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ARTICLESleep duration varies as a function of glutamate  
and GABA in rat pontine reticular formation

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*Department of Anesthesiology, University of Michigan, Ann Arbor, Michigan, USA***Abstract**

The oral part of the pontine reticular formation (PnO) is a component of the ascending reticular activating system and plays a role in the regulation of sleep and wakefulness. The PnO receives glutamatergic and GABAergic projections from many brain regions that regulate behavioral state. Indirect, pharmacological evidence has suggested that glutamatergic and GABAergic signaling within the PnO alters traits that characterize wakefulness and sleep. No previous studies have simultaneously measured endogenous glutamate and GABA from rat PnO in relation to sleep and wakefulness. The present study utilized *in vivo* microdialysis coupled on-line to capillary electrophoresis with laser-induced fluorescence to test the hypothesis that concentrations of glutamate and

GABA in the PnO vary across the sleep/wake cycle. Concentrations of glutamate and GABA were significantly higher during wakefulness than during non-rapid eye movement sleep and rapid eye movement sleep. Regression analysis revealed that decreases in glutamate and GABA accounted for a significant portion of the variance in the duration of non-rapid eye movement sleep and rapid eye movement sleep episodes. These data provide novel support for the hypothesis that endogenous glutamate and GABA in the PnO contribute to the regulation of sleep duration.

**Keywords:** capillary electrophoresis, *in vivo* microdialysis, NREM sleep, REM sleep.

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Glutamatergic signaling contributes to the regulation of sleep and wakefulness. In numerous brain regions, levels of glutamate are highest during wakefulness or rapid eye movement (REM) sleep (Azuma *et al.* 1996; Kodama *et al.* 1998; Kodama and Honda 1999; Léna *et al.* 2005; Lopez-Rodriguez *et al.* 2007; John *et al.* 2008; Dash *et al.* 2009). One exception is the thalamus, where levels of glutamate are highest during non-REM (NREM) sleep (Kekesi *et al.* 1997). The effects of glutamate receptor agonists on traits of sleep and wakefulness are dependent on glutamate receptor subtype (Lai and Siegel 1991; Datta *et al.* 2002), agonist concentration (Datta *et al.* 2001a,b), and brain region (Alam and Mallick 1994; Cape and Jones 2000; Boissard *et al.* 2002; Wigren *et al.* 2007; Pal and Mallick 2009). The oral part of the pontine reticular formation (PnO) is a component of the ascending reticular activating system and plays a role in sleep cycle control (Brown *et al.* 2010). The PnO receives glutamatergic projections from the amygdala (Fung *et al.* 2011), the laterodorsal tegmental and pedunculopontine tegmental nuclei (Lai *et al.* 1993), and data from immunohistochemical studies suggest that the PnO contains glutamatergic neurons (Kaneko *et al.* 1989). Administration of glutamate receptor agonists into the PnO excites PnO

neurons in cat (Greene and Carpenter 1985) and rat (Stevens *et al.* 1992), indicating that functional glutamatergic signaling occurs within the PnO.

GABAergic signaling also regulates sleep and wakefulness. Dependent upon brain region, levels of endogenous GABA are highest during REM sleep (Nitz and Siegel 1997a,b), NREM sleep (Nitz and Siegel 1996; Kekesi *et al.* 1997), or wakefulness (Kekesi *et al.* 1997; Vanini *et al.* 2011). GABAergic signaling also causes an increase in wakefulness (Mallick *et al.* 2001) or REM sleep (Nitz and Siegel 1997a; Mallick *et al.* 2001; Vanini *et al.* 2007; Pal and Mallick 2009) in a brain-region dependent manner. Within the PnO, GABA promotes wakefulness and inhibits REM

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**Abbreviations used:** CE, capillary electrophoresis; EMG, electromyogram; LIF, laser-induced fluorescence; NREM, non-rapid eye movement; PnO, pontine reticular formation, oral part; REM, rapid eye movement.

sleep (Camacho-Arroyo *et al.* 1991; Xi *et al.* 1999; Sanford *et al.* 2003; Marks *et al.* 2008; Watson *et al.* 2008; Flint *et al.* 2010). GABAergic projection neurons from the thalamus, hypothalamus, basal forebrain, and ventral lateral periaqueductal gray terminate in the PnO (Boissard *et al.* 2003; Rodrigo-Angulo *et al.* 2008; Sapin *et al.* 2009). Each of these brain regions regulates sleep and wakefulness. The rostral brainstem also contains a column of GABAergic neurons that connects both sides of the PnO, and these neurons may modulate sleep and wakefulness (Liang and Marks 2009).

Measuring levels of glutamate and GABA across states of sleep and wakefulness presents an analytical challenge because of the short duration of rodent sleep episodes and analytical methods that require relatively large sample volumes (3–10  $\mu$ L). To overcome these limitations, the present experiments took advantage of capillary electrophoresis with laser-induced fluorescence detection (CE-LIF), which has the capacity to rapidly measure analytes from small sample volumes. With the use of *in vivo* microdialysis coupled on-line to CE-LIF, the present study measured concentrations of glutamate, GABA, aspartate, taurine, serine, and glycine. These measures were used to test the hypothesis that concentrations of glutamate and GABA in rat PnO vary significantly across states of wakefulness, NREM sleep, and REM sleep. Concentrations of aspartate, taurine, serine, and glycine were not predicted to show state specific changes, as there is no existing evidence that any of these amino acids functions in the pontine reticular formation to modulate sleep and wakefulness.

## Experimental procedures

### Chemicals, solutions, and materials

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride, potassium chloride, and calcium chloride were purchased from Fisher Scientific (Pittsburgh, PA, USA). All solutions were made with 18.2 M $\Omega$  MilliQ water (Millipore Corporation, Billerica, MA, USA). Ringer's solution consisted of 147 mM NaCl, 1.22 mM CaCl<sub>2</sub>, 2.68 mM KCl, and 1.01 mM MgSO<sub>4</sub> at pH 6.0. Electrophoresis buffer contained 10 mM sodium tetraborate and 0.9 mM hydroxypropyl- $\beta$ -cyclodextrin (0.8 molar substitution) at pH 9.5. Derivatization solution was comprised of 42.6 mM *o*-phthalaldehyde, 148.6 mM  $\beta$ -mercaptoethanol, 9 mM sodium tetraborate, 0.81 mM hydroxypropyl- $\beta$ -cyclodextrin and 10% (vol/vol) methanol at pH 9.5. Fused silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA).

### Animals, surgery, and conditioning

All experiments were approved by the University of Michigan Committee on Use and Care of Animals and adhered to guidelines established by the Guide for the Care and Use of Laboratory Animals, Eighth Edition (National Academies Press, Washington, DC, 2011). Male, adult, Crl:CD\*(SD) (Sprague–Dawley) rats

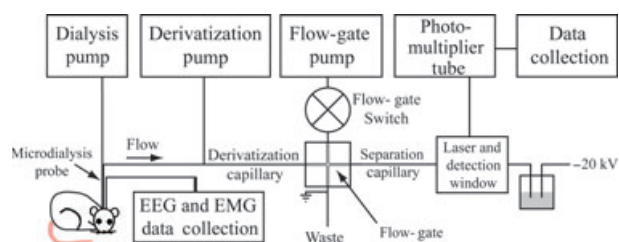
weighing 235–250 g were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in a temperature and humidity controlled environment. Rats were kept on a 12 h light/dark cycle (lights on at 0600), had *ad libitum* access to food and water, and were allowed a minimum of 1 week for adjustment to the housing facilities before undergoing any procedures.

Detailed descriptions of surgical methods for implanting a guide tube for microdialysis and electrodes for recording the cortical EEG and electromyogram (EMG) have been published (Watson *et al.* 2007, 2008; Brevig *et al.* 2010). Briefly, isoflurane (2.5% for induction; 1.5–2.0% for maintenance; delivered in 100% O<sub>2</sub>) was used to anesthetize rats during surgery. Three electrodes for recording the cortical EEG were positioned above the primary and secondary motor cortices. Two electrodes for recording the EMG were inserted into dorsal neck muscles, and one indifferent electrode was placed between the skin and muscle layers above the spine. Three anchor screws were implanted into the skull to hold the head cap in place. A guide tube (8IC312GP0XXC; Plastics One Inc., Roanoke, VA, USA) containing a stylet (8IC312DCXXC, Plastics One, Inc.) was aimed (Paxinos and Watson 2007) 1 mm above the PnO (from bregma: 8.4 mm posterior, 1.2 mm lateral, 8.4 mm ventral). The EEG and EMG electrode leads, electrode pedestal, and guide tube were cemented in place with dental acrylic. Rats were given 3 days to recover from surgery and an additional 2 weeks for conditioning before being used for experiments. During the recovery period, rats were conditioned to handling, being housed in a Ratum experiment cage (Bioanalytical Systems Inc, West Lafayette, IN, USA), and being tethered to the six-channel cable (used for recording EEG and EMG signals) for at least 8 h/day. This conditioning paradigm has been successfully used previously (Watson *et al.* 2007, 2008; Brevig *et al.* 2010). For each experiment, rats were placed in the Ratum bowl the afternoon before the start of the experiment.

### *In vivo* microdialysis and CE-LIF

Microdialysis probes were constructed according to a previously described method (Pettit and Justice 1989). Briefly, two lengths of fused silica capillary were offset by 1 mm, bound together with a small drop of cyanoacrylate, and fed through a guide tube connector (8IC313ICPNIT, Plastics One Inc.). The tips of the capillaries were then encased in a semi-permeable membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) that was plugged on one end with polyimide resin (Alltech Associates, Deerfield, IL, USA). The regenerated cellulose membrane had a molecular weight cut-off of 18 kDa. The membrane was bonded to the capillaries and all non-active areas of the microdialysis probe (from tip to connector) were coated with polyimide resin. The final maximum dimensions of each dialysis probe were 1 mm active length with an outer diameter of 240  $\mu$ m. Once screwed into position on the guide tube, the microdialysis membrane extended 1 mm below the bottom of the guide tube. The microdialysis probe was perfused at a flow rate of 0.4  $\mu$ L/min for all experiments.

Coupling *in vivo* microdialysis on-line to CE-LIF has been described previously (Lada *et al.* 1997; Bowser and Kennedy 2001; Smith *et al.* 2004) and is schematized in Fig. 1. Briefly, the outlet of the microdialysis probe was connected on-line to a CE-LIF system. Derivatization solution and dialysate were mixed in a 1 : 2.5 ratio. Derivatized dialysate was then injected electrokinetically onto the



**Fig. 1** Schematic of the capillary electrophoresis (CE) instrument. Drawing on the lower left indicates placement of a microdialysis probe in the brain of an awake rat. Dialysate from the probe flows through the derivatization capillary into a flow-gate interface and then is injected onto the separation capillary. Analytes migrate along the separation capillary and through a detection window, where a laser beam induces fluorescence.

separation capillary via the flow-gate interface. As the analytes migrated along the separation capillary, they passed through a transparent window of the capillary. A laser was focused through this window to induce fluorescence. Emitted fluorescence was collected by a photo-multiplier tube. The current from the photo-multiplier tube was amplified and then collected by software written using LabVIEW™ 8.0 (National Instruments, Austin, TX, USA). Concentrations of amino acids were quantified using the Cutter analysis program (Shackman *et al.* 2004).

#### Recording and analysis of sleep and wakefulness

EEG and EMG electrodes were connected to a Model 1700 differential AC amplifier (A-M Systems Inc., Sequim, WA, USA) via a six-channel cable. The amplified signal was then sent to a Micro 1401 mkII data acquisition unit [Cambridge Electronic Design Ltd. (CED), Cambridge, UK] for signal processing and storage (on a personal computer) by Spike2 version 5.14 software (CED). EEG and EMG signals were analyzed in 10-s bins for classification as wakefulness, NREM sleep, or REM sleep. Wakefulness displayed low amplitude, mixed frequencies in the EEG and moderate to high muscle tone in the EMG. NREM sleep was characterized by the presence of delta waves (0.5–4.0 Hz) or spindles (8.5–14.0 Hz) in the EEG and a decrease (compared to wakefulness) in the amplitude of the EMG. REM sleep was characterized by a predominant peak in the theta range (4.5–8.0 Hz) of the EEG and a further reduction of EMG amplitude. Two investigators, one of whom was blinded to the experimental conditions, scored every 10-s bin of each recording as wakefulness, NREM sleep, or REM sleep. A minimum of 98% agreement between the two investigators was achieved for each sleep record.

#### Experiments

At the beginning of each experiment, the computer collecting data from the CE-LIF system and the computer collecting data from the EEG and EMG recording system were synchronized to a National Institute of Standards and Technology Internet time server to ensure correct time alignment between the data files. The time for the dialysate to travel from the microdialysis membrane to the detection window was calculated prior to each experiment. The mean  $\pm$  SEM travel time was 8 min 40 s  $\pm$  56 s. For each experiment, a microdialysis probe was inserted into the implanted guide tube

2 h before the start of sample collection. This allowed adequate time for concentrations of amino acids to stabilize. This pre-experiment insertion time was determined by preliminary experiments (data not shown) and conformed to or exceeded pre-experiment insertion times previously published for measures of glutamate and GABA (Smith *et al.* 2004; Watson *et al.* 2007, 2008; Ferrario *et al.* 2008). Sample collection began between 1400 and 1600. At the end of each experiment, the rat was placed in its home cage and returned to its homeroom. Rats ( $n = 10$ ) were allowed to sleep *ad libitum* prior to and during the experiment.

#### Histological verification of microdialysis sites

Each rat was used for one experiment. At least 3 days after the experiment, rats were deeply anesthetized and decapitated. Brains were quickly removed, frozen, and placed in a Leica model CM3050S cryostat (Leica Microsystems, Nussloch, Germany). Serial, coronal sections (40  $\mu$ m) were mounted on chrom-alum coated slides, fixed with hot paraformaldehyde vapor, and stained with cresyl violet. All sections containing the microdialysis site were digitized and compared to a stereotaxic atlas of the rat brain (Paxinos and Watson 2007) for determining the three-dimensional coordinates (in mm) of the dialysis site. Only experiments in which the microdialysis membrane was localized to the PnO were included in the data analysis.

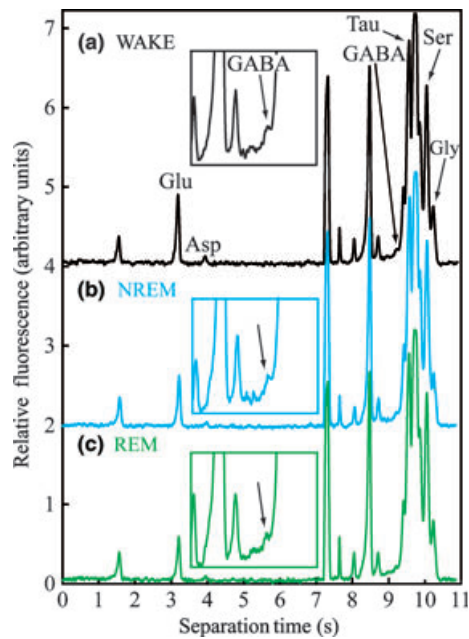
#### Statistics

Statistical analyses were performed in consultation with the University of Michigan Center for Statistical Consultation and Research. Descriptive and inferential statistics were performed using GB-Stat™ PPC 6.5.6 (Dynamic Microsystems Inc., Silver Springs, MD, USA) and SAS version 9.2 (SAS Institute, Cary, NC, USA). Neurochemical data were analyzed using a linear mixed-model, repeated-measures ANOVA followed by Tukey-Kramer multiple comparisons tests. Data comparing episode duration for wakefulness, NREM sleep, and REM sleep to the duration of the decrease in amino acid concentration were analyzed using simple regression ANOVA.

## Results

#### Concentrations of glutamate and GABA decreased significantly during sleep

A total of 4672 electropherograms were collected for this study. The conservative statistical approach constrained the degrees of freedom to the total number of rats used ( $n = 10$ ). Electropherograms (Fig. 2) were obtained during polygraphically identified states of wakefulness, NREM sleep, and REM sleep (Fig. 3a). During every experiment, rats cycled spontaneously between these three behavioral states (Fig. 3b). The mean  $\pm$  SEM percent of recording time spent in each state for all rats was 54.2  $\pm$  3.7 for wakefulness, 38.1  $\pm$  3.1 for NREM sleep, and 7.7  $\pm$  1.8 for REM sleep. Histological analysis (Fig. 4) confirmed that samples of all analytes were collected from the PnO. The mean  $\pm$  SEM stereotaxic coordinates for all 10 dialysis sites were 8.2  $\pm$  0.1 mm posterior to bregma, 1.3  $\pm$  0.1 mm lateral to the midline, and 8.9  $\pm$  0.1 mm ventral to the skull surface.

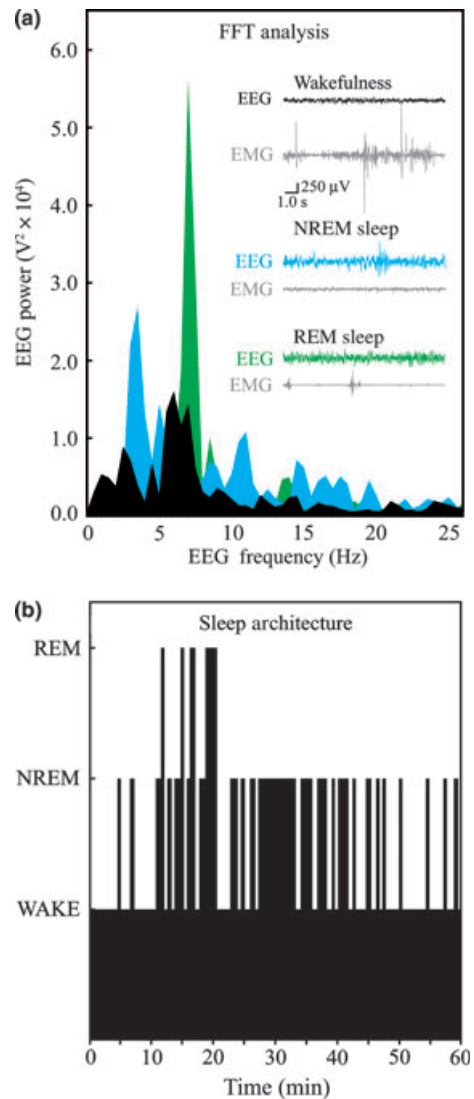


**Fig. 2** Electropherograms collected from the same rat during episodes of (a) wakefulness (WAKE), (b) NREM sleep, and (c) REM sleep. Glutamate (Glu), aspartate (Asp), GABA, taurine (Tau), serine (Ser), and glycine (Gly) were separated and detected within 11 s of sample injection. The inset over each electropherogram magnifies the GABA peak (arrow).

Figure 5 summarizes the group data, based on 2447 amino acid measures obtained during wakefulness, 1963 measures collected during NREM sleep, and 262 measures taken during REM sleep. The mean  $\pm$  SEM dialysate concentrations ( $\mu\text{M}$ ) of each amino acid during wakefulness were: glutamate =  $1.948 \pm 0.018$ , aspartate =  $0.522 \pm 0.003$ , GABA =  $0.428 \pm 0.003$ , taurine =  $7.442 \pm 0.055$ , serine =  $10.665 \pm 0.083$ , and glycine =  $4.942 \pm 0.046$ . Repeated-measures ANOVA revealed significant changes in the concentration of glutamate (Fig. 5a;  $F = 31.2$ ;  $df = 2, 10$ ;  $p < 0.0001$ ) and GABA (Fig. 5b;  $F = 15.27$ ;  $df = 2, 10$ ;  $p = 0.0009$ ) across the sleep/wake cycle. As predicted, concentrations of aspartate, taurine, serine, and glycine did not vary significantly across states.

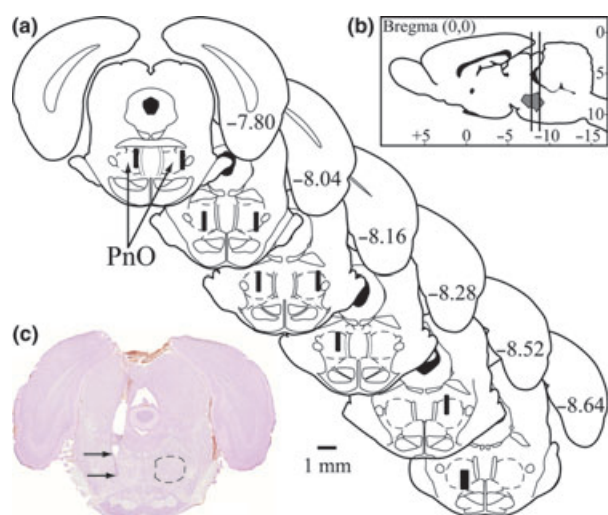
#### Duration of sleep episodes varied as a function of the decrease in the concentrations of glutamate and GABA

To determine if decreases in concentrations of glutamate and GABA accounted for the duration of behavioral state episodes, the duration of wakefulness, NREM sleep, and REM sleep episodes was plotted versus the duration of the decrease in glutamate or GABA concentrations (Fig. 6). Points on the line of identity ( $y = x$ ) indicate episodes in which glutamate or GABA concentrations were decreased during the entire episode. Points on the  $y$ -axis ( $x = 0$ )



**Fig. 3** Electrographic identification of sleep and wakefulness. (a) Representative 10-s recordings of cortical EEG and electromyogram (EMG) as well as a fast Fourier Transform of the respective EEG signals are shown for wakefulness (black), NREM sleep (blue), and REM sleep (green). (b) A time-course plot of sleep architecture during a typical experiment. The height of the black bars indicates the behavioral states of wakefulness (lowest bars), NREM sleep (intermediate height bars), or REM sleep (highest bars). Data shown in (a) and (b) were obtained from the same rat.

indicate that the concentrations of glutamate or GABA did not decrease at any time during the state episode. Points falling between the  $y$ -axis and the line of identity indicate episodes during which glutamate or GABA decreased for part of the episode, but not for the entire duration of the episode. For each plot shown in Fig. 6, the duration of the decrease in glutamate or GABA accounted for a significant ( $p < 0.0001$ ) amount of variance in the duration of the behavioral state.



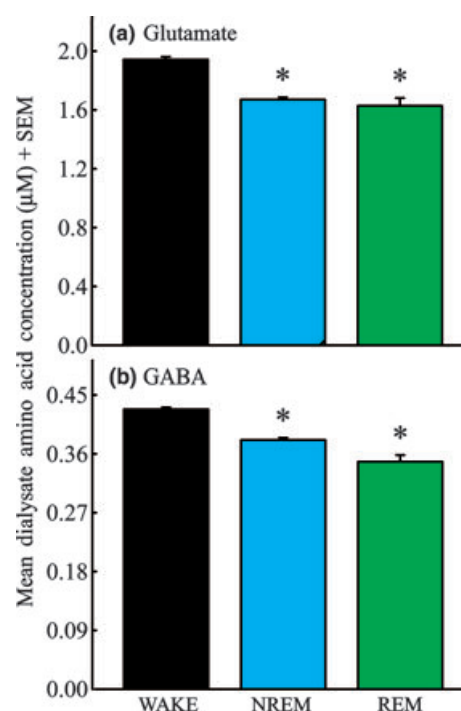
**Fig. 4** Collection of analytes was localized to the oral part of the pontine reticular formation (PnO). (a) Schematic drawings of rat brainstem show the location of each microdialysis membrane ( $n = 10$ ) as a black cylinder (drawn to scale). (b) A sagittal schematic of the rat brain has vertical lines to indicate the anterior-to-posterior span of the microdialysis sites shown in part (a). The shaded area between the vertical lines indicates the boundaries of the PnO. The schematics were modified from a rat brain atlas (Paxinos and Watson 2007). (c) A typical, cresyl violet-stained section with the upper arrow pointing to the location of the bottom of the guide tube and the lower arrow pointing to the deepest part of the microdialysis site in the PnO. Dotted line on the right side indicates the boundary of the PnO, according to Paxinos and Watson (2007).

#### Episode ratios for glutamate and GABA decreased below waking levels during NREM sleep and REM sleep

Episode ratio was defined as the number of episodes with concentrations of amino acid greater than the average concentration during wakefulness divided by the number of episodes with concentrations of amino acid less than the average concentration during wakefulness. Figure 7 plots the episode ratio of glutamate and GABA for wakefulness, NREM sleep, and REM sleep. Ratio values greater than 1.0 indicate that more than half of the episodes were associated with concentrations of glutamate and GABA above 100% of control (average concentrations during wakefulness). NREM sleep and REM sleep showed a predominance of episodes characterized by concentrations of glutamate and GABA below 100%, as evidenced by ratio values at or below 0.6. For glutamate, the episode ratio decreased by 58% during NREM sleep and 67% during REM sleep compared to wakefulness. The episode ratios for GABA during NREM sleep and REM sleep both decreased by 50% compared to wakefulness.

#### Discussion

*In vivo* microdialysis coupled on-line to CE-LIF was used to demonstrate that concentrations of glutamate and GABA in

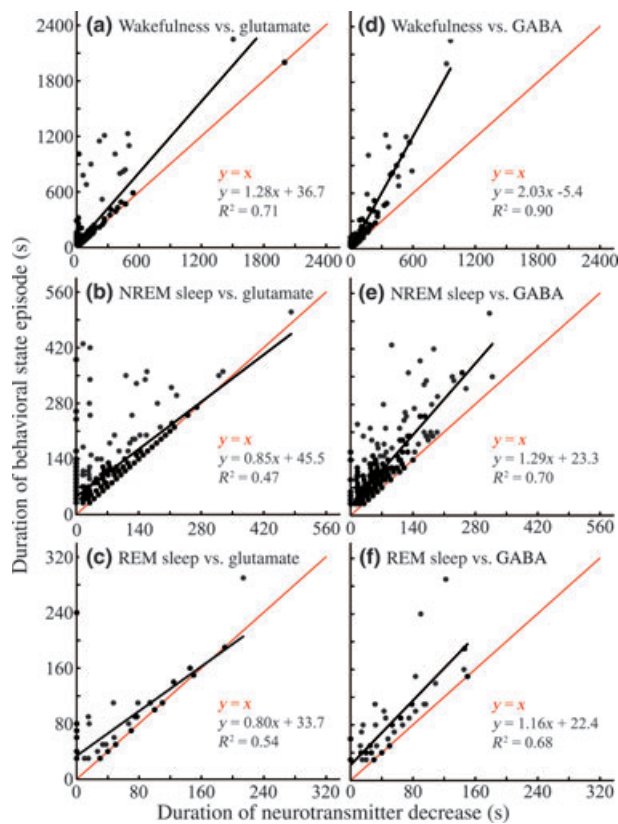


**Fig. 5** Concentrations of glutamate and GABA decreased significantly below waking levels during sleep. Asterisks indicate a significant ( $p < 0.05$ ) decrease compared to wakefulness. (a) The concentration of glutamate significantly decreased by 14.2% during NREM sleep and by 16.4% during REM sleep. (b) The concentration of GABA decreased significantly during NREM sleep (–11.0%) and REM sleep (–18.7%). There was no significant difference in concentrations of glutamate or GABA between NREM sleep and REM sleep.

the PnO were greater during wakefulness than during NREM sleep and REM sleep (Fig. 5). A significant amount of the variance in the duration of episodes for each state was accounted for by the duration of the decrease in glutamate and GABA (Fig. 6). Episode ratios for NREM sleep and REM sleep (Fig. 7) revealed that most sleep episodes were associated with decreases in concentrations of glutamate and GABA. The significance of these findings is discussed below.

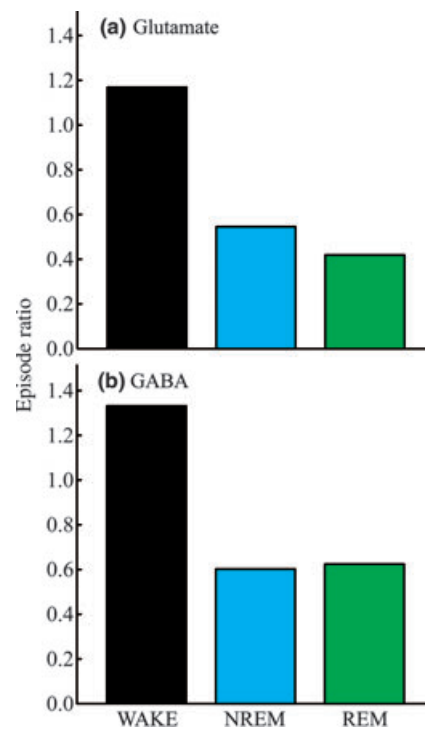
#### State-specific concentrations of GABA and glutamate

Studies using cat have shown that state-specific changes in GABA concentrations are brain-region specific. In locus coeruleus (Nitz and Siegel 1997b) and dorsal raphe nucleus (Nitz and Siegel 1997a), concentrations of GABA are significantly higher during REM sleep than during NREM sleep or wakefulness. In the posterior hypothalamus (Nitz and Siegel 1996) and thalamus (Kekesi *et al.* 1997), however, concentrations of GABA are highest in NREM sleep. In the thalamus (Kekesi *et al.* 1997) concentrations of GABA are also significantly lower during REM sleep than during wakefulness.



**Fig. 6** Duration of behavioral state episode varied as a function of duration of the decrease in concentrations of glutamate and GABA. Each plot indicates that for both glutamate (a–c) and GABA (d–f), the lower-level phenotype of duration of amino acid decrease (abscissa) accounted for a significant amount of the variance in the higher-level phenotype of behavioral state duration (ordinate). Black line plots  $y$  as a function of  $x$ ; red line plots the line of identity. (b, c, f) Slopes of the black line near or less than the slope of the line of identity (red line) indicate that the longer the decrease in amino acid concentration, the longer the duration of NREM sleep (b) or REM sleep (c and f).

The present study revealed for the first time in rat that the concentration of GABA in the PnO is significantly decreased during both NREM sleep and REM sleep compared to concentrations measured during wakefulness (Fig. 5). Similarly, in the pontine reticular formation of cat, the concentration of GABA is significantly decreased below waking levels during both REM sleep (Vanini *et al.* 2011) and the loss of wakefulness caused by the general anesthetic isoflurane (Vanini *et al.* 2008). There are species differences between cat and rat with respect to NREM sleep-specific changes in GABA. The concentration of GABA in cat pontine reticular formation is not significantly different between wakefulness and NREM sleep (Vanini *et al.* 2011), whereas the present study revealed that the concentration of GABA in rat PnO is significantly lower during NREM sleep than during wakefulness (Fig. 5b). The reasons for these species differences are not understood. Different sleep responses to administering



**Fig. 7** Episode ratios of glutamate and GABA decreased during sleep. Each plot indicates the state-specific episode ratio of amino acid concentrations during wakefulness (WAKE), NREM sleep, and REM sleep for glutamate (a) and GABA (b).

GABA<sub>A</sub> receptor antagonists (Xi *et al.* 1999; Sanford *et al.* 2003) and muscarinic cholinergic receptor agonists (Lydic and Baghdoyan 2008) into the pontine reticular formation of rat and cat are well documented.

The present findings are consistent with reports demonstrating that changing GABAergic signaling within rat PnO alters states of sleep and wakefulness. Increasing endogenous GABA by blocking GABA uptake with nipecotic acid causes a significant increase in wakefulness and a significant decrease in NREM sleep and REM sleep (Watson *et al.* 2008). Decreasing GABA levels in the PnO by selectively inhibiting GABA synthesis with 3-mercaptopropionic acid significantly decreases wakefulness and significantly increases NREM sleep and REM sleep (Watson *et al.* 2008). These GABA-induced alterations in sleep and wakefulness likely occur via the GABA<sub>A</sub> receptor. Microinjecting muscimol, a GABA<sub>A</sub> receptor agonist, into the pontine reticular formation of mouse (Flint *et al.* 2010), rat (Camacho-Arroyo *et al.* 1991), and cat (Xi *et al.* 1999) increases wakefulness. Furthermore, microinjection of bicuculline, a GABA<sub>A</sub> receptor antagonist, into the pontine reticular formation increases REM sleep (Camacho-Arroyo *et al.* 1991; Xi *et al.* 1999; Sanford *et al.* 2003; Marks *et al.* 2008; Flint *et al.* 2010). These data support the interpretation that endogenous GABA within the PnO promotes wakefulness.

The present results demonstrate for the first time that the concentration of glutamate in the PnO changes in a state-specific manner (Figs 5, 6, and 7). Previous reports measuring glutamate by HPLC have shown no state-specific changes in the concentration of glutamate within the pontine reticular formation (Kodama *et al.* 1998; Hasegawa *et al.* 2003). Using CE-LIF, the present study has shown that the concentration of glutamate within the PnO is significantly higher during wakefulness than during NREM sleep and REM sleep. Pharmacological data also indicate that glutamatergic transmission contributes to the regulation of sleep and wakefulness. Systemic administration of ketamine, an NMDA receptor antagonist, disrupts the normal sleep cycle of cat and creates a dissociated behavioral state (Lydic and Baghdoyan 2002).

Local administration of glutamate or glutamate receptor antagonists to the pontine reticular formation has been shown to alter certain traits (acetylcholine levels, respiratory rate, startle response, and REM sleep latency) that characterize states of sleep and wakefulness. Administration of NMDA receptor antagonists to the pontine reticular formation decreases acetylcholine release and breathing in cat (Lydic and Baghdoyan 2002), and acetylcholine release in cat pontine reticular formation is greatest during REM sleep (Leonard and Lydic 1997). In the caudal portion of rat pontine reticular formation,  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid/kainate and NMDA receptors attenuate the fear-potentiated or acoustic startle response (Krase *et al.* 1993; Fendt *et al.* 1996). Direct administration of glutamate to rat pedunclopontine tegmental nucleus also decreases startle response by exciting inhibitory pedunclopontine tegmental neurons that project to the caudal part of the pontine reticular formation (Simon *et al.* 2011). Microinjection of glutamate into cat pontine reticular formation shows a trend for decreasing REM sleep latency (Baghdoyan *et al.* 1989). Together, these data support the interpretation that glutamatergic signaling within sleep-regulating regions of the pons modulates behavioral state.

Microinjection of glutamate into the pontine peri-locus coeruleus  $\alpha$  or nucleus magnocellularis of decerebrate cat causes muscle atonia (Lai and Siegel 1988), suggesting that the concentration of endogenous glutamate in the PnO might increase during REM sleep. However,  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid/kainate receptor agonists suppress muscle tone and NMDA receptor agonists increase muscle tone when these drugs are administered directly into the pontine peri-locus coeruleus  $\alpha$  and nucleus magnocellularis of decerebrate cat (Lai and Siegel 1991). This finding suggests that within these brain regions, the effects of glutamate on muscle atonia are receptor-subtype specific, and does not support or oppose the present finding of a decrease in the concentration of endogenous glutamate within the PnO during NREM sleep and REM sleep. Furthermore, the dialysis sites in the present study are not homologous to the sites used in the glutamate microinjection studies (Lai and

Siegel 1988, 1991). The present study measured glutamate in an area ventral to the region in which glutamate caused atonia in the cat. Additional studies are needed to determine the synaptic mechanisms by which glutamatergic signaling within the PnO regulates sleep and wakefulness.

#### Comparison of methods for quantifying changes in amino acid concentrations across behavioral states

Biosensors (John *et al.* 2008; Dash *et al.* 2009) as well as *in vivo* microdialysis coupled to HPLC (Azuma *et al.* 1996; Kekesi *et al.* 1997; Kodama *et al.* 1998; Kodama and Honda 1999; Lopez-Rodriguez *et al.* 2007) or CE (Léna *et al.* 2005) have been used to measure state-specific changes in glutamate concentration. Changes in the concentration of GABA across states of wakefulness and sleep have been measured using HPLC (Nitz and Siegel 1996, 1997a,b; Kekesi *et al.* 1997; Vanini *et al.* 2011). The main advantage of using biosensors to monitor glutamate is temporal resolution. Glutamate biosensors have sampling rates ranging from 1 to 4 s (John *et al.* 2008; Dash *et al.* 2009). The main disadvantages of biosensors are that they are currently limited to monitoring a single analyte and biosensors are not available for every analyte of interest. HPLC offers the advantage of monitoring multiple analytes simultaneously, but has a disadvantage in the time domain. The mass detection limits associated with HPLC measurements require sampling times ranging from 1 to 10 min. In animal models with long-duration sleep episodes, such as cat, these sampling times are adequate (Nitz and Siegel 1996, 1997a,b; Kekesi *et al.* 1997; Kodama *et al.* 1998; Kodama and Honda 1999; Lopez-Rodriguez *et al.* 2007; Vanini *et al.* 2011). For animal models with short-duration sleep episodes, these sampling times are inadequate. Rodents have relatively short-duration (less than 1 min) episodes of NREM sleep and REM sleep. Thus, achieving adequate sample volumes involves either not distinguishing between NREM sleep and REM sleep (Azuma *et al.* 1996), mixing samples from multiple sleep episodes, or discarding samples comprised of less than 80% of one state. If microdialysis is coupled off-line to CE (Léna *et al.* 2005), the sample volume requirements (2  $\mu$ L) and sampling times (2 min) are similar to HPLC. However, if *in vivo* microdialysis is coupled on-line to CE-LIF, the advantages of HPLC (multiple analytes) and biosensors (fast sampling) are achieved. The present study used *in vivo* microdialysis coupled on-line with CE to measure changes in concentrations of amino acids across states of sleep and wakefulness. With the current CE system, analyte separation time was 11 s (Fig. 2). When sample loading and injection times are included, it was possible to simultaneously measure concentrations of six amino acids once every 15 s. Thus, sampling was performed on a time scale that approaches the time resolution of biosensors while the ability to quantify multiple analytes comprising each sample was retained.

## Limitations and conclusions

The strengths and limitations of microdialysis have been reviewed elsewhere (Watson *et al.* 2006), and include the inability to identify the cellular source(s) of glutamate and GABA. Sources can include neurons, glia, and cellular metabolism (van der Zeyden *et al.* 2008). GABAergic input to the PnO arises from multiple sources, including the hypothalamus (Rodrigo-Angulo *et al.* 2008), thalamus (Rodrigo-Angulo *et al.* 2008), basal forebrain (Rodrigo-Angulo *et al.* 2008), amygdala (Fung *et al.* 2011), contralateral PnO (Liang and Marks 2009), and ventral lateral periaqueductal gray (Boissard *et al.* 2003; Sapin *et al.* 2009). Signaling by glutamate and GABA includes synaptic and volume transmission (Del Arco *et al.* 2003), because both of these amino acids function as neurotransmitters and as gliotransmitters (Angulo *et al.* 2008; Halassa and Haydon 2010). Evidence suggests that glutamate and GABA released inside the synapse may not spill out into the extracellular space; instead, diffusion of synaptic glutamate and GABA may be tightly controlled by glia ensheathing the synapse (Del Arco *et al.* 2003). The organization and interaction between pre-synaptic, post-synaptic, and astrocytic membranes has led to the idea of the tripartite synapse (Del Arco *et al.* 2003). In this model, neurotransmitters that spill out of the synaptic cleft activate receptors on astrocytes. Astrocytes, in turn, release transmitters into the synapse to regulate synaptic signaling or into the extracellular space to regulate volume transmission (Del Arco *et al.* 2003). In addition to gliotransmission and neurotransmission, extrasynaptic glutamate and GABA may be derived from cellular metabolism. Because there are multiple sources of extrasynaptic glutamate and GABA, determining the source of glutamate or GABA measured by any technique is a challenge. The multiple sources of glutamate and GABA may help explain the findings (Fig. 6) that certain episodes of NREM sleep and REM sleep were associated with increases in glutamate or GABA.

In conclusion, although the sources of sampled glutamate and GABA are not known, the present data demonstrate that during normal sleep, glutamatergic and GABAergic tone in the pontine reticular formation decreases. Furthermore, these state-specific decreases were selective for GABA and glutamate, and did not occur for amino acids in the pontine reticular formation that are not thought to play a major role in the regulation of sleep and wakefulness, including taurine, serine, glycine, and aspartate. These data provide novel support for the hypothesis that endogenous glutamate and GABA in the PnO contribute to the regulation of sleep-episode duration.

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