Cottam et al., 1993). The ADK inhibitors 5-iodotubercidin, 5′-amino-5′-deoxyadenosine, 5′-deoxy-5-iodotubercidin, as well as novel classes of ADK inhibitors such as 4-(*N*-phenylamino)-5-phenyl-7-(5′-deoxyribofuranosyl)pyrrolo[2,3-day]pyrimidine (GP683), were shown to inhibit seizures in the maximal electroshock (MES) model in rats (Wiesner et al., 1999). Among those pyrrolo[2,3-day]pyrimidine nucleoside analogs, the 5′-amino-5′-deoxy analogs of 5-bromo- and 5-iodotubercidin exhibited the highest potency and efficacy in the MES model (Ugarkar et al., 2000b). Although none of those compounds met a safety, efficacy, and side effect profile suitable for further drug development (Ugarkar et al., 2000a), substitution of the tubercidin molecule with aromatic rings at the N4 and C5 positions yielded highly potent ADK inhibitors with efficacy in the MES model and reduced side effects (Ugarkar et al., 2000a). Potency of nucleoside ADK inhibitors was significantly enhanced (e.g., 10-fold compared with 5′-deoxy-5′-aminoadenosine) in 6,8-disubstituted purine nucleosides (Bookser et al., 2005a). Since cytotoxicity was found to be due to phosphorylation at the 5′-position of the ribose base, l-xylofuranosyl analogs of tubercidin were synthesized, which could no longer be phosphorylated due to their altered stereochemical orientation; the lead compound GP790 of those α-l-lyxofuranosyl nucleosides displayed prominent anti-inflammatory activity in a rat paw swelling model (Ugarkar et al., 2003). Likewise, erythrofuranosyltubercidin analogs were resistant to phosphorylation, and the orally bioavailable lead compound GP3966 was shown to exhibit broad-spectrum analgesic properties in dogs (Boyer et al., 2005). Diaryltubercidins such as GP3269 were orally active in the rat formalin paw model; however, the utility of this compound class was limited due to poor water solubility. To improve water solubility while retaining ADK inhibition potency a new compound class was generated by replacing the hydrophobic C4-phenylamino substituent with a hydrophilic glycinamide group. Although drugs from this compound class showed strong oral efficacy in pain models in the rat and marmoset monkey (ED_{50} estimated at 0.9 mg/kg) without evidence of side effects such as ataxia, sedation, or emesis, one compound caused lethal toxicity in the rat formalin paw model. Therefore, work on this series of compounds was discontinued (Bookser et al., 2005b).

C. Nonnucleoside Adenosine Kinase Inhibitors. Nonnucleoside ADK inhibitors lack ribose or cyclopentane rings and are either built on pyridopyrimidine cores or on alkynylpyrimidine cores, which were shown to reduce pain and inflammation in a variety of animal models (Cowart et al., 2001; Zheng et al., 2001; Gfesser et al., 2003; Gomtsyan et al., 2002, 2004; Gomtsyan and Lee, 2004). A virtual screening approach led to the discovery of a different class of nonnucleoside ADK inhibitors based on 2-aryl oxazolo-pyrimidines, which were further optimized to yield a variety of highly potent derivatives (Fig. 3) (Bauser et al., 2004). Earlier classes of nonnucleoside ADK inhibitors tended to cause locomotor side effects, a problem that was remedied by introducing polar 7-substituents of pyridopyrimidine derivatives (Zheng et al., 2003). Improved analgesic properties were achieved by the introduction of 5,6,7-trisubstituted 4-aminopyrido[2,3-day]pyrimidines as a novel class of nonnucleoside ADK inhibitors (Perner et al., 2003). In contrast, 6,7-disubstituted 4-aminopyrido[2,3-day]pyrimidines displayed only modest potency to inhibit ADK in intact cells (Perner et al., 2005). From the class of 4-amino-5,7-disubstituted pyridopyrimidines, which had been considered for clinical drug development, 5-(3-bromophenyl)-7-(6-morpholin-4-4-ylpyridin-3-yl)pyrido[2,3-day]pyrimidin-4-ylamine (ABT-702) has most widely been studied. ABT-702 (Fig. 3) was shown to have an EC_{50} of 1.7 nM and was equally effective on long and short isoforms of ADK from different organs and species (Jarvis et al., 2000). It was shown to be orally active and efficacious in reducing acute somatic nociception (ED_{50}: 65 μmol/kg p.o.) in the mouse hot-plate assay. It also dose-dependently reduced nociception in the phenyl-*p*-quinone-induced abdominal constriction assay (Jarvis et al., 2000) and was shown to be efficacious in a wide range of pain- and inflammation-related tests, including carrageenan-induced thermal hyperalgesia, the formalin test of persistent pain, and models of nerve injury-induced and diabetic neuropathic pain. Therapeutic effects were reversed by blocking adenosine receptors, indicating that the therapeutic effects were based on a rise in

adenosine (Kowaluk et al., 2000; Suzuki et al., 2001). Although 4-amino-5,7-disubstituted pyridopyrimidines were characterized as potent ADK inhibitors, compounds with a nitrogen atom in position C_7 of the heterocyclic ring, however, were shown to have mutagenic properties in the Ames assay (Matulenko et al., 2005).

D. Pronucleotides. A series of 6-(het)aryl-7-deazapurine pronucleotides was recently synthetized and shown to exhibit cytostatic activity. Interestingly, several of these pronucleotides strongly inhibited human ADK; however, the mechanistic implications of this finding have not been investigated further (Spacilova et al., 2010).

E. Substrates of Adenosine Kinase. A different pharmacological application makes use of the capability of ADK to phosphorylate nucleoside-based prodrugs into their active derivatives. This strategy has been employed to develop potential anticancer drugs. Thus, it was found that the proapoptotic effects of N^6-substituted derivatives of adenosine are related to their intracellular conversion into corresponding mononucleotides by ADK (Mlejnek and Dolezel, 2005). Vidarabine (9-β-D-ribofuranosyladenine or AraA) is an analog of adenosine containing D-arabinose instead of D-ribose and was originally considered as an anticancer drug (LePage et al., 1973). However, AraA also exhibits antiviral activity (Bryson et al., 1974) and was the first antiviral nucleoside to be licensed for the treatment of herpes virus infections in humans (Whitley et al., 1976). AraA needs to be phosphorylated to its 5′-triphosphate to be effective as an inhibitor of herpes virus replication (Balzarini and De Clercq, 1990). ADK converts AraA to its 5′-monophosphate, which is then further converted to its antiviral and cytotoxic 5′-triphosphate derivative (Chan and Juranka, 1981; Chan and Guttman, 1985).

V. Physiology and Pathophysiology

A. Lessons from Genetically Modified Organisms

Whereas important insights into the biochemistry of ADK were gained from mutant cell lines, the complex physiologic and pathophysiological roles of ADK were largely derived from genetic manipulations of ADK. Of note are genetic manipulations in mice and in the plant *A. thaliana* (mouse-ear cress). Together these studies demonstrate that ADK expression needs to be tightly controlled to maintain normal physiologic function. Studies on ADK from parasites will be discussed in a later section of this review.

1. Constitutive Deletion of Adenosine Kinase. A homozygous constitutive disruption of the *Adk*-gene was first accomplished in the mouse via a standard gene targeting approach (Boison et al., 2002b). Homozygous mutants were characterized by early postnatal mortality. Three causes of death were identified, as follows. 1) Mutant pups were affected by deficits in thermoregulation. When separated from their mothers at a room temperature of 22°C the body temperature of $Adk^{-/-}$ mutants dropped to 24°C within 15.6 minutes in contrast to wild-type littermates, which took 26.3 minutes to reach the same temperature (Boison et al., 2002b). Adenosine is known to regulate thermoregulation through A_1R- and A_{2A}R-dependent mechanisms (Jonzon et al., 1986; Zarrindast and Heidari, 1993; Fredholm et al., 2011b) and cooler pups were more likely to be culled by their mothers than normothermic littermates. 2) Mutant pups developed intermittent periods of apnea up to two times per hour and up to 20 seconds in duration, which contributed to lethal outcome during the first days after birth (Boison et al., 2002b). Periods of apnea in the mutant pups is consistent with increased activation of adenosine receptors in brain stem, which contribute to the control of respiratory function (Aoki et al., 2004; Wilson et al., 2004). 3) From postnatal day 4 onward, $Adk^{-/-}$ mutants developed microvesicular hepatic steatosis and failed to thrive as evidenced by significantly reduced weight gain and early death: 35% of the mutants died within the first 4 days of life, 53% between postnatal day 5 and 8, and only 12% survived up to 14 days. At postnatal day 7 a brightly colored yellow liver (Fig. 4) could visually be detected beneath the skin (Boison et al., 2002b). Metabolite analysis from liver samples revealed 2.3-fold elevated SAH and SAM and a 35% decrease in ATP in the homozygotes, whereas heterozygous mutants appeared to be normal. Increased SAH and SAM indicate disruption of the transmethylation pathway and demonstrate that constant removal of adenosine by ADK is necessary for the maintenance of transmethylation reactions (Boison et al., 2002b). Since liver is both the organ with the highest expression levels of ADK and the organ in which 80% of all transmethylation reactions take place, it is tempting to conclude that liver ADK plays a major role in the maintenance of transmethylation (Boison et al., 2002b). Because of the prominent liver pathology and early death of most mutants, further insight into the role of ADK in other organ systems could not be derived.

Interestingly, a genetic disruption of the *Adk* gene in *A. thaliana* led to a remarkably similar phenotype (Moffatt et al., 2002). Affected plants were characterized by major developmental abnormalities, including small growth with rounded, wavy leaves and a compact, bushy appearance. Importantly, the lack of adenosine salvage in the ADK-deficient plants led to elevated SAH and resulted in the inhibition of SAM-dependent transmethylation reactions. The authors of this study concluded that adenosine must be steadily removed by ADK to prevent feedback inhibition of SAH hydrolase and maintain SAM utilization and recycling (Moffatt et al., 2002).

2. Transgenic Overexpression of Adenosine Kinase. To investigate the role of ADK in the control of brain activity, a mouse model was developed containing a

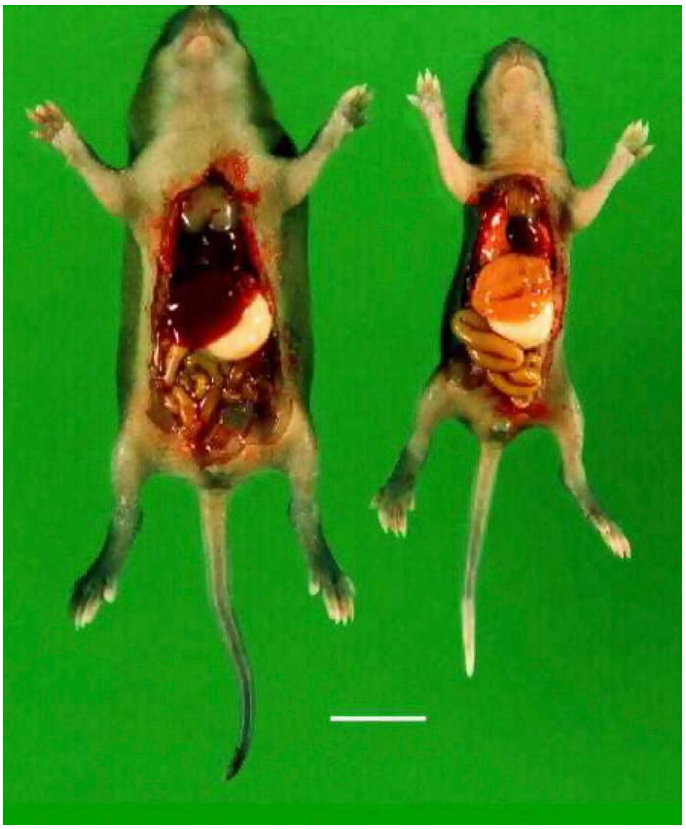

Fig. 4. Genetic disruption of ADK leads to hepatic steatosis. Wild-type mouse (left) and $Adk^{-/-}$ mouse (right) prepared at postnatal day 7. Note the reduced body size of the mutant and the yellow discoloration of the liver. Scale bar: 1 cm.

loxP-flanked *Adk* transgene encoding the short cytoplasmic isoform of ADK under the control of a human ubiquitin promoter within the $Adk^{-/-}$ background (Adk-tg) (Fedele et al., 2005). Adk-tg mice displayed increased brain ADK activity and constitutive overexpression of transgenic ADK throughout the brain, with particularly high levels in hippocampal pyramidal neurons. Consequently, the Adk-tg mice were characterized by various abnormalities in brain function. Brain ADK expression levels were found to 1) critically affect the basal concentration of ambient adenosine as evaluated by microelectrode biosensors, 2) determine the degree of tonic adenosine-dependent synaptic inhibition and hippocampal plasticity, 3) modulate the age-dependent effects of brain derived neurotrophic factor on hippocampal synaptic transmission, and 4) influence $GABA_A$ receptor-mediated currents in CA3 pyramidal neurons (Diógenes et al., 2012). Physiologically, overexpression of ADK in the brain of Adk-tg mice resulted in frequent electrographic seizures at a rate of about four seizures per hour (Fedele et al., 2005; Li et al., 2007a, 2008b). Furthermore, the animals displayed increased susceptibility to stroke- or seizure-induced neuronal cell death (Pignataro et al., 2007a; Li et al., 2008a,b; Shen et al., 2011), indicating that overexpression of ADK, resulting in a decreased concentration of endogenous adenosine, rendered the brain more vulnerable to seizures and to neuronal cell death. Behaviorally, Adk-tg mice were resistant to amphetamine induced hyperlocomotion (Yee et al., 2007; Shen et al., 2012) and displayed severe learning deficits in the Morris water maze task and in Pavlovian conditioning (Yee et al., 2007). Adenosine is known to be an important regulator of sleep physiology (Bjorness and Greene, 2009; Huang et al., 2011; Porkka-Heiskanen and Kalinchuk, 2011; Schmitt et al., 2012). Consequently, disruption of adenosine homeostasis by overexpression of ADK altered sleep physiology, with Adk-tg mice being awake more than 58 minutes more per day than wild-type mice and spending significantly less time in rapid eye movement (REM) sleep (Palchykova et al., 2010). In addition, ADK expression in brain stem might play an important role in addictive behavior, since morphine withdrawal behavior was significantly diminished in Adk-tg mice (Wu et al., 2013). Together, these data suggest that ADK expression in the brain is crucial for the regulation of a multitude of behaviors that depend on maintenance of adenosine homeostasis.

3. Brain-Specific Alterations of Adenosine Kinase Expression in Mice. In a first attempt to gain region-specific insights into the role of ADK expression on brain function, an *Emx1-Cre* transgene (Iwasato et al., 2004) was bred into the Adk-tg line to delete the loxP-flanked Adk-tg gene within the entire dorsal telencephalon. The resulting fb-Adk-def mice were characterized by a forebrain-selective reduction of ADK expression (Li et al., 2008b), increased levels of adenosine in the cerebral cortex (Shen et al., 2011), and resistance to acute seizures, after the excitotoxin kainic acid was injected into the amygdala (Li et al., 2008b). The animals were also resistant to seizure- or stroke-induced neuronal cell loss, indicating a strong neuroprotective effect of raised adenosine levels in the cortex (Li et al., 2008b; Shen et al., 2011). Importantly, fb-Adk-def mice were also resistant to the development of epilepsy in a mouse model of intra-amygdaloid kainic acid-induced epileptogenesis, suggesting for the first time that adenosine might have antiepileptogenic properties (Li et al., 2008b). Behaviorally, fb-Adk-def mice showed profound impairment in spatial working memory and enhanced motor responses to *N*-methyl-D-aspartate receptor blockade (Singer et al., 2012). More work is needed to identify the role of ADK in specific brain areas, and new lines of conditional *Adk*-mutants are needed to address pertinent region-specific questions.

B. Adenosine Kinase Mutations in Humans

Six human patients have been described recently with mutations in *Adk* that prevent the expression of functional protein (Bjursell et al., 2011). All mutations caused disruptions in the methionine cycle resulting in

hypermethioninemia, inhibition of transmethylation, and severe liver pathology reminiscent to changes found in *Adk*$^{-/-}$ mice (Bjursell et al., 2011). In the neonatal period, affected infants failed to thrive and the children were affected by severe developmental delay and encephalopathy. Epileptic seizures developed in all six children with an age of onset between 10 and 35 months (Bjursell et al., 2011). This seizure phenotype is not consistent with the general anticonvulsant role of ADK reduction. However, developmental or epigenetic effects contributing to this seizure phenotype cannot be excluded and warrant further investigation. One girl died during sleep at the age of 10 years and 9 months (Bjursell et al., 2011), an event that might be related to sudden unexpected death in epilepsy (SUDEP) and insufficiencies in metabolic adenosine clearance (Shen et al., 2010). This human condition validates results obtained from transgenic animals and demonstrates that ADK is a crucial enzyme for the maintenance of normal body functions.

C. Human Neuropathology

In the adult brain, ADK is predominantly expressed in astrocytes (Studer et al., 2006). Many neurologic conditions are associated with inflammatory processes and the development of astrogliosis, which is a macroglial response characterized by astroglial cell proliferation and hypertrophy (Pekny and Nilsson, 2005). As demonstrated in different animal models of neurologic disease, overexpression of ADK appears to be a general response to astroglial activation (Boison, 2012b; Boison et al., 2010). These findings prompted the investigation of specimens surgically resected from the human brain and of human post mortem samples. Importantly, ADK was significantly overexpressed in surgically resected tissue from patients with mesial temporal lobe epilepsy (Aronica et al., 2011; Masino et al., 2011). In addition, ADK was found to be overexpressed in human astrocytic tumors and related to tumor-associated epilepsy (de Groot et al., 2012). These histopathological findings demonstrate an association of overexpression of ADK with human epilepsy and support data from transgenic animals showing a tight link between ADK expression levels and seizure susceptibility.

D. Role of Adenosine Kinase in Brain Development

ADK expression undergoes a remarkable shift during early postnatal brain development in rodents (Fig. 5) (Studer et al., 2006). After birth, ADK expression is largely limited to the expression of the long isoform in the nuclei of neurons. During the first 14 days of postnatal brain development ADK expression gradually shifts from neurons to astrocytes and from expression of the long nuclear isoform to the short cytoplasmic isoform (Studer et al., 2006). By postnatal day 21, the brain shows the adult expression pattern of ADK, with ADK expression largely restricted to the cytoplasmic isoform (Fedele et al., 2005) and to expression in astrocytes (Studer et al., 2006). The only neurons in the adult brain that maintain high expression levels of ADK are neurons from the olfactory bulb, whereas dentate granular neurons maintain a low level expression of the nuclear isoform of ADK into adulthood (Gouder et al., 2004; Studer et al., 2006). During early postnatal development of the hippocampal formation, the nuclear expression of neuronal ADK is gradually phased out as the cells mature (Fig. 5) (Studer et al., 2006). Since ADK is a metabolic clearance enzyme necessary for the maintenance of transmethylation reactions, it is tempting to speculate that the expression of the nuclear isoform of ADK in immature or developing neurons might be implicated in epigenetic functions based on interaction with DNA methylation pathways. This transient neuronal expression profile of ADK could therefore play important roles for brain plasticity and development.

E. Role of Adenosine Kinase in Specific Organ Systems and Pathologies

ADK controls specific organ functions through a combination of adenosine receptor-dependent and -independent mechanisms. Any change in adenosine

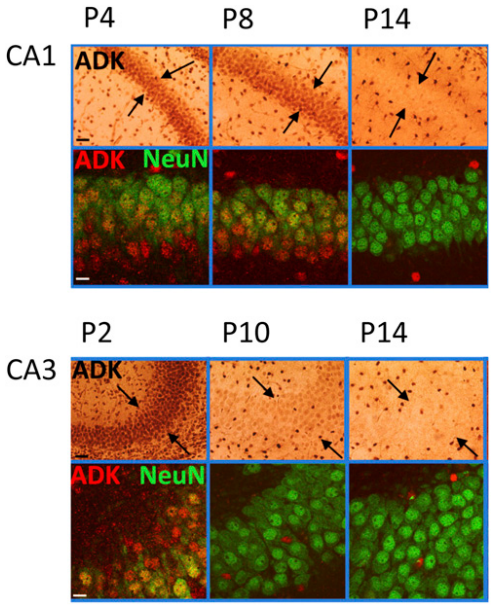

Fig. 5. ADK expression changes during early postnatal brain development of the mouse. Top, first row: ADK immunohistochemistry (brown) shows strong ADK labeling in cell bodies of CA1 pyramidal cells at P4 and P8 but not at P14. Arrow pairs denote outer and inner boundaries of stratum pyramidale. Top, second row: Confocal imaging of immunofluorescence for ADK (red) and the neuronal marker NeuN (green) shows colocalization of ADK and NeuN in CA1 pyramidal cell bodies at P4 and P8 (yellow). Black scale bar: 75 μm; white scale bar: 12 μm. Bottom: Corresponding immunohistochemical characterization of the CA3 area, which loses neuronal ADK expression earlier than CA1.

homeostasis will affect activation of all adenosine receptors simultaneously and the resulting effects on organ function are more likely based on changes in network homeostasis rather than a specific receptor subtype. If not mentioned otherwise, the net effects described below are based on a combination of adenosine receptor-dependent and -independent mechanisms.

1. Liver. Liver is the organ with the highest expression levels of ADK (Fedele et al., 2005; Cui et al., 2011) and the organ in which 80% of all transmethylation reactions of the human body take place (Mato et al., 2008). The biochemistry of transmethylation reactions has been discussed at an earlier place in this review. Importantly, ADK expression in liver is a requirement to maintain the metabolic clearance of adenosine and thus the flow of transmethylation reactions. As discussed above, disruption of ADK expression has dire consequences for the liver: hepatic steatosis develops, a pathology shared between $Adk^{-/-}$ mice and human patients with ADK deficiency (Boison et al., 2002a; Bjursell et al., 2011). Thus, ADK deficiency in the liver is likely to affect the availability of many methylated compounds, such as choline or related metabolites in fat metabolism.

2. Pancreas. In pancreas, the nuclear isoform of ADK is specifically expressed in β-cells, whereas α-cells and fibroblasts express exclusively the cytoplasmic isoform of ADK (Annes et al., 2012). These findings suggest a specific role of nuclear ADK for β-cell function. Using a lentiviral approach to infect cultured β-cells with an RNAi targeted to ADK, it was shown in mixed cultures containing infected, uninfected, and control-infected cells that both types of control cells exhibited the same basal proliferation rate, whereas cells that received the ADK-directed siRNA demonstrated a 2.5-fold increase in their proliferation rate (Annes et al., 2012). These findings demonstrate that the nuclear isoform of ADK attenuates the proliferation of β-cells in a cell-autonomous manner.

3. Heart. Adenosine exerts a variety of cardioprotective effects, which are largely based on the activation of A_1Rs (Hedqvist and Fredholm, 1979). Those cardioprotective effects include protection against ischemia/reperfusion injury (Mubagwa and Flameng, 2001; Peart and Headrick, 2007), reduction of oxidative stress (Narayan et al., 2001; Reichelt et al., 2009), and attenuation of hypertrophy and heart failure (Liao et al., 2003; Lu et al., 2008; Xu et al., 2008). Since the antihypertrophic effects of adenosine cannot completely be abrogated by genetic deletion or pharmacological blockade of the adenosine receptors (Lu et al., 2008; Fassett et al., 2011), the intracellular metabolism of adenosine in cardiomycytes might play a critical role. Interestingly, cardiomyocytes preferentially express the nuclear isoform of ADK (Fassett et al., 2011), indicating an intracellular function of adenosine in cardiomyocyte physiology. In line with this notion, blockade of ADK with either RNAi or ADK inhibitors (iodotubercidin or ABT-702) completely reversed the antihypertrophic effects of external adenosine or its analog 2-chloroadenosine (Fassett et al., 2011). These results support an inhibitory role of ADK on cell growth of cardiomyocytes. Analysis of cell signaling pathways identified Raf-dependent signaling to the mTOR/p70Sk complex as an important contributor to cardiomyocyte hypertrophy, which can be disrupted by adenosine through a mechanism dependent on ADK (Fassett et al., 2011). However, whether this interaction with the mTOR pathway is direct or indirect, e.g., via changes in DNA methylation, has not been identified. In conclusion, as in β-cells, these studies suggest an important role of nuclear ADK in the regulation of cell proliferation.

4. Brain. Endogenous adenosine has long been known to regulate excitability within the brain (Dunwiddie, 1980; Dunwiddie et al., 1981). Consequently, dysregulation of ADK expression and resulting disruption of adenosine homeostasis is implicated in a wide range of neurologic and neuropsychiatric pathologies. Although developmental changes in ADK expression have been documented in the developing brain (Studer et al., 2006), functional implications of ADK expression have only been studied in the adult brain to date.

a. Cell type specificity of ADK expression. In the adult brain, ADK expression is largely restricted to astrocytes (Studer et al., 2006). As mentioned earlier, notable exceptions are neurons from the olfactory bulb, which maintain high levels of ADK expression into adulthood (Gouder et al., 2004). In addition, the cell bodies and nuclei of dentate granular neurons maintain low levels of nuclear ADK expression (Li et al., 2008b). The functional implications of neuronal ADK expression in the adult brain remain to be determined.

b. Isoform specificity of ADK expression. In the adult brain, expression of the short cytoplasmic isoform of ADK dominates quantitatively as determined in Western blots, which separate the two isoforms (Cui et al., 2009; Fedele et al., 2005). Cytoplasmic ADK spreads throughout the astroglial network and gives the impression of a ubiquitous ADK background (Gouder et al., 2004; Studer et al., 2006). Widespread distribution of ADK immunoreactivity is in line with regulation of the adenosine concentration in brain tissue. Indeed, engineered changes in cytoplasmic ADK expression in mouse brain were shown to be sufficient to alter the tissue concentration of adenosine (Shen et al., 2011). Conversely, the nuclear isoform of ADK shows distinct expression in the nuclei of astrocytes and to a lesser degree in dentate granular cell neurons (Studer et al., 2006; Li et al., 2008b), whereas neurons from the olfactory bulb have high expression levels of both

isoforms of ADK. Interestingly, the nuclear expression of ADK is seen in cell types that maintain plastic behavior into adulthood, such as astrocytes or cells from the granular cell layer of the dentate gyrus, whereas terminally differentiated cells, such as most neurons, lack ADK expression. The nuclear expression of ADK is thus consistent with cell-autonomous effects of adenosine, which might be related to an epigenetic role of ADK as regulator of DNA methylation. A possible epigenetic role of nuclear ADK would be consistent with the expression of nuclear ADK in plastic cell types and findings from β-cells of the pancreas and from cardiomyocytes, in which ADK was shown to have prominent effects on cell proliferation (Fassett et al., 2011; Annes et al., 2012). This is an exciting possibility that warrants further investigation.

c. Epilepsy. Epilepsy is a chronic seizure disorder that affects about 1% of the population. It is widely believed that most forms of epilepsy are acquired and result from a precipitating injury, which can be a traumatic injury to the brain, a stroke or period of hypoxia, a viral infection, or febrile seizure (Pitkanen and Lukasiuk, 2011; Vezzani et al., 2011; Aronica and Vezzani, 2012). Importantly, inflammatory processes as well as microglial and astroglial activation play important roles in the development of epilepsy. In particular, reactive gliosis, a fairly common morphologic and biochemical conversion of astrocytes into a pathologically hyperactive state is a pathologic hallmark of epilepsy. Since astrocytes form complex astroglial networks (Giaume et al., 2010), any disruption of astrocyte function in epilepsy, such as structural, biochemical, and metabolic changes, is expected to disrupt network homeostasis within the brain on a global scale. Synaptic levels of adenosine are largely controlled by an astrocytic sink for adenosine, which is based on astroglial expression of ADK (Boison et al., 2010). Although neurons, which mostly lack ADK, can constitute a major source for the direct release of adenosine (Lovatt et al., 2012), astrocytes can release ATP as the metabolic precursor of adenosine (Pascual et al., 2005). Reuptake of adenosine into the astrocyte is mediated via two types of equilibrative nucleoside transporters and driven by metabolic clearance of adenosine via phosphorylation into AMP by ADK (Fig. 6). Following an insult to the brain, astrocytic ADK expression undergoes a biphasic response: acute downregulation of the enzyme within hours as an acute neuroprotective response (Gouder et al., 2004; Pignataro et al., 2008) is followed by astrogliosis and associated overexpression of ADK within days or weeks (Gouder et al., 2004; Li et al., 2007a, 2012). Consequently, ADK was found to be upregulated and causing adenosine deficiency in epileptogenic sclerotic tissue in a variety of rodent models of epilepsy (Fig. 7) as well as in human specimens resected from patients with temporal lobe epilepsy and hippocampal sclerosis (Li et al., 2008b; Aronica et al., 2011). By use of a mouse model of CA3-selective astrogliosis it was shown that spontaneous recurrent seizures were both temporally and spatially related to the astrogliotic focus and to the area of overexpressed ADK. Since similar seizures were triggered by transgenic overexpression of ADK in brain, increased metabolic clearance of adenosine via increased expression of ADK in astrocytes is a likely contributing mechanism for seizure generation in epilepsy (Li et al., 2008b). In addition, homeostatic functions of the adenosine system appear to play a crucial role in epileptogenesis. Both transgenic animals with forebrain-selective reduction of ADK, as well as recipients of adenosine releasing stem cell-derived infrahippocampal grafts, showed a significant attenuation of astrogliosis following a kainic acid-induced status epilepticus, failed to increase ADK expression, and most importantly, did not develop any spontaneous seizures following an adequate trigger for epileptogenesis (Li et al., 2008b). These findings indicate that astroglial ADK is a promising target for the prediction and prevention of seizures in epilepsy

d. Traumatic brain injury. Astrogliosis and associated overexpression of ADK has also been identified in a rat model of severe traumatic brain injury (TBI) induced by a lateral fluid percussion injury. Interestingly, the injured animals developed epileptiform bursts associated with astrogliosis and overexpression of ADK well before the development of clinical epilepsy (Lusardi et al., 2012).

e. Central apnea. Adenosine homeostasis in the brain stem is implicated in the regulation of respiratory

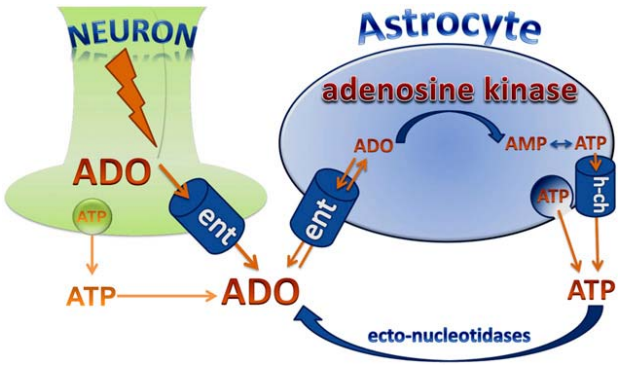

Fig. 6. Astrocytes constitute a sink for the metabolic clearance of adenosine in the brain. Whereas neurons are capable of releasing adenosine directly, astrocytes can release ATP via vesicular release and/or by direct release through hemichannels (h-ch). Extracellular ATP is rapidly degraded into adenosine (ADO) by a series of ectonucleotidases. Adenosine can also be released directly via equilibrative nucleoside transporters (nt). Intracellular adenosine levels are largely controlled by adenosine kinase, which phosphorylates adenosine into AMP. Small changes in adenosine kinase activity rapidly translate into major changes in adenosine. Intracellular astrocytic adenosine kinase is considered to be a metabolic reuptake system for adenosine. Only selected mechanisms and pathways are shown; for details please refer to main text.

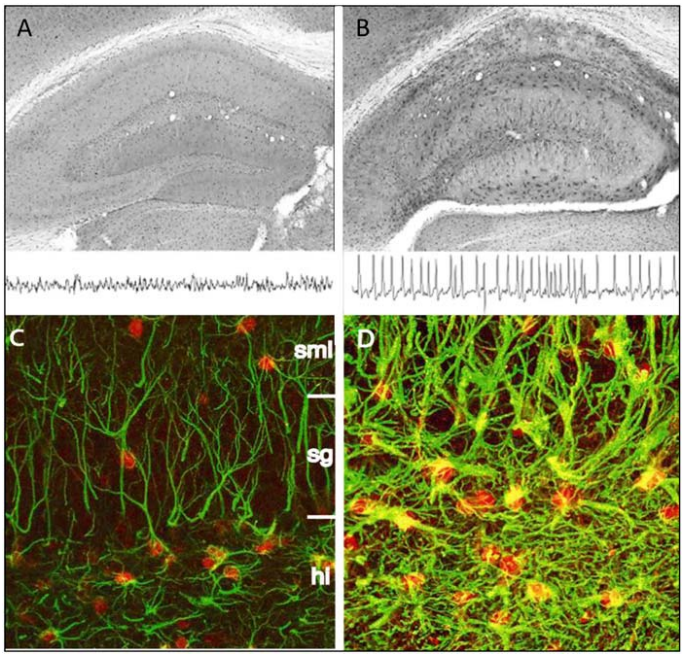

Fig. 7. Astrogliosis and overexpression of ADK in a mouse model of temporal lobe epilepsy. (A and B) Brains from kainic acid (KA)-treated mice were taken at 4 weeks after either intrahippocampal KA or saline injection. Transverse brain sections of the KA-injected brain hemisphere were stained for ADK-immunoreactivity. Note prominent overexpression of astrogliosis in association with spontaneous seizures activity in B. (C and D) Colocalization of ADK and GFAP immunofluorescence, as seen by confocal laser scanning microscopy. Transverse brain sections of a KA-injected animal taken 4 weeks after the injection and those from a naive control animal were double stained for ADK (red) and the astrocyte marker GFAP (green). Optical sections were digitized at high magnification and superimposed for display. (C) Dentate gyrus of a control animal. Note that the cell bodies of individual astrocytes (green processes) are stained for ADK (red). (D) Dentate gyrus of a KA-injected animal. Note the massive gliosis characterized by the swelling of cell bodies, the enlargement of astrocytic processes, and the expansion of ADK-immunoreactivity into the processes (colocalization of ADK and GFAP, yellow). *sp,* Stratum pyramidale; *sml,* stratum moleculare; *sg,* stratum granulosum.

function by A_1 and A_{2A}Rs (Fredholm, 1984; Lagercrantz et al., 1984). Remarkably, suppression of respiratory function is a major cause of death following a severe TBI, and high levels of adenosine in the cerebrospinal fluid were associated with acute lethal outcome in human victims of a severe TBI (Clark et al., 1997). Consequently, a combination of an excessive injury-related surge in adenosine, in combination with deficiencies in metabolic clearance of brain stem adenosine by ADK, would constitute a major risk factor for the development of lethal apnea. This hypothesis was tested in a rat model of severe TBI with an acute mortality rate of 46.7%. As expected, the acute mortality was found to be related to prolonged apnea. To determine whether excessive adenosine receptor activation contributed to lethal outcome, a subset of rats was treated with a 25 mg/kg concentration of the nonselective adenosine receptor antagonist caffeine intraperitoneally within 1 minute of the injury. Importantly, a single acute injection of caffeine was shown to completely prevent TBI-induced mortality when given immediately following the TBI, demonstrating that excessive adenosine contributed to lethal outcome (Lusardi et al., 2012). As in TBI, sudden unexpected death in epilepsy (SUDEP) has been associated with respiratory suppression (Langan, 2000; So, 2008). To address the hypothesis whether seizure-induced adenosine release, in combination with deficient metabolic adenosine clearance, might be a sufficient cause for SUDEP, seizures in mice were triggered in combination with pharmacologically (combination of ADK and adenosine deaminase inhibitor) induced deficiency in metabolic adenosine clearance. The combination of impaired adenosine clearance with kainic acid-induced seizures triggered sudden death in all animals. However, caffeine, when given after seizure onset, significantly increased the survival time in affected animals (Shen et al., 2010). Together, the TBI and SUDEP studies suggest that the capacity for metabolic adenosine clearance in brain stem by ADK might critically determine vulnerability to lethal apnea. In line with these findings, it is important to note that sudden infant death syndrome (SIDS) is a condition frequently characterized by lethal respiratory suppression and familial occurrence (Oren et al., 1987; Harper et al., 2000). Interestingly, a retrospective study has demonstrated the incidence of hepatic steatosis in about 10% of a total of 418 SIDS cases (Boles et al., 1998). Given the occurrence of apnea and hepatic steatosis in $Adk^{-/-}$ mice, it is tempting to speculate that inborn deficiencies in ADK might contribute at least to a subset of SIDS cases, a possibility that warrants further investigation.

f. Stroke. ADK expression levels critically determine the brain's vulnerability to the effects of a stroke. Thus, transgenic overexpression of ADK in *Adk-tg* mice led to a 3-fold increase in infarct volume compared with wild-type control animals, when exposed to 15 minutes of middle cerebral artery occlusion (MCAO) followed by 24 hours of reperfusion, whereas all *Adk-tg* mice died following 60 minutes of MCAO, a condition in which wild-type animals routinely survive (Pignataro et al., 2007a). In contrast, transgenic fb-Adk-def mice with increased ADK expression in striatum (164%) and reduced ADK expression in cortical forebrain (65%) demonstrate increased striatal infarct volume (126%) but reduced cortical infarct volume (27%) after 60 minutes of MCAO and 23 hours of reperfusion compared with wild-type controls. These findings indicate that ADK expression levels in the CNS determine cerebral injury levels by regulating the availability of adenosine activating the neuroprotective function of the A_1R (Shen et al., 2011). These findings were further corroborated using an adeno associated virus-based strategy to modify ADK expression in astrocytes. Mice receiving intrastriatal injections of virus that carried either *Adk*-sense or -antisense constructs to overexpress or knockdown ADK in vivo were characterized by

increased (126%) or decreased (51%) infarct volume, respectively, when subjected to MCAO (Shen et al., 2011). Together, these data define ADK as a possible therapeutic target for modulating the degree of stroke-induced brain injury.

Ischemic preconditioning is a phenomenon in which tolerance to injury develops based on the experience of a preceding noninjurious challenge (Dirnagl et al., 2009). Thus, mice subjected to 15 minutes of MCAO followed 72 hours later by 60 minutes of MCAO display robust protection of the affected brain hemisphere (Stenzel-Poore et al., 2003). Because of its neuroprotective capabilities, adenosine is a logical candidate to mediate ischemic tolerance (Williams-Karnesky and Stenzel-Poore, 2009). To investigate whether adenosine might play a role in protecting the hippocampus after focal ischemia, Adk-tg mice were subjected to transient MCAO. Although the hippocampus of wild-type mice was consistently spared from injury after 60 minutes of MCAO, hippocampal injury became evident in Adk-tg mice after only 15 minutes of MCAO. To determine whether downregulation of endogenous ADK might qualify as a candidate mechanism mediating endogenous neuroprotection, ADK expression in wild-type mice was evaluated various time points after an MCAO. Although ADK expression was found to be reduced brain wide up to 1 day following 60 minutes of MCAO (Fig. 8), a significant reduction of ADK expression was also found in the ipsilateral hippocampus after 15 minutes of MCAO and 3 hours of reperfusion (Pignataro et al., 2008). Moreover, abrogation of lipopolysaccharide (LPS)-induced ischemic preconditioning in Adk-tg mice indicated that ADK activity negatively regulates LPS-induced tolerance to stroke (Shen et al., 2011). Thus, transient downregulation of hippocampal ADK after a stroke might be an endogenous neuroprotective mechanism of the brain.

g. Sleep. Sleep and the intensity of sleep are enhanced by adenosine and its receptor agonists, whereas antagonists such as caffeine or theophylline induce wakefulness (Huang et al., 2011; Porkka-Heiskanen and Kalinchuk, 2011). In rodents, adenosine metabolic enzymes, including ADK, undergo diurnal changes, with higher enzymatic activities usually observed during the active period of the animals (Alanko et al., 2003; Mackiewicz et al., 2003). In line with a role of ADK in sleep modulation, sleep was found to be profoundly altered in Adk-tg mice (Palchykova et al., 2010). The mutant animals displayed a profound reduction in electroencephalogram power in low frequencies in all vigilance states and a reduction in the 6–11 Hz theta activity in REM sleep and in waking. Adk-tg mice also slept about 1 hour less per day compared with wild-type control mice, and REM sleep duration was reduced by 20%. In Adk-tg mice, the effects of sleep deprivation on slow-wave activity and energy were significantly reduced (Palchykova et al., 2010). These findings are in line with pharmacological data, in which the ADK inhibitor ABT-702 caused a significant shift in the slow-wave sleep and REM sleep ratio in rats (Radek et al., 2004). Together, these data demonstrate that levels of ADK activity in the brain critically determine important parameters of sleep physiology.

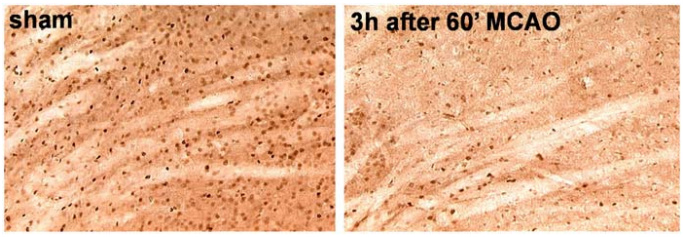

Fig. 8. Downregulation of ADK in a mouse model of cerebral stroke. Brains from mice were taken 3 hours after 60 minutes of middle cerebral artery occlusion (right) or a sham surgery and stained for ADK immunoreactivity. Images show part of the striatum ipsilateral to the stroke.

h. Cognition. As an upstream regulator of major neurotransmitter systems, including glutamatergic neurotransmission (Sebastiao and Ribeiro, 2009b; Ribeiro and Sebastiao, 2010; Diógenes et al., 2012), adenosine is a prime candidate for the modulation of cognitive processes. Importantly, transgenic overexpression of ADK in the brain of mice (Adk-tg mice) caused prominent cognitive impairment on several levels (Yee et al., 2007; Singer et al., 2012). The motor stimulant effect of MK-801 was potentiated in Adk-tg mice suggesting *N*-methyl-D-aspartate receptor hypofunction (Yee et al., 2007). In line with this finding Adk-tg mice displayed severe learning deficits in the domains of reference memory, working memory, and associative learning (Yee et al., 2007). The link between overexpression of ADK and cognitive impairment might be of pathologic relevance for neurologic conditions in which overexpression of ADK has either been confirmed (epilepsy) or suspected (Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis) (Boison, 2007; Boison et al., 2010). Interestingly, these conditions share 1) astrogliosis as a histopathological hallmark (Schiffer et al., 1996; Blumcke et al., 1999; Renkawek et al., 1999; Ala et al., 2000; Yamanaka et al., 2008) and 2) cognitive impairment as comorbidity or key pathologic feature (Palop and Mucke, 2009; Aarsland and Kurz, 2010; Rusina et al., 2010; Bell et al., 2011). Thus, astrogliosis-associated overexpression of ADK might be causally involved in the development of cognitive comorbidities spanning a wide range of neurologic conditions.

i. Schizophrenia. The adenosine hypothesis of schizophrenia postulates that hypofunction of adenosine signaling may contribute to the pathophysiology of schizophrenia (Boison et al., 2012) and constitutes a novel concept to integrate the dopaminergic hyperfunction

(Seeman, 1987) and the glutamatergic hypofunction (Gordon, 2010) hypotheses of schizophrenia. Presynaptically, adenosine regulates the release of both glutamate and dopamine largely via A_1Rs (Thompson et al., 1993; Wu and Saggau, 1997), whereas the output of glutamatergic and dopaminergic neurotransmission might be regulated postsynaptically by the proposed heterodimerization of A_{2A} receptors with glutamate or dopamine receptors (Franco et al., 2007; Fuxe et al., 2007, 2010). Although this interaction remains hypothetical, it provides an attractive mechanism whereby adenosine could act as an upstream regulator of glutamatergic and dopaminergic neurotransmission. Consequently, any disruption in adenosine homeostasis would lead to imbalances in both glutamatergic and dopaminergic neurotransmission. In line with this notion, adenosine-deficient Adk-tg mice show altered locomotor behavior in response to both dopaminergic (amphetamine) and glutamatergic (MK-801) stimulants (Yee et al., 2007; Shen et al., 2012). In addition, Adk-tg mice display attentional deficits, which are a characteristic hallmark of schizophrenia (Yee et al., 2007; Shen et al., 2012). If adenosine deficiency is implicated in the expression of symptoms that are of relevance for schizophrenia, then therapeutic adenosine augmentation should be beneficial for the treatment of schizophrenia. Indeed, blockade of ADK by ABT-702 exerted potent antipsychotic-like activity in wild-type mice, whereas local cell-based adenosine augmentation in striatum restored responsiveness to amphetamine in Adk-tg mice, whereas the same manipulation of the hippocampus reversed the working memory deficits of the mutants (Shen et al., 2012). These findings suggest that ADK plays a critical role as upstream regulator of several molecular pathways implicated in the pathophysiology of schizophrenia.

5. Cochlea. Adenosine plays important roles in the auditory system, in particular in protecting the cochlea from oxidative stress (Vlajkovic et al., 2009). An otoprotective role of adenosine is supported by findings showing that A_1R activation can prevent cochlear injury caused by acoustic trauma or by ototoxic drugs (Vlajkovic et al., 2009). Therefore, adenosine metabolic enzymes, such as ADK, have emerged as attractive targets for controlling oxidative stress in the cochlea (Vlajkovic et al., 2009). In the adult cochlea of the rat, ADK immunoreactivity was mostly localized to the nuclear or perinuclear region of spiral ganglion neurons, to lateral wall tissue, and to epithelial cells lining the scala media (Vlajkovic et al., 2010). Like in the brain, ADK expression was subject to highly coordinated expression changes during early postnatal development of the cochlea (Vlajkovic et al., 2010), implicating a putative role of ADK in the regulation of developmental processes. Therapeutically, the chronic application of the ADK inhibitor ABT-702 (1.5 mg/kg twice per week for 3 or 6 months) was shown to attenuate the development of age-related hearing loss in C57Bl/6 mice (Vlajkovic et al., 2011). At the age of 9 months, when nontreated control mice exhibited significant loss of hair cells and hearing capability, ABT-702-treated mice showed better hearing thresholds in auditory brain stem responses as reflected in lower threshold shifts at 10 and 16 kHz. Importantly, the treated animals were also characterized by increased hair cell survival in the apical cochlea (Vlajkovic et al., 2011). Although ADK inhibition was shown to be a promising therapeutic approach for the attenuation of age-related hearing loss, ABT-702 treatment was not able to prevent the development of sound-induced hearing loss (Vlajkovic et al., 2010).

6. Diabetes. In diabetes mellitus, adenosine homeostasis is critically altered in several tissues. Thus it was shown that the cytosolic activity (V_{max}) of ADK was decreased by 40 to 50% in kidney, heart, and liver of rats, in which diabetes mellitus was induced by streptozotocin (Pawelczyk et al., 2000). In line with these findings, *Adk* transcript levels were found to be reduced by up to 50% in the same organs and as early as 24 hours following the induction of diabetes (Pawelczyk et al., 2000). Reduced ADK expression and reversed transport of adenosine from cells into the extracellular space were also found to be associated with suppressed proliferation of diabetic T lymphocytes, an effect linked to excessive A_{2A}R stimulation (Sakowicz-Burkiewicz et al., 2006). In the brain of diabetic rats, binding densities of the neuroprotective A_1 receptors were found to be reduced by 36%, whereas those of the facilitatory A_{2a}R increased by 83% in total hippocampal membranes (Duarte et al., 2006). Thus, increased adenosine signaling in the diabetic brain in combination with a shift in adenosine receptor expression patterns might be an explanation for the development of diabetic encephalopathy and the protective effects of caffeine (Duarte et al., 2007,2009). Conversely, insulin-treatment was found to restore expression and activity levels of *Adk* transcripts and ADK protein, respectively (Sakowicz-Burkiewicz et al., 2006). Restoration of ADK expression by insulin in rat lymphocytes was mediated by activation of the mitogen-activated protein kinase pathway (Pawelczyk et al., 2003). As already discussed above, the nuclear isoform of ADK plays a key role in regulating the proliferation of β-cells in the pancreas. In particular, the therapeutic inhibition of nuclear ADK in β-cells might constitute a promising therapeutic avenue to increase the number of insulin-producing cells in diabetic conditions, in which glucose-based mechanisms in the control of β-cell replication fail (Porat et al., 2011; Annes et al., 2012).

7. Arthritis. Homeostasis of adenosine receptor signaling is of crucial importance in the regulation of inflammation and the release of proinflammatory cytokines (Hasko et al., 2008; Cronstein, 2010; Ernst

et al., 2010). The A_{2A} and A_3Rs in particular play key roles in the regulation of inflammatory pathways in a variety of conditions including arthritis (Morello et al., 2006; Hasko et al., 2008). While A_{2A} and A_3 receptors were shown to be upregulated in patients with rheumatoid arthritis (Varani et al., 2009, 2010, 2011), adenosine was shown to suppress elevated levels of the proinflammatory cytokines TNF-α and IL-1β in patients with rheumatoid arthritis (Forrest et al., 2005; Varani et al., 2010). It is now well accepted that adenosine exerts potent anti-inflammatory effects via activation of A_{2A} and A_3 receptors. Therefore, A_{2A} and A_3 receptor agonists (Yan et al., 2003; Akkari et al., 2006; Flogel et al., 2012) and ADK inhibitors (Cronstein et al., 1995; Boyle et al., 2001) constitute rational therapeutic strategies for the treatment of arthritis.

8. Colitis. Ulcerative colitis is an inflammatory bowel disease (IBD) that causes long-lasting inflammation in part of the digestive tract. As discussed above, the homoeostasis of adenosine receptor signaling is also of critical significance for the chronic inflammatory reactions in IBD (Hasko et al., 2008). Limited oxygen availability and inflammation in mucosal membranes lead to increased production of adenosine from degradation of ATP and ADP; increased expression of adenosine receptors, in particular the A_{2B} receptor; reduced uptake of extracellular adenosine, and decreased metabolic clearance of adenosine (Eltzschig et al., 2009). Recent data demonstrate that enhanced adenosine signaling via the A_{2B}R attenuated mucosal inflammation, permeability, and tissue injury during intestinal ischemia or experimental colitis, whereas suppression of macrophage activation was also involved in the beneficial effects of A_{2B}R activation (Eltzschig et al., 2009; Hasko et al., 2009). Therefore, therapeutic modulation of adenosine signaling appears to be a rational approach for the treatment of IBD.

9. Cancer. The role of the adenosine/ADK regulatory system in cancer may depend on the type of cancer. Several studies report a cytotoxic role of extracellular adenosine. Thus, extracellular adenosine was shown to induce apoptosis in MCF-7 human breast cancer cells, an effect that was shown to be intracellular and independent of adenosine receptor activation (Tsuchiya et al., 2012). Mechanistically, it was shown that adenosine promoted the translocation of apoptosis-inducing factor-homologous mitochondrion-associated inducer of death from the cytosol into the nucleus (Tsuchiya et al., 2012). An earlier study suggested that the cytotoxic effects of adenosine on breast cancer cells might be based on conversion of adenosine to AMP (ADK dependent) followed by activation of nucleoside kinase and activation of the mitochondrial/intrinsic apoptotic pathway (Hashemi et al., 2005). An intracellular adenosine receptor-independent apoptotic effect on astrocytoma cells was found to depend on intracellular activation of an adenosine analog (Ceruti et al., 2000). A cytotoxic role for adenosine was also demonstrated in human gastric cancer cells, with a mechanism based on conversion of adenosine to AMP and activation of the intrinsic apoptotic pathway through AMP kinase activation (Saitoh et al., 2004). In addition, A_3 receptor activation might be of benefit for the treatment of colorectal cancer with clinical trials on the way (Yan et al., 2003). In line with a cytotoxic role of adenosine, *Adk*-gene expression was found to be significantly higher in human patient-derived colorectal cancer tissue than in healthy control tissue (Giglioni et al., 2008; Vannoni et al., 2004a,b), suggesting that either more efficient metabolic clearance of cytotoxic adenosine might provide an advantage for tumor cells or that a general increase in purine metabolic enzymes might permit accelerated purine metabolism to support the growth of cancerous tissue. In contrast to those findings, ADK activity was found to be reduced in hepatoma cells, suggesting that increased adenosine might provide a selective advantage for hepatic cancers. Although increased adenosine has been linked to cytotoxic and apoptotic effects in several cancer types, it needs to be stressed that increased levels of adenosine also inhibit immune responses and inflammatory responses and stimulate angiogenesis, effects that might benefit tumor growth on a physiologic level. More work needs to be done to fully understand the role of adenosine in cancer biology, in particular regarding epigenetic ramifications of adenosine regulation, and to investigate whether ADK might constitute a therapeutic target for the treatment of cancer.

VI. Therapeutic Applications of Adenosine Kinase-Based Interventions

A. Strategies to Alter Adenosine Kinase Activity

1. Pharmacology. Pharmacological approaches to harness the therapeutic potential of adenosine augmentation are based on nucleoside and nonnucleoside ADK inhibitors, which have been discussed in preceding sections. The major advantage of this approach, in contrast to more selective adenosine receptor agonists, is that ADK inhibitors can potentiate an *endogenous* stress response of the body and potentiate the actions of *endogenous* adenosine in a site- and event-specific manner (Kowaluk et al., 1998; Britton et al., 1999; Wiesner et al., 1999; Kowaluk and Jarvis, 2000). An additional advantage of ADK inhibitors is the rise of the tissue concentration of adenosine, which will not only lead to the increased activation of all subtypes of adenosine receptors but also to adenosine receptor-independent effects, including epigenetic changes due to interference of adenosine with DNA methylation. Therefore, in contrast to receptor-specific

ligands, ADK inhibitors are capable of affecting complex networks synergistically on multiple different levels, taking advantage of the multimodal activity of an endogenous homeostatic regulator of network function. However, therapeutic adenosine augmentation by systemic ADK inhibition might not be a viable therapeutic option due to liver toxicity (Boison et al., 2002b) and the occurrence of brain hemorrhage in some of the preclinical studies (McGaraughty et al., 2005). Therefore, localized or focal therapeutic approaches might be better suited to harness the therapeutic potential of adenosine in a more refined way.

2. Gene Therapy. One strategy to targeted local or even cell-type selective adenosine augmentation is gene therapy. In contrast to conventional gene therapies in which a transgene is added, the therapeutic goal here is to use gene therapy to reduce expression of the *endogenous Adk* gene. This can best be achieved using antisense approaches (Boison, 2010) to knock down gene expression. Two studies have used this approach to knock down ADK expression in models of seizures and stroke (Shen et al., 2011; Theofilas et al., 2011). Both studies are based on the same AAV8-based vector, which expresses an *Adk* cDNA in antisense orientation under the control of an astrocyte specific gfaABC$_1$D promoter (Lee et al., 2008). When injected into the hippocampus of Adk-tg mice with spontaneous electrographic seizures, recipients of the *Adk* antisense virus had a substantial unilateral decrease in seizure activity ipsilateral to the virus injection site with 0.6 ± 0.6 seizures/hour compared with 5.8 ± 0.5 seizures/hour on the contralateral (noninjected) side (Theofilas et al., 2011). Similarly, injection of the antisense virus into the striatum of mice decreased their infarct volume to 51% of control, when these animals were subjected to 60 minutes of MCAO to model a stroke (Shen et al., 2011). Together, these studies constitute a proof of principle that a gene therapy targeting ADK, restricted to a specific brain area (hippocampus or striatum) and to a specific cell type (astrocyte), can have potent therapeutic effects based on augmenting the anticonvulsive and neuroprotective properties of adenosine. More work needs to be done to evaluate whether anti-ADK gene therapies are effective in clinically relevant models of temporal lobe epilepsy.

3. Cell Therapy. A different approach for the local augmentation of adenosine signaling is to first delete the *Adk* gene in cultured cells to induce therapeutic adenosine release and then to transplant the cells into a host to therapeutically exploit locally enhanced levels of adenosine. The first successful cell therapy approach was achieved in baby hamster kidney (BHK) cells in which the *Adk* gene had been disrupted by a combination of chemical mutagenesis and selection for ADK deficiency; importantly, disruption of the *Adk* gene was more effective in inducing cellular adenosine release than disruption of the adenosine deaminase gene (Huber et al., 2001). When encapsulated into semipermeable polymer fibers and transplanted into the ventricular system of epileptic rats that were kindled in the hippocampus, ADK-deficient BHK cells releasing about 40 ng adenosine per 10^5 cells per day almost completely suppressed any seizures in an A_1R-dependent manner, whereas animals receiving control implants with wild-type cells continued to display their preimplantation seizure behavior (Huber et al., 2001). Unfortunately, seizure suppression was limited to 2 weeks due to the reduced longevity of the encapsulated cells. To develop a more versatile cell-based system for seizure control, both alleles of the *Adk* gene were disrupted in mouse embryonic stem (ES) cells by homologous recombination with a gene targeting construct; the $Adk^{-/-}$ ES cells yielded glial populations with an adenosine release of up to 40 ng/10^5 cells/h comparable to the amounts of adenosine released from BHK cells (Fedele et al., 2004). When differentiated into neural precursor cells and grafted into the infrahippocampal fissure of rats, the $Adk^{-/-}$ cell grafts profoundly suppressed kindling epileptogenesis (Li et al., 2007b). More importantly, when grafted into the infrahippocampal fissure of mice 24 hours after a status epilepticus, the same cells prevented the development of epilepsy (Li et al., 2008b). Recipients of the $Adk^{-/-}$ cells were characterized by attenuated astrogliosis, almost normal ADK expression levels, and complete lack of any seizures, whereas recipients of wild-type cells, or sham-treated control animals developed astrogliosis with overexpressed ADK as well as spontaneous electrographic seizures at a rate of about 4 seizures/hour (Li et al., 2008b). In a different approach, the same cells were transplanted into the striatum of mice 7 days prior to the onset of a stroke, modeled by 60 minutes of MCAO. After 23 hours of reperfusion, recipients of the $Adk^{-/-}$ cells were characterized by a significant reduction in infarct volume. Neuroprotection was strongest in adenosine-releasing glial precursor cell recipients, which were characterized by an 85% reduction of the infarct area. Graft-mediated neuroprotection correlated with a significant improvement of general and focal neurologic scores (Pignataro et al., 2007b). In an attempt to engineer human stem cells for therapeutic adenosine release, human mesenchymal stem cells were infected with a lentivirus engineered to express a micro RNA directed against *Adk*. This RNAi approach resulted in a reduction of ADK to 20% of its normal levels and triggered the release of about 1 ng adenosine/10^5 cells/h (Ren et al., 2007). When transplanted into the infrahippocampal fissure of mice, these implants reduced acute seizure-induced cell death (Ren et al., 2007) and led to a partial suppression of epileptogenesis (Li et al., 2009). This partial therapeutic effect is most

likely due to the 40 times lower amounts of adenosine released by those cells compared with the engineered ES cells that completely lacked any ADK expression. Together, these reports demonstrate that disruption of ADK expression in cells is a promising therapeutic strategy to augment adenosine signaling at a local site within the brain, with potent therapeutic effects resulting in neuroprotection, seizure suppression, and, ultimately, prevention of epileptogenesis.

4. Ketogenic Diet. A high-fat, low-carbohydrate ketogenic diet is a metabolic intervention that provides effective seizure control in many forms of pharmacoresistant epilepsy, particularly in children (Neal et al., 2008; Yellen, 2008; Freeman, 2009; Kossoff and Rho, 2009; Kossoff et al., 2009). Despite its clinical use for over 80 years, the mechanisms underlying the therapeutic actions of a ketogenic diet have remained enigmatic. A ketogenic diet forces the brain to use ketones instead of glucose as primary energy source, and it is those metabolic changes that are thought to underlie the therapeutic effects of this type of dietary intervention (Bough et al., 2006; Kalapos, 2007; Ma et al., 2007; Bough, 2008; Yellen, 2008). A large body of evidence supports the notion that a ketogenic diet leads to increased adenosine signaling in the brain (Masino and Geiger, 2008, 2009; Masino et al., 2009, 2012). Indeed, it was recently shown that a ketogenic diet reduced the expression of ADK in mice (Masino et al., 2011). In support of increased adenosine signaling, a ketogenic diet suppressed seizures in adenosine deficient Adk-tg mice, but not in A_1R-deficient mice, demonstrating that functional A_1R activation is necessary for the antiepileptic effects of the diet (Masino et al., 2011). Apart from seizure control, ketogenic diets have also been shown to be beneficial in experimental paradigms of pain and inflammation, a therapeutic outcome compatible with increased adenosine signaling (Ruskin et al., 2009).

5. Transcriptional Repression. In an innate response to hypoxia, vasculature reacts with a rise in adenosine (Berne, 1963; Berne et al., 1974). Although functional inhibition of ADK in response to hypoxia has been reported previously (Decking et al., 1997), a recent study reported transcriptional repression of the *Adk* gene resulting in a 85% reduction of the endothelial *Adk* transcript (Morote-Garcia et al., 2008). It was further shown that transcriptional repression of the *Adk* gene was dependent on hypoxia inducible factor 1-α (HIF-1α) and that repression of ADK led to an attenuation of vascular leakage in vitro and in vivo (Morote-Garcia et al., 2008). This transcriptional mechanism appears to be an evolutionary conserved strategy to directly couple adenosine homeostasis to a system that can sense an environmental condition of critical importance for the bioenergetic equilibrium of a cell.

B. Applications in Preclinical Studies

On the basis of the rationale outlined in preceding sections, therapeutic adenosine augmentation is of value in a variety of pathogenic conditions. Importantly, therapeutic adenosine augmentation is uniquely suited to synergistically modify disrupted networks via an *endogenous* upstream regulator through activation of four adenosine receptor-dependent pathways, but also through additional epigenetic and bioenergetic mechanisms. In the following preclinical examples, therapeutic gain through ADK manipulation is illustrated.

1. Diabetes. One approach in diabetes therapy is promotion of β-cell replication, which normally is under the control of glucose (Porat et al., 2011). However, it is the responsiveness of β-cell replication to excess glucose, which fails in the diabetic condition. Therefore, promotion of β-cell replication independent of glucose constitutes an important therapeutic goal. By using a small-molecule screening platform to identify molecules that increase β-cell replication, Annes and colleagues (2012) recently identified a class of ADK inhibitors that specifically promoted β-cell replication in a cell type-selective manner. ADK inhibitor-dependent β-cell replication was blocked by the phosphoinositide kinase inhibitor wortmannin and the mammalian target of rapamycin (mTOR) inhibitor rapamycin, suggesting involvement of the phosphoinositide kinase/mTOR pathway. In line with this finding, ADK inhibition resulted in the increased phosphorylation status of ribosomal protein S6, a downstream target of the mTOR pathway. Intriguingly, mTOR is a cytoplasmic and nuclear kinase (Zhang et al., 2002), which might be of relevance for the presence of nuclear ADK in β-cells. In vivo, the ADK inhibitor ABT-702 resulted in a robust increase in β-cell proliferation, and this effect was shown to be specific to β-cells since the replication rate of exocrine cells or that of hepatocytes was not altered by ABT-702 (Annes et al., 2012). Although ADK inhibitors show promising potential in increasing β-cell proliferation, and thus the number of insulin secreting cells, it remains to be demonstrated whether ADK inhibitors can improve responsiveness to glucose in a model of diabetes.

2. Epilepsy. Adenosine augmentation therapies make rational therapeutic use of an endogenous anticonvulsant and neuroprotectant of the brain with the potential to not only suppress seizures, but also to prevent epileptogenesis (Boison, 2009, 2012a). ADK inhibitors are capable of raising the levels of endogenous adenosine and, as originally proposed, to potentiate an endogenous adenosine response, such as the well-documented seizure-induced adenosine release, in a site- and event-specific manner (Kowaluk et al., 1998; Kowaluk and Jarvis, 2000; McGaraughty et al., 2001b, 2005). Since pathologic overexpression of ADK has

been demonstrated within epileptogenic brain areas (Gouder et al., 2004; Li et al., 2008b; Aronica et al., 2011; Boison, 2012b) and since overexpression of ADK alone can trigger electrographic seizures (Etherington et al., 2009; Li et al., 2008b, 2012; Theofilas et al., 2011), the scientific rationale for the use of ADK inhibitors in epilepsy therapy is strong. Importantly, the ADK inhibitor 5-ITU was shown to suppress seizures in mice that were resistant to conventional antiepileptic drugs (Gouder et al., 2004), suggesting that ADK inhibitors might be effective in pharmacoresistant epilepsy. Moreover, acute seizures induced by injection of bicuculline into the prepiriform cortex of rats were shown to be blocked by subsequent injection of the ADK inhibitors 5′-amino-5′-deoxyadenosine or 5-ITU, but not by the ADA inhibitor 2′-deoxycoformycin, suggesting that the anti-ictogenic activity of ADK inhibition is superior to ADA inhibition (Zhang et al., 1993). Among a wide spectrum of ADK inhibitors that have subsequently been developed for seizure control (Ugarkar et al., 2000a,b), GP-3269 showed enhanced oral bioavailability (60%) and extended plasma half-life (>4 hour), attenuation of the seizure response in the rat maximum electroshock (MES) and kindling models, and lack of profound cardiovascular side effects (Erion et al., 1997; McGaraughty et al., 2005). Despite an improved cardiovascular side effect profile, the brain wide therapeutic modulation of ADK might not be a therapeutic option due to psychiatric and cognitive effects (Yee et al., 2007; Boison et al., 2012; Shen et al., 2012). As discussed in preceding sections, in vivo or ex vivo gene therapies targeting ADK may provide a promising alternative to restrict therapeutic effects to an identifiable epileptogenic focal area with demonstrated adenosine dysfunction. Thus, infrahippocampal grafts of cells engineered to lack *Adk* were shown not only to suppress seizures but also to prevent epileptogenesis in a variety of experimental paradigms in which an epileptic state was created in rats or mice by repeated suprathreshold electrical stimulation (kindling) or by status epilepticus-induced brain injury (Huber et al., 2001; Li et al., 2007b, 2008b, 2009; Ren et al., 2007).

3. Pain. Several states of pathologic pain, in particular neuropathic pain, appear to share common mechanisms with epilepsy and, not surprisingly, antiepileptic drugs are frequently highly effective in the treatment of chronic pain (Horga de la Parte and Horga, 2006; Malawska and Kulig, 2008). As in epilepsy, adenosine provides potent inhibition to hyperexcitable neuronal circuits resulting in profound antinociceptive effects of adenosine (Lynch et al., 2003; Sawynok and Liu, 2003). Consequently, ADK inhibitors have been considered as a very attractive target for the treatment of various pain states, and proof of feasibility studies with prototypes of ADK inhibitors have demonstrated efficacy in several animal models of nociception (Keil and DeLander, 1994; Poon and Sawynok, 1995, 1998; Sawynok and Liu, 2003). Unfortunately, short half lives in vivo, poor bioavailability, lack of pharmacological selectivity, and potential to form cytotoxic metabolites limited further preclinical testing of those prototype inhibitors (Cottam et al., 1993; Wiesner et al., 1999; Ugarkar et al., 2000a). Therefore, most subsequent studies have focused on the structurally novel nucleoside (A-134974), nonnucleoside (ABT-702), and carbocyclic (A-286501) ADK inhibitors (Fig. 3). These compounds were shown to be orally active and to alleviate acute nociception, neuropathic allodynia, chemogenic nociception, and inflammatory thermal hyperalgesia (Kowaluk et al., 2000; McGaraughty et al., 2001a; Jarvis et al., 2002b). Importantly, all drugs selectively attenuated inflammatory hyperalgesia selectively in the inflamed hindpaw consistent with the notion that ADK inhibitors increase adenosine concentration preferentially at sites of injury or trauma. These drugs also showed a remarkable improvement in their therapeutic window compared with adenosine receptor agonists. Although a 10- to 16-fold separation between ED_{50} values for motor depressant and antihyperalgesic actions was noted for the newer ADK inhibitors, there was significantly less separation for directly acting adenosine receptor agonists, with the largest effect ratio being only 4.3 (Jarvis et al., 2002a). Likewise, the cardiovascular side effect profile of the ADK inhibitors was improved compared with direct adenosine receptor agonists (Kowaluk et al., 2000; Jarvis et al., 2002b). The antinociceptive effects of ADK inhibitors are primarily based on a spinal site of action. Intrathecal administration of A-134974 was more effective (ED_{50} = 6 nmol) than intracerebroventricular (ED_{50} = 100 nmol) or intraplanar (ED_{50} > 300 nmol) injection in its antihyperalgesic effects, whereas supraspinal activity of the drug was associated with motor depressant effects (McGaraughty et al., 2001a). As in epilepsy, the challenge to implement ADK-based therapies is restriction of treatment to the pathogenetic area responsible for pain generation. Restriction of treatment to a spinal site, e.g., by anti-ADK gene therapy, might provide local benefit without side effects.

4. Inflammation. As outlined in a preceding section, adenosine is an endogenous anti-inflammatory agent. Consequently, ADK inhibitors hold promise for the treatment of a large spectrum of inflammatory conditions. Thus, ADK inhibitors were shown to provide benefit in a rat model of pleuritis (Cottam et al., 1993) and in inflammatory pain models in the rat (Poon and Sawynok, 1998; Kowaluk et al., 2000; McGaraughty et al., 2001a; Suzuki et al., 2001). The ADK inhibitor GP-515 dose-dependently inhibited carrageenan-induced rat paw swelling and reduced cutaneous neutrophil invasion and vascular leakage in a rat skin lesion model; the latter effects were shown to

be A_2 receptor dependent (Rosengren et al., 1995). Furthermore, GP-515 affected carrageenan-induced inflammation in air pouches induced in BALB/c mice. Importantly, adenosine concentrations in pouch exudates were found to be increased. The anti-inflammatory effects of GP-515 were abrogated after the injection of ADA into the pouch, indicating that enhanced adenosine signaling induced by ADK inhibition was responsible for the therapeutic effect. GP-515 also reduced leukocyte counts and TNFα concentrations in the exudate (Cronstein et al., 1995). Suppression of TNFα production by GP-515 was further demonstrated in LPS-stimulated peripheral blood mononuclear cells (Eigler et al., 2000). Subsequently, GP-515 was shown to improve clinical and histologic outcome in a murine model of dextran sulfate sodium-induced colitis, a well-accepted model of inflammatory bowel disease (Siegmund et al., 2001). In addition, colon shortening, an indirect parameter for the degree of inflammation, was reduced as was the weight of the spleen. Mechanistically, GP-515 suppressed interferon-γ synthesis in LPS-induced splenocytes isolated from the colitis mice. In addition, CD69 expression, a marker for immune activation, was found to be reduced in the GP-515-treated colitis mice (Siegmund et al., 2001).

5. Cerebral Stroke. To capitalize on the neuroprotective potential of endogenous adenosine, three independent studies demonstrated a protective effect of ADK inhibitors on the infarct volume in a rat focal ischemia model (Miller et al., 1996; Jiang et al., 1997; Tatlisumak et al., 1998). When given 30 minutes before the onset of middle cerebral artery occlusion, 5′-deoxy-5-iodotubercidin reduced the infarct volume by 34 to 57% depending on the experimental paradigm and dose used (Miller et al., 1996; Jiang et al., 1997). More importantly, 5′-deoxy-5-iodotubercidin and GP-683 reduced the infarct volume up to 44% when given 30 to 360 minutes after the MCAO (Miller et al., 1996; Jiang et al., 1997; Tatlisumak et al., 1998). However, GP-683 treatment at a dose of 2 mg/kg was associated with a nonstatistically significant increase in mortality (Tatlisumak et al., 1998). Surprisingly, 5-iodotubercidin failed to protect against cerebral ischemic injury in gerbils in a temporary bilateral carotid artery occlusion model (Phillis and Smith-Barbour, 1993). In line with the neuroprotective effect of ADK inhibition, focal treatment strategies were realized by stem cell therapy and gene therapy (Pignataro et al., 2007b; Shen et al., 2011). Thus, a focal intrastriatal implant of ADK-deficient ES cell-derived glial progenitor cells led to an 85% reduction of the infarct volume in mice, when the cells were transplanted 7 days prior to 60 minutes of MCAO (Pignataro et al., 2007b). Likewise, an AAV-based gene therapy virus engineered to knock down ADK in astrocytes was shown to reduce infarct volume in mice by 51% when injected 4 weeks prior to the artery occlusion (Shen et al., 2011). Although preventative in nature and therefore not translatable to a clinical stroke scenario, these studies demonstrate that glial interventions and local ADK treatment approaches can provide significant neuroprotective effects in a mouse focal ischemia model.

6. Hearing Loss. Only two studies tested the otoprotective potential of the ADK inhibitor ABT-702. Although ABT-702 failed to restore hearing thresholds after exposure to traumatic noise (Vlajkovic et al., 2010), the chronic treatment with ABT-702 (1.5 mg/kg intraperitoneally twice a week) attenuated hair cell loss and age-related hearing loss in C57BL/6 mice (Vlajkovic et al., 2011). It remains to be determined why ABT-702 had an opposite outcome in those studies.

7. Schizophrenia. The adenosine hypothesis of schizophrenia predicts a deficiency of endogenous adenosine signaling in schizophrenia, which would synergistically affect dopaminergic and glutamatergic neurotransmission (Boison et al., 2012). In line with this hypothesis, adenosine-deficient Adk-tg mice display altered locomotor responses to both glutamatergic and dopaminergic psychostimulants. In addition, these animals display a wide spectrum of phenotypes in the affective and cognitive domains (Yee et al., 2007; Boison et al., 2012; Shen et al., 2012). If adenosine deficiency is implicated in the pathophysiology of schizophrenia, then ADK inhibition should provide benefit. Indeed, ABT-702 was recently shown to exhibit antipsychotic-like activity in a prepulse inhibition (PPI) paradigm in mice (Shen et al., 2012), which is a widely accepted measure of sensorimotor gating deficits believed to underlie sensory flooding and cognitive fragmentation in schizophrenia (Braff et al., 2001a,b). PPI disruption in rodents can be induced with the dopaminergic agonist apomorphine and is a well-established model of schizophrenia with predictive validity for antipsychotic drugs (Swerdlow et al., 2008). Furthermore, enhancement of basal PPI is considered to be a marker of antipsychotic action, since antipsychotics increase PPI in drug-naive animals (Singer et al., 2009). A recent dose response analysis (2.5, 5, and 10 mg/kg ABT-702) revealed that ABT-702 increased PPI in drug-naive animals independent of dose. In addition, ABT-702 at a dose of 5 mg/kg was found to be effective in reversing the PPI-disruptive effect of apomorphine (2 mg/kg). Together, these data demonstrated that ADK inhibition enhanced basal PPI in drug naive wild-type mice and exerted antipsychotic-like efficacy in a pharmacologically induced animal model of schizophrenia (Shen et al., 2012).

8. Cardioprotection. Adenosine is not only a potent neuroprotectant in the brain but also a powerful cardioprotectant, but the underlying protective mechanisms might be different (Przyklenk and Whittaker,

2005; Peart and Headrick, 2007; Gomes et al., 2011; McIntosh and Lasley, 2012). Whereas adenosine homeostasis in the brain is largely under the control of ADK (Pak et al., 1994; Boison et al., 2010), in the rabbit heart ADA inhibition was found to have a more profound adenosine augmenting effect than ADK inhibition (Manthei et al., 1998). In an initial study, ADK inhibition with 5-ITU or ADA inhibition with erythro-9-(2-hydroxy-3-nonyl)adenine, but not a combination of both drugs, was shown to improve functional recovery in the ischemic-reperfused mouse heart (Peart et al., 2001). Interestingly, ADK inhibition was shown to attenuate the cardioprotective effects of exogenous adenosine, suggesting that cardioprotection involves purine salvage through ADK (Peart et al., 2002). A mitochondrial ATP-sensitive K^+ channel blocker was shown to abrogate the cardioprotective effects of adenosine when coinfused with 5-ITU, demonstrating that conversion of adenosine to AMP, and thus ADK activity, might play a critical role in the cardioprotective mechanisms of adenosine (Peart et al., 2003). Together, these data suggest that both ADA or ADK inhibition can limit injury during ischemia-reperfusion via adenosine receptor activation. However, cardioprotection via either enzyme inhibitor appears to require an alternative purine-salvage pathway to be functional, and this pathway was reduced in aged hearts, which are increasingly susceptible to ischemic damage (Willems and Headrick, 2005). Subsequently, those findings were reproduced in a rat model of myocardial infarction (Peart and Gross, 2005). It was shown that the ADK inhibitor GP-515 induced vascular endothelial growth factor expression in cultured rat myocardial myoblasts, an effect that was completely blocked by the addition of ADA, which, when given alone, led to a decrease in baseline vascular endothelial growth factor expression (Gu et al., 2000). Since ADA—in contrast to brain—is highly expressed in the heart (Barankiewicz et al., 1997), ADK inhibitors might not be useful adenosine augmenting agents to promote cardioprotection. The interaction between ADA and ADK in adenosine metabolism of the heart might also be a reason why ADK inhibitors (for CNS applications) have fewer cardiac side effects than direct adenosine receptor agonists (Kowaluk et al., 2000). More recently, it was shown that the ADK inhibitors 5-ITU and ABT-702 as well as RNAi directed against *Adk* prevented the antihypertrophic effects of adenosine on cardiomyocytes, suggesting that ADK activity is needed to provide those antihypertrophic cardioprotective effects (Fassett et al., 2011). In conclusion, given the antihypertrophic role of nuclear ADK in cardiomyocytes and given the complexity of interactions between ADK and ADA in regulating cardiac adenosine homeostasis, ADK inhibition might not be a useful approach for cardioprotection.

9. Sepsis. Since ADK inhibitors are potent anti-inflammatory agents, they may also be of therapeutic benefit in septic shock. The ADK inhibitor GP-515 significantly decreased mortality in two models of septic shock induced by either lethal i.v. injection of endotoxin or induction of bacterial peritonitis (Firestein et al., 1994). It was shown that the protective effect of GP-515 was adenosine receptor dependent and that decreased neutrophil accumulation in the lungs and reduced TNFα levels in plasma were involved (Firestein et al., 1994). It was further shown that ADK inhibition prevented hypoxia-induced vascular leakage (Morote-Garcia et al., 2008). Since only few studies have addressed ADK as potential target for the treatment of sepsis, more work is needed to judge therapeutic usefulness.

10. Cartilage Protection. The anti-inflammatory properties of ADK inhibitors are likewise of therapeutic value in cartilage protection. The ADK inhibitor ITU was shown to attenuate cartilage damage induced by either IL-1β or by LPS in an in vitro cartilage explant model; depending on the model system used, ADK inhibition inhibited glycosaminoglycan release, prostaglandin E_2 release, or NO production (Petrov et al., 2005) and was shown to be more effective in the prevention of NO formation than ADA inhibition (Tesch et al., 2002). Similarly, ABT-702 significantly decreased cartilage destruction in a rat adjuvant arthritis model, a therapeutic effect that was associated with suppression of collagenase and stromelysin gene expression (Boyle et al., 2001).

C. New Therapeutic Concepts and Future Trends

The examples presented above demonstrate a general therapeutic utility of ADK inhibitors in augmenting the multiple beneficial cytoprotective and anti-inflammatory effects of *endogenous* adenosine. However, despite very promising results, in particular in the areas of inflammation, pain, and epilepsy, clinical ADK inhibitor development has largely been abandoned since around 2005 due to risks of toxicity and intolerable side effects. Since then, as outlined in earlier sections of this review, our knowledge on the role and function of *endogenous* ADK has increased tremendously leading to the re-definition of ADK as a promising therapeutic target. New solutions to challenging hurdles in ADK-based therapy development and newly defined functions of *endogenous* ADK will lead to a new era of ADK-based therapeutics. Several new trends and novel functions of ADK are noteworthy (Fig. 9):

1. Local therapies: The identification of ADK overexpression in many neuropathological conditions with causal implications in pathogenesis and pathophysiology (Boison, 2008; Boison et al., 2010) redefines ADK as a rational therapeutic

target. The realization that ADK dysfunction is restricted to certain brain areas (e.g., hippocampus) and cell types (astrocytes) provides the therapeutic rationale to develop region- and cell type-specific therapeutic interventions with the goal to normalize ADK function within an affected brain area. This goal could most effectively be achieved via gene therapies targeting ADK in a region- and cell type-specific manner (Boison, 2009). Local therapeutic interventions would also circumvent challenges and side effects, which hampered the clinical development of systemic ADK inhibitors.

2. Adenosine receptor-independent mechanisms: Historically, ADK inhibitor development has aimed at achieving increased adenosine receptor activation as a consequence of an increased concentration of endogenous adenosine (Kowaluk et al., 1998; Kowaluk and Jarvis, 2000; McGaraughty et al., 2005). However, a recent study suggests a novel use of ADK inhibitors that is independent of increased adenosine receptor activation. Importantly, ADK inhibitors were shown to promote the proliferation of β-cells in the pancreas through an adenosine receptor-independent mechanism (Annes et al., 2012).
3. Role of nuclear isoform of ADK: Tissue levels of adenosine and the concentration of extracellular adenosine, which determine the degree of adenosine receptor activation, are thought to depend on the cytoplasmic isoform of ADK (Studer et al., 2006; Shen et al., 2011). However, the nuclear isoform of ADK might play key roles in the regulation of cell proliferation, as demonstrated in β-cells of the pancreas and in cardiomyocytes (Fassett et al., 2011; Annes et al., 2012). On the basis of a tightly controlled developmental expression profile of ADK in neonatal rodent brain, a role of the nuclear isoform of ADK in the control of neurogenesis can also be postulated (Studer et al., 2006). Obviously, the nuclear isoform of ADK might play an important role in cancer biology. Thus, nuclear ADK emerges as a novel therapeutic target to capitalize on specific nuclear functions of ADK such as interaction with the mTOR pathway (Fassett et al., 2011; Annes et al., 2012). However, it will be a challenge to develop novel therapeutics to selectively target the nuclear isoform of ADK.
4. Epigenetic role of ADK: Adenosine is an obligatory end product of transmethylation reactions, including DNA methylation (Finkelstein and Martin, 1986; Mato et al., 2008). If adenosine is not constantly removed by ADK, adenosine accumulates and reverses the direction of the SAH-hydrolase reaction, resulting in increased levels of SAH (Moffatt et al., 2002; Boison et al., 2002b). SAH in turn is known to inhibit DNA methyltransferases through substrate inhibition (James et al., 2002). Since direct DNA methyltransferase inhibitors, such as azacytidine and decitabine are highly toxic (Weisman et al., 1985; Yogelzang et al., 1997), ADK inhibitors might find new uses as DNA regulating agents, e.g., in cancer therapy.
5. Regulation of cell proliferation: Through a combination of epigenetic and additional adenosine receptor-independent mechanisms, such as interaction with the mTOR pathway, ADK regulating agents might find new uses as therapeutics to affect cell proliferation. The use of ADK inhibitors to stimulate β-cell proliferation is one promising step in this direction.

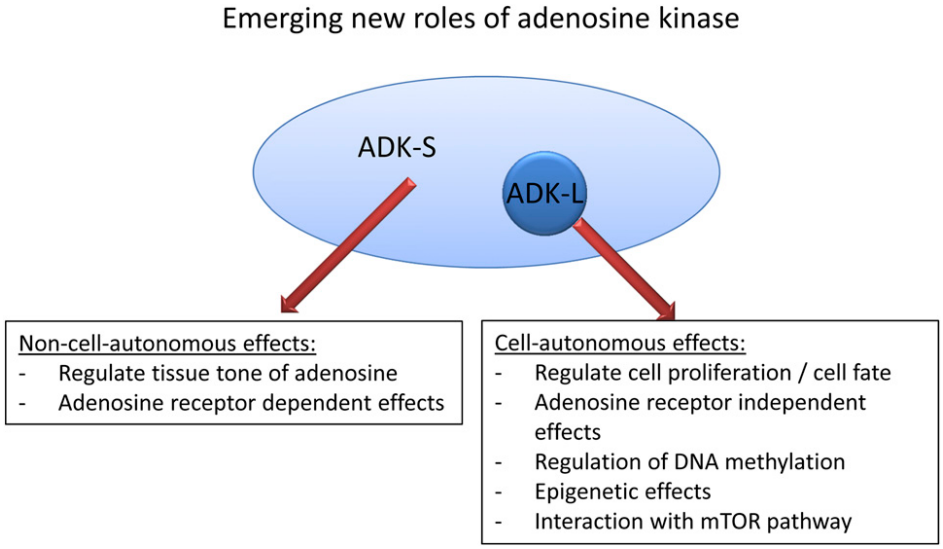

Fig. 9. Emerging new roles of adenosine kinase. Summary of key findings; for details please refer to main text.

VII. Implications for Human Pathogens

Prokaryotic and eukaryotic microorganisms usually have distinct adaptations in their nucleoside and nucleotide metabolism that differ from their hosts. Those differences can be exploited to develop antiparasitic drugs, which are specific for the parasites but do not affect the host. In particular, purine salvage pathways play important roles for parasites, which frequently lack pathways for the de novo synthesis of purines. Parasitic ADK therefore plays important roles in adenosine salvage, a feature that can be exploited therapeutically. In the following sections parasitic ADK and therapeutic avenues will briefly be discussed.

A. Mycobacterium tuberculosis. *Mycobacterium tuberculosis* is a pathogenic bacterial species and the causative agent of most cases of tuberculosis. *M. tuberculosis Adk* was the first bacterial ADK to be cloned and characterized and shown to exhibit a >12-fold enhanced affinity for the antimycobacterial prodrug 2-methyladenosine compared with human ADK (Long et al., 2003). Recent crystallographic data from recombinant *M. tuberculosis* ADK and structural differences to human ADK are expected to yield information guiding the design of more potent and selective antimycobacterial agents (Wang et al., 2005; Reddy et al., 2007). Identification of structure-activity relationships allowed the identification of highly selective substrates (i.e., prodrugs of toxic metabolites), such as 2-aza-adenosine, 8-aza-9-deaza-adenosine, 2-fluoro-adenosine, carbocyclic-adenosine, 8-aza-carbocyclic-adenosine, or 9-[α-l-lyxofuranosyl]-adenine and potent inhibitors of purine salvage, such as *N*1-benzyl-adenosine, 2-fluoro-adenosine, 6-cyclopentyloxy-purine riboside, 7-iodo-7-deaza-adenosine, or 5′-amino-5′-deoxy-adenosine (Long and Parker, 2006; Long et al., 2008). In particular, a new class of halogenated 3-deaza-adenosine analogs was shown to be 10-fold better substrates for *M. tuberculosis* ADK compared with human ADK, a finding that may lead to a new class of antitubercular agents (Long et al., 2007).

B. Trypanosoma brucei. *Trypanosoma brucei* is an extracellular eukaryotic parasite that causes sleeping sickness. It lacks de novo purine synthesis and therefore depends on adenosine taken up from the host's blood by high affinity transporters (Vodnala et al., 2008). Although *T. brucei* ADK is not essential for the survival of the parasite (Luscher et al., 2007), the combination of the high affinity of *T. brucei* ADK for adenosine and the existence of efficient adenosine transporters result in a strong purine salvage system in *T. brucei*, which, potentially, should render the parasite more sensitive than mammalian cells to antimetabolites such as AraA that need to be phosphorylated by ADK to be transformed into their cytotoxic form. Indeed, it was recently shown that AraA inhibited parasite proliferation in an ADK-dependent manner by affecting nucleotide levels and inhibition of nucleic acid synthesis (Vodnala et al., 2008).

C. Leishmania donovanii. *Leishmania donovanii* is a purine-auxotrophic parasitic protozoan that causes visceral leishmaniasis, also known as kala-azar. The stage-specific differential activity pattern of the parasitic ADK made the enzyme an attractive target for chemotherapeutic intervention. *L. donovanii* ADK can be inhibited by very low concentrations of the adenosine analogs tubercidin and 6-methylmercaptopurine riboside, whereas ADK-deficient promastigotes were shown to survive and grow in the presence of 20 μM tubercidin (Iovannisci and Ullman, 1984; Datta et al., 1987). The biochemistry of *L. donovanii* ADK has been studied intensively, and regulatory mechanisms governing the activity of *L. donovanii* ADK might be instructive to understand ADK regulation in more complex mammalian systems, however, distinct differences also exist. Similar to mammalian ADK, *L. donovanii* has a kinetic mechanism of a sequential Bi-Bi reaction, with AMP and ADP acting as enzyme regulators in vivo (Bhaumik and Datta, 1988); however, immunologically, the enzyme was found to be distinct from mammalian ADK (Bhaumik and Datta, 1989). In contrast to mammalian ADK, the protozoan enzyme binds adenosine exclusively through the catalytic site and is therefore not inhibited by its own substrate at high adenosine concentrations (Bhaumik and Datta, 1992). *L. donovanii* ADK enzyme is an aggregation-prone protein and its activity is regulated by an aggregation-disaggregation cycle, in which a *L. donovanii* cyclophilin disaggregates the aggregated form and thus stabilizes the active form of the enzyme (Chakraborty et al., 2002), whereas the aggregated inactive form was found to be stabilized by ADP (Sen et al., 2006). Stress-induced translocation of the cyclophilin from the lumen of the endoplasmic reticulum to the cytosol was found to be implicated in the regulation of ADK activity (Sen et al., 2007). To date, possible protein interaction partners have not been identified for mammalian ADKs; however, regulation of ADK by an aggregation-disaggregation cycle is an intriguing mechanism allowing ADK to respond rapidly and reversibly to changes in environmental conditions.

D. T. gondii. *T. gondii* is a purine auxotroph intracellular parasitic protozoan that causes toxoplasmosis in humans. ADK-mediated phosphorylation of salvaged adenosine provides the major route of purine acquisition of the parasite. Consequently, *T. gondii* ADK represents a promising target for the rational design of antiparasitic compounds. Enzyme inhibition was observed with the purine nucleoside analogs AraA, 4-nitrobenzylthioinosine, N^6-(*p*-methoxybenzoyl)adenosine, tubercidin, and iodotubercidin; ADK-deficient mutants of *T. gondii* were resistant to these drugs

(Iltzsch et al., 1995; Darling et al., 1999; Sullivan et al., 1999). Recombinant expression and crystallographic analysis of *T. gondii* ADK revealed structural differences compared with human ADK, resulting in a major change in the orientation of the two domains and changes in substrate binding properties; these structural differences might form the basis for the rational design of ADK inhibitors that are selective for *T. gondii* ADK (Cook et al., 2000; Recacha et al., 2000; Schumacher et al., 2000; Zhang et al., 2006, 2007). 6-Benzylthioinosine was identified as a prototype subversive substrate for *T. gondii* ADK and used as a lead to develop a new class of compounds shown to be selectively toxic to the parasites but not their host (Yadav et al., 2004; Rais et al., 2005). In particular, due to their increased internal flexibility, the 7-deaza-6-benzylthioinosine analogs were shown to exhibit improved binding to the hydrophobic pocket of the *T. gondii* enzyme (Kim et al., 2008). Consequently, those agents showed a selective antitoxoplasmic effect in wild-type parasites, whereas ADK-deficient mutants were resistant to the drug (Al Safarjalani et al., 2008, 2010). A different approach was taken in identifying short inhibitory RNAs or double-stranded RNAs targeting the expression of *T. gondii Adk* gene, an approach that might eventually lead to the development of novel therapeutics or vaccines (Yu et al., 2008, 2009).

E. Cryptosporidium parvum. *Cryptosporidium parvum* is one of several protozoan species that cause cryptosporidiosis, a parasitic disease of the mammalian intestinal tract. *C. parvum* is a purine auxotroph and the sole route for purine salvage by the parasite is ADK. Overexpression and purification of recombinant *C. parvum* has recently been achieved, and initial data suggest that 4-nitro-6-benzylthioinosine, a compound with therapeutic promise against the related parasite *T. gondii*, also inhibits *C. parvum* ADK (Galazka et al., 2006).

F. Anopheles gambia and Plasmodium falciparum. *Anopheles gambia* is the most common vector for the transmission of *Plasmodium falciparum* in Africa, a purine auxotroph parasite that causes malaria. *P. falciparum* does not have ADK by itself and therefore requires access to host- or vector-derived purines to survive. *A. gambia* ADK was recently cloned, expressed, and characterized; remarkably, *A. gambia* ADK has the highest affinity for adenosine (K_m = 8.1 nM) of any known ADKs. The ability to salvage adenosine by ADK separates the insect host from the parasite and provides a rationale for metabolic and inhibitor design studies to investigate targets in host-parasite interactions (Cassera et al., 2011).

VIII. Conclusions and Outlook

Adenosine is a ubiquitous energy metabolite that fulfills many beneficial functions in most organ systems. Early attempts to harness the therapeutic potential of adenosine were based on the development of adenosine receptor-specific ligands. However, because of the widespread distribution of the receptors, systemic side effects precluded the clinical development of many of the most potent drugs. A breakthrough came with the realization that ADK inhibitors could potentiate the beneficial effects of endogenous adenosine in a site- and event-specific manner and thereby prevent widespread side effects of systemic adenosine receptor drugs. Promising preclinical data in the areas of inflammation, pain, and epilepsy led to intense drug development efforts, mostly between 1995 and 2005. According to a press release from 1996, GP-3269 is the only ADK inhibitor studied in phase I studies in humans, but data have not been disclosed. Subsequently, limitations of ADK inhibitors became evident, and drug development efforts have largely been stalled. Limitations included liver toxicity, and, according to a preliminary report, the development of GP-3966 was halted due to CNS hemorrhage in rats and dogs. The advent of new molecular tools and the report of unexpected basic research findings during that past 10 years have resulted in a recent surge of interest in ADK. An almost-abandoned old target has been completely reinvented and is likely to lead to unprecedented new therapeutic opportunities in many different areas (Table 1). Of note are:

1. Overexpression of ADK has been identified in several pathologies, in which resulting adenosine deficiency plays a key role in pathophysiological mechanisms. The identification of pathologic ADK overexpression provides a strong scientific rationale to target ADK therapeutically.
2. New developments suggest that ADK might be exploitable therapeutically by antisense gene therapy, which might be a unique strategy to target ADK in a region- and cell type-specific manner.
3. Novel roles of ADK, in particular of the nuclear isoform of ADK, have been identified, which could be exploited to modulate cell proliferation therapeutically with ADK regulating agents.
4. A possible role of ADK in regulating epigenetic functions of the cell is likely to lead to novel applications of ADK-regulating agents as "epigenetic medicines."
5. Exciting developments in the area of bacteriology and parasitology suggest that ADK in purine auxotroph organisms differs significantly enough from human ADK to allow therapeutic exploitation of those differences to develop new classes of antiparasitic drugs.

Perhaps the most exciting future therapeutic perspective is the availability of ADK modulating agents that can be used to regulate the availability of the

TABLE 1
Therapeutic opportunities of ADK manipulation: summary of major findings and new trends

Goal	Method	Outcome
Adenosine augmentation	Systemic ADK inhibitors	Seizure suppression Antinociception Anti-inflammatory action Neuroprotection Attenuation of age-related hearing loss Antipsychotic-like activity
Adenosine augmentation	Ketogenic diet	Seizure suppression
Adenosine augmentation	$Adk^{-/-}$ stem cell transplantation	Neuroprotection Seizure suppression Prevention of epilepsy
Adenosine augmentation	Anti-ADK gene therapy	Neuroprotection Seizure suppression
Cell proliferation	ADK inhibitors	Stimulation of β-cell proliferation
Parasitology	Prodrug activation via ADK	Parasite toxicity

homeostatic bioenergetic network regulator adenosine. In targeting ADK, the unique opportunity exists to modulate an entire network not only based on activation of multiple adenosine receptor-dependent pathways but also based on adenosine receptor-independent biochemical, bioenergetic, and epigenetic mechanisms. This multimodal approach is a novel therapeutic concept that differs from conventional pathway-centric drug development efforts. The realization that adenosine homeostasis—and consequently network function—is disrupted in many pathologic conditions offers the unique therapeutic opportunity to use ADK-regulating agents to reconstruct or restore network homeostasis in disease. Those novel concepts may indeed redefine ADK as a therapeutic target and may offer new hopes to finding cures for intractable diseases.

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