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Glutamate spillover augments GABA synthesis and release from axodendritic synapses in rat hippocampus

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Abstract

Tight coupling between GABA synthesis and vesicle filling suggests that the presynaptic supply of precursor glutamate could dynamically regulate inhibitory synapses. Although the neuronal glutamate transporter Excitatory Amino Acid Transporter 3 (EAAT3) has been proposed to mediate such a metabolic role, highly efficient astrocytic uptake of synaptically released glutamate normally maintains low extracellular glutamate levels. We examined whether axodendritic inhibitory synapses in *stratum radiatum* of hippocampal area CA1, which are closely positioned among excitatory glutamatergic synapses, are regulated by synaptic glutamate release via presynaptic uptake. Under conditions of spatially and temporally coordinated release of glutamate and GABA within pyramidal cell dendrites, blocking glial glutamate uptake enhanced quantal release of GABA in a transporter-dependent manner. These physiological findings correlated with immunohistochemical studies revealing expression of EAAT3 by interneurons and uptake of D-aspartate into putative axodendritic inhibitory terminals only when glial uptake was blocked. These results indicate that spillover of glutamate between adjacent excitatory and inhibitory synapses can occur under conditions when glial uptake incompletely clears synaptically released glutamate. Our anatomical studies also suggest that perisomatic inhibitory synapses, unlike synapses within dendritic layers of hippocampus, are not capable of glutamate uptake and therefore transporter-mediated dynamic regulation of inhibition is a unique feature of axodendritic synapses that may play a role in maintaining a homeostatic balance of inhibition and excitation.

Introduction

Regulation of synaptic vesicle filling is recently gaining recognition as a fundamental mechanism of synaptic plasticity (Edwards, 2007). Release of single vesicles does not appear to saturate post-synaptic GABA_A receptors at inhibitory synapses (Nusser et al., 2001), including those on pyramidal neurons in hippocampal area CA1 (Hajos et al., 2000), allowing for modulation of synaptic strength due to changes in vesicle content. GABA synthesis and vesicle filling are tightly coupled; GABA that is newly synthesized from glutamate is packaged preferentially over preformed GABA (Jin et al., 2003). Therefore, the supply of glutamate to synaptic terminals plays an important role in the regulation of vesicular GABA content. However, the mechanisms that control the glutamate supply to inhibitory synaptic terminals are only beginning to be understood.

Glutamate can be taken up directly from the extracellular space by neurons or converted from glutamine intracellularly. Recent electrophysiological data demonstrate that uptake of extracellular glutamate (Hartmann et al., 2008; Mathews and Diamond, 2003) and glutamine (Fricke et al., 2007; Liang et al., 2006) by membrane transporters on inhibitory neurons of the hippocampus dynamically regulates vesicle filling and synaptic strength via GABA metabolism. Several lines of evidence are consistent with a role for Excitatory Amino Acid Transporter 3 (EAAT3) in supplying glutamate to inhibitory synaptic terminals. EAAT3 mRNA is expressed in GABAergic neurons of the hippocampus (Berger and Hediger, 1998; Kugler and Schmitt, 1999), and EAAT3 immunoreactivity is present on inhibitory synaptic terminals (He et al., 2000; Rothstein et al., 1994). Moreover, antisense knockdown of EAAT3 in rat brain *in vivo* decreases tissue GABA levels and impairs new GABA synthesis (Sepkuty et al., 2002).

Despite the mounting evidence for an important role of presynaptic EAAT3 in regulating inhibitory transmission, its precise function has not been determined. Glutamate uptake is unlikely to play a constitutive role in GABA synthesis because extracellular glutamate normally is maintained at very low levels due to the efficiency of astrocytic uptake using EAAT2 and, to a lesser extent, EAAT1 (Lehre and Danbolt, 1998). We hypothesize that temporally- and spatially-limited fluctuations in extracellular glutamate can dynamically regulate GABA synthesis and inhibitory synaptic strength at terminals expressing EAAT3. This regulation may constitute a feedback mechanism to meet a higher demand for GABA in response to increased excitation at the microcircuit level. The hippocampus has unique anatomical features that may favor such an interaction due to the close proximity of GABAergic and glutamatergic synapses along the dendrites of pyramidal neurons (Megias et al., 2001). Although excitatory synapses are located on dendritic spines while inhibitory synapses are on shafts, there often are no intervening glial processes that would create a barrier to the spillover of glutamate between these synapses (Lehre and Danbolt, 1998).

Neither the expression pattern of EAAT3 on interneurons nor the ability of synaptically released glutamate to regulate GABA metabolism in neighboring synapses has been explored previously. In this study, we asked whether synaptically-released glutamate can enhance neurotransmitter GABA synthesis and synaptic strength selectively within the inhibitory terminals of area CA1 originating from interneurons of the axodendritic class, as compared with those from axosomatic interneurons, whose terminals are distant from excitatory synapses. We also tested whether hippocampal dendritic inhibitory synapses possess a unique ability to take up glutamate due to their expression of glutamate transporters. We provide evidence that temporally and spatially-specific enhancement of GABAergic transmission by excitatory synapses occurs in the rodent hippocampus. We conclude that this unique form of intersynaptic crosstalk is due not only to the anatomical proximity of synapses but also to a selective capability for glutamate uptake in dendritic versus somatic inhibitory synapses.

Methods

Brain slice preparation

Seven to 14 day-old Sprague Dawley rats were deeply anesthetized with isoflurane and decapitated under a protocol approved by Vanderbilt University's Institutional Animal Care and Use Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Hippocampi were removed and cut into 400 μ M sections (Vibratome Company, St. Louis, MO) in an ice-cold oxygenated solution containing (in mM): sucrose 75, NaCl 87, KCl 2.5, MgCl₂ 7, CaCl₂ 0.5, NaH₂PO₄ 1.25, glucose 25, and NaHCO₃ 25. Slices were transferred to a chamber containing artificial cerebrospinal fluid (ACSF), which contained (in mM): NaCl 119, CaCl₂ 2.5, KCl 2.5, MgCl₂ 1.3, Na₂PO₄ 1, NaHCO₃ 26.2 and glucose 11 (pH 7.4, 290

mOsm), and bubbled with 95% O₂/5% CO₂. Slices were incubated at 35°C for 30 minutes, then at room temperature for 30 minutes before recording.

Electrophysiology

Slices were constantly perfused at approximately 2 mL/min with ACSF bubbled with 95% O₂/5% CO₂. Patch pipettes with resistances of 3–4 MΩ were filled with a solution containing (in mM): cesium chloride 130, HEPES 10, EGTA 10, lidocaine N-ethyl bromide (QX-314) 1, Na⁺-ATP 0.2, and Mg²⁺-ATP 2 (pH 7.4, 290 mOsm). Using an upright fixed stage microscope (Zeiss, Thornwood, NY) pyramidal neurons in area CA1 were identified under video observation using infrared differential interference contrast imaging. Whole cell recordings were obtained from pyramidal neurons at a holding potential of −60 mV with a MultiClamp 700A amplifier (Molecular Devices Corporation, Sunnyvale, CA). Currents were filtered at 2 kHz, sampled at 5 kHz (20 kHz for EPSCs) and digitally stored for offline analysis. Access resistance was monitored throughout the experiment and data were discarded if change was more than 20%. A bipolar stainless steel electrode was placed in *stratum radiatum* (constant current stimulation, 25–100 μA, 100 μs duration) where monosynaptic inhibitory post-synaptic currents (IPSCs) could be evoked simultaneously with excitatory post-synaptic currents (EPSCs) from the Schaffer collateral fibers. The currents became pure IPSCs after addition of 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM) and dizocilpine maleate (MK-801, 5 μM) to block postsynaptic glutamate receptors. Evoked IPSCs were abolished in the presence of the GABA_A antagonist SR95531 (data not shown).

To measure quantal release from stimulated inhibitory terminals, the normal ACSF was replaced with ACSF in which CaCl₂ was replaced with an equimolar concentration of SrCl₂ (SrACSF; also containing 10 μM DNQX + 5 μM MK-801). Slices were perfused with SrACSF until stimulation produced only desynchronized small IPSCs (SrIPSCs; ~5 min). Stimuli were delivered every 6 seconds. In some experiments, spontaneous mIPSCs were recorded in ACSF containing 0.5 μM tetrodotoxin (TTX; Tocris Bioscience, Ellisville, MO). For examination of evoked SrEPSCs, DNQX and MK-801 were omitted and SR95531 (5 μM) was included in the SrACSF. All electrophysiology experiments were performed at room temperature, as we have shown no significant differences in the rapid transporter-mediated regulation of quantal size between room temperature and 34°C (Fricke et al., 2007; Mathews and Diamond, 2003).

Data analysis

For SrIPSC or mIPSC analysis, individual events meeting threshold criteria were detected using MiniAnalysis software (Synaptosoft, Fort Lee, NJ) and visually inspected to exclude artifacts. Those single events arising from a stable baseline were used for analysis. In SrACSF, the events occurring 1 second after the stimulation were collected for analysis (ignoring the first 50–100 msec following the stimulus which may contain multiple overlapping events). At least 100 events that could be identified as unitary quanta were recorded from each cell in each condition, and the median amplitude was calculated. Each experiment was performed in multiple cells ('n'; range 3–9) prepared from at least 3 animals and the mean of the medians ± standard deviation was reported for each condition. To assess the drug effects, changes in median amplitudes during drug application were reported as a percent change from baseline ± SEM. Data were tested for normality (Kolmogorov-Smirnov test) and, unless otherwise noted, passed the normality test and were compared using a paired t-test.

Immunohistochemistry in fixed brain sections

Fourteen day-old Sprague-Dawley rats were deeply anesthetized with sodium pentobarbital. Transcardiac perfusion with 0.9% saline followed by a solution of 4% paraformaldehyde (freshly prepared, pH 7.2) was performed. Brains were post-fixed overnight at 4°C. Coronal sections (40 μm) were cut using a freezing microtome (Leica Microsystems, Germany).

Immunohistochemistry was performed with free-floating sections blocked in 4% milk then incubated overnight at 4°C in mouse monoclonal anti-EAAT3 antibody (Invitrogen/Chemicon, Carlsbad, CA). A study characterizing this antibody indicated that it recognized rat as well as human and mouse EAAT3, and its reactivity in rat brain was blocked by preadsorption with the control peptide (Shashidharan et al., 1997). An optimal antibody dilution of 1:1000 was determined after evaluation of immunostaining over a range of dilutions (1:250 to 1:5000). Permeabilization with detergent (Triton, Tween or saponin), inclusion of 4% milk during the primary antibody incubation or incubation for longer periods (up to 72 hours) reduced the staining intensity. For secondary detection, sections were incubated in biotinylated anti-mouse IgG (1:1000, Jackson ImmunoResearch, West Grove, PA) for one hour at room temperature, then with avidin–biotin amplification reagent with horseradish peroxidase (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA). Detection of horseradish peroxidase activity was achieved with the 3,3'-diaminobenzidine reaction or, in some studies, using tyramide amplification (TSA kit #4, Invitrogen) for confocal microscopy. Sections used for fluorescent imaging were pretreated with Tris–glycine (pH 7.4) prior to primary antibody incubation. In agreement with manufacturer's claim that Vectastain "Elite" ABC provides 5–10 times greater detection sensitivity, use of the regular Vectastain ABC reagent kit reduced the signal such that interneurons were not detectable. Double labeling with mouse monoclonal anti-GAD65 antibody (1:500, Invitrogen/Chemicon) was performed as a sequential reaction after permeabilization with 0.2% Triton X-100. Then sections were incubated in Cy2 anti-mouse IgG (1:500, Jackson ImmunoResearch) for one hour at room temperature. Immunostained sections were air-dried overnight then dehydrated and coverslipped using DPX mounting medium. Double fluorescent microscopy was performed using a Zeiss LSM 510 META confocal microscope. For all antisera, omission of primary antibodies confirmed lack of specific cellular labeling (data not shown). Tissue from EAAT3 knockout mouse (Peghini et al., 1997) was generously provided by Dr. Jeffrey S. Diamond (NINDS, NIH). These sections were incubated with unconjugated anti-mouse IgG Fab fragments prior to addition of the EAAT3 primary antibody to block endogenous IgG to minimize nonspecific staining.

D-aspartate uptake and immunohistochemistry in brain slices

Acute hippocampal slices were prepared as described above except thickness was 150 μ m. Slices were incubated with D-aspartate (250 μ M) in oxygenated ACSF at 35°C for 30 minutes then washed in ice cold ACSF. In some experiments either DHK (500 μ M, Tocris) or THA (1 mM, Sigma Aldrich) were included in the ACSF. After washing, slices were immediately fixed in 0.625% glutaraldehyde and 1% formaldehyde for one hour at room temperature then overnight at 4°C in a 1:10 dilution of the fixative. Immunohistochemistry was performed as above using rabbit polyclonal anti-D-aspartate (1:1500, US Biological) and monoclonal anti-GABA (1:200, Sigma Aldrich). Cy3 anti-rabbit (1:1000, Jackson) and Cy2 anti-mouse (1:500, Jackson) secondary antibodies were used and imaging was performed with a Zeiss laser scanning confocal microscope (LSM 510). Each experiment was performed in multiple slices from each of at least three animals.

Results

Spatially- and temporally-specific enhancement of inhibition by glutamate spillover

Acute changes in vesicular filling at inhibitory synapses in hippocampal area CA1 are manifest as changes in the amplitudes of quantal events ((Fricke et al., 2007; Liang et al., 2006; Mathews and Diamond, 2003). We have previously demonstrated that quantal size at hippocampal inhibitory synapses is bidirectionally controlled by glutamate uptake via high affinity transporters, including enhancement by exogenously applied glutamate (Mathews and Diamond, 2003). In this study we asked whether synaptically-released glutamate could augment quantal size in adjacent inhibitory synapses. To examine this question, we took

advantage of the unique synaptic organization of hippocampal area CA1, where different classes of inhibitory neurons form synapses either within the dendritic trees of pyramidal cells (axodendritic inhibitory terminals) where they lie adjacent to afferent glutamatergic terminals, or on the cell bodies of pyramidal neurons (axosomatic terminals), which are devoid of glutamatergic synapses. We stimulated the glutamatergic fibers of the Schaffer collateral pathway in *stratum radiatum* and simultaneously evoked GABA release from local inhibitory terminals (Figure 1A) to study the effect of temporally and spatially constricted co-release of glutamate and GABA. After replacing extracellular calcium with strontium and blocking glutamate (AMPA and NMDA) receptors, evoked synaptic events were desynchronized (Goda and Stevens, 1994) and GABAergic quanta could be resolved. The amplitudes of quantal events evoked in SrACSF (SrIPSCs) were indistinguishable from those of mIPSCs recorded in TTX in the same preparation (median amplitudes were 34.7 ± 6.4 pA in SrACSF versus 35.2 ± 7.7 pA in TTX; $p = 0.9$; $n = 6$ and 7 cells respectively).

Dihydrokainate (DHK), a selective inhibitor of the major astrocytic glutamate transporter EAAT2 (Arriza et al., 1994), impairs the clearance of synaptically-released glutamate and increases the amount of glutamate that “spills over” between adjacent synapses (Asztely et al., 1997). In the presence of DHK ($300 \mu\text{M}$), the median amplitudes of evoked SrIPSCs were increased by $33 \pm 7\%$ ($p = 0.01$, $n = 5$ cells; Figure 1C,F) and the entire distribution of SrIPSC amplitudes was shifted toward larger values (Figure 1D). To demonstrate that the effect of inhibiting EAAT2 was due to increased glutamate spillover and enhanced presynaptic uptake (Figure 1B), we tested whether DL-threo- β -hydroxyaspartic acid (THA), a non-selective EAAT inhibitor that blocks both EAAT3 and EAAT2, could reverse the effect of DHK on SrIPSC amplitudes. In the additional presence of THA ($300 \mu\text{M}$), DHK had no effect on evoked quanta ($98 \pm 7\%$ of control; $p = 0.85$; $n = 3$; Figure 1E,F). To test whether the effect of DHK was due to an increase in the concentration of GABA in the synaptic cleft, we examined the effect of a low affinity antagonist of the GABA_A receptor, (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA), on evoked SrIPSCs in the absence and presence of DHK. Because TPMPA’s rate of dissociation from postsynaptic receptors is rapid relative to the duration of the GABA transient in the synaptic cleft, we hypothesized that TPMPA would have a lower efficacy in DHK if the cleft concentration of GABA were increased. TPMPA at its approximate EC₅₀ ($500 \mu\text{M}$; Ragozzino et al., 1996) reduced SrIPSCs in the absence of DHK by $44 \pm 3\%$ ($n = 3$). However, in the presence of DHK, the effect of TPMPA on SrIPSCs was attenuated ($25 \pm 3\%$; $n = 3$; $p < 0.04$ compared with controls; data not shown), consistent with our conclusion that DHK increases synaptic vesicle content of GABA in synapses adjacent to active excitatory terminals.

In contrast to SrIPSCs evoked from inhibitory terminals simultaneously with glutamate from adjacent Schaffer collateral terminals, quanta released spontaneously when action potentials were blocked with TTX (i.e. traditional mIPSCs) have a random temporal and spatial distribution and occur with low frequency. The amplitudes of mIPSCs were unaltered by DHK ($92 \pm 6\%$; $p = 0.2$; $n = 7$ cells; Figure 2A,D). To address the possibility that a higher frequency of vesicular release during stimulation in SrACSF compared with spontaneous release in TTX accounts for the differential effect of DHK, we used ACSF containing high K⁺ (10 mM and 20 mM KCl) to depolarize terminals and increase the frequency of spontaneous release by ~ 2 - and 10 -fold, respectively. We found no effect of DHK on quantal size in 10 mM K^+ ($98 \pm 6\%$; $p = 0.4$; $n = 3$ cells; Figure 2B,D) or 20 mM K^+ ($96 \pm 6\%$; $p = 0.3$; $n = 3$ cells; Figure 2D). To additionally test whether the effects we observed in SrACSF were related in some way to the absence of Ca²⁺ or to the presence of Sr²⁺, we measured the effect of DHK on events that occurred spontaneously in SrACSF (i.e. without stimulation). Similar to our findings in TTX, we found no effect of DHK on the amplitudes of these spontaneously released SrIPSCs ($105 \pm 17\%$ of control; $p = 0.8$; $n = 4$; Figure 2C,D). Therefore, blocking uptake of synaptically-

released glutamate with DHK selectively enhances quantal size in the subset of inhibitory synapses adjacent to stimulated excitatory synapses.

We considered the possibility that DHK increased the quantal release of glutamate rather than enhancing its spillover. We repeated the experiment depicted in Figure 1A in the presence of the GABA antagonist SR95531 (5 μ M) instead of glutamate antagonists to determine the size of evoked quantal EPSCs (SrEPSCs). DHK (300 μ M) did not alter the median amplitude of SrEPSCs ($97 \pm 3\%$ of control; $p = 0.5$; $n = 4$ cells; data not shown).

EAAT3 expression and functional glutamate uptake patterns in CA1 interneurons

To determine whether the effect of glutamate spillover on vesicular content of GABA correlated with glutamate transporter expression on inhibitory interneurons, we examined the expression pattern of EAAT3 in hippocampal area CA1. Using a monoclonal antibody against an N-terminal extracellular domain of EAAT3 (Shashidharan et al., 1997), we found a pattern of immunoreactivity on pyramidal cell cell bodies and dendrites in area CA1 that has been described previously (He et al., 2000; Shashidharan et al., 1997). We also observed EAAT3-immunoreactivity on a population of non-pyramidal interneurons located in layers above and below the pyramidal cell layer (Figure 3A,B), which includes the cell bodies of origin of the axodendritic synapses (Freund and Buzsaki, 1996). Because EAAT3 expression in hippocampal interneuron cell bodies has not been reported previously, we investigated the specificity of the antibody. Neither interneurons nor pyramidal neurons exhibited EAAT3 immunoreactivity when stained under the same conditions in tissue from the EAAT3 knockout mouse (Figure 3C).

Because previous ultrastructural studies using electron microscopy have demonstrated expression of EAAT3 on presynaptic inhibitory terminals (He et al., 2000; Rothstein et al., 1994), and our results suggested selective regulation of dendritic synapses via presynaptic glutamate uptake, we sought to determine the expression pattern of EAAT3 on inhibitory synapses. We performed double immunofluorescent studies to ask whether EAAT3 colocalized with GAD65, a presynaptic marker for inhibitory terminals (Esclapez et al., 1994). Despite using two sequential signal amplification steps (avidin-biotin and tyramide), we did not observe significant EAAT3 immunoreactivity on GAD65-positive puncta (Figure 4). We considered two possible explanations for our negative result: 1) transporter expression at presynaptic sites is below the detection limits of our immunohistochemical techniques, or 2) EAAT3-mediated glutamate uptake does not occur directly at the synaptic terminals but rather may occur at cell bodies where EAAT3 is detected immunohistochemically. We hypothesized that glutamate uptake at cell bodies would not permit the rapid regulation of GABA quantal size by extracellular glutamate due to local excitatory synaptic activity that we observed in our physiological experiments.

D-aspartate uptake in presynaptic terminals of hippocampal interneurons

D-aspartate is a non-metabolized substrate for the EAATs that is actively taken up by and concentrated in synaptic terminals (Danbolt, 2001). Exogenous application of D-aspartate to hippocampal slices appears concentrated in ultrastructurally-confirmed synaptic terminals (predominantly excitatory) relative to astrocytes, possibly because synaptic terminals are better preserved during fixation and sectioning (Gundersen et al., 1993) or because this compartment preferentially accumulates the substrate (Furness et al., 2008). We took advantage of this technique to visualize the relative distribution of D-aspartate uptake into inhibitory terminals as a proxy for transport activity within a neuroanatomical context.

In *stratum radiatum* of hippocampal area CA1, exogenously-applied D-aspartate localized to varicosities among the dendrites of pyramidal neurons (Figure 5A). This pattern of uptake is

mediated by high affinity glutamate transporters (EAATs) because the labeling was abolished by co-incubation of D-aspartate with the non-selective EAAT inhibitor THA (500 μ M; data not shown), in agreement with previous studies (Furness et al., 2008). Co-labeling of the hippocampal slices with an antibody against GABA revealed numerous GABA-positive varicosities surrounding pyramidal cell bodies and along their proximal dendrites, corresponding to the locations of GABAergic synaptic terminals (Figure 5C). Only a small number of GABA-labeled sites demonstrated D-aspartate uptake (Figure 5A,C), in agreement with previous findings that most of the uptake into presynaptic terminals occurs at excitatory synapses under these conditions (Furness et al., 2008; Gundersen et al., 1993).

Because DHK inhibits astrocytic glutamate uptake and enhances quantal size when local synapses are active, we hypothesized that it would increase the amount of exogenous D-aspartate that reaches inhibitory synaptic terminals and enhance uptake at those sites. While we did not stimulate our slices, we incubated in D-aspartate for 30 minutes, which would allow us to assess the slow accumulation of this non-metabolized substrate into inhibitory synapses when astrocytic uptake was blocked by DHK. In agreement with our hypothesis, in the presence of DHK there was a several-fold increase in the number of putative GABAergic terminals that were also labeled for D-aspartate within the proximal region of *stratum radiatum* (Figure 5B). Importantly, D-aspartate did not appear to concentrate in interneuron cell bodies, despite the presence of EAAT3 on somata in our immunohistochemical studies. In contrast to the dendritic terminals, perisomatic GABA-positive varicosities were never co-labeled with D-aspartate, even in the presence of DHK (Figure 5C,D), suggesting that perisomatic synapses do not possess presynaptic glutamate transporters. Therefore, while the D-aspartate uptake experiments do not demonstrate spillover of synaptically-released substrate, they support the hypothesis that astrocytic and neuronal EAATs compete for substrate within *stratum radiatum* and that the inhibitory terminal glutamate transporter exhibits the pharmacological profile of EAAT3 (i.e. DHK-insensitive and THA-sensitive).

Discussion

Glutamate spillover occurs between excitatory synapses in the CA1 region of the hippocampus (Huang and Bergles, 2004), particularly within the dendritic processes in *stratum radiatum* where there is a relative lack of intervening astrocytic processes (Lehre and Danbolt, 1998; Ventura and Harris, 1999). Nevertheless, astrocytes express glutamate transporters at very high densities that prevent diffusion beyond adjacent synapses and prevent toxic accumulation of glutamate in the extracellular space. Spillover between excitatory and inhibitory synapses in the hippocampus has also been described (Jiang et al., 2001; Semyanov and Kullmann, 2000) that results in modulation of GABA release via presynaptic receptor activation. Our study demonstrates that astrocytic glutamate transporters (i.e. EAAT2) limit glutamate diffusion between adjacent excitatory and inhibitory synapses in *stratum radiatum*. Without astrocytic uptake, synaptically-released glutamate spills over to inhibitory terminals and rapidly upregulates GABA synaptic vesicle content and inhibitory synaptic strength.

Our results demonstrate a novel mechanism of heterosynaptic regulation of inhibitory synapses that takes advantage of presynaptic control of quantal size by GABA metabolism. Presynaptically localized high affinity glutamate transporters allow low micromolar concentrations of extracellular glutamate to be concentrated into inhibitory synaptic terminals where GAD65 is positioned to synthesize GABA at the surface of synaptic vesicles (Figure 1B). Our previous work using uptake inhibitors and exogenous glutamate demonstrated bidirectional control of GABA vesicle content that was manifest as changes in quantal size (Mathews and Diamond, 2003). We now demonstrate two important new findings related to this novel mechanism of plasticity. First, individual inhibitory synapses that are located near glutamatergic terminals are selectively regulated by glutamate spillover. Evidence for this

anatomical specificity was demonstrated by the enhancement of SrIPSCs by simultaneous stimulation of excitatory and inhibitory synapses in the presence of the astrocytic glutamate uptake inhibitor, DHK. Second, this presynaptic regulation occurs only at dendritic inhibitory terminals where a close relationship between excitatory and inhibitory synapses exists. D-aspartate immunohistochemistry revealed that DHK enhances uptake into dendritic inhibitory terminals in *stratum radiatum* but not perisomatic synapses on CA1 pyramidal neurons. We conclude that somatic terminals, in addition to their greater distance from excitatory terminals, are not capable of glutamate uptake and therefore are not likely to be subject to dynamic regulation by extracellular glutamate levels.

EAAT3 is the likely presynaptic glutamate transporter

Our electrophysiological results showing rapid and selective modulation of inhibitory synapses supports previous ultrastructural studies indicating that EAAT3 is expressed at inhibitory terminals (He et al., 2000; Rothstein et al., 1994), and suggest that expression of EAAT3 is confined to terminals within the dendritic layers of hippocampus. Although we observed expression of EAAT3 on hippocampal interneurons throughout area CA1, we were unable to detect its expression at GABAergic terminals with standard immunohistochemical techniques, possibly because the number of presynaptic transporters is relatively low. Application of exogenous D-aspartate in the presence of DHK revealed robust uptake into putative GABAergic terminals in *stratum radiatum*. Although EAAT2 expression has been reported on excitatory pre-synaptic terminals (Chen et al., 2002; Chen et al., 2004), DHK would be expected to prevent, not enhance, D-aspartate uptake if EAAT2 were the presynaptic transporter at inhibitory terminals. Furthermore, reversal of the effect of DHK with a non-selective EAAT inhibitor, THA, argues that EAAT3 and not indirect metabolism (i.e. glutamine) mediates the observed physiological effect. Moreover, our results are in agreement with a previous study utilizing molecular manipulation of EAAT3 expression in which decreased GABA synthesis and tissue content were observed following chronic administration of EAAT3 antisense oligonucleotides (Sepkuty et al., 2002). Consistent with our findings, Sepkuty et. al found that DHK enhanced GABA synthesis in hippocampal tissue. Taken together, these data indicate that EAAT3 is responsible for our observed glutamate-mediated enhancement of inhibitory synapses.

Potential role of spillover-mediated enhancement of inhibition

Previous studies investigating heterosynaptic regulation in the hippocampus used high frequency stimulation of Schaffer collateral fibers to induce glutamate spillover. These studies have demonstrated that activation of presynaptic glutamate receptors on local inhibitory terminals occurs under conditions in which sufficiently high extrasynaptic glutamate levels are reached. Under these circumstances, the probability of GABA release was increased or decreased via activation of presynaptic kainate or metabotropic glutamate receptors, respectively (Jiang et al., 2001; Semyanov and Kullmann, 2000), indicating that a multiplicity of mechanisms serve to regulate inhibitory neurotransmission under conditions of increased excitatory drive. In our studies, we resolved individual quanta, the amplitudes of which are not influenced by release probability, in an effort to isolate the effects of glutamate spillover on vesicle content. Clearly, effects of glutamate spillover on spontaneous or evoked IPSCs would be more complex depending on a variety of factors including the spatial and temporal pattern of glutamate release and the concentrations achieved. Still, if DHK were exerting a direct effect on presynaptic glutamate receptors (eg. kainate receptors) or non-specific effects due to elevated extracellular glutamate levels within the slice, we would expect to see effects on spontaneously released quanta (mIPSCs and spontaneous SrIPSCs).

Our study was performed in slices from young rats (postnatal day 7 to 14), a time during which numerous developmental changes are occurring, including formation of new synapses.

Expansion of astrocytic processes and developmentally regulated glutamate transporter expression (Furuta et al., 1997) contribute to a five-fold increase in the rate of glutamate clearance in area CA1 from postnatal day 14 to adulthood (Diamond, 2005). Therefore, there are likely to be significant developmental changes in the heterosynaptic regulatory mechanism we have described.

Our results demonstrate mechanism of synaptic plasticity that differentially modulates dendritic over perisomatic synapses, which are known to play markedly different roles in the hippocampus. Somatic synapses exert powerful inhibitory effects and due to their extensive axonal branching are able to synchronize large populations of pyramidal neurons (Cobb et al., 1995). In contrast, dendritic inhibitory synapses control the excitatory influence of the major CA1 input pathways and are crucial to maintaining a balance of excitation and inhibition. Accordingly, axosomatic interneurons may regulate seizure generation (Cossart et al., 2001; Kohling et al., 2000), while dendritic inhibition is deficient in experimental epilepsy of hippocampal origin (Cossart et al., 2001; El-Hassar et al., 2007). Therefore, the regulation of inhibition at dendritic synapses would be expected to play an important role in the pathophysiology of seizures. Indeed, knockdown of EAAT3 in hippocampus results in spontaneous seizures (Sepkuty et al., 2002), suggesting that EAAT3 plays a crucial role in preventing the onset of seizures. Studies of human epileptic hippocampus demonstrate elevated extracellular glutamate levels compared with the contralateral normal hippocampus (Cavus et al., 2005; During and Spencer, 1993), and glutamate levels rise further prior to onset of spontaneous seizures (During and Spencer, 1993). Hyperactivity of glutamatergic circuits chronically as well as hypersynchronous activity at seizure onset may result in enhanced spillover and compensatory augmentation of inhibitory synapses.

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Figure 1. Enhanced spillover of glutamate augments inhibitory neurotransmission in *stratum radiatum* of area CA1. A) Schematic representation of experimental method to stimulate glutamate release from Schaffer collateral terminals simultaneously with GABA release from local inhibitory terminals. B) Schematic diagram of adjacent glutamatergic and GABAergic synaptic terminals in *stratum radiatum*. Normally glutamate is taken up into astrocytes (shaded cell) via EAAT2. In the presence of dihydrokainate (DHK), glutamate may spill over to adjacent inhibitory terminals to be taken up via EAAT3 and undergo metabolism to enhance vesicular GABA content. C) Representative average quantal responses evoked in SrACSF before (black trace) and during application of DHK (gray trace). D) Cumulative probability histograms

showing shift in the amplitude distribution of SrIPSCs in the presence of DHK (gray curve) compared with control (black curve). SrIPSCs from individual cells were normalized to the median amplitude and distributions from all cells were averaged. E) Representative average quantal responses evoked in SrACSF before (black trace) and during co-application of DHK and DL-threo-hydroxyaspartate (THA, gray trace). Scale bars represent 10 pA, 10 msec for C and E. F) Summary of effect of DHK on evoked quantal size (median amplitude) alone and in presence of THA. * indicates $p < 0.01$ compared with control amplitudes.

Figure 2.

Inhibiting astrocytic uptake of glutamate does not augment quantal size in the absence of coordinated stimulation of neighboring glutamatergic and GABAergic terminals. DHK has no effect on quantal size as determined in the presence of TTX (A), TTX + high potassium to increase frequency (B, 10 mM KCl) or occurring spontaneously in SrASCF (C). All figures are representative average current traces from representative cells; black trace is averaged control mIPSC and grey trace is averaged mIPSC in the presence of DHK. Scale bars are 10 pA, 10 msec. A summary of all conditions is shown in (D). Number of cells indicated for each bar; error bars represent SEM.

Figure 3.

Immunohistochemical labeling of EAAT3 in area CA1. Staining of pyramidal cell bodies in *stratum pyramidale* (*st. pyr.*) and their proximal dendrites in *stratum radiatum* (*st. rad.*) as well as diffuse staining in the distal dendritic regions is observed in A and B. Numerous non-pyramidal cell bodies are also stained in *stratum oriens* (*st. oriens*; A) and *st. rad.* (B) as indicated by arrows. A few astrocytic processes were also labeled (example marked by an asterisk) as previously described (Conti et al., 1998). Scale bars = 50 μ m. (C) Specificity of the EAAT3 antibody was examined in the EAAT3 knockout mouse. The image is displayed at higher power in differential interference contrast to show the outlines of unstained cells in

area CA1. Neurons in *stratum pyramidale* are unlabeled. Arrows indicate the locations of two unlabeled non-pyramidal interneurons in *stratum oriens*.

Figure 4.

Lack of colocalization of EAAT3 immunoreactivity to GABAergic synaptic terminals in *stratum radiatum* area CA1. A) EAAT3 immunoreactivity localizes over pyramidal cell bodies in *stratum pyramidale* (*st. pyr.*) and dendrites in *stratum radiatum* (*st. rad.*). B) GAD65 immunoreactivity occurs in varicosities throughout both layers. C) Merged image shows essentially no colocalization of EAAT3 and GAD65. Asterisks indicate the location of three pyramidal cell bodies. Scale bar = 10 μ m.

Figure 5.

D-aspartate uptake into putative GABAergic synaptic terminals is enhanced when astrocytic uptake is blocked with DHK. Merged images show D-aspartate (red) and GABA (green) immunoreactivity in the absence (A,C) and presence (B,D) of DHK (500 μ M). Uptake of exogenous D-aspartate occurs in varicosities throughout *stratum radiatum* (*st. rad.*) of area CA1 appears similar in the absence and presence of DHK (A,B). Labeling of GABA is concentrated in numerous varicosities surrounding pyramidal cell bodies in *stratum pyramidale* (*st. pyr.*) and their dendrites in *st. rad.* Colabeling of varicosities with D-aspartate and GABA is increased several-fold in the presence of DHK. Higher power views demonstrate a lack of D-aspartate immunoreactivity in perisomatic GABA-positive varicosities both in control (C) and DHK (D), but a large number of GABA-positive varicosities are co-labeled with D-aspartate in the proximal dendrites in DHK (D).