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# Modulation of epileptiform activity by glutamine and system A transport in a model of post-traumatic epilepsy

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**Epileptic activity arises from an imbalance in excitatory and inhibitory synaptic transmission. To determine if alterations in the metabolism of glutamate, the primary excitatory neurotransmitter, might contribute to epilepsy we directly and indirectly modified levels of glutamine, an immediate precursor of synaptically released glutamate, in the rat neocortical undercut model of hyperexcitability and epilepsy. We show that slices from injured cortex take up glutamine more readily than control slices, and an increased expression of the system A transporters SNAT1 and SNAT2 likely underlies this difference. We also examined the effect of exogenous glutamine on evoked and spontaneous activity and found that addition of physiological concentrations of glutamine to perfusate of slices isolated from injured cortex increased the incidence and decreased the refractory period of epileptiform potentials. By contrast, exogenous glutamine increased the amplitude of evoked potentials in normal cortex, but did not induce epileptiform potentials. Addition of physiological concentrations of glutamine to perfusate of slices isolated from injured cortex greatly increased abnormal spontaneous activity in the form of events resembling spreading depression, again while having no effect on slices from normal cortex. Interestingly, similar spreading depression like events were noted in control slices at supraphysiological levels of glutamine. In the undercut cortex addition of methylaminoisobutyric acid (MeAIB), an inhibitor of the system A glutamine transporters attenuated all physiological effects of added glutamine suggesting that uptake through these transporters is required for the effect of glutamine. Our findings support a role for glutamine transport through SNAT1 and/or SNAT2 in the maintenance of abnormal activity in this in vitro model of epileptogenesis and suggest that system A transport and glutamine metabolism are potential targets for pharmacological intervention in seizures and epilepsy.**


## Introduction

An underlying imbalance between excitation and inhibition is thought to mediate seizure activity. It follows that alterations in metabolism of glutamate, the primary excitatory neurotransmitter, are likely to have marked effects on epileptiform activity. The most direct mechanism for the recycling of neurotransmitters is presynaptic reuptake followed by repackaging in synaptic vesicles (Krantz et al., 1999), but presynaptic plasma membrane glutamate transporters are absent from the vast majority of excitatory neurons indicating that such a mechanism is not used for recycling synaptically released glutamate (Danbolt, 2001). Instead, the glutamate backbone appears to transit through a glutamate–glutamine shuttle between neurons and glia. In this pathway, released glutamate is cleared from the synapse by high affinity excitatory amino acid transporters (EAATs) on the glial plasma membrane (Chaudhry et al., 2002a; Hamberger et al., 1979a; Laake et al., 1995). The high level of expression of these transporters in the perisynaptic space, their high affinity and their ionic coupling leads to low resting levels of the glutamate in the cleft and minimizes the diffusion of the neurotransmitter (Chaudhry et al., 1995; Nicholls and Attwell, 1990; Zerangue and Kavanaugh, 1995; Zhou and Sutherland, 2004). Once cleared from the synapse, glutamate is metabolized to glutamine by the glial-specific enzyme glutamine synthetase (GS). The glutamine is then released from glial cells and taken up by neurons where it is converted to glutamate through the activity of mitochondrial phosphate activated glutaminase (PAG) and repackaged into synaptic vesicles for rerelease. The relevance of this pathway in glutamate recycling is supported by marked reduction in synaptically released glutamate by inhibition of glutamine synthetase or glutaminase (Conti and Minelli, 1994; Rothstein and Tabakoff, 1984). Unlike glutamate, glutamine does not appear to have specific neuronal receptors or to be directly involved in neuronal signaling. Indeed the glutamine concentration in the cerebrospinal fluid is several hundred millimolar (Fishman, 1992; Lerma et al., 1986), many fold higher than any other amino acid, making for an ideal extracellular intermediate in the pathway.

Glutamine also plays a role in the metabolism of the primary inhibitory neurotransmitter, GABA. Although the majority of GABA is taken up by GABAergic neurons, approximately one-

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fifth is taken up by astrocytes (Schousboe, 2000). The metabolism of GABA through a transamination reaction within astrocytes leads to the production of glutamate (Cooper et al., 2003). Glutamate can then be metabolized to glutamine by the activity of glutamine synthetase, and since the same molecular mechanisms that mediate the transfer of glutamine from glia to glutamatergic neurons are also present in GABAergic neurons and the surrounding astrocytes, a similar cycle can occur (Liang and Coulter, 2004; Patel et al., 2001).

Although glutamine is the primary precursor for synaptically released glutamate, the molecular mechanisms mediating intercellular movement in the glutamine–glutamate shuttle are incompletely characterized. It has recently been suggested the SNAT3/SN1 and SNAT5/SN2 transporters that cotransport glutamine and $Na^+$ in exchange for a $H^+$ facilitate electroneutral efflux of glutamine from glial cells (Chaudhry et al., 1999; Cubelos et al., 2005; Mackenzie and Erickson, 2004). SNAT1 and SNAT2 transporters that are structurally related to SNAT3 and SNAT5 cotransport glutamine and $Na^+$ and are pH-sensitive but do not move protons (Chaudhry et al., 2002b; Reimer et al., 2000). The electrogenic nature of SNAT1/2 activity and the coupling to the $Na^+$ gradient, enable the generation of large transmembrane glutamine gradients across the neuronal plasma membrane. These transporters can thus function to move glutamine generated in glial cells sequentially into neurons, but their role in the glutamine–glutamate shuttle remains controversial (Conti and Melone, 2006; Rae et al., 2003).

To maintain the increased rate of neurotransmitter release associated with epileptiform activity, an increase in the rate of synthesis of glutamate is necessary. A likely mechanism to achieve this is an increased rate of recycling through an upregulation of the shuttle. The role of glutamate–glutamine shuttle in epilepsy, however, is complex and controversial. Data from a study of human epileptic tissue indicate a decrease in the shuttle in the hippocampi of patients with temporal lobe epilepsy (Petroff et al., 2002) and levels of glutamine synthesis are reduced in temporal lobe tissue from such patients (Eid et al., 2004). Further, methionine sulfoximine (MSO), an inhibitor of glutamine synthetase induces seizures in animals (Rowe and Meister, 1970). However, in vitro studies with MSO have demonstrated inhibition of epileptiform activity in hippocampal slices treated with bicuculline (Bacci et al., 2002) and this inhibition is reversed by the addition of glutamine, suggesting that the specific disruption of the glutamine–glutamate shuttle is responsible for the anti-epileptic effect. In the same model, addition of MeAIB, a system A transporter inhibitor also reduced epileptiform activity, while the PAG inhibitor 6-diazo-5-oxo-L-norleucine (DON) has been shown to attenuate seizures in an animal model (Chung and Johnson, 1984).

Partial neocortical isolates ("cortical islands" or "undercuts") with intact pial vasculature are an established model of post-traumatic cortical hyperexcitability (Echlin and Battista, 1963; Hoffman et al., 1994). In cortical slices prepared from lesioned rat brain the hyperexcitability is characterized by delayed, stimulus-induced polyphasic epileptiform potentials. These potentials are initiated through activity of layer V neurons which have altered electrophysiological properties and anatomical connections (Hoffman et al., 1994; Prince and Tseng, 1993; Salin et al., 1995). To determine if changes in metabolism of glutamine might contribute to the electrophysiological abnormalities seen in the neocortical undercut, we have carried out molecular, biochemical and electrophysiological measurements. We have found that compared to naïve cortex, slices from injured cortex take up exogenously added glutamine more readily than control slices and likely do so through the upregulation of neuronal system A transporters. In addition, we find that exogenous glutamine enhances the abnormal evoked and spontaneous activity characteristic of the injured neocortex and that this effect can be blocked by inhibition of system A transport.

## Methods

### *Cortical undercut lesions*

All experiments were carried out according to protocols approved by the Stanford Institutional Animal Care and Use Committee. Lesions were made as previously described (Graber and Prince, 2006; Hoffman et al., 1994). Adult rat at ages P21–25 were anesthetized with ketamine/rompun and a small bone window centered on the coronal suture removed, exposing a portion of the frontoparietal cortex unilaterally, but leaving the dura intact. A 30-gauge needle, bent at a right angle 2.5–3 mm from the tip, was inserted tangentially through the dura, just beneath the pial vessels. The insertion was made parasagittally ~1–2 mm from the interhemispheric sulcus. The needle was lowered to a depth of 2 mm and then rotated through 180° to produce a contiguous white matter lesion. The needle was then elevated to a position just under the pia, making a second transcortical cut, and removed. The skull opening was covered with sterile plastic wrap (Saran Wrap), and the skin sutured. Animals were monitored until complete surgical recovery, and given buprenorphine post-surgically for analgesia. Lesioned animals were reanesthetized for slice experiments 2–6 weeks later.

### *Preparation of sections for in situ and immunohistochemistry*

Rats were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were blocked and post-fixed in 4% paraformaldehyde for 24 h and cryoprotected in a mixture of 30% sucrose/4% paraformaldehyde. Coronal slices were sectioned on a sliding microtome at a thickness of 30 μm and collected in cold 4% paraformaldehyde for *in situ* hybridization and immunohistochemistry or in 0.1 M phosphate buffer for Nissl staining and immunocytochemical staining.

### *In situ hybridization*

In situ hybridization with RNA probes generated with digoxigenin-labeled UTP was carried out as previously described (Reimer et al., 2000). Briefly sense and antisense probes corresponding to coding sequence nucleotides 750–1274 for SNAT1; 746–1327 for SNAT2; 395–925 for SNAT3 and 368–858 for SNAT5 were generated with digoxigenin-labeled UTP For hybridization paraformaldehyde fixed slices were treated with proteinase K washed and fixed again in paraformaldehyde, washed, treated with triethanolamine–HCl pH 8.0 with acetic anhydride, washed and then blocked in hybridization buffer lacking the probe for 3–4 h at 65°C. Hybridization buffer was removed and fresh hybridization buffer with 1 μg/ml of probe was

placed over the sections. Slides were incubated at 65°C for 16–20 h in a humidified chamber, washed and probed with an alkaline phosphatase conjugated anti-digoxigenin antibody (Roche). After washing the slices were incubated in 1 μl/ml of NBT (Nitro blue tetrazolium chloride—Roche) and 3.5 μl/ml of BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt—Roche) and allowed to develop in the dark for between 2 and 24 h, depending on the abundance of the RNA. Once an adequate level was reached the slides were washed twice in $H_2O$ to remove substrates, then fixed in formaldehyde for 60 min at room temperature. Coverslips were mounted with Aquamount (Polysciences).

*Generation of SNAT1 and SNAT2 antibodies*

To generate SNAT1 and SNAT2 specific antibodies GST fusion proteins with the amino terminal regions of the proteins (amino acids 1–63 for SNAT1 and 1–65 for SNAT2) were made using PCR with oligonucleotide primers designed to maintain the open reading frame after subcloning into the GST vector PGEX-3X (Pharmacia Biotech). The fusion proteins were produced in bacteria and purified as previously described (Chaudhry et al., 1998). The purified protein was submitted for commercial custom antibody production (Zymed) and IgY was purified with a commercial kit (Pierce) according to the manufacturer's instructions.

*Immunofluorescence*

The floating sections were washed in PBS, then blocked in 5% normal goat serum (NGS)/0.3% Triton X-100/PBS and incubated overnight at 4°C in 1% NGS/0.3% TX-100/PBS containing primary antibody. Both SNAT1 and SNAT2 antibodies were diluted 1:2000. After extensive washing, sections were incubated in fluorescently labeled secondary antibodies (Pierce) at a dilution of 1:200–400 for 45 min, then washed extensively in blocking buffer. The sections were rinsed twice in PBS, mounted in medium (Vectashield; Vector), and coverslipped.

*Western blots*

Samples of homogenized brain tissue containing 30μg of protein were mixed with sample buffer and separated on a 10% SDS-polyacrylamide gel. The resulting gels were transferred to a PVDF membrane that was then immunoblotted with immune serum diluted in 1× PBS containing 0.1% Tween-20 and 1% nonfat dry milk after blocking in 1× PBS containing 0.1% Tween-20 and 5% nonfat dry milk, followed by hybridization with HRP linked secondary antibody (Pierce). Detection of hybridization was done with enhanced chemiluminescence (Amersham) and exposure of the blot to Kodak XOMAT film for 1 to 20 min.

*Preparation of brain slices*

Neocortical slices were prepared as previously described (Kumar and Huguenard, 2001). Briefly, rats were anesthetized (50 mg/kg pentobarbital, i.p) and decapitated. The brain was removed and transferred into ice cold low NaCl slicing solution containing (in mM): 234 sucrose, 11 glucose, 24 $NaHCO_3$, 2.5 KCl, 1.25 $NaH_2PO_4$, 10 $MgSO_4$, and 0.5 $CaCl_2$ bubbled with 95%$O_2$/5% $CO_2$. The whole brain was glued to a cover slip and 400-μm coronal slices were made using a vibratome (TPI). Slices were incubated in ACSF containing (in mM): 126 NaCl, 26 $NaHCO_3$, 2.5–5 KCl, 1.25 $NaHPO_4$, 2 $MgCl_2$, 2 $CaCl_2$, and 10 glucose at 32°C for 1 h prior to recording. The solution was continuously bubbled with 95% $O_2$/5% $CO_2$ (Kumar and Huguenard, 2001).

*MeAIB uptake and amino acid analysis*

For amino acid analysis slices (400 μm thick) were incubated in a humidified oxygenated interface holding chamber with 3 ml of nominally $Mg^{++}$-free ACSF without or with 500 μM glutamine. After incubation for 2 h at 37°C, slices were rapidly washed in ice-cold phosphate-buffered saline and then transferred to a 1.5 ml conical tube with 75% aqueous ethanol heated to 80°C. After vortexing for 20-s samples were incubated 80°C for an additional 5 min and then transferred to ice. Samples were homogenized with 4 cycles of 15 1-s pulses with a tip sonicator (Branson). A volume of 1 μl was set aside for protein measurement and the remainder was centrifuged for 30 min at 15,000×*g* to remove insoluble material. The extracts were dried overnight in a Sorvall SpeedVac and submitted for amino acid analysis (UC Davis Molecular Structure Facility, Davis, CA).

For MeAIB uptake experiments coronal sections (400 μm) containing the undercut were incubated in oxygenated ACSF at 32°C for 1 h then transferred to 10 ml ACSF with 20 nCi/ml $H^3$-MeAIB (American Radiolabeled Chemicals) in a 50 ml conical tube preheated to 37°C in a water bath. After 10-min incubation with gentle agitation and oxygenation with 95% $O_2$/5% $CO_2$, the slices were washed immediately with two changes of cold ACSF on ice. The cortex above the area of undercut was then removed using a scalpel blade and the same region of the cortex on the uninjured contra lateral side was removed as a control and the tissues frozen. Tissues were homogenized in a 1.5 ml tube using a macerating rod with 100 μl PBS then a 5 μl sample was taken to estimate protein quantity using a Bradford assay. The homogenized tissues were lysed with addition of 250 μl 1% SDS, incubated for 10 min at room temperature then mixed in 3 ml scintillation fluid (CytoScint, ICN) for analysis on Beckman LS6000SC gamma counter. The final $^3H$ counts were adjusted for protein quantity.

*Field recordings*

Slices were placed in an interface recording chamber partially submerged in ACSF and superfused continuously (~2.0 ml/min) with ACSF equilibrated with 95% $O_2$, 5% $CO_2$, with temperature maintained at 35°C. Extracellular field potentials were recorded from Layer V of the neocortex by using glass micropipettes (~1 MΩ) filled with ACSF. A bipolar stimulating electrode was placed to stimulate the layer VI/white matter boundary at the frequencies indicated. Each pulse consisted of a 50-μs constant current stimulation. For experiments with MeAIB, osmolality was adjusted by diluting by 3% with water. This modest dilution is expected to have negligible effects on extracellular ion concentrations. As undercut slices showed a wide range of initial activity, those showing either no polyphasic (epileptiform) activity or responses that were insensitive to changes in stimulation frequency were excluded from analysis.

For experiments analyzing frequency-dependent activation of polyphasic activity slices were stimulated at 5-s intervals with varying current ranging from 0 to 300 μA in 10 μA increments to

identify the range that gave rise to polyphasic fields. The mid range current was selected and for slices showing approximately 20–30% polyphasic frequency at 5-s interval, the frequency was recorded at each ISI for at least 50 stimuli from 2.5-, 5-, 10-s intervals and 20 for 15-s interval. For slices in which there was a graded response in success rate to increasing the ISI from 2.5 to 15 s, glutamine was added and after 15-min recordings were again made as above. Experiments with MeAIB were done in an analogous fashion.

## Results

### *Glutamine uptake is increased in the undercut cortex*

The glutamine–glutamate shuttle model predicts that glutamine released from glia and taken up by neurons is the metabolic precursor of synaptically released glutamate. To determine if an increased facility to synthesize and release glutamate might contribute to the hyperexcitability of epileptic tissue, we sought to determine if glutamine and glutamate metabolism are altered in the cortical undercut model of chronic epileptic tissue. As a first step in assessing potential changes in amino acid metabolism in epileptic tissue we measured steady-state amino acid levels in stimulated control and cortical undercut slices in the presence and absence of exogenously applied glutamine. Slices were incubated for 2 h at 35°C in a humidified oxygenated interface holding chamber with $Mg^{++}$-free ACSF without or with 500 μM glutamine. To measure levels of free amino acids the tissues were then homogenized in aqueous ethanol and the extracts were submitted for amino acid analysis. The data demonstrate significant increases in glutamine concentration normalized to protein in response to added glutamine for both control and undercut slices (Fig. 1). However, a much larger increase occurs in the undercut slices (1.5-fold in controls, 2.6 in undercut). Although changes in glutamate and GABA levels were also detected in the undercut slice, these changes were not statistically significant.

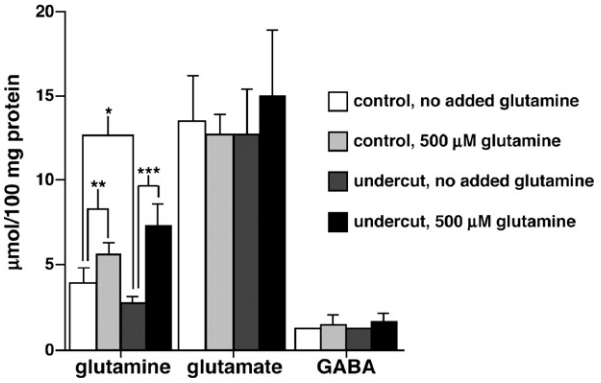


Fig. 1. Exogenous glutamine leads to increased tissue glutamine concentration in control and undercut slices. Amino acid levels were measured in ethanol extracts from control ($n=6$) and undercut slices ($n=6$) incubated in oxygenated interface chambers for 2 h in ACSF with or without 0.5 mM glutamine. Results demonstrate that in the absence of added glutamine, tissue levels of the amino acid are significantly lower ($p<0.05$*) in undercut samples than in controls. There are also statistically significant increases ($p<0.03$** for control and $p<0.0003$*** for undercut) in glutamine levels in both control and undercut tissue in response to exogenous glutamine with a 50% increase in glutamine content in control slices and a 160% increase in undercut slices. Differences in glutamine concentrations in the control versus undercut slices treated with glutamine are statistically significant ($p<0.05$), but glutamate and GABA levels are not significantly altered in either undercut or control slices in response to glutamine.

### *System A and system N transporters are upregulated in undercut cortex*

Anatomical and functional studies have suggested that system N and system A transporters may be involved in glutamine release of from glial cells and uptake into neurons respectively. To determine if the increase in glutamine concentration in the undercut slices might be mediated by increased expression of these transporters we examined mRNA levels of SNAT3 and SNAT5, glial system N transporters, and two system A transporters SNAT1 and SNAT2. We used digoxigenin-labeled antisense RNA probes to identify cells expressing the mRNAs for the transporters. Expression of SNAT3 was increased in the tissue immediately surrounding the lesion of the undercut cortex and we saw no difference in SNAT5 expression (data not shown). GFAP expression increased in similar spatial pattern to that of SNAT3, suggesting that the increase in SNAT3 expression may be related to reactive gliosis. The number of cells expressing high levels of SNAT1 (Fig. 2A) and especially SNAT2 (Fig. 2B) mRNA appear to be increased in the undercut cortex, but in a pattern different from that of SNAT3. The levels of mRNA for both transporters appear to be upregulated in neurons of the deep cortical layers, and not in the tissue immediately surrounding the lesion.

Levels of mRNA may not reflect protein expression. Indeed SNAT2 mRNA has been reported in neurons and glia, but immunostaining suggests little protein expression in glial cells compared to neurons (Gonzalez-Gonzalez et al., 2005; Reimer et al., 2000). Thus to confirm that increases in mRNA expression are associated with increases in transporter protein levels, and to localize the protein, we used immunostaining to assess SNAT1 and SNAT2 in the undercut cortex. Antibodies specific to the rat SNAT1 and SNAT2 proteins were generated by using GST fusion proteins containing the amino terminal regions of the proteins (amino acids 1–63 for SNAT1 and 1–65 for SNAT2) as immunogens in chickens. Western blots of HEK293T cells expressing the recombinant proteins demonstrate that the antibodies are specific with no cross reactivity (data not shown). Indirect fluorescent immunostaining of cortex in undercut animals demonstrate a qualitative increase in SNAT1 and SNAT2 expression compared to contralateral control cortex (Fig. 3A). There appears to be a greater increase in SNAT2 expression and this is supported by western blot analysis of samples isolated from 3 control and undercut cortex samples (Fig. 3B). The increased immunostaining for both proteins appears to be restricted to neurons, and predominantly in the deep cortical layers, similar to increases seen in the mRNA expression for the transporters.

To confirm that the increased expression of SNAT1 and SNAT2 proteins is associated with an increase in the transport activity of these proteins we examined the uptake of methylamino isobutyric acid (MeAIB), an amino acid analog with a specificity for system A transporters (Christensen et al., 1965). Using a radiotracer uptake assay (see Methods) we found a 1.27±0.17-fold increase in MeAIB uptake in undercut cortex compared to the contralateral control cortex ($p<0.001$, $n=8$ slices from 3 animals, data not shown). This parallels our findings with steady-state glutamine levels and is consistent with increased glutamine uptake in the undercut cortex resulting from and upregulation of SNAT1 and/or SNAT2 expression and activity.

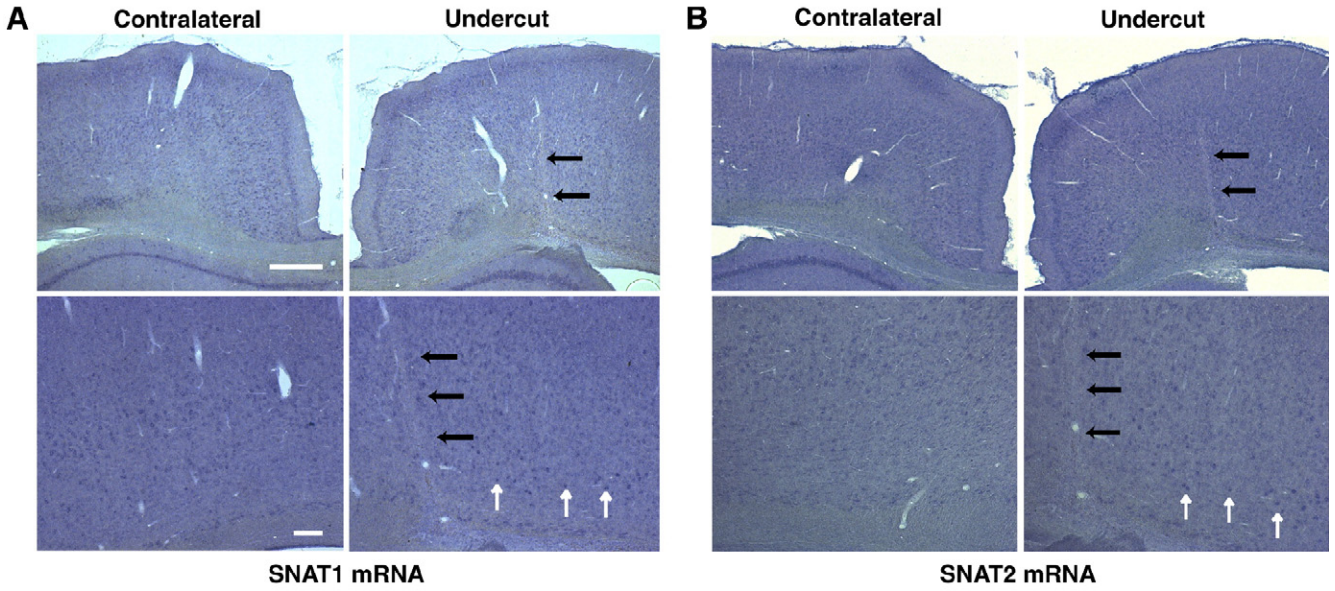


Fig. 2. System A and system N transporters are upregulated in undercut cortex. Digoxigenin-labeled probes for mRNAs of the system A transporters SNAT1 (A) and SNAT2 (B) were used to assess expression of the transporter genes by in situ hybridization. Examination of cortex adjacent to the undercut lesion (right panels, black arrows) demonstrates an increased signal in deep cortical layers (white arrows) when compared to the contralateral control cortex (left panels) for both transporter mRNAs. The scale bars indicate 500 μm for the low power images and 100 μm for high power images.

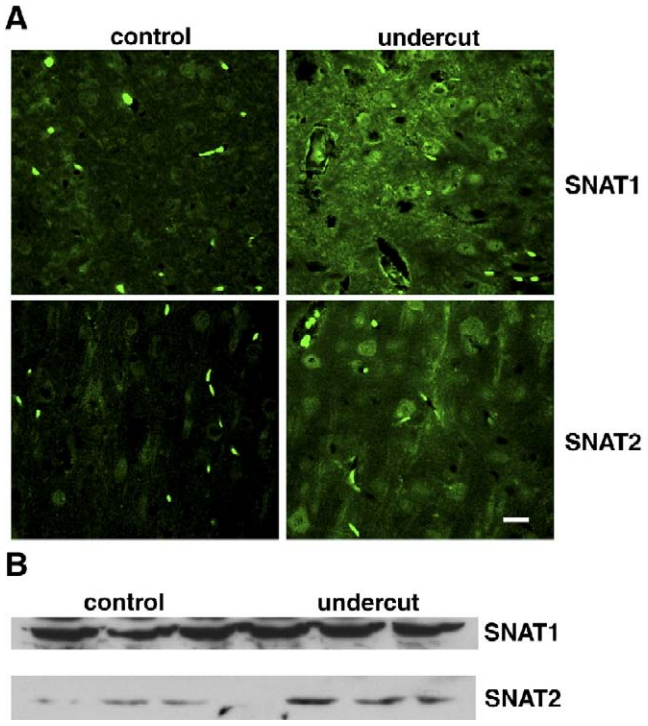


Fig. 3. SNAT1 and SNAT2 protein are increased in the undercut cortex. Immunostaining with SNAT1 and SNAT2 specific antibodies (A) demonstrates a marked increase in SNAT1 (upper panels) and SNAT2 (lower panels) immunoreactivity in layer V neurons of undercut cortex (right panels) compared to contralateral control cortex in the same sections (left panels). With the same antibodies Western blotting of protein extracts from 3 control (left) and 3 undercut cortex samples each from individual animals demonstrate a more marked increase in SNAT2 (lower panel) compared to SNAT1 (upper panel). For immunofluorescence images scale bar indicates 20 μm.

*Glutamine increases the size of evoked field potentials in undercut and control cortex*

Since glutamine is the immediate metabolic precursor of synaptically released glutamate, it follows that the addition of exogenous glutamine may alter the electrophysiological characteristics of rat brain slices. Further, the increased expression and activity of SNAT1 and SNAT2 in undercut cortex suggests that the injured cortex may be prone to augmented hyperexcitability in the presence of glutamine. To determine if addition of glutamine alters evoked responses in control and/or undercut cortex, we measured the field potentials triggered by stimuli given every 30 s in control and undercut cortex (Fig. 4). In the absence of glutamine field potentials are markedly larger and more complex in the undercut compared to the control cortex as previously reported (Prince and Tseng, 1993). With the addition of glutamine a graded increase in the size of the short latency primary component of the evoked potentials was seen in the control cortex with increasing concentrations of glutamine through 1 mM (twice the physiologically relevant concentration). In the undercut cortex an increase in the size of the primary evoked potentials occurs in response to 250 μM glutamine, but higher concentrations, led to generation of spreading depression events (see below) that precluded measurement of stable evoked potentials.

*Exogenous glutamine facilitates late polyphasic potentials in undercut cortex*

Late polyphasic potentials (Fig. 5A, success) are typically seen following stimulation of the underlying white matter in undercut but not naïve cortex (Prince and Tseng, 1993). The likelihood that these potentials will occur in the undercut cortex is influenced by the rate of stimulation such that the percent of stimuli that lead to these late potentials is reduced as stimulation rate is increased (Prince and Tseng, 1993). To determine if exogenous glutamine can influence

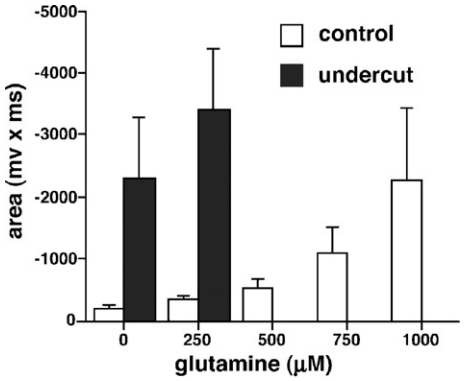


Fig. 4. Glutamine increases the size of primary evoked potentials in control and undercut cortex. Measurements of mean primary evoked response area (mV × ms) from control and undercut slices demonstrate larger potentials in the undercut slices in the absence of glutamine. In both control and undercut slices addition of glutamine leads to increases in the size of evoked potentials. At concentrations of 500 μM and above glutamine induced spreading depression like events in undercut cortex and precluded an appropriate analysis. Data were collected from four independent slices for each group. The primary evoked response was defined as that occurring within 50 ms of the stimulus.

the refractory period of late polyphasic potentials, we determined the proportion of stimuli leading to polyphasic potentials (referred to as successes) over a range of interstimulus intervals (ISI) in the presence and absence of exogenous glutamine (Fig. 5B). In standard ACSF ISIs between 2.5 and 15 s typically yield a graded range of success rates with longer ISIs associated with a higher likelihood of polyphasic potentials. With the addition of glutamine, we found an increase in the success rate at a given ISI resulting in a leftward shift of the ISI versus success rate plot. This led to a decrease in the ISI predicted to give a 50% success rate (50% ISI) from 8.2 s for ACSF to 5.9 s for ACSF with glutamine. Due to difference in intrinsic properties from slice to slice, the success rate at a given ISI can be quite varied. We therefore did a paired analysis of the 50% ISI for each slice treated without and with glutamine. In this analysis the 50% ISI was consistently and significantly reduced (Fig. 5C, Student's *t*-test, $p < 0.01$).

*MeAIB attenuates the effect of exogenous glutamine in the undercut cortex*

The increased expression of SNAT1 and SNAT2 and increased MeAIB uptake in the lesioned cortex suggest that the effect of glutamine on the electrophysiology may be mediated by these transporters. To address this we examined the effect of MeAIB on the incidence of late polyphasic potentials in undercut cortex. Addition of MeAIB had a dramatic effect on polyphasic potentials, leading to a marked reduction in the frequency in all slices, even at prolonged ISIs (Fig. 5B).

An additional effect of exogenous glutamine that was evident was a marked increase in the frequency of spreading depression like events characterized by large (several mV) and prolonged (several tens of seconds) field potentials (e.g., Fig. 6D). In the absence of exogenous glutamine these events occurred rarely, but with addition of 500 μM glutamine spreading depression events were regularly observed (Fig. 6A) in undercut slices. To determine if this effect of glutamine might also be sensitive to the effects of MeAIB, the

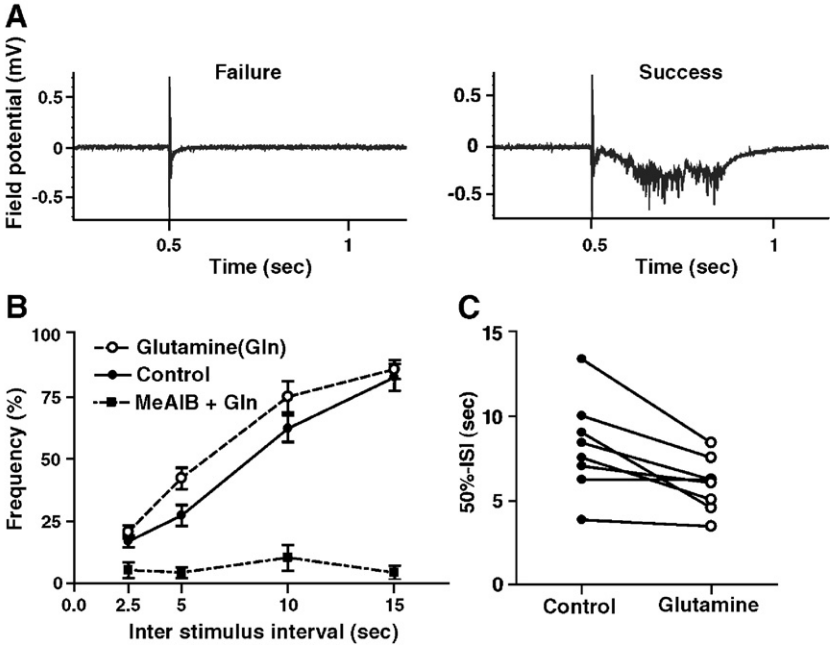


Fig. 5. Glutamine and MeAIB have opposing effects on the likelihood of polyphasic potentials in undercut cortex. (A) Sample evoked potentials from undercut cortex field recordings demonstrate a simple monophasic response either without (failure, left) or with (success, right) a late polyphasic response. (B) Incidence of polyphasic events were determined in undercut slices in response to varying interstimulus intervals (ISIs) and addition of glutamine or glutamine and MeAIB. Slices were exposed to either 250 μM glutamine or 250 μM glutamine with 10 mM MeAIB in the ACSF ($n = 5$ from 4 animals). Addition of glutamine increased the incidence of polyphasic events causing a leftward shift in the curve indicating a decrease the refractory period. By contrast, addition of MeAIB almost completely blocked polyphasic activity. (C) The interval evoking 50% successes (50% ISI) was consistently shortened by 250 μM glutamine, as shown in this paired analysis. The 50% ISI decreased from 8.2 s to 5.9 s. Student's two-tailed *t*-test $p < 0.01$, $n = 8$ slices from 4 animals.

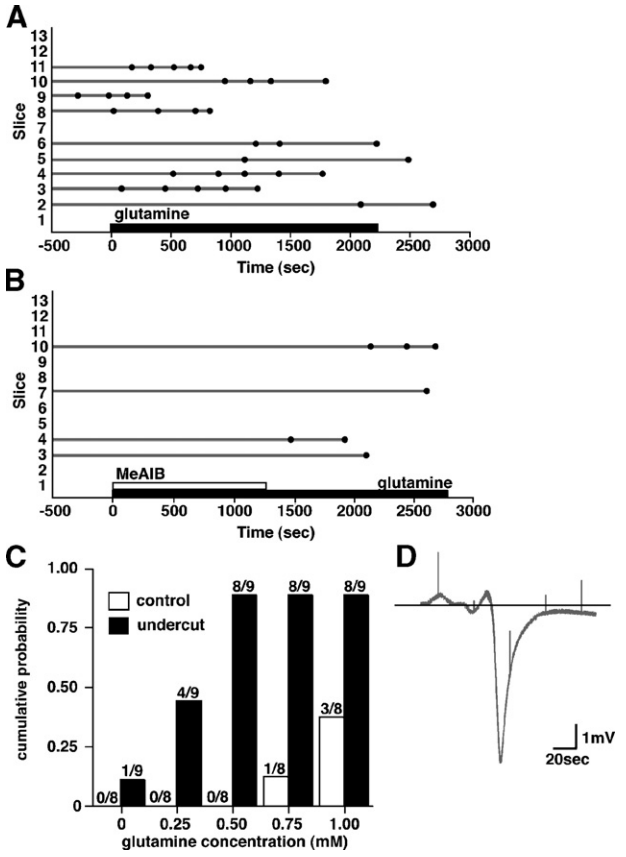


Fig. 6. Glutamine induces spreading depression like events, which are attenuated by MeAIB. (A) Analysis of undercut slices demonstrates frequent large negative field potentials (e.g., panel D) after the addition of 500 μM glutamine in 9 of 13 undercut slices from 8 animals. (B) This effect is attenuated by the addition of MeAIB (5 mM) with no events in any of 13 slices during 20-min recording periods. After washout of MeAIB in the continued presence of 500 μM glutamine, 4 slices exhibit at least one large depolarization event. Each slice was recorded for a total of 45 min after initial exposure to glutamine. Cumulative event analysis demonstrates spreading depression like events in control and undercut cortex, with events appearing more frequently as glutamine concentration is increased. Field recordings were monitored for 10 min at each glutamine concentration. (D) Sample tracing of a spreading depression event depicts typical negative potential lasting ~30 s. Brief transient events are stimulus artifacts associated with evoked synaptic potentials.

incidence of these events was determined in the presence of 5 mM MeAIB in addition to glutamine. With MeAIB treatment no large field potentials were detected in a total of 13 slices, each monitored for 20 min (Fig. 6B). During a 20-min MeAIB washout period (500 μM glutamine, no MeAIB) large spontaneous field potentials were noted in 4 slices, indicating that the slices were capable of exhibiting such activity. Interestingly, spreading depression-like events were also seen in naïve cortex, but only at supraphysiological concentrations of glutamine (Fig. 6C).

## Discussion

Glutamine is the most abundant amino acid in cerebrospinal fluid and, as an intermediate in the glutamine–glutamate cycle, it is likely to influence the synaptic release of the excitatory neurotransmitter glutamate. While a shift towards excitability is thought to underlie epilepsy the role of the glutamine and the glutamine–glutamate cycle in epilepsy is unclear. Here using an established model of post-traumatic hyperexcitability, we present data indicating that glutamine metabolism is altered in the injured cortex and that availability of glutamine can enhance the abnormal excitability characteristic of this model.

Although the concentration of glutamine in cerebrospinal fluid is in the range of several hundred micromolar (Fishman, 1992; Lerma et al., 1986), it is not typically included in the ACSF used in slice electrophysiology. For preparations in which glutamate release is enhanced this may be of concern. Indeed it has been previously shown that there is time-dependent loss of glutamine and to a lesser extent glutamate and GABA in hippocampal slices incubated in ACSF and that addition of exogenous glutamine can prevent this loss (Kapetanovic et al., 1993). Similar to these results we found that perfusion with exogenous glutamine increases glutamine content in neocortical slices. We also found that the effect of exogenous glutamine was more dramatic in the undercut cortex. We did not, however, see any changes in the glutamate or GABA levels in response to exogenous glutamine. This may be due, in part, to the fact that our slices were incubated in low $Mg^{++}$ ACSF, so increased glutamate and GABA synthesis from exogenous glutamine may have led to an increase in release, without any significant change in tissue concentrations.

Several transport systems for glutamine exist on neurons and glia (Broer and Brookes, 2001). In the glutamine–glutamate cycle glutamine is released from glial cells and taken up by neurons. The electroneutral system N transporters are readily capable of bidirectional flux and can thus mediate the glial release of glutamine while the system A transporters are electrogenic and can generate large glutamine concentration gradients across the neuronal plasma membrane (Mackenzie and Erickson, 2004). The localization of the system A transporters SNAT1 and SNAT2 to neuronal cell bodies and metabolic labeling experiments coupled with system A transporter inhibition with MeAIB have led to suggestions that SNAT1 and SNAT2 may not be the primary neuronal glutamine transporters in the glutamine–glutamate cycle (Conti and Melone, 2006; Rae et al., 2003). However, in an acute slice model of hyperexcitability, MeAIB significantly attenuated epileptiform activity (Bacci et al., 2002), suggesting that system A transporters may play a role in states of sustained glutamate release. Our finding that SNAT1 and SNAT2 expression are markedly increased in the undercut cortex is consistent with a role in an upregulated capacity to release glutamate.

The ability of glutamine to increase the size of evoked potentials in control and undercut cortex suggests that glutamine may be feeding in to the glutamine–glutamate cycle to generate more glutamate for release. This implies that under conditions normally used to study electrophysiology in slices, glutamine is deficient even for naïve slices. Perfusion of slices, particularly if they are stimulated to produce intense response, likely leads to a loss of glutamine and glutamate that are extracellular as they transit through the cycle (Kapetanovic et al., 1993). Addition of glutamine to perfusate permits continued glutamate synthesis and release even while much of the released glutamate is washed out. An alternative explanation for the effect of glutamine is that it is leading to a partial depolarization of the neurons as previously reported (Chaudhry et al., 2001). However, MeAIB, which also leads to a partial depolarization of neurons through the same mechanism, appears to have effects opposite those of glutamine.

In the undercut slices as the frequency of stimuli is increased, the likelihood that a polyphasic epileptiform potential will be generated is decreased (Prince and Tseng, 1993). The mechanism for this refractoriness is unclear, but it is likely that some recovery period is necessary for neurons to reform and refill synaptic vesicles. Since with long ISIs robust polyphasic potentials can be seen with nearly every stimulus in the absence of exogenous glutamine, the neocortical networks must be capable of sustained epileptiform firing. This suggests that the glutamine and glutamate are not completely washed out with perfusion, or that alternative pathways for glutamate synthesis can keep up with a slower rate of release. Our finding that addition of glutamine increases the frequency of successful polyphasic potentials over a range of ISIs suggests that exogenous glutamine can enhance synthesis and release of glutamate. It also suggests that glutamine–glutamate availability may be a limiting factor for excitatory neurotransmission under conditions of sustained and rapid firing. Further, our finding that the system A transporter inhibitor MeAIB markedly reduces the frequency of polyphasic potentials even at very prolonged ISIs suggests that in the absence of exogenously applied glutamine neuronal uptake of glutamine contributes to glutamate release. It is likely that even with perfusion the glutamine–glutamate cycle is still partially intact.

Spreading depression is a well documented but poorly understood phenomenon that has been implicated in migraines, stroke, head trauma and even epilepsy (Somjen, 2001). While the implications of these events are unclear, it has been suggested that they can induce neuronal damage. In undercut cortex, spreading depression like events occurred in some slices following addition of glutamine. We found that increases in glutamine produced progressively larger increases in the incidence of spreading depression events while MeAIB completely blocks this effect. These findings suggest that, as with the effect of glutamine on evoked potentials, system A transporters are necessary. This again suggests that glutamine may be feeding into the glutamine–glutamate cycle to enhance the synthesis and release of glutamate.

In characterizing the effects of glutamine on the electrophysiology of cortex in the undercut model of post traumatic hyperexcitability we have found that glutamine can enhance the phenotype of this model. The ability of MeAIB to block the effects of glutamine strongly suggests that system A transporters are necessary for the effect of glutamine. This is further supported by the upregulation of SNAT1 and SNAT2 in the injured cortex. It has been demonstrated that the glutamine–glutamate shuttle influences GABA synthesis and release as well (Liang and Coulter, 2004; Sepkuty et al., 2002). Our findings of increased excitability in response to glutamine suggest that in the undercut model the effect of glutamine on the synthesis of glutamate and GABA may be skewed towards glutamate.

Although blocking the glutamine–glutamate shuttle can inhibit synthesis of glutamate and GABA, the shuttle also serves as the primary mechanism for clearing glutamate from synapses (Danbolt, 2001; Eid et al., 2004; Nicholls and Attwell, 1990; Petroff et al., 2002; Storm-Mathisen et al., 1986; Zhou and Sutherland, 2004). Thus blocking the shuttle at different steps may have profoundly different effects on excitability. Indeed this may explain how in temporal lobe epilepsy glutamine synthetase, a main component of the shuttle appears to be down regulated (Eid et al., 2004) while inhibition of the shuttle in animals and slices can attenuate seizures. While the role of system A transporters in normal physiology remains unclear, our findings strongly suggest that these transporters may be important in the setting of post-traumatic hyperexcitability and suggest that inhibiting these transporters may be a rational approach to seizure management in the setting of post-traumatic epilepsy.

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