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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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Abstract

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 increases 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).

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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-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, slice 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 120–135° 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. An additional transcortical lesion was placed ~2 mm lateral and parallel to the parasagittal cut in a similar manner. 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 wk later.

Preparation of sections for in situ and immunohistochemistry

Rats were anaesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Brains were blocked and postfixed in 4% paraformaldehyde for 24 hours 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 histochemistry or in 0.1M 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 hours at 65°C. Hybridization buffer was removed and fresh hybridization buffer with 1µg/ml of probe was placed over the sections. Slides were incubated at 65°C for 16–20 hours in a humidified chamber, washed and probed with an alkaline phosphatase conjugated anti-digoxigenin antibody (Roche). After washing the slices were incubated in 1µl/ml of NBT (Nitro blue tetrazolium chloride - Roche) and 3.5µl/ml of BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt - Roche) and allowed to develop in the dark for between 2–24 hours, depending on the abundance of the RNA. Once an adequate level was reached the slides were washed twice in H₂O to remove substrates, then fixed in formaldehyde for 60 minutes at room temperature. Coverslips were mounted with Aquamount (Polysciences, Warrington, PA).

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, South San Francisco) and IgY was purified with a commercial kit (Pierce Rockford, IL) 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 minutes, washed extensively in blocking buffer. The sections were rinsed twice in PBS, mounted in medium (Vectashield; Vector, Burlingame, CA), 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 1X PBS containing 0.1% Tween-20 and 1% nonfat dry milk after blocking in 1X 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 minutes.

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 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, and 0.5 CaCl₂ bubbled with 95% O₂/5% CO₂. The whole brain was glued to a cover slip and 400 µm coronal slices were made using a vibratome (TPI, St. Louis, MO). Slices were incubated in ACSF containing (in mM): 126 NaCl, 26 NaHCO₃, 2.5–5 KCl, 1.25 NaHPO₄, 2 MgCl₂, 2 CaCl₂, and 10 glucose at 32°C for 1 hour prior to recording. The solution was continuously bubbled with 95% O₂/5% CO₂ (Kumar and Huguenard, 2001).

MeAIB uptake and amino acid analysis

For amino acid analysis slices (400 μ m thick) were incubated in a humidified oxygenated interface holding chamber with 3 ml of nominally Mg⁺⁺ free ACSF without or with 500 μ M glutamine. After incubation for 2 hrs 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 seconds samples were incubated 80°C for an additional 5 minutes and then transferred to ice. Samples were homogenized with 4 cycles of 15 one second pulses with a tip sonicator (Branson). A volume of 100 μ l was set aside for protein measurement and the remainder was centrifuged for 30 minutes at 15,000g 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 μ m) containing the undercut were incubated in oxygenated ACSF at 32°C for 1h then transferred to 10ml ACSF with 20nCi/ml H³-MeAIB (American Radiolabeled Chemicals) in a 50ml conical tube preheated to 37°C in a water bath. After 10min incubation with gentle agitation and oxygenation with 95% O₂/5% CO₂, 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 1.5ml tube using a macerating rod with 100 μ l PBS then a 5 μ l sample was taken to estimate protein quantity using a Bradford assay. The homogenized tissues were lysed with addition of 250 μ l 1% SDS, incubated for 10min at room temperature then mixed in 3ml scintillation fluid (CytoScint, ICN) for analysis on Beckman LS6000SC gamma counter. The final H³ 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 % O₂, 5 % potentials were recorded CO₂, with temperature maintained at 35°C. Extracellular field from Layer V of the neocortex by using glass micropipettes (~1 M Ω) filled with ACSF. A concentric bipolar stimulating electrode was placed to stimulate the underlying white matter at the frequencies indicated. Each pulse consists of a 50 μ s constant current stimulation. For experiments with 10mM 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 polyphasic frequency slices were stimulated at 5 second intervals with varying current ranging from 0~300 μ A in 10 μ A increments to identify the range that gave rise to polyphasic fields. The mid range current was selected and for slices showing approximately 20–40% polyphasic frequency at 5 sec interval, the frequency was recorded at each ISI for at least 50 stimuli from 2.5, 5, 10, and 15sec. For slices in which there was a graded response in success rate to increasing the ISI from 2.5 to 15 sec, glutamine was added and recordings were again made at ISIs of 2.5, 5, 10 and 15 sec for at least 50 stimuli. 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 hours at 35°C in a humidified oxygenated interface holding chamber with Mg⁺⁺ free ACSF without or with 500µM 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 (figure 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.

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 (Figure 2A) and especially SNAT2 (Figure 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 (figure 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 (figure 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.

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 first 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 seconds in control and undercut cortex (figure 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 μ M 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 (figure 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 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 (figure 5B). In standard ACSF ISIs between 2.5 and 15 seconds typically yield a graded range of success rates with longer ISIs associated with success rates of ~70–100%. 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 vs success rate plot. This led to a decrease in the ISI predicted to give a 50% success rate (50%-ISI) from 8.2 seconds for ACSF to 5.9 seconds 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 (figure 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 (figure 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 10s of seconds) field potentials (e.g. figure 6D). In the absence of exogenous

glutamine these events occurred rarely, but with addition of 250 μ M glutamine spreading depression events were regularly observed (figure 6A) in undercut slices. To determine if this effect of glutamine might also be sensitive to the effects of MeAIB, the incidence of these events was determined in the presence of 10mM MeAIB in addition to glutamine. With MeAIB treatment no large field potentials were detected in a total of 13 slices, each monitored for 20 minutes (figure 6B). During a 15 min MeAIB washout period (250 μ M 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 (figure 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 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 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 synthesis of glutamate from 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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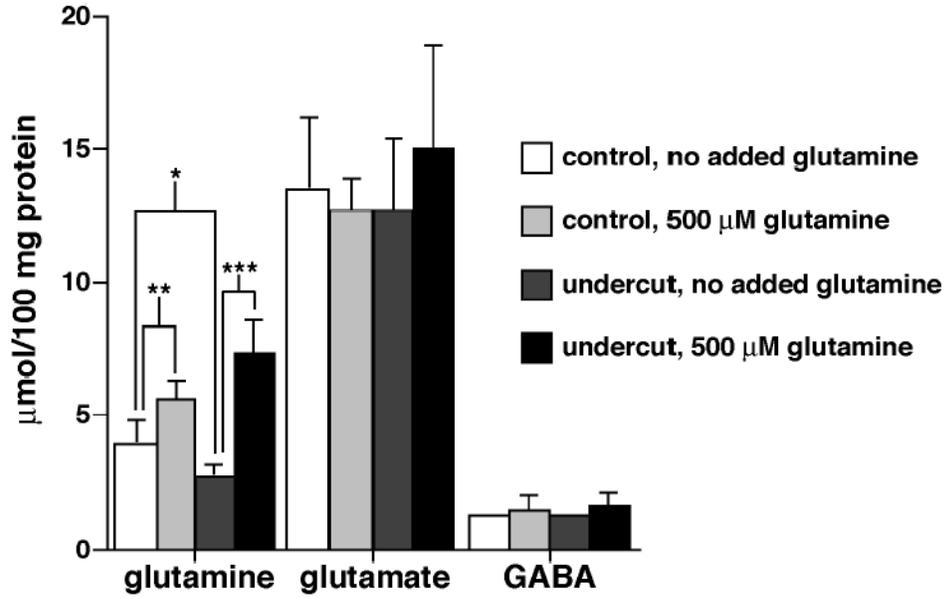


Figure 1. Exogenous glutamine leads to an glutamine concentration in control and undercut slices Amino acid levels were measured in ethanol extracts from control (n=6) and undercut slices (n=6) incubated in oxygenated interface chambers for 2 hours in ACSF with or without 0.5 mM glutamine. Results demonstrate 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.

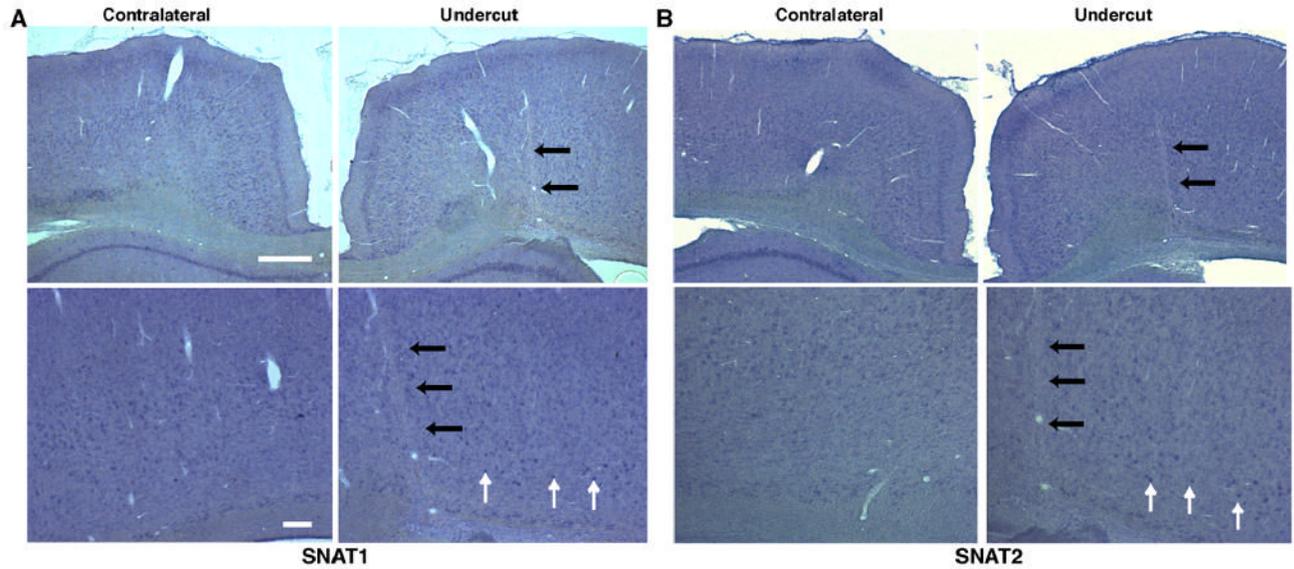


Figure 2. System A and System N transporters are upregulated in undercut cortex
 Digoxigenin labeled probes for mRNAs of the system A transporters SNAT1 (A) and SNAT2 (B) were used to assess expression of the transporter genes by in situ hybridization. Examination of cortex adjacent to the undercut lesion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

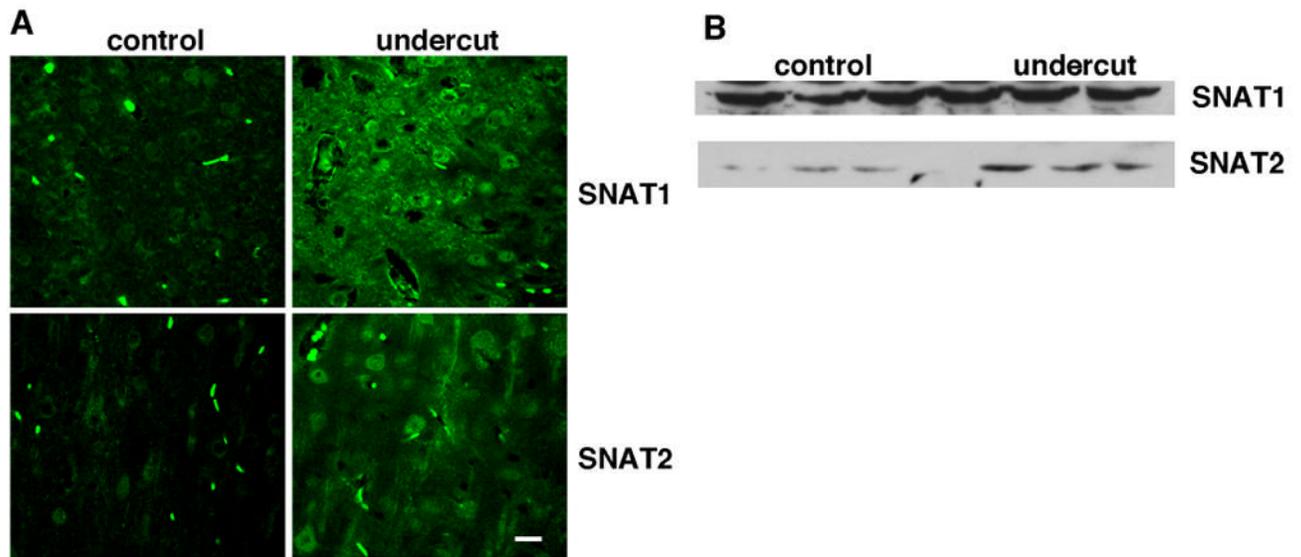


Figure 3. SNAT1 and SNAT2 protein are increased in the undercut cortex

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 μ m.

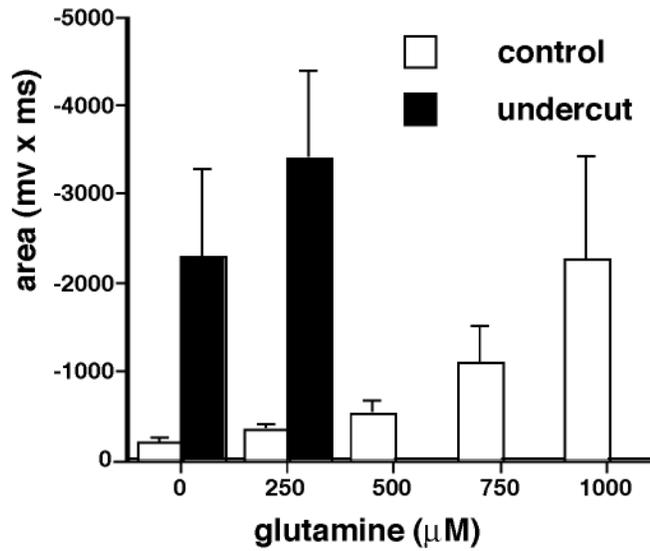


Figure 4. Glutamine increases the size of primary evoked potentials in control and undercut cortex Measurements of mean primary evoked response area ($\text{mV} \times \text{ms}$) from control and undercut slices demonstrate larger potentials in the undercut slices in the absence of glutamine. Addition of glutamine leads to increases for both with the size of the potentials increasing with glutamine concentrations of up to 1mM . At concentrations of $500\mu\text{M}$ 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 $50\mu\text{s}$ of the stimulus.

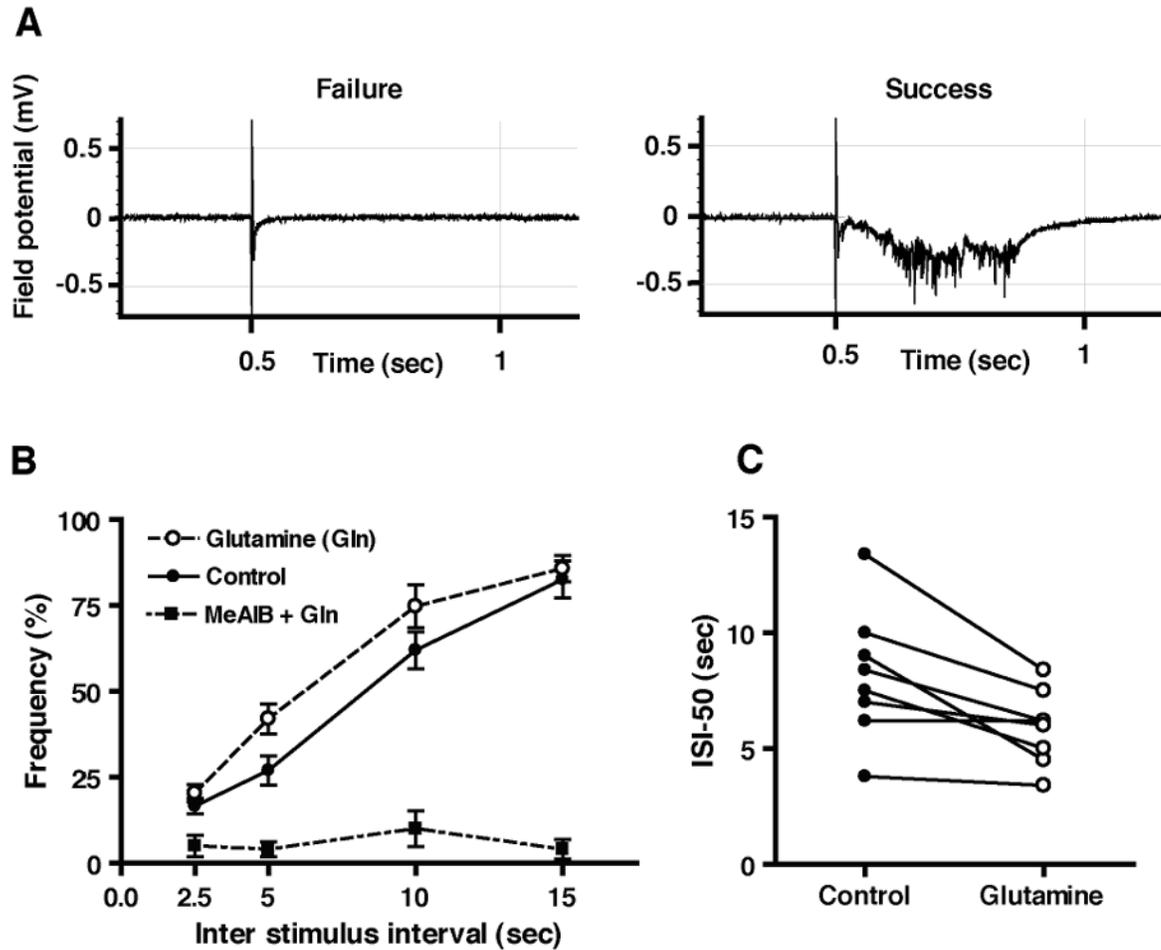


Figure 5. Glutamine and MeAIB have opposing effects on the likelihood of polyphasic potentials in undercut cortex

Sample evoked potentials from undercut cortex field recordings (A) demonstrate a simple monophasic response either without (failure, left) or with (success, right) a late polyphasic response. B) Incidence of polyphasic events in undercut slices in response to varying interstimulus intervals (ISIs). Slices were exposed to either 250 μ M glutamine or 250 μ M glutamine with 10mM 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 (ISI-50) was consistently shortened by 250 μ M glutamine, as shown in this paired analysis. The ISI-50 decreased from 8.2 seconds to 5.9 seconds. Student's two-tailed t-test $p < 0.01$, n=8 slices from 4 animals.

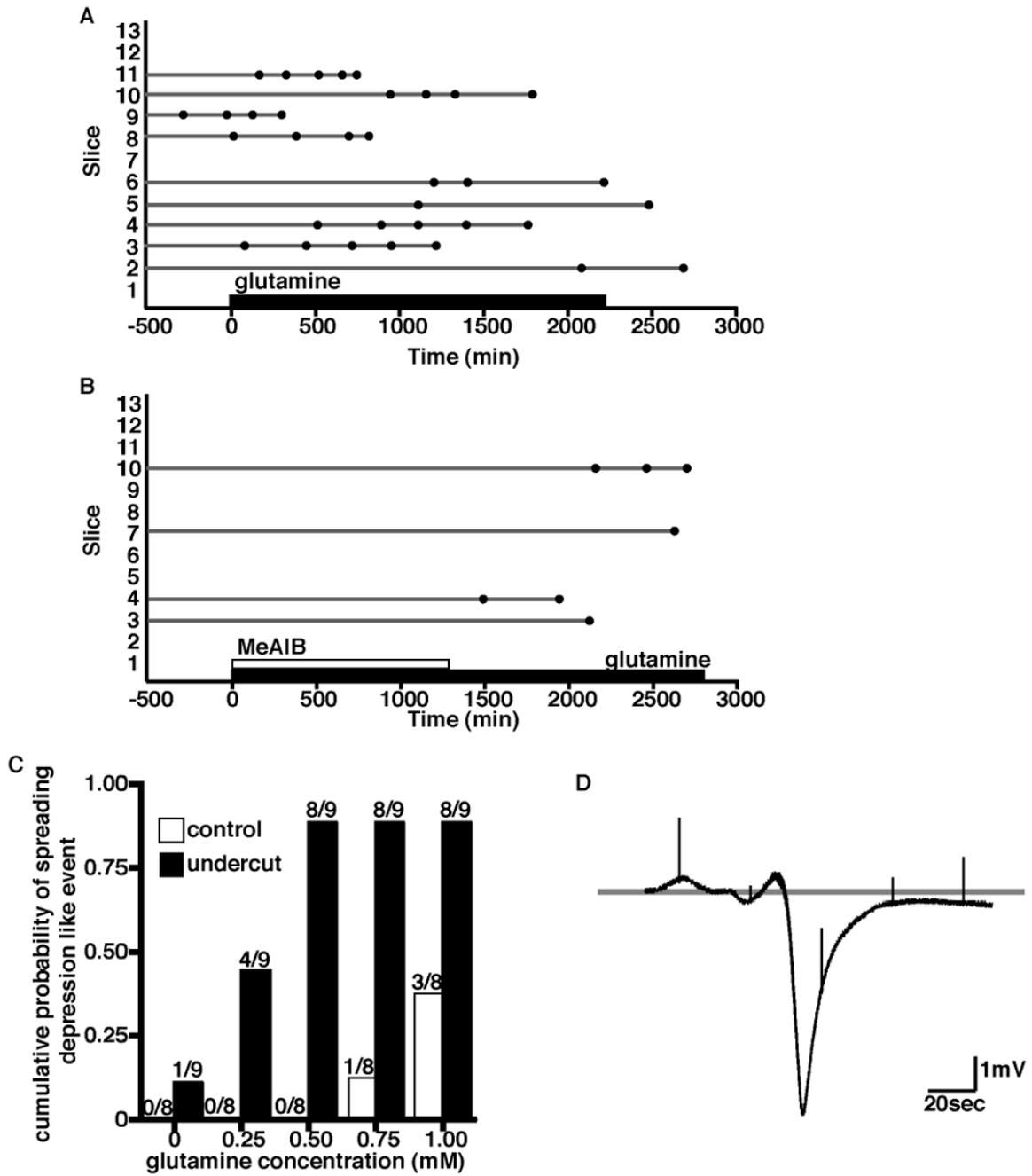


Figure 6. Glutamine induces spreading depression like events, which are attenuated by MeAIB

A) Analysis of undercut slices demonstrates frequent large negative field potentials (e.g. panel D) after the addition of 250 μ M glutamine in 9 of 13 undercut slices from 8 animals. B) This effect is attenuated by the addition of MeAIB (10mM) with no events in any of 13 slices during 20 minute recording periods. After washout of MeAIB in the continued presence of 250 μ M glutamine, 4 slices exhibit at least one large depolarization event. Each slice was recorded for a total of 45 minutes 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 sec. Brief transient events are stimulus artifacts associated with evoked synaptic potentials.