Maintenance of Thalamic Epileptiform Activity Depends on the Astrocytic Glutamate-Glutamine Cycle

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The generation of prolonged neuronal activity depends on the maintenance of synaptic neurotransmitter pools. The astrocytic glutamate-glutamine cycle is a major mechanism for recycling the neurotransmitters GABA and glutamate. Here we tested the effect of disrupting the glutamate-glutamine cycle on two types of neuronal activity patterns in the thalamus: sleep-related spindles and epileptiform oscillations. In recording conditions believed to induce glutamine-scarcity, epileptiform oscillations displayed a progressive reduction in duration that was partially reversible by the application of exogenous glutamine (300 µM). Blocking uptake of glutamine into neurons with α-(methylamino) isobutyric acid (MeAIB; 5 mM) caused a similar reduction in oscillation duration, as did blocking neuronal GABA synthesis with 3-mercaptopropionic acid (MPA; 10 µM). However, comparable manipulations did not affect sleep spindles. Together, these results support a critical role for the glutamate-glutamine cycle in providing the neurotransmitters necessary for the generation of epileptiform activity, and suggest potential therapeutic approaches that selectively reduce seizure activity but maintain normal neuronal activity.

Keywords: Thalamus, Oscillations, Glutamate-Glutamine Cycle, Absence Epilepsy
**INTRODUCTION**

Reliable synaptic transmission is supported by neuronal pools of neurotransmitter. The possibility of transmission failure resulting from depletion of neurotransmitter pools is reduced by mechanisms that replenish neurotransmitters, including *de novo* synthesis and recycling of previously released transmitters (Hertz 2006). The most direct neurotransmitter recycling mechanism relies on presynaptic neurons to internalize released neurotransmitters from the extracellular space (Krantz et al. 1999).

Indirect mechanisms that recycle the neurotransmitters glutamate and γ-aminobutyric acid (GABA) also exist. Specifically, high affinity transporters take up GABA and glutamate into astrocytes (Schousboe 2000; Chaudhry et al. 2002; Hamberger et al. 1979; Laake et al. 1995), where they are ultimately converted via GABA decarboxylase and glutamine synthetase into glutamine (Cooper et al. 2003; Bak et al. 2006). Astrocytic system-N transporters efflux glutamine to the extracellular space where it is then taken up into neurons by system-A transporters (Chaudhry et al. 2002; Fricke et al. 2007). Neuronal glutamine is then converted into glutamate by phosphate-activated glutaminase (Kvamme 1998; Bak et al. 2006). In inhibitory neurons, glutamate is further converted into GABA by glutamate decarboxylase (Liang et al. 2006) before being packaged into vesicles.

Evidence is emerging that the glutamate-glutamine cycle is critical for the proper maintenance of synaptic activity. Pharmacological disruption of the glutamate-glutamine cycle in the hippocampus and cortex reduces vesicular glutamate and GABA levels (Rae et al. 2003; Laake et al. 1995; Liang et al. 2006) and blocks epileptiform activity (Tani et al. 2006; Bacci et al. 2002). However, previous studies have also shown that glutamate-
glutamine cycle disruption does not affect all types of neuronal activity generated in the hippocampus (Liang et al. 2006; Kam and Nicoll 2007). These contradictory results suggest that the importance of the glutamate-glutamine cycle as a neurotransmitter recycling mechanism is activity and structure dependent.

One structure where the maintenance of synaptic transmission is likely to depend on astrocytic neurotransmitter recycling is the thalamus, a subcortical sensory processing structure. Previous studies have shown that plasmalemmal GABA and glutamate transporters are localized exclusively on astrocytes in the thalamus (Vitellaro-Zuccarello et al. 2003; De Biasi et al. 1998; Danbolt 2001), precluding significant neurotransmitter reuptake by presynaptic neurons. This anatomical specificity suggests that astrocytes play a dominant role in recycling thalamic neurotransmitters and that perturbation of the glutamate-glutamine cycle would greatly affect thalamic activity.

The thalamus is involved in generating both sleep-related and epileptic activity (Steriade et al. 1993; Steriade and Llinas 1988; Huguenard and Prince 1994; McCormick and Bal 1997). Under normal conditions, the thalamus can generate 6-14 Hz spindles, oscillations that are expressed predominantly during sleep. These oscillations are generated by networks of reciprocally connected glutamatergic thalamocortical (TC) neurons and GABA-ergic reticular (RT) neurons. During some forms of epilepsy, these thalamocortical networks become dysfunctional and are involved in generating the oscillatory spike-wave discharges associated with absence seizures (Huguenard and Prince 1994; McCormick and Bal 2007). Activity patterns resembling both sleep spindles and spike-wave oscillations can be generated in vitro (von Krosigk et al. 1993; Jacobsen et al. 2001; McCormick 2002). Specifically, spindle-like activity in ferret and rat
thalamic brain slices can be transformed into highly synchronous, 2-4 Hz epileptiform oscillations by bath application of bicuculline methiodide (BMI), a GABA<sub>δ</sub> receptor antagonist that increases thalamic dependence on GABA<sub>δ</sub>-mediated currents (von Krosigk et al. 1993; Huguenard and Prince 1994; McCormick 2002; Kleinman-Weiner et al. 2009).

Here, we determine the dependence of spindle-like and epileptiform activity on the glutamate-glutamine cycle. We take advantage of a previously-reported time-dependent depletion of glutamine that occurs during <i>in vitro</i> experiments. Specifically, glutamine, and to a lesser extent glutamate and GABA, is gradually depleted from brain slices due to diffusion from the tissue into the bathing medium (Kapetanovic 1993). Here we assess the functional relevance of such depletion. We report that under conditions matching those previously reported (Kapetanovic 1993), epileptiform, but not non-pathological spindle-like activity, exhibits a progressive, activity-dependent rundown which is rescued by exogenous application of glutamine. Furthermore, this glutamine-induced recovery is reversed following pharmacological blockade of either system-A transporters or blockade of GABA synthesis. Together, these results demonstrate a dependence of thalamic epileptiform oscillations on the neurotransmitter recycling sustained by the astrocytic glutamate-glutamine cycle.

**MATERIALS AND METHODS**

*Slice Preparation:* Experiments involving the use of animals were conducted in compliance with the rules set forth by the Stanford Institutional Animal Care and Use Committee. Sprague-Dawley rat pups of both sexes, aged 11-15 days were anesthetized
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(50 mg/kg/sodium pentobarbital, intraperitoneal injection) and decapitated. Brains were removed and transferred to chilled (4 °C), oxygenated slicing solution (in mM, 234 sucrose, 11 glucose, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, and 0.5 CaCl₂, 310 mOsm). Horizontal slices (400 µM) containing somatosensory thalamus were made as previously described (Huguenard and Prince 1994) using a Leica VT1200 microtome. Slices were then incubated in warm (32-33 °C) artificial cerebrospinal fluid (ACSF, in mM, 10 glucose, 26 NaHCO₃, 2.5 KCl, 1.25 NaHPO₄, 1 MgSO₄, 2 CaCl₂ and 120 NaCl, 298 mOsm) for 1 hour and subsequently incubated at room temperature (23 °C).

Slice Recordings: Slices were placed in an interface chamber for recording and perfused with warm, oxygenated ACSF at a rate of 2 ml/minute. Extracellular multiunit field recordings were made using monopolar tungsten microelectrodes (50-100 kΩ, FHC, Bowdoin, ME) placed in the thalamic reticular nucleus (containing RT neurons) and the ventrobasal somatosensory relay nucleus (containing TC neurons). Signals were amplified 10,000 times and bandpass filtered between 100 Hz and 3 kHz. Electrical stimuli were delivered to the internal capsule with a pair of tungsten microelectrodes (50-100 kΩ, FHC, Bowdoin, ME). The stimuli were 100 µsec in duration, 50 V in amplitude, and delivered once every 30 seconds.

Pharmacology: Epileptiform oscillations were induced by adding bicuculline methiodide (BMI, 10 µm, Tocris Bioscience, Ellisville, MO) to the ACSF solution (von Korisgk et al. 1993; Huguenard and Prince 1994; McCormick 2002; Kleinman-Weiner et al. 2009). Unless otherwise noted, slices were exposed to drugs for approximately 30 minutes. L-glutamine (hereafter referred to as glutamine; 300 µM, Sigma, St. Louis, MO; Hagenfeldt et al. 1984; Lerma et al. 1986) was added to ACSF following 30 minutes of stimulation.
For some experiments, a higher concentration of glutamine (600 µM, Sigma, St. Louis, MO) was applied 30 minutes after application of 300 µM glutamine. D-glutamine (300 µM, Sigma, St. Louis, MO) a stereoisomer of glutamine, was used during control experiments. For experiments testing the activity-dependence of rundown, slices were stimulated for 2.5 minutes, left un-stimulated for 25 minutes and then stimulated for an additional 2.5 minutes. α-(methylamino) isobutyric acid (MeAIB, 5 mM, Sigma, St. Louis, MO; Bröer and Brookes 2001; Liang et al. 2006) was used to block system-A transporters. D-mannitol was added to control solutions for MeAIB experiments in order to equalize changes in osmolarity (final osmolarity, 305 ± 1 mOsm). 3-mercaptopropionic acid (MPA, Sigma, St. Louis, MO, Engel et al. 2001; Murphy et al. 1998; Netopilová et al. 1997; Lindgren 1983; Yamauchi et al. 1989) was used to block GAD (glutamate decarboxylase) at 10 µM, a concentration twofold higher than the Ki (Netopilová et al. 1995). For experiments testing the effect of GAD blockage on epileptiform activity in the absence of glutamine, MPA was applied after approximately 22 minutes. During experiments testing the effect of GAD blockage on epileptiform activity in the presence of glutamine, glutamine was added to the ACSF after 7-13 minutes, followed 18-22 minutes later by the application of MPA.

**Data Analysis:** Data were collected with an Axon Instruments DigiData 1200 and pClamp 8 software. Data were analyzed with Matlab scripts (MathWorks, Natick, MA) designed to quantify burst activity during thalamic oscillations. Briefly, voltage deflections with a slope 3 times greater than background noise were defined as action potentials. Bursts of action potentials were then defined as a minimum number of spikes separated from the previous spike by a maximum time interval. Oscillations were defined
as groups of bursts containing a minimum number of bursts with a maximum allowable
time between bursts. Because the properties of spindle and BMI-induced activity are
different, the parameters used to quantify the two types of oscillations were distinct. For
spindle activity, a burst was defined as at least 4 spikes with interspike intervals of less
than 5 ms, with an oscillation defined as at least 4 bursts with a maximum interburst
interval of 400 ms. For BMI-induced activity, a burst was defined as at least 4 spikes with
interspike intervals of less than 15 ms. BMI-induced oscillations were defined as
containing at least 4 bursts, with a maximum interburst interval of 1 s. The data we report
reflect the average of 5 sweeps during a given condition. RM ANOVA refers to the
Repeated Measures ANOVA on Ranks test. Linear fits and correlation values (Pearson
product-moment correlation coefficients) were generated in Origin 7 (OriginLab
Corporation, Northhampton, MA).

RESULTS
Evoked Spindle oscillations are not affected by glutamine-scarce conditions.
Kapetanovic et al. (1993) demonstrated a significant, time-dependent loss of
glutamine content from brain slice preparations when incubated in the absence of
exogenous glutamine. We tested whether such a loss of endogenous glutamine could
provide a novel, non-pharmacological model of glutamate-glutamine cycle disruption in
the thalamus. In horizontal rat thalamic slices, electrical stimulation of the internal
capsule evokes spindle-like oscillations evident in RT and VB nuclei (Jacobsen et al.
2001). These oscillations consist of 6-14 Hz waxing and waning activity and are similar
to spindle oscillations recorded in vivo (Steriade et al. 1993). In the absence of exogenous
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Glutamine, spindle activity evoked at regular intervals (30 s) displayed no significant change in duration (initial: 4.1 ± 2.9 s; late initial (after 30 min): 3.8 ± 2.7 s; n = 7; p > 0.05; RM ANOVA, Fig. 1). Furthermore, subsequent exposure of the slice to physiological amounts of glutamine (300 µM; Hagenfeldt et al. 1984; Lerma et al. 1986) failed to alter evoked spindle activity (glutamine: 3.7 ± 2.6 s; wash: 3.6 ± 2.3 s; n = 7; p > 0.05, RM ANOVA; Fig. 1). Presuming our experiments replicate the glutamine depletion shown by Kapetanovic et al. (1993), these results demonstrate that physiological, spindle-like activity is not susceptible to either rundown by glutamine depletion or to changes induced by glutamine restoration, suggesting that the glutamate-glutamine cycle is not necessary for the maintenance of such activity.

BMI-evoked oscillations display rundown of activity.

Following application of BMI to a thalamic slice, 6-14 Hz spindle oscillations are converted into highly synchronous 2-4 Hz epileptiform oscillations (von Krosigk et al. 1993; Jacobsen et al. 2001; Kleinman-Weiner et al. 2009). Unlike spindle activity, we observed a progressive time-dependent decrease in the duration of epileptiform oscillations. After evoking epileptiform responses every 30 seconds for 30 minutes, the duration of the evoked oscillations decreased by an average of 31% (initial: 6.4 ± 3.2 s; late initial (after 30 mins): 3.9 ± 1.3 s; n = 17 slices; p < 0.0001, RM ANOVA; Fig. 2a, 3c). We refer to this decrease in duration as oscillation “rundown”. Rundown was usually characterized by two phases, an early rapid decrease over the first minute or so, followed by a more gradual depression over 30 minutes. As oscillation frequency was unchanged (p > 0.05, RM ANOVA), the oscillation rundown was primarily due to a decrease in the number of bursts evoked per stimulus (initial: 18.3 ± 8.7 bursts; rundown: 12.0 ± 3.6
The decrease in duration was positively correlated with the initial oscillation duration (R=0.69; n=17; p<0.005, Pearson Correlation; Fig. 2c), indicating that slices with stronger initial activity exhibit greater rundown.

The positive correlation between initial duration and percent rundown was likely not skewed by changes in slice glutamine concentration associated with prolonged incubation time between slice preparation and recording. First, the initial oscillation duration observed in a slice was not correlated with time elapsed between the preparation of the slice and the start of the experiment (i.e. incubation duration, not shown, R=-0.192; n=17; p>0.05, Pearson Correlation). Moreover, the decrease in duration observed in a slice was not correlated with incubation duration (R=-0.185; n=17; p>0.05, Pearson Correlation; Fig. 3d).

To directly test the activity-dependence of rundown we evoked epileptiform responses every 30 seconds for 2.5 minutes, halted stimulation for 25 minutes and then resumed stimulation for an additional 2.5 minutes. Both the initial oscillations (i.e those in the first 2.5 minutes, Figure 3A1,B black symbols), and those recorded after 25 minutes without stimulation (Figure 3A2, B, grey symbols) showed an early decrease in duration over the first minute or so, followed by relatively stable oscillations at a slightly decreased level. The activity free period was not associated with changes in either the first oscillation in each series, or the later oscillations (overall average duration of initial 5 sweeps: 6.9 ± 1.8 s; post-rest duration, 5 sweep average: 6.3 ± 1.2 s; n=5; p>0.05, Signed Rank Test; Fig. 3c).

Glutamine partially restores oscillation activity lost during rundown.
If the observed rundown of oscillatory activity was due to the loss of glutamine in brain slices as described by Kapetanovic et al. 1993, then supplementation of rundown slices with glutamine should reverse the loss and restore activity. Application of 300 µM glutamine partially restored evoked oscillation duration to 81% of pre-rundown oscillation duration (initial: 6.4 ± 3.2 s; rundown: 3.9 ± 1.3 s; glutamine: 4.7 ± 1.9 s; n=17; p<0.0001, RM ANOVA; Fig. 4). As was the case during oscillation rundown, the glutamine-dependent rescue of oscillation duration was primarily caused by a change in the number of bursts per oscillation (rundown: 12.0 ± 3.6 bursts; glutamine: 14.7 ± 5.3 bursts; p<0.005, RM ANOVA). Subsequent washout of exogenous glutamine yielded a decrease in oscillation duration to levels approximating the initial rundown (wash: 4.2 ± 1.8 s; 106% of rundown oscillation duration; p<0.0001, RM ANOVA; Fig. 4). Oscillation frequency did not significantly change during any of the three experimental conditions (p>0.05, RM ANOVA). The partial rescue of oscillation duration by 300 µM glutamine was not further enhanced by the application of a higher concentration of glutamine (600 µM; initial: 8.8 ± 2.6 s; rundown: 4.2 ± 0.9 s; 300 µM glutamine: 5.7 ± 1.5 s; 600 µM glutamine: 6.2 ± 1.7 s; n=5; p>0.05 300 µM versus 600 µM glutamine; p<0.005 all other conditions, RM ANOVA; Figure 4).

*D*-glutamine does not rescue rundown epileptiform oscillation activity.

To control for non-specific effects of glutamine, we exposed thalamic slices to an inert stereoisomer of glutamine, *D*-glutamine, which is transported into neurons (Pow and Crook, 1996) but accumulates in presynaptic neurons because it is not converted into excitatory glutamate by phosphate-activated glutaminase (Brown et al. 2008). To test the effect of *D*-glutamine on rundown, we stimulated slices in glutamine-free conditions to
promote rundown and then exposed slices to D-glutamine. Unlike L-glutamine, D-
glutamine had no effect on rundown oscillations (300 µM, initial: 7.3 ± 4.5 s; rundown:
4.0 ± 1.2 s; D-glutamine: 4.0 ± 1.1 s; wash: 4.1 ± 1.5 s; n = 6; p > 0.05, RM ANOVA; Fig.
4).

MeAIB reverses the rescue of epileptiform oscillations

Current models of the astrocytic glutamate-glutamine cycle ascribe glutamine
uptake into neurons to system-A transporter activity (Chaudhry et al. 2002; Fricke et al.
2007). Blockade of these transporters reduces the amount of recycled neurotransmitter
(Armano et al. 2002). MeAIB is a competitive inhibitor of system-A transporters (Bröer
and Brookes, 2001). In order to isolate the effects of MeAIB, we included 300 µM
 glutamine in the ACSF in order to prevent rundown. Subsequent application of MeAIB
onto glutamine-supplemented slices reduced the duration of the evoked oscillations by
24% (Glutamine: 15.0 ± 2.5 s; MeAIB: 11.1 ± 2.2 s; n = 6; p < 0.05 Signed Rank Test; Fig.
5).

MPA reverses the rescue of epileptiform oscillations.

The glutamine supplied by astrocytic glutamate-glutamine cycle is used to
replenish two distinct neurotransmitters: glutamate and GABA. Individually blocking the
recycling of these neurotransmitters could reveal distinct roles of glutamate and GABA in
maintaining epileptiform activity. In GABAergic neurons, GABA is converted from
 glutamate with the enzyme glutamate decarboxylase (Martin and Tobin 2000), which can
be blocked by 3-mercaptopropionic acid (MPA) (Engel et al. 2001; Netopilová et al.
1995; Murphy et al. 1998). Prior to block of GABA synthesis, rundown of evoked
oscillations was induced in thalamic slices and then rescued through the application of
300 µM glutamine (Fig. 6b). The subsequent application of 10 µM MPA onto these glutamine-supplemented slices reversed glutamine-rescue of evoked oscillatory activity (17% reduction in oscillation duration by MPA; Control 3.8 ± 1.2s; Glutamine: 5.2 ± 2.3s; MPA: 4.4 ± 2.2s; n= 6; p<0.01, RM ANOVA; Fig. 6). In the absence of glutamine, MPA application would be expected to induce negligible effects. Indeed, MPA does not significantly reduce the duration of rundown oscillations in slices that had not been supplemented with glutamine (Rundown: 8.8 ± 2.4s; MPA 8.3 ± 2.6s; n=8; p>0.05, RM ANOVA).

DISCUSSION

The functional relevance of the glutamate-glutamine cycle in recycling neurotransmitter and supporting neuronal activity has been studied in multiple neuronal systems, including hippocampus and neocortex (Rae et al. 2003; Laake et al. 1995; Liang et al. 2006; Tani et al. 2006; Kam and Nicoll 2007; Bacci et al. 2002). Here we assess glutamate-glutamine cycle dependence in the thalamus, a brain structure that generates two types of network oscillations: sleep-related spindles and seizure-related spike-wave discharges. We demonstrate that thalamic epileptiform oscillations, but not spindle activity, are dependent on the glutamate-glutamine cycle.

Glutamine is a major precursor of glutamate and GABA (Peng et al. 1993; Lebon et al. 2002; Tapia and González 1978; Paulsen et al. 1988; Patel et al. 2001). A critical step in the glutamate-glutamine cycle is the astrocytic release of glutamine into the extracellular space (Chaudhry et al. 2002). We developed a model of glutamate-glutamine cycle disruption based upon depletion of extracellular glutamine. *In vivo*, the
extracellular space contains 200-500 μM glutamine (Hagenfeldt et al. 1984; Lerma et al. 1986). Perfusing brain slices with glutamine-free ACSF causes diffusion of extracellular glutamine out of the tissue and into the bathing medium (Kapetanovic et al. 1993). Over time, this diffusion results in a pronounced decrease in glutamine tissue concentrations (90% lost after 3 hours) and to a lesser extent, neurotransmitters such as glutamate and GABA (45% and 25% respectively lost after 3 hours; Kapetanovic et al. 1993). Although we did not directly measure glutamine tissue concentrations, our recording conditions were comparable to those used by Kapetanovic et al (1993), and are likely to induce comparable decreases in tissue concentrations of glutamine, glutamate, and GABA. Glutamine depletion is expected to act as a potent disruptor of the glutamate-glutamine cycle and to yield functionally relevant changes in neuronal activity.

**Thalamic dependence on the glutamate-glutamine cycle is activity-dependent**

Despite the proposed disruption of the glutamate-glutamine cycle, the duration and frequency of thalamic spindle activity was not affected by prolonged exposure to glutamine-free ACSF. Spindle activity is hypothesized to represent sparse network oscillations, with only a small percentage of thalamic neurons participating during any given cycle of the oscillation (Huguenard and McCormick 2007; von Krosigk et al. 1993; Steriade and Llinas 1988). Specifically, each spindle oscillation is thought to be generated by a dynamically changing sub-population of thalamic neurons (Steriade and Llinas 1988; von Krosigk et al. 1993), an effect mediated by the inhibitory connections between RT neurons which tend to decrease network synchronization (for review, Huguenard and McCormick 2007). If true, then such sparseness provides individual neurons with periods of inactivity during which they may replenish neurotransmitter
stores. Indeed, previous reports in the hippocampus have shown that alternate sources of glutamate and GABA may be sufficient to support low levels of synaptic activity (Liang et al. 2006; Matthews and Diamond 2008). Alternatively spindles may be maintained by the glutamine and neurotransmitter concentrations which survive diffusion (Kapetanovic et al. 1993).

In contrast to spindles, epileptiform activity in the thalamus appears highly dependent on the glutamate-glutamine cycle. BMI disrupts thalamocortical networks, wherein reduced intra-RT inhibition and enhanced RT excitability transform sparse spindle activity into robust epileptiform oscillations (von Krosigk et al. 1993; Huguenard and Prince 1994; McCormick and Bal 1997; Kleinman-Weiner et al. 2009). We hypothesized that the enhanced activity of epileptiform oscillations might unmask glutamate-glutamine cycle dependence. Indeed, BMI-treated slices displayed progressive rundown of epileptiform oscillations evoked in glutamine-free ACSF. Rundown was associated with a decrease in the number of action potential bursts evoked by a stimulus, rather than with changes in oscillation frequency. This distinction indicates that during rundown the thalamic network is activated for shorter periods of time but is not intrinsically modified. Oscillation rundown was partially reversed by adding glutamine to the ACSF, a manipulation previously shown to restore tissue glutamine and neurotransmitter levels (Kapetanovic et al. 1993). This rescue of epileptiform activity was maintained only if exogenous glutamine was constantly supplied, suggesting that glutamine diffusion out of brain slices is a continuous process.

Our results suggest that functionally relevant neurotransmitter losses are activity-dependent. Firstly, we observed that oscillation rundown was greatest in the most active
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slices. Consistent with this observation, rundown could be prevented by halting stimulation for 25 minutes. During incubation, glutamine rapidly diffuses from the extracellular space into the bathing medium (Kapetanovic at al. 1993). As we did not observe any correlation between duration of slice incubation and percent rundown, our results suggest that extracellular glutamine concentration changes that occur after the required 1 hour slice incubation are not functionally relevant. However, the correlation between initial oscillation duration and the percent decrease in oscillation duration suggests an activity-dependent depletion of neurotransmitter stores separate from the extracellular contents lost into the bathing medium during incubation. A likely candidate is intracellular neurotransmitter stores, which may be maintained if synaptic activity remains low during the incubation period. If so, repeated stimulation of activity during experiments would likely result in the release of neurotransmitter stores into the extracellular space followed by astrocytic conversion to glutamine, which may diffuse out of the slice. Such diffusion would deprive neurons of a source of neurotransmitter replenishment and thus deplete neurotransmitter stores, reducing the ability of the slice to generate prolonged oscillations. More active slices would release more neurotransmitter, yielding greater glutamine loss and greater rundown.

Previous reports support the hypothesis that the functional significance of the glutamate-glutamine cycle is dependent on the level of activity. In hippocampal CA1 pyramidal neurons, exogenous glutamine application did not affect the amplitude or frequency of spontaneous miniature inhibitory post synaptic currents (mIPSCs) or miniature excitatory post synaptic currents (mEPSCs; Kam and Nicoll 2007). Similarly, glutamate-glutamine cycle disruption does not affect the amplitudes of evoked inhibitory
post-synaptic potentials (IPSPs) in the CA1 pyramidal neurons of relatively inactive slices (Liang et al. 2006). However, GABAergic IPSP amplitudes are reduced when glutamate-glutamine cycle disruption is paired with burst stimulation at physiologically relevant rates (Liang et al. 2006). Furthermore, glutamate-glutamine cycle blockade reduces the frequency of spontaneous epileptiform activity in the hippocampus (Bacci et al. 2002) and reduces the likelihood of evoking epileptiform activity in the neocortex (Tani et al. 2006). Together, these results support the hypothesis that higher activity rates display greater dependence on the glutamate-glutamine cycle- a hypothesis that our study further supports.

*Roles for GABA and glutamate recycling in maintenance of epileptiform oscillations*

In the thalamus, both glutamate and GABA recycling appear to depend on the exogenous glutamine supplied by the astrocytic glutamate-glutamine cycle (Vitellaro-Zuccarello et al. 2003; De Biasi et al. 1998; Danbolt 2001). Therefore, disruptions to the glutamate-glutamine cycle should affect both neurotransmitter concentrations. MeAIB, a competitive inhibitor of system-A transporter-mediated neuronal glutamine uptake (Bröer and Brookes 2001) progressively shortened oscillations by an average of 24% in slices that were provided with exogenous glutamine. This finding supports the hypothesis that extracellular glutamine is a critical substrate for glutamate and/or GABA, and that blocking uptake of exogenous glutamine into neurons diminishes the thalamic circuit’s ability to maintain prolonged epileptiform oscillations.

However, glutamatergic and GABA-ergic synapses have distinct vesicle refilling and release properties (Moulder et al. 2007; Li et al. 2005), suggesting that glutamate-glutamine cycle disruptions may not equally affect glutamate and GABA synaptic
function. To address this issue, we aimed to selectively block transmitter production. Selectively blocking glutamate synthesis poses a technical challenge because glutamate is a GABA precursor (Martin and Tobin 2000; Liang et al. 2006) and, therefore, manipulations that target glutamate stores indirectly affect GABA stores.

The functional relevance of GABA can be examined by selectively blocking the synthesis of GABA from glutamate using MPA (Engel et al. 2001; Netopilová et al. 1995; Murphy et al. 1998), which reduced oscillation duration by an average of 17% in the presence of glutamine. The significant reduction of oscillations that occurs in MPA indicates that selective disruption of GABA production in RT neurons is sufficient to affect epileptiform activity.

Might decreases in glutamate levels following glutamine depletion also contribute to the rundown of epileptiform activity? The slightly greater rundown caused by MeAIB compared to MPA could reflect the fact that maintenance of epileptiform activity requires the recycling of both GABA and glutamate via the glutamate-glutamine cycle. The lack of an MPA effect in the absence of glutamine suggests that glutamine depletion results in decreased glutamate levels, especially those relevant for synaptic GABA recycling. Whether similar depletion of glutamate is relevant for synaptic pools of glutamate itself remains to be determined, but seems likely.

However, the smaller rundown caused by MPA compared to MeAIB could result from incomplete block of GABA synthesis. At high concentrations (\(>50 \mu M\)) MPA is reported to have nonspecific effects, including inhibition of the catabolic enzyme GABA transaminase (Lamar 1970; Loscher and Vetter 1985). Therefore in our experiments, we
used 10 μM MPA, a relatively low concentration that is nevertheless double the reported
Kᵢ for GAD (Netopilová et al., 1995), and thus likely to produce robust inhibition.

Survival of thalamic activity despite glutamate-glutamine cycle disruption

Interestingly, we observed two forms of activity that persist despite presumed
glutamine-depletion: (a) spindle-like activity and (b) post-rundown epileptiform activity.
The maintenance of these two activity types suggests that weaker/shorter patterns of
activity are resistant to glutamine depletion. Contributory mechanisms might include
alternative pathways such as non system-A glutamine uptake, synthesis of glutamate
from glucose (Hertz 2006) and/or synthesis of GABA from glutamate sources not
associated with astrocytic metabolism (Mathews and Diamond 2003), whose
contributions might be sufficient to maintain neurotransmitter at concentrations that can
support low levels of activity. Furthermore, the possibility exists that previously
unidentified neurotransmitter transporters on thalamic neurons might allow maintenance
of some activity.

Targeting the glutamate-glutamine cycle for selective seizure relief

Previous studies have demonstrated that the role of the astrocytic glutamate-
glutamine cycle in supporting neuronal activity is highly structure- and activity-
dependent (Rae et al. 2003; Laake et al. 1995; Liang et al. 2006; Tani et al. 2006; Kam
and Nicoll 2007; Bacci et al. 2002). Here, we demonstrate that in the thalamus, the
functional significance of the astrocytic glutamate-glutamine cycle is also highly activity-
dependent. We show that glutamine depletion or pharmacological disruption of the
astrocytic glutamate-glutamine cycle restricts the ability of the thalamic circuit to
maintain intense, long lasting 2-4 Hz epileptiform activity. While it is unknown whether
the intact brain can experience local glutamine depletion, our study nonetheless identifies disruption of the glutamate-glutamine cycle as a potential *in vivo* therapeutic tool. Indeed, considering the maintenance of normal neuronal activity during disruption of the glutamate-glutamine cycle, it may be possible to provide therapeutic approaches that target the cycle to selectively reduce epileptiform activity.
ACKNOWLEDGEMENTS

We would like to thank Max Kleiman-Weiner, Hiroaki Tani, and all the members of the Huguenard Lab for technical guidance and discussions.
GRANTS

M.P.B was funded through an Epilepsy Foundation Fellowship. This research was supported by NIH grants NS12151 and NS034774.
REFERENCES


Glutamate-Glutamine Cycle Supports Epileptiform Activity


Glutamate-Glutamine Cycle Supports Epileptiform Activity


Glutamate-Glutamine Cycle Supports Epileptiform Activity

Ventura R, Harris KM. Three-dimensional relationships between hippocampal

Vitellaro-Zuccarello L, Calvaresi N, De Biasi S. Expression of GABA transporters
GAT-1 and GAT-3, in the cerebral cortex and thalamus of the rat during postnatal

Von Krosigk M, Bal T, McCormick DA. Cellular Mechanisms of a Synchronized

Yamauchi R, Amatsu M, Okada Y. Effect of GABA (γ-aminobutyric acid) on
neurotransmission in inferior colliculus slices from the guinea pig. Neuroscience
FIGURE LEGENDS

**Figure 1: Spindle activity is not affected by rundown.**
A. Sample traces show spindles evoked under different experimental conditions at the times indicated. Stimulus is indicated by black dot. B. Population data from 7 slices depicting mean ± standard error duration of spindle responses in the conditions noted in A. No time- or glutamine-dependent changes were observed (p>0.05, RM ANOVA).

**Figure 2: Epileptiform oscillations display rundown of their duration.**
A. Sample traces of initial oscillations, and oscillations evoked after approximately 30 minutes. Stimulus is indicated by black dot. B. The time course of evoked oscillation duration shows activity-dependent rundown towards a plateau. Plot shows average response duration at ½ minute intervals (n=17 slices, error bars indicate standard error). C. The degree of rundown is correlated with the original oscillation duration (R=0.69; n=17; p<0.005, Pearson Correlation, Line shows linear fit).

**Figure 3: Oscillation rundown is activity-dependent.**
A1, 2. Sample traces of initial oscillations and oscillations evoked after a 25 minute stimulation-free rest period. Stimulus indicated by black dot. B. The time course of evokes oscillation duration wshows no rundown following a 25 minute rest period. Plot shows average response duration at ½ minute intervals (n=5, error bars indicate standard error). C. Averaged durations at Initial (0-2.5 min) and Post-rest (27.5-30 min) time bins show no rundown of activity when stimulation is halted for 25 minutes between
stimulation periods. D. The degree of rundown is not correlated with the incubation duration (R=-0.185, n=17, p>0.05, Pearson Correlation, Line shows linear fit).

**Figure 4: Exogenous glutamine (300 µM) partially and reversibly rescues evoked oscillations.**

A, B. Sample traces (A) and time course of a typical experiment (B) show rundown of oscillation duration, the rescue effect of exogenous glutamine, and the secondary rundown which occurs following wash of glutamine. Stimulus is indicated by black dot and glutamine application by black bar. C. Averaged durations at Initial (00:00-02:30), Rundown (27:30-30:00), Glutamine (57:30-60:00), and Wash (87:30-90:00) time bins show rundown and the reversible effect of glutamine (C1, 300 µM; *p<0.0001, RM ANOVA; error bars indicate standard error), but not D-glutamine (C2, 300 µM; *p>0.05, RM ANOVA; error bars indicate standard error) on oscillation duration. C3. Averaged durations at Initial (0-2.5 min), Rundown (27.5-30 min), 300 µM Glutamine (57.5-60 min) and 600 µM Glutamine (87.5-90 min) time bins show no additional rescue of oscillation duration with an increased glutamine concentration (n=5; p>0.05, RM ANOVA).

**Figure 5: Blocking uptake of glutamine into neurons causes oscillation rundown.**

A, B. Sample traces (A) and the time course of a typical experiment (B) show a reduction in oscillation duration following application of the system-A transporter antagonist, MeAIB, in the presence of glutamine. Stimulus is indicated by black dot, glutamine application by black bar and MeAIB application by grey bar. C. Averaged durations, at
times of equilibrium glutamine (0-2.5 min) and MeAIB (17.5-20.83 min after MeAIB application) effect, show that MeAIB causes a reduction in oscillation duration even in the presence of exogenous glutamine (*p<0.05, Signed Rank Test, error bars indicate standard error).

Figure 6: Blocking the conversion of glutamate into GABA reverses the glutamine-dependent rescue of evoked oscillations.

A, B. Representative traces (A) and the time course of a typical experiment (B) show glutamine-dependent rescue of rundown followed by a secondary run down in the presence of MPA, a glutamate decarboxylase inhibitor. Stimulation is indicated by black dot, glutamine application by black bar, and MPA application by grey bar. C. Averaged durations at times of equilibrium control (0-2.5 min), glutamine (22.5-25 min) and MPA (60-62.5 min) effect show the rescue effect of glutamine, and the reversal of that effect by application of MPA (*p<0.01, RM ANOVA, error bars indicate standard error).
Bryant, AS et al., Figure 1
Bryant, AS et al., Figure 2
Bryant, AS et al., Figure 3
A

Rundown; 28.5 min
Glutamine; 57.5 min
Wash; 88.5 min

B

Oscillation Duration (s)

C1

C2

C3

Glutamine

Bryant, AS et al., Figure 4
A

Control; 1 min
Glutamine; 23.5 min
MPA; 60.5 min

2 s
200 µV

B

Oscillation Duration (s)

Time (min)

0 10

Glutamine

MPA

C

Oscillation Duration (s)

Control
Glutamine
MPA

Bryant, AS et al., Figure 6