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SHORT COMMUNICATION

Glial and light-dependent glutamate metabolism in the suprachiasmatic nuclei

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The suprachiasmatic nuclei, the main circadian clock in mammals, are entrained by light through glutamate released from retinal cells. Astrocytes are key players in glutamate metabolism but their role in the entrainment process is unknown. We studied the time dependence of glutamate uptake and glutamine synthetase (GS) activity finding diurnal oscillations in glutamate uptake (high levels during the light phase) and daily and circadian fluctuations in GS activity (higher during the light phase and the subjective day). These results show that glutamate-related astroglial processes exhibit diurnal and circadian variations, which could affect photic entrainment of the circadian system.

Keywords: Astrocytes, circadian, glutamate uptake, glutamine synthetase, SCN

INTRODUCTION

The central circadian clock in mammals is located in the hypothalamic suprachiasmatic nuclei (SCN) (Lehman et al., 1987; Moore & Eichler, 1972; Morin et al., 1989; Ralph et al., 1990; Sujino et al., 2003), which are strongly modulated and entrained by light (Aschoff, 1960; Pittendrigh, 1981). In the presence of light, a specific type of retinal ganglion cells (melanopsin-positive) release glutamate in the ventral area of the SCN *via* the retino-hypothalamic tract (RHT) (Golombek & Rosenstein, 2010). Within the SCN, astrocytes and neurons (Guldner, 1983) respond to glutamate leading to phase shifts and the entrainment process (Meijer et al., 1993).

All known glutamate receptors are present in the SCN including ionotropic-NMDA receptor (NMDAR), AMPA receptor (AMPAR), kainite receptor (KAR) and the metabotropic receptors (mGluRs) (Gannon & Rea, 1994). Light-induced phase shifts of circadian rhythms are blocked by glutamate antagonists *in vivo* and in hypothalamic brain preparations containing the SCN, while both glutamate and NMDA phase shift circadian rhythms and induce c-FOS expression in the nuclei (Abe et al., 1991, 1992). NMDA induces photic-like phase shifts when administered in the SCN both *in vivo* and *in vitro*, causing delays when applied during the early night and advances when applied in the late night. NMDAR is considered to be key players in the entrainment process (Colwell & Menaker, 1992), while mGluR was shown to modulate the SCN response (Haak, 1999; Haak et al., 2006; Meijer et al., 1988; Mintz et al., 1999; Shibata et al., 1994).

Astrocytes can release, take up and respond to glutamate (Kettenmann & Steinhäuser, 2005; Verkhratsky & Kirchhoff, 2007a,b; Volterra & Meldolesi, 2005); they play a critical (and almost exclusive) role in the clearance of extracellular glutamate through uptake and degradation (Schousboe & Waagepetersen, 2005). The glutamate released to the extracellular space is removed by astroglial cells *via* glutamate transporters (both GLAST and GLT-1) and subsequent conversion to glutamine through the activity of glutamine synthetase (GS, an enzyme which is absent in neurons) (Norenberg & Martinez-Hernandez, 1979). Glutamate transporter (GluT) is also expressed in the SCN (Spanagel et al., 2005) but no circadian rhythms of glutamate uptake were reported (Beaule et al., 2009). Astrocytes also exhibit molecular circadian rhythms *in vitro* that can be synchronized by serum shocks, temperature pulses and VIP (Marpegan et al., 2009, 2011; Prolo et al., 2005). Immunoreactivity to glial fibrillary acidic protein (GFAP), a structural protein of glial cells, exhibits daily rhythms within the SCN (Lavialle et al., 2001; Leone et al., 2006).

As the effects of light on the mammalian circadian system are achieved mostly through retina-derived

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glutamate release in the ventral SCN, we hypothesized that the presence of light would lead to high astrocytic glutamate metabolism, while darkness will lead to lower glutamate metabolism within the SCN, resulting in a daily rhythm with glutamate metabolism being high during the day and low during the night.

We tested if glutamate metabolism oscillates throughout the day in the rodent SCN and whether this variation is dependent on the light cycle. We found diurnal variations in both glutamate uptake and glutamine synthetase activity, suggesting that SCN astroglial cells can be key players in glutamate mechanisms involved in photic circadian entrainment.

MATERIALS AND METHODS

Animals

Adult (3–5 months old) and young (15 days) male mice (*Mus musculus*, C57Bl/6J background) were raised in our colony, housed under a 12:12-h light:dark photoperiod (LD, with light onset at 8 AM and lights off at 8 PM) with food and water *ad libitum*. Time is expressed as *zeitgeber* time (ZT), with ZT12 defined as the time of lights off. For constant dark conditions (DD), animals were kept 2 days in DD and circadian time 12 (the start of subjective night) was considered to be the same than the previous ZT. All animal experiments were carried out in accordance with the NIH Guide for the care and use of laboratory animals; and all experimental protocols were performed according to international ethical standards (Portaluppi et al., 2010).

Enucleation

Mice were anesthetized (ketamine/xylazine) and eyes were removed from their cavity as previously described by Lavialle et al. (2001). Animals were monitored under anesthesia and then returned to their cage for a 2-week recovery. Running-wheel activity was monitored to estimate culling time for the experiments.

Glutamate uptake in crude synaptosomal fractions

L-[^{3}H]-glutamate uptake was assessed in crude SCN synaptosomal preparations with minor modifications to the previously described protocol (Moreno et al., 2005). Animals were sacrificed at two different time points for each condition: midpoint of the day/subjective day (ZT7/CT7), midpoint of the night/subjective night (ZT19/CT19) and brains were quickly removed and placed in a glass dish on ice. Hypothalamic sections, 2 mm thick, were cut and the region containing the SCN was punched out. The influx of L-[^{3}H]-glutamate was assessed in a crude synaptosomal fraction of SCN punches. SCN tissue was homogenized in 0.32 M sucrose containing 1 mM $MgCl_2$ and centrifuged at 900 *g* for 10 min at 4 °C. Nuclei-free homogenates were further centrifuged at 30 000 *g* for 20 min. The pellet was immediately resuspended in HEPES-Tris buffer, containing 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose adjusted to pH 7.4 with Tris base. Samples were then incubated with L-[^{3}H]-glutamate (200 000–500 000 dpm/tube, specific activity 17.25 Ci/mmol) for 10 min at 37 °C. Uptake was stopped by addition of 4 ml of ice-cold HEPES-Tris buffer. The mixture was immediately poured onto Whatman GF/B filters under vacuum. The filters were washed twice with 4 ml aliquots of ice-cold buffer, and the radioactivity on the filters was counted in a liquid scintillation counter. Non-specific uptake into synaptosomes was assessed by adding an excess of glutamate (10 mM) (Moreno et al., 2005).

Glutamate uptake in the SCN

Glutamate uptake in the SCN was measured with minor modifications to a previously described protocol (Beaule et al., 2009). Young (15–25 day old) mice were anesthetized with CO_2 and decapitated at ZT10–11. Their brains were then dissected and glued to a vibroslicer plate submerged in cold oxygenated HBSS (Hank's Balanced Salt Solution, from Sigma). Slices (300 μm) containing the SCN were collected and 2 mm punches were obtained from the SCN area. These punches were placed on a square piece of Millicell-CM organotypic culture membrane placed on top of a Millicell insert (Millipore, 0.4 μm pore size, PICM0RG50). The insert was placed in a 35 mm sterile Petri dish (Falcon BD Biosciences, CA) containing 1 ml of DMEM (Dulbecco's Modified Eagle Medium, Sigma) containing 2% B27 supplement (50X, Life Technologies) and 2% L-Glutamine (Life Technologies), and kept in a cell culture incubator at 37 °C for 36 h before measurements. The explants with their Millicell membrane were transferred to a 48-well culture plate and incubated for 30 min at 37 °C in 300 μl of uptake buffer (5 mM Tris base, 10 mM HEPES, 140 mM NaCl, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM K_2HPO_4, 10 mM dextrose and 1 mM methionine sulfoximine) for 30 min. Methionine sulfoximine was added to inhibit glutamine synthetase activity. After pre-incubation, the buffer was replaced for uptake buffer supplemented with radioactive glutamate for 10 min at 37 °C (1 μM or 10 μM with 0.2 μCi L-[3H]-glutamate −55 Ci/mmol-, GE Healthcare, Pittsburgh, PA). To test for nonspecific glutamate uptake, 100 μM L-2,4-*trans*-PDC (L-*trans*-pyrrolidine-2,4-dicarboxylic acid, Tocris Bioscience, Ellisvill, MO) was added to the uptake buffer of some cultures to block high-affinity transporters. In all cultures, we terminated uptake with four 2-min buffer rinses (identical to uptake buffer except that NaCl was replaced by 140 mM LiCl) at 4 °C. We lysed the cells immediately after the fourth wash with 0.1 M NaOH and divided the lysate to measure glutamate levels (100 μl of cell lysate) and protein concentrations (30 μl cell lysate). Glutamate levels were measured by liquid scintillation counting (Wallac 1410 Liquid Scintillation Counter) by 3 ml of liquid scintillation cocktail (Ecoscint made by MP Biochemicals, Solon, OH). Protein concentration for each sample was measured in duplicate against a

Bovine Serum Albumin (BSA) standard using the Bradford Assay (Fisher Thermo Scientific, Waltham, MA).

Western blotting

To assess daily variations on GS expression, animals were sacrificed at each ZT (3, 9, 15, 21), brains were quickly removed on ice and placed at −80 °C. Hypothalamic sections 2 mm thick were cut and the SCN region was punched out and homogenized in 50 mM Tris/HCl buffer (pH 7.4), with 1% NP-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM NaF, a protease inhibitor cocktail (AEBSF, E-64, bestatin, aprotinin and leupeptin) and 1 mM sodium ortovanadate (all drugs from Sigma-Aldrich, St. Louis, MO). SCN protein blots (30 µg run in 9% SDS-PAGE gels and transferred to Hybond nitrocellulose membranes from Amersham Bioscience, Piscataway, NJ) were incubated for 12 h at 4 °C with specific mouse antibodies anti-GS (BD Transduction Laboratories, 1/2500 in Tris-HCl 20 mM buffer with 0.1% Tween-20 – TTBS). Immunoreactivity was assessed using a secondary antibody coupled to horseradish peroxidase and visualized with the ECL kit (Amersham). Blots were stripped (according to Amersham Bioscience's protocol) and re-incubated with rabbit antibody anti-actin (Sigma, diluted 1/2000 in TTBS, incubated for 12 h at 4 °C). Densitometry of immunoreactive bands was analyzed by means of one-way ANOVA followed by Tukey's test.

GS activity measurement

SCN tissue was obtained as described for glutamate uptake in synaptosomes. Each SCN was homogenized in 75 µl of 10 mM potassium phosphate, pH 7.2. Glutamine synthetase activity was measured as previously described (Moreno et al., 2005) with minor modifications. Reaction mixtures contained 50 µl of SCN homogenates and 50 µl of a stock solution (100 mM imidazole-HCl buffer, 40 mM $MgCl_2$, 50 mM β-mercaptoethanol, 20 mM ATP, 100 mM glutamate and 200 mM hydroxylamine, adjusted to pH 7.2). Tubes were incubated for 15 min at 37 °C. The reaction was stopped by adding 200 µl of ferric chloride reagent (0.37 M $FeCl_3$, 0.67 M HCl and 0.20 M trichloroacetic acid). Samples were placed for 5 min on ice. Precipitated proteins were removed by centrifugation, and the absorbance of the supernatants was read at 535 nm against a blank. Under these conditions, 1 µmol of γ-glutamyl hydroxamic acid gives an absorbance of 0.340. Glutamine synthetase-specific activity was expressed as µmoles of γ-glutamyl hydroxamate per hour per milligram of protein (Moreno et al., 2005).

Statistical analysis

According to the experimental designs, data were analyzed using Student's *t* test, one-way ANOVA and two-way ANOVA, as specified in the Results section.

RESULTS

Glutamate uptake exhibits daily but not circadian variations in mouse SCN

Glutamate uptake was measured in SCN crude synaptosomal extracts from mice at two different time points in LD and DD conditions (Figure 1A). In LD, glutamate uptake was compared between ZT7 (daytime) and ZT19 (night time) and, in DD between the resting phase at CT7 (subjective day) and active phase at CT19 (subjective night). We found higher glutamate uptake during the light phase (Student's *t* test, $p<0.05$) than the dark phase in LD and no differences between the subjective night and subjective day in DD (Student's *t* test, $p>0.05$). This daily rhythm indicates that glutamate uptake in the SCN is influenced by the presence of a light/dark cycle.

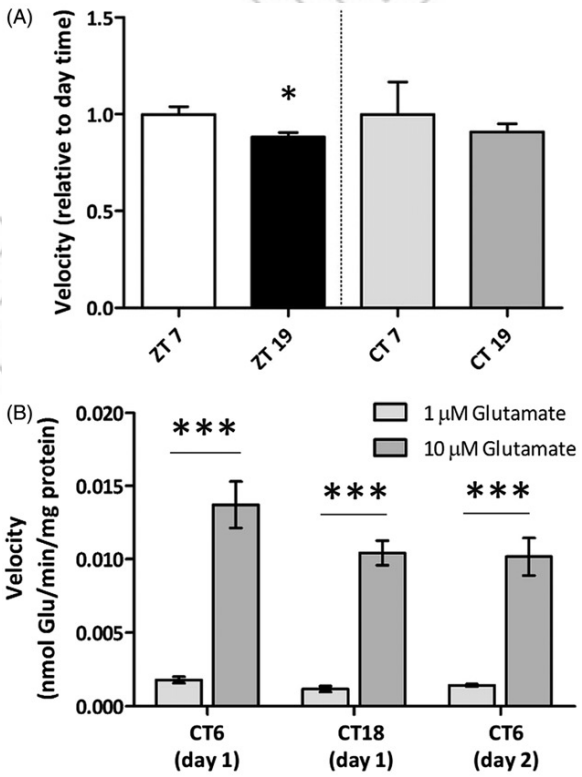

FIGURE 1. Glutamate uptake in the SCN. (A) Glutamate uptake was measured in SCN synaptosomal crude extracts from animals in LD and DD. Uptake velocity was higher during the day in LD ($n=3$ SCN per time point; two-tail Student's *t* test, $^*p<0.05$) but did not vary significantly in DD ($n=3$ SCN per time point; two-tail Student's *t* test, $p>0.05$). (B) Glutamate uptake was not circadian in SCN slices. Glutamate uptake was reliably about 10 times higher for 10 µM glutamate than 1 µM glutamate, but did not differ with circadian time at either concentration ($n=3$–4 SCN per time point). Two-way ANOVA showed a significant main effect of glutamate concentration ($p<0.01$) and no effect for circadian time ($p>0.05$), with no significant interaction ($p>0.05$). *Post hoc* comparisons using the Bonferroni test revealed that glutamate uptake velocity was significantly higher for 10 µM glutamate than 1 µM glutamate for each CT ($^{***}p<0.001$). Data are plotted as mean ± SEM.

The glutamate uptake assay was specific and reflected glial-dependent activity. Glutamate uptake in SCN explants was dose-dependent with significantly increased uptake at higher glutamate concentrations and was significantly inhibited in the presence of 100 μM *trans*-PDC (a high-affinity glutamate transporter inhibitor) (Supplementary Figure 1). However, glutamate uptake did not change under constant dark conditions at any of the tested doses (two-ways ANOVA followed by a Bonferroni *post-hoc* test, $p<0.01$ for glutamate concentration, $p>0.05$ for circadian time) (Figure 1B).

Glutamine synthetase activity exhibits daily and circadian rhythms in the mouse SCN

We observed strong constitutive GS expression in protein extracts from mouse SCN at all time-points (Figure 2A, one-way ANOVA, $p>0.05$). However, we found significant day–night variations for GS activity under both LD and DD (Figure 2B). Higher levels of GS activity were found during the day or subjective day under LD and DD, respectively (Student's *t* test, $^*p<0.05$).

To test if the variations of GS activity were locally controlled within the SCN or a consequence of retinal input, we evaluated GS activity in extracts from enucleated mice. We found a variation in GS activity, but contrary to the results for LD and DD, we observed a higher GS activity during the subjective night as compared to the subjective day (Student's *t* test, $^*p<0.05$). We conclude that SCN GS activity is elevated during the day by the circadian clock and modulated by the eye.

DISCUSSION

Our results indicate that astroglial glutamate metabolism in the mouse SCN is modulated along the day, depending on input from the eyes. Specifically, we find that glutamate uptake in the SCN is enhanced by daytime light and degraded by enhanced daytime GS activity. Input from the eyes reduces GS activity, especially at night. Moreover, the circadian clock modulates GS activity, but not glutamate uptake.

The high levels of glutamate uptake by SCN synaptosomes and GS activity by SCN glia during the light phase are consistent with mechanisms aimed at reducing glutamate levels in the SCN following light-induced release from the retina. Enhanced glutamate metabolism during the light phase could result, for example, from glutamate-induced translocation of GluT to the plasma membrane or *via* glutamate receptor regulation (Duan et al., 1999; Gegelashvili et al., 1996; Hertz, 2006). The absence of evidence for diurnal variations of GS expression suggests that glutamate conversion to glutamine is regulated by modulation of enzymatic activity and not by changes in GS protein levels throughout the day.

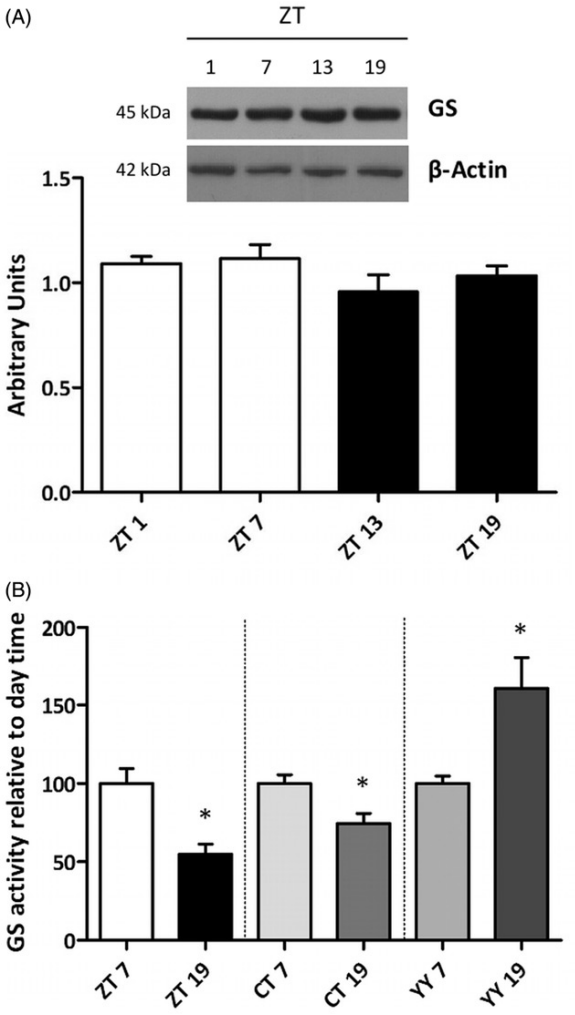

FIGURE 2. GS abundance and activity in the mouse SCN. (A) Glutamine synthetase protein levels did not vary in the mouse SCN under LD conditions. The upper panel shows a representative Western blot for GS and β-actin. Control relative values from different experiments indicated no significant differences between ZTs (one way ANOVA, $p>0.05$). (B) Glutamine synthetase activity in the mouse SCN under different environmental conditions. GS activity was significantly lower at night in LD (ZT19) ($^*p<0.01$ Student's *t* test, $n=9$ SCN per time point) and at subjective night in DD (CT19) ($^*p<0.01$ Student's *t* test, $n=20$ SCN per time point). In enucleated mice (YY), GS activity was higher during the subjective night (YY19) ($^*p<0.01$ Student's *t* test, $n=9$). Data are shown as mean ± SEM.

Diurnal variations in glutamate uptake can explain the differences found between glutamate- and NMDA-induced phase-response curves, which have been previously reported (Ding et al., 1994; Mintz et al., 1999; Shibata et al., 1994). While NMDA changes the phase of circadian oscillations both *in vivo* and *in vitro*, glutamate induces a photic-PRC *in vitro* but not *in vivo* (Ding et al., 1994). A possible explanation is that *in vivo* glutamate is quickly removed from the extracellular space after its release or infusion by EAAT, but since NMDA is not a target of transporters, it can phase-shift

the clock without being affected by GluT. *In vitro*, glutamate induces phase shifts only at high concentrations (the SCN are resistant to glutamate concentrations which are excitotoxic for other brain regions, such as 1 mM (Meijer et al., 1993) and this could also be a consequence of glutamate uptake (in the SCN the uptake is about three times higher than in cortex tissue (data not shown)). Finally, the co-administration of a GluT inhibitor (directly in the SCN) potentiates light-induced phase delays during the early subjective night (Kallingal & Mintz, 2006): again, if glutamate uptake is inhibited, glutamate can be available in the synaptic space during a longer time and/or at higher concentration.

We tested a second hypothesis regarding whether diurnal variations in glutamate metabolism are lost under constant darkness, a condition where glutamate levels should be very low in the SCN due to the absence of light-induced retinal signals. We found that glutamate uptake was not circadian either in synaptosomes from animals sacrificed in DD or in SCN slices *in vitro* (Figure 1). However, GS activity did exhibit a circadian variation (Figure 2). Therefore, the presence of light and/or modulatory processes originating from the eye itself are modifying glutamate metabolism in the SCN: glutamate uptake is light-dependent while GS activity is under circadian control, as was previously described for GFAP expression in the SCN (Lavialle et al., 2001; Lee et al., 2003; Yamazaki et al., 2002). Finally, the phase of the circadian rhythm of GS activity was different in the absence of retinal innervation. Previous work showed that the eye influences SCN circadian rhythms in DD (Beaule & Amir, 2003; Lavialle et al., 2001; Lee et al., 2003; Yamazaki et al., 2002). The interaction between the retina and hypothalamic oscillators might represent mechanisms for the modulation of neuronal and glial activity, which deserve to be explored further. Beyond retinal derived glutamate, other non-glutamatergic signals participate in SCN entrainment (Beaule et al., 2009; Hannibal et al., 2008) and can be affecting it through the modulation of glutamate metabolism (Figiel & Engele, 2000).

In order to have a complete understanding of the role of astroglial glutamate metabolism in the SCN, it is necessary to extend the results of this work to other time-points along the day and to use higher resolution real-time techniques to understand how the neurons and astrocytes within the SCN interact eliminating confounding effects of extra-suprachiasmatic areas.

In summary, our results suggest that different variables related to glutamate metabolism in the SCN are under diurnal, circadian and/or retinal modulation. Since this excitatory neurotransmitter represents the main neurochemical input to the biological clock, these variations are certainly important for the understanding of how the circadian oscillator keeps track of internal and external time.

DECLARATION OF INTEREST

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Supplementary material available online
Supplementary Figure 1