Neuroscience 303 (2015) 211–219

DISRUPTED SLEEP–WAKE REGULATION IN TYPE 1 EQUILIBRATIVE NUCLEOSIDE TRANSPORTER KNOCKOUT MICE

T. KIM,[a†] V. RAMESH,[a‡] M. DWORAK,[a¶] D.-S. CHOI,[b] R. W. MCCARLEY,[a] A. V. KALINCHUK[a] AND R. BASHEER[a*]

[a] *VA Boston Healthcare System and Harvard Medical School, 1400 V.F.W. Parkway, West Roxbury, MA 02132, United States*

[b] *Department of Molecular Pharmacology and Experimental Therapeutics, Molecular Neuroscience Program, Mayo Clinic College of Medicine, Rochester, MN 55905, United States*

Abstract—The type 1 equilibrative nucleoside transporter (ENT1) is implicated in regulating levels of extracellular adenosine ($[AD]_{ex}$). In the basal forebrain (BF) levels of $[AD]_{ex}$ increase during wakefulness and closely correspond to the increases in the electroencephalogram (EEG) delta (0.75–4.5 Hz) activity (NRδ) during subsequent non-rapid eye movement sleep (NREMS). Thus in the BF, $[AD]_{ex}$ serves as a biochemical marker of sleep homeostasis. Waking EEG activity in theta range (5–9 Hz, Wθ) is also described as a marker of sleep homeostasis. An hour-by-hour temporal relationship between the Wθ and NRδ is unclear. In this study we examined the relationship between these EEG markers of sleep homeostasis during spontaneous sleep–wakefulness and during sleep deprivation (SD) and recovery sleep in the ENT1 gene knockout (ENT1KO) mouse. We observed that baseline NREMS amount was decreased during the light period in ENT1KO mice, accompanied by a weak correlation between Wθ of each hour and NRδ of its subsequent hour when compared to their wild-type (WT) littermates. Perfusion of low dose of adenosine into BF not only strengthened the Wθ–NRδ relationship, but also increased NREMS to match with the WT littermates suggesting decreased $[AD]_{ex}$ in ENT1KO mice. However, the SD-induced $[AD]_{ex}$ increase in the BF and the linear correlation between the EEG markers of sleep homeostasis were unaffected in ENT1KO mice suggesting that during SD, sources other than ENT1 contribute to increase in $[AD]_{ex}$. Our data provide evidence for a differential regulation of wakefulness-associated $[AD]_{ex}$ during spontaneous *vs* prolonged waking. Published by Elsevier Ltd. on behalf of IBRO.

Key words: adenosine, basal forebrain, sleep deprivation, sleep homeostasis, NREM delta, wake-theta.

INTRODUCTION

Levels of extracellular adenosine $[AD]_{ex}$ are partly regulated by nucleoside transporters, the bidirectional equilibrative nucleoside transporters (ENT1, 2, 3 and 4) driven by chemical gradient (Baldwin et al., 2004), and the concentrative nucleoside transporters (CNT1, 2, and 3) driven by sodium (and proton) electrochemical gradients (Gray et al., 2004). ENT1 is implicated in the regulation of $[AD]_{ex}$ in basal forebrain (BF) (Basheer et al., 2004; Brown et al., 2012). Blocking AD uptake within BF by ENT1 inhibitor, nitrobenzylthioinosine (NBMPR), increases $[AD]_{ex}$ and sleep (Porkka-Heiskanen et al., 1997). In rats, a decrease in NBMPR binding following acute sleep deprivation (SD) is attributed to the SD-induced increase in $[AD]_{ex}$ (Alanko et al., 2003). Besides the transport of $[AD]_{ex}$ via transporters, breakdown of extracellular adenosine triphosphate $[ATP]_{ex}$ released in response to increased neuronal activity as a cotransmitter from neurons or by gliotransmission, contribute to increased $[AD]_{ex}$ (Dunwiddie and Masino, 2001). Both, decreased re-uptake by transporters, and increased release/breakdown of gliotransmission-derived ATP are implicated in the regulation of $[AD]_{ex}$ in BF (Alanko et al., 2003; Halassa et al., 2009).

AD acts as a somnogenic factor in the BF consisting of cortically-projecting wake-active neurons (Basheer et al., 2004; Porkka-Heiskanen and Kalinchuk, 2011; Brown et al., 2012). Levels of $[AD]_{ex}$ are higher during wakefulness when compared to sleep (Porkka-Heiskanen et al., 1997; McKenna et al., 2003; Murillo-Rodriguez et al., 2004). SD further increase the $[AD]_{ex}$ within 3 h in BF and by 5 h in the frontal cortex (Kalinchuk et al., 2011). In a recent study we reported that the SD-induced increase in BF $[AD]_{ex}$ depends on the neuronal induction of inducible nitric oxide synthase-dependent nitric oxide, not observed during spontaneous wakefulness, suggesting a potential difference in the mechanism of $[AD]_{ex}$ regulation during spontaneous wakefulness and SD (Kalinchuk et al., 2006b; Kalinchuk et al., 2010).

*Corresponding author. Tel: +1-857-203-6181; fax: +1-857-203-5592.

E-mail address: Radhika_Basheer@hms.harvard.edu (R. Basheer).

† Current address: Department of Psychiatry, School of Medicine, Kyung Hee University Hospital at Gangdong, Seoul, South Korea

‡ Current address: Systems Neurophysiology Research, Specialty Care, Springfield, PA, 19010, United States

¶ Current address: Institute for Neuroscience, German Sport University, Cologne, Germany

Abbreviations: BF, basal forebrain; EEG, electroencephalogram; ENT1, type 1 equilibrative nucleoside transporter; ENT1KO, ENT1-gene knock-out; EMG, electromyogram; HSR, homeostatic sleep response; KO, knockout; ML, mediolateral; NBMPR, nitrobenzylthioinosine; NREMS, non-rapid eye movement sleep; REM, rapid eye movement; RT-PCR, Real Time Polymerase Chain Reaction; RS, recovery sleep; SD, sleep deprivation; WT, wild-type.

http://dx.doi.org/10.1016/j.neuroscience.2015.06.037
0306-4522/Published by Elsevier Ltd. on behalf of IBRO.

The increases in $[AD]_{ex}$ occurs during wakefulness. However, to date, its homeostatic effects are evaluated by the increases in the electroencephalogram (EEG) delta activity (0.75–4.5 Hz, NRδ) during subsequent non-rapid eye movement sleep (NREMS). In humans and rodents waking EEG activity in theta range (5–9 Hz, Wθ) is also described as a marker of sleep homeostasis (Cajochen et al., 1995; Aeschbach et al., 1997; Finelli et al., 2000; Vyazovskiy and Tobler, 2005; Kalinchuk et al., 2015). A positive correlation exists between the rise rate of Wθ and the NRδ in NREMS (Finelli et al., 2000). In this study using an ENT1-gene knock-out (ENT1KO) mouse model (Choi et al., 2004), we sought to examine the role of ENT1 in spontaneous sleep–wake regulation. We observed that ENT1 is key in regulating a linear relationship between the hourly Wθ and the NRδ of the subsequent hour. We also examined the role of ENT1 on SD-induced increase in $[AD]_{ex}$ in the BF and homeostatic sleep response (HSR). We observed that SD-induced HSR was unaffected in ENT1KO mice with concurrent increase in BF $[AD]_{ex}$. To our knowledge, this is the first study to measure $[AD]_{ex}$ in the BF of mice by microdialysis and to demonstrate that $[AD]_{ex}$ regulates the Wθ- NRδ relationship.

EXPERIMENTAL PROCEDURE

Animals

The ENT1KO mice were generated as described by Choi et al. (2004). Briefly, the exons 2–4 of ENT1 gene were deleted in mice with C57BL/6J × 129X1/SvJ genetic background. The controls were the wild-type (WT) littermates of the knockout (KO) mice. Male adults (3–4 months old) mice were used in the study. Mice were housed in standard Plexiglas cages (room temperature 23.5–24.0 °C; 12 h light:12 h dark cycle, lights on at 07:00 A.M., ZT 0) with food and water provided *ad libitum*. Animal care and handling procedures were approved by the Association for Assessment and Accreditation of Laboratory Animal Care and Use Committee at Veterans Affairs Boston Healthcare System, Harvard University, and National Institutes of Health.

EEG electrode and microdialysis cannula implantation

For EEG recordings, mice were implanted with EEG and electromyogram (EMG) electrodes under general anesthesia (1–3% isoflurane inhalation). EEG electrodes (stainless-steel screws) were implanted epidurally over the frontal [anteroposterior (AP), +1.0 mm from Bregma; mediolateral (ML), 1.0 mm] and parietal (AP, +1.0 mm from lamda; ML, 1.0 mm) cortices. EMG recording electrodes (silver wires covered with Teflon) were implanted into neck muscles. A unilateral guide cannula for microdialysis probes (CMA 7 Guide Cannula, CMA Microdialysis, Solna, Sweden) was inserted targeting the dorsal margin of BF including substantia innominata, horizontal limb of diagonal band, and the magnocellular preoptic nucleus (AP, 0.0 mm; ML, 1.5 mm; dorsoventral, 5.0 mm) (Franklin and Paxinos, 2008). EEG, EMG electrodes and one guide cannula were attached to a microconnector and fixed to the skull with dental cement. After surgery, analgesic (Ketofen, 5 mg/kg) was given intramuscularly and each mouse was housed in individual cages and allowed to recover from surgery for one week.

EEG recording and analyses

One week after surgery, the mice were transferred to the recording cages in a sound-attenuated room for habituation with attached EEG recording cables for two days. Baseline EEG was monitored for a 24-h period starting at 7:00 A.M. The EEG/EMG signals were amplified and sampled at 100 Hz. EEG recordings (acquisition using Grass Gamma, version 4.3) were manually scored using the Rat Sleep Stager (version 3.2) in 10-s epochs for non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep, and wakefulness. Time spent in each vigilance state was calculated and compared between WT littermates and ENT1KO mice. The frequency and duration of NREMS and REM episodes were analyzed to determine the difference of sleep quality in ENT1KO mice. Two established homeostatic sleep markers, EEG activity in NRδ (Borbely, 1982) and Wθ (Finelli et al., 2000; Vyazovskiy and Tobler, 2005; Kalinchuk et al., 2015), were determined using Fast Fourier Transform. Both NRδ and Wθ were normalized to its own baseline 24 h mean values of NRδ and Wθ, respectively. To determine the relationship between the increases in Wθ with increasing duration of wakefulness, we performed correlation analysis between the time awake within each hour and the Wθ values for the same hour. Furthermore, to examine the relationship between the two homeostatic markers, we performed an hour-to hour analysis between previous hour Wθ and following hour NRδ.

Adenosine perfusion

Microdialysis probes (CMA 7, membrane length and outer diameter 1 mm and 0.24 mm, respectively, CMA Microdialysis, Solna, Sweden) were inserted into the guide cannula targeting BF and were connected to the programmable microinjection pump (CMA/100, CMA microdialysis), 12 h before the perfusion experiment. Decreased adenosine tone in ENT1 mice has been reported using an indrect measurement of a reduction in A1 receptor-mediated inhibition of glutamate excitatory postsynaptic currents (EPSCs) (Choi et al., 2004; Nam et al., 2013). To determine if the decreased $[AD]_{ex}$ levels underlie the differences in Wθ–NRδ relationship during the light period in ENT1KO mice, we perfused either artificial cerebrospinal fluid (aCSF, control) or 100 μM AD into the BF. Previous studies showed that a 2–3-fold increase in $[AD]_{ex}$ during SD resulted in 50% increase in time spent in NREM sleep during the recovery period (Porkka-Heiskanen et al., 1997; Basheer et al., 1999; Kalinchuk et al., 2006a,b). Similar (50%) increase in NREM sleep was also observed following reverse microdialysis of 300 μM AD into BF (Porkka-Heiskanen et al., 1997; Basheer et al., 1999; Kalinchuk et al., 2008). Our criteria