



## Adenosine has two faces: Regionally dichotomous adenosine tone in a model of epilepsy with comorbid sleep disorders

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### ABSTRACT

**Objective:** Adenosine participates in maintaining the excitatory/inhibitory balance in neuronal circuits. Studies indicate that adenosine levels in the cortex and hippocampus increase and exert sleep pressure in sleep-deprived and control animals, whereas in epilepsy reduced adenosine tone promotes hyperexcitability. To date, the role of adenosine in pathological conditions that result in both seizures and sleep disorders is unknown. Here, we determined adenosine tone in sleep and seizure regulating brain regions of Kv1.1 knockout (KO) mice, a model of temporal epilepsy with comorbid sleep disorders.

**Methods:** 1) Reverse phase-high performance liquid chromatography (RP-HPLC) was performed on brain tissue to determine levels of adenosine and adenine nucleotides. 2) Multi-electrode array extracellular electrophysiology was used to determine adenosine tone in the hippocampal CA1 region and the lateral hypothalamus (LH).

**Results:** RP-HPLC indicated a non-significant decrease in adenosine (~50%,  $p = 0.23$ ) in whole brain homogenates of KO mice. Regional examination of relative levels of adenine nucleotides indicated decreased ATP and increased AMP in the cortex and hippocampus and increased adenosine in cortical tissue. Using electrophysiological and pharmacological techniques, estimated adenosine levels were ~35% lower in the KO hippocampal CA1 region, and 1–2 fold higher in the KO LH. Moreover, the increased adenosine in KO LH contributed to lower spontaneous firing rates of putative wake-promoting orexin/hypocretin neurons.

**Interpretation:** This is the first study to demonstrate a direct correlation of regionally distinct dichotomous adenosine levels in a single model with both epilepsy and comorbid sleep disorders. The weaker inhibitory tone in the dorsal hippocampus is consistent with lower seizure threshold, whereas increased adenosine in the LH is consistent with chronic partial sleep deprivation. This work furthers our understanding of how adenosine may contribute to pathological conditions that underlie sleep disorders within the epileptic brain.

### 1. Introduction

Persistent disturbances in sleep architecture and quality are commonly associated with many neurological and psychiatric disorders, including epilepsy (St. Louis, 2011; Krause et al., 2017). Adenosine is a neuromodulator that elicits antiseizure and somnogenic actions. Adenosine tone has an inhibitory effect on excitatory synapses via the adenosine 1 receptor (A<sub>1</sub>R) throughout the brain (Dunwiddie, 1980). During temporal lobe seizures, adenosine levels rise which suppresses further seizure activity (Winn et al., 1980). However, in cases of chronic

epilepsy this inhibitory mechanism is weakened or lacking as seen in animal models and humans with lower adenosine tone (Rebola et al., 2003; Masino et al., 2011; Boison, 2016; Porkka-Heiskanen et al., 1997). As a somnogen, adenosine levels have been reported to rise in specific cortical, temporal, and subcortical brain regions when wake periods exceed physiological durations (Porkka-Heiskanen et al., 2000; Basheer et al., 2004; Weber and Dan, 2016). Rising adenosine levels are thought to promote sleep by inhibiting neurons involved in wakefulness and cognition; thus, decreased levels may be responsible for sleep disruption (Boison and Aronica, 2015). For individuals with both epilepsy

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and sleep disruption, a logical, yet untested, hypothesis is that a uniformly reduced adenosine tone underlies both conditions (Boison, 2016).

Here we determined the adenosine tone in  $K_v1.1$  knockout (KO) mice, a model of epilepsy with comorbid sleep disorders. KO mice lack the  $\alpha$ -subunit of the  $K_v1.1$  voltage-gated delayed rectifier potassium channel, have a severe epilepsy phenotype with multiple types of seizures, including frequent generalized tonic-clonic seizures, and present pathology similar to temporal lobe epilepsy (Smart et al., 1998; Wenzel et al., 2007; Simeone et al., 2016). We have recently reported that KO mice also have sleep-disorder symptoms consistent with involvement of the orexin/hypocretin system, including disrupted sleep architecture and insufficient amounts of non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep (Roundtree et al., 2016).

Orexin/hypocretin neurons are a subset of wake-promoting neurons located in the lateral hypothalamus (LH). Severe seizures propagate to the LH in KO mice, and the LH displays signs of chronic pathology (including increased blood-brain permeability, astrogliosis and impaired mitochondrial function) and increased orexin/hypocretin protein levels (Roundtree et al., 2016). Orexin/hypocretin neurons express  $A_1R$  and their inhibition is known to promote sleep (Chemelli et al., 1999; Liu and Gao, 2007). Thus, we hypothesized that adenosine concentrations would be lower in KO hippocampus and LH providing a common etiology for ictogenesis in the hippocampus and for the sleep disorders seen in the LH of KO mice. Contrary to our hypothesis, our data indicate regionally divergent adenosine tones in KO brain indicating a hitherto unrecognized complexity in the regulation of adenosine nucleotides during complex and chronic neurological disorders.

## 2. Methods

### 2.1. Animals

C3HeB/FeJ  $K_v1.1$  knockout (KO) and wild-type (WT) littermates were bred, reared, genotyped, and housed as previously described (Roundtree et al., 2016). Mice were entrained to a strict 12 h light/dark cycle with access to food and water ad libitum. Lights on occurred at zeitgeber time (ZT) 00:00 h. For all electrophysiology experiments, mice were sacrificed between 03:00–04:00 ZT to control for diurnal changes in adenosine levels in the brain. Adult male and female mice (5–9 weeks old) were used for these experiments. All experiments conformed to NIH guidelines in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals and were approved by Creighton University's Institutional Animal Care and Use Committee.

### 2.2. Reversed-phase high pressure liquid chromatography (RP-HPLC) tissue extraction

For RP-HPLC experiments, mice were euthanized using a Muromachi Microwave Fixation System (Model MMW-05, Muromachi Kikai Co., LTD, Tokyo, Japan) in order to quickly ( $< 1$  s) raise the brain temperature above  $85^\circ\text{C}$  and inactivate enzymatic activity. This is necessary in order to prevent post-mortem ATP/ADP/AMP metabolism by nucleotidase activity and accumulation of adenosine as a technical artifact. Mice were initially anesthetized using isoflurane and placed in a restrainer with a water jacket and euthanized (3.5 kW and 0.89 s); (Delaney and Geiger, 1996). The whole brain was removed and examined for proper fixation then immediately placed in 0.1 M sulfuric acid and homogenized on ice. Tris (0.1 M) base was added to homogenized tissue in sulfuric acid followed by 1 M NaOH to neutralize the homogenate. The neutralized homogenate was centrifuged at 15 rcf for 15 min at  $4^\circ\text{C}$ . Supernatant was collected for RP-HPLC (Akula et al., 2008). Adenosine concentrations from whole brains were normalized to protein concentration as determined using the Bradford method performed on a sample of each homogenate.

### 2.3. HPLC analysis of whole brain tissue

Conversion of adenosine to the  $N^6$ -etheno derivative was accomplished by a previously published method (Haink et al., 2003). Briefly, standards or the extraction solution (8 mice total 50  $\mu\text{L}$ ) was added to a freshly prepared mixture of chloroacetaldehyde ( $\sim 50\%$  in water from Aldrich) and sodium acetate buffer (1 M, pH 4.5) (11.2/138.8, v/v, 150  $\mu\text{L}$ ). After heating at  $60^\circ\text{C}$  for 1 h, the mixture was cooled in ice water to stop the reaction, filtered through a 0.45  $\mu\text{m}$  membrane and used without dilution for RP-HPLC analysis. RP-HPLC was performed on a Waters 626 instrument equipped with a 600S controller, a 474 scanning fluorescence detector and a 717plus autosampler. Analysis of etheno-derivatized standards and extracts was performed by a previously published method modified to separate  $N^6$ -ethenoadenosine from interfering peaks derived from mouse brain (Bhatt et al., 2012). Separation was achieved on a Waters XTerra MS  $C_{18}$  column ( $3 \times 50$  mm,  $5\mu\text{m}$ ), equipped with a  $C_{18}$  guard cartridge (Phenomenex, Torrance, CA), eluted with mixtures of buffer A (tetrabutylammonium hydrogensulfate (5.7 mM) and  $\text{KH}_2\text{PO}_4$  (30.5 mM) adjusted to pH 5.8 with 2 M KOH) and buffer B (acetonitrile/buffer A, 2/1, v/v) at 1.5 mL/min. Samples of the derivatizing mixture (50  $\mu\text{L}$ ) were loaded on to the column, which had been previously equilibrated with buffer A. The column was eluted by increasing the percentage of buffer B in the eluent to 40% over 1 min, holding buffer B at 40% for 1.4 min before reducing the percentage of buffer B back to 0% over 0.1 min. The column was then washed with buffer A for 2.5 min before the next sample was loaded. The eluent was continuously monitored for fluorescence employing an excitation wavelength of 280 nm and an emission wavelength of 410 nm (Bhatt et al., 2012).  $N^6$ -ethenoadenosine eluted from the column after 1.7 min. Quantification of adenosine amounts was determined using external standards (25–1000 nM,  $r^2 = 0.9996$ ).

### 2.4. HPLC analysis of adenine nucleotides in specific brain regions

Determination of the concentration of adenine nucleotides followed Levitt et al. (1984). Microwaved fixed brain tissues were homogenized (9 mice total,) in 75% MeOH/0.15 M NaCl to 0.5 mg/mL W/V. The homogenate was combined with 0.15 M NaCl and  $\text{CHCl}_3/\text{MeOH}$  (2:1 v/v) containing 0.01% BHT. Samples were vortexed for 2 min and phases were allowed to separate for 30 min on ice. After separation, samples were centrifuged for 5 min at 7.8 kg. The lower phase was removed for nonpolar metabolite analysis while the upper, aqueous phase was saved for polar metabolite analysis. To compare adenine nucleotide concentrations, the aqueous portion was converted to its etheno adduct. The aqueous extract was dried under nitrogen then resuspended in a 10:1 (v/v) mixture of Krebs buffer (in mM): 113 NaCl, 4.8 KCl, 2.5  $\text{CaCl}_2$ , 1.2  $\text{KH}_2\text{PO}_4$ , 1.2  $\text{MgSO}_4$ , 25  $\text{NaHCO}_3$  and 5.5 glucose) and chloroacetaldehyde. Samples were heated to  $80^\circ\text{C}$  for one hour then cooled to room temperature for HPLC analysis. HPLC consisted of a Shimadzu LC-20AB binary pump connected to a Shimadzu RF-20Axs fluorescent detector. Five  $\mu\text{L}$  of the derivatized samples were injected onto an Agilent Eclipse AAA  $4.6 \times 150$  mm column at a flow rate of 0.8 mL/min. Mobile phase A consisted of 0.1% formic acid and mobile phase B consisted of 0.1% formic acid in acetonitrile, and the following gradient was used to separate the adenosine molecules: initial conditions of 0% B was held for 5 min, followed by increases to 4.7% B over 2 min, 11.7% B over 8 min, 35% B over 5 min, 95% B over 5 min then held for 5 min at 95% B. The column was then returned to initial conditions and held for 5 min. Etheno detection was achieved using an excitation wavelength of 300 nm and an emission wavelength of 420 nm. The adenylate energy charge (AEC) was calculated with the following equation:

$$\text{Energy charge} = \frac{[\text{ATP}] + 1/2[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

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