Published in final edited form as:

J Neurosci Res. 2009 September ; 87(12): 2812–2820. doi:10.1002/jnr.22092.

Simultaneous Efflux of Endogenous D-Ser and L-Glu From Single Acute Hippocampus Slices During Oxygen Glucose Deprivation

Daniel L. Kirschner^{1,2,★}, Ann L. Wilson¹, Kelly L. Drew^{1,2}, and Thomas K. Green^{1,2}

¹Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska

²Department of Chemistry and Biochemistry, University of Alaska Fairbanks, Fairbanks, Alaska

Abstract

D-serine and L-glutamate play crucial roles in excitotoxicity through N-methyl-D-aspartate receptor coactivation, but little is known about the temporal profile of efflux during cerebral ischemia. We utilized a newly designed brain slice microperfusion device coupled offline to capillary electrophoresis laser-induced fluorescence to monitor dynamic efflux of endogenous D-ser and L-glu in response to oxygen glucose deprivation (OGD) in single acute hippocampus slices. Efflux profiles with 2-min temporal resolution in response to 24-min OGD show that efflux of D-ser slightly precedes efflux of L-glu by one 2-min sampling interval. Thus both coagonists are available to activate NMDA receptors by the time when glu is released. The magnitude of D-ser efflux relative to baseline values is, however, less than that for L-glu. Peak efflux during OGD, expressed as pre-OGD baseline values, was as follows: D-ser 254% ± 24%, L-glu 1,675% ± 259%, L-asp 519% ± 128%, and L-thr 313% ± 33%. L-glutamine efflux was shown to decrease significantly in response to OGD. The microperfusion/CE-LIF approach shows several promising attributes for studying endogenous chemical efflux from single, acute brain slices.

Keywords

D-serine; L-glutamate; excitotoxicity; microperfusion; stroke; ischemia; cardiac arrest; capillary electrophoresis

Stroke is the third leading cause of death in the United States and the leading cause of adult long-term disability (Rosamond et al., 2008). Much of the resulting brain damage caused by stroke is due to a cascade of excitotoxic events occurring as a result of insufficient blood supply to the brain. Similar excitotoxicity is responsible for much of the high morbidity and mortality in patients suffering from postcardiac arrest syndrome (Neumar et al., 2008). In the central nervous system (CNS), the excitotoxic cascade is initiated by the onset of energy deficit, which forces cell depolarization and subsequent neurotransmitter release. High levels of L-glutamate (L-glu), the dominant excitatory neurotransmitter in the CNS, efflux from neurons and glia, resulting in excitotoxicity (Lipton, 1999). The N-methyl-D-aspartate receptor (NMDAR) is a glutamatergic ligand gated calcium/sodium ion channel that is responsible for the majority of excitotoxic cell death that occurs as a result of L-glu released during cerebral ischemia (Arundine and Tymianski, 2004).

The NMDAR is unique among neurotransmitter receptors in its requirement for coagonist activation (Kleckner and Dingledine, 1988; Curras and Pallotta, 1996). D-serine (D-ser), an endogenous amino acid, is synthesized and released from neurons (Kartvelishvily et al.,

★Correspondence to: Daniel L. Kirschner, 900 Yukon Drive, Fairbanks, AK 99775. fsdlk2@uaf.edu.

2006) and glia (Oliet and Mothet, 2006) and is colocalized in brain regions having high concentrations of NMDAR. D-ser binds with high nanomolar affinity to the strychnine-insensitive binding domain (coagonist site, “glycine site”) on the obligatory NR1 subunit of the NMDAR. Recent evidence implicates D-ser as the essential coagonist of NMDA receptor-induced neurotoxicity in several brain regions (Katsuki et al., 2004; Shleper et al., 2005).

L-glu is released rapidly within the first few minutes of cerebral ischemia in vivo and in vitro, suggesting that it is an early event in the neuroxic cascade. Simultaneous efflux of D-ser would be necessary for excitotoxicity via NMDAR activation, but little is known about the early time course of D-ser release. Lo and colleagues (1998) quantified several transmitters, including D-ser, during 2 hr of focal ischemia with 10-min temporal resolution in vivo. Recently, we developed a sensitive enantioselective capillary electrophoresis laser-induced fluorescence (CE-LIF) technique to assay D-ser, L-glu, and several other amino acids in biological samples (Kirschner et al., 2007). The focus of the current work was to couple our CE-LIF approach to a newly designed in vitro microperfusion device described herein that would allow for more rapid monitoring of dynamic changes in D-ser and L-glu efflux from single acute hippocampus slices in response to modeled ischemia. Here we apply our microperfusion technique to study L-glu and D-ser temporal response with 2-min sampling during oxygen glucose deprivation (OGD), an in vitro model of cerebral ischemia. By using the microperfusion/CE-LIF technique, we demonstrate parallel increases in D-ser and L-glu efflux during OGD. To our knowledge, our data represent the fastest temporal resolution of endogenous D-ser efflux during modeled cerebral ischemia.

Materials and Methods

General

All chemicals were purchased from Aldrich Chemical Company (St. Louis, MO) unless otherwise noted. Sulfated β -cyclodextrin was purchased from Fluka (Buchs, Switzerland; lot No. 1148973). Naphthalene-2,3-dicarboxaldehyde was obtained from Invitrogen (Carlsbad, CA). Buffers and reagents were prepared regularly every 1–2 weeks and stored at 4°C when not in use. Microchambers were soaked and perfusion tubing was flushed for 1 hr with 70% ethanol prior to each experiment.

Microperfusion Chamber

Acute slice chambers were constructed in-house at the University of Alaska Fairbanks Engineering Department machine shop (Fig. 1a–d). The chambers were machined from polycarbonate and incorporate a beveled leak-free lid and chamber body. The chamber body houses inlet and outlet ports connected via a small channel to an oval 2-mm-deep recessed microchamber (dimensions $\sim 9 \times 5$ mm) specifically designed to accommodate 400- μ m-thick acute hippocampus slices from rats. The microchambers were engineered with a microchannel support in the well bottom to increase fluid exchange around the acute tissue preparation. The microchannels were machined ~ 400 μ m wide, 300 μ m deep in a waffle pattern shown in Figure 1c. A polycarbonate lid was machined with a 1-mm-deep beveled oval flange to form a watertight (O-ring free) seal with the chamber body. This design yields a sealed chamber 700 μ m deep with an additional 300- μ m microchannel support. The sealed chamber has an estimated volume of ~ 35 μ l without tissue in place. The lid is secured to the base with four stainless screws. Four chambers were mounted in parallel to a stainless-steel tray submerged in a thermistor-controlled StableTemp water bath (Cole Parmer Instrument Company, Vernon Hills, IL) kept at a constant temperature. All connections were made with 1/16-in.-O.D. 100- μ m-I.D. PEEK tubing with flanged fittings (outlet port used 150- μ m-I.D. PEEK tubing). Perfusion medium for each chamber was administered via a 10-stage syringe pump (Harvard Apparatus, Holliston, MA), with medium passing first through a four-port diagonal flow

switching valve (Upchurch Scientific, Oak Harbor, WA) for administering treatments, before entering the water bath, where it was preheated prior to passing through the microchamber inlets. The chamber outlet tubing exited the water bath, where samples were manually collected dropwise.

Acute Hippocampus Slices

Adult male Sprague Dawley rats (Simonson, Gilroy, CA; age 3–4 months, 250–400 g) were used for all experiments. Procedures were in accordance with The Institutional Animal Care and Use Committee of the University of Alaska Fairbanks. Transverse 400- μ m acute hippocampus slices were prepared as follows. Rapidly dissected hippocampi were embedded in agar and sliced with a Vibratome 1000 Plus sectioning system (The Vibratome Company, St. Louis, MO). Slicing was performed at $\sim 2^{\circ}\text{C}$ in oxygenated HEPES-buffered artificial cerebral spinal fluid (HEPES aCSF containing in mM: 120 NaCl, 20 NaHCO_3 , 6.88 HEPES acid, 3.3 HEPES sodium salt, 5.0 KCl, 2.0 MgSO_4 , pH 7.30–7.40). Slices were transferred from HEPES-buffered aCSF to a Brain Slice Keeper (Scientific Systems Design Inc., Mississauga, Ontario, Canada) and allowed to recover in oxygenated aCSF (aCSF continuously bubbled with 95% O_2 , 5% CO_2 containing in mM: 120 NaCl, 25 NaHCO_3 , 10 glucose, 3.3 KCl, 1.2 NaH_2PO_4 , 2.4 MgSO_4 , 1.8 CaCl_2 , pH 7.30–7.40) for 1 hr at room temperature prior to placement in individual microchambers. Slices were then transferred individually to chambers and lids sealed. The apparatus containing four parallel chambers was submerged in a bath at 36°C ($\pm 0.2^{\circ}\text{C}$), and acute slices were perfused at a flow rate of 7 $\mu\text{l}/\text{min}$. Sampling began 20 min after submerging the sealed chambers to allow adequate time for stabilization of neurochemical efflux. Perfusate was collected at 2-min intervals during pretreatment (basal), treatment, and the first 20 min of reperfusion. After 20 min of reperfusion, samples were collected every 3 min. Treatment consisted of a 24-min exposure to OGD solution (glucose-free aCSF equilibrated with 95% N_2 5% CO_2 for a minimum of 1 hr until pH stabilized in the range of 7.3–7.4). The PO_2 in OGD solution varied from 0 to 2.9 mmHg with an average for six determinations of 1.1 mmHg as measured with a miniature Clark-style electrode (Instech Laboratories, Plymouth Meeting, PA). In control slices, treatment was administered by switching to standard aCSF. Collected fractions were kept at -80°C until they were analyzed by chiral CE-LIF.

CE-LIF Analysis

Perfusate was derivatized as described previously (Kirschner et al., 2007) to form highly fluorescent cyanobenz[f]isoindole amino acids (CBI-amino acids). Briefly, 1 μl thawed perfusate was reacted with 1 μl 2 mM naphthalene 2,3-dicarboxaldehyde (NDA) in methanol and 1 μl NaCN (5.5 mM) in 60 mM sodium tetraborate. Cyanide solution contained 3 μM D-amino adipic acid as internal standard. Samples were reacted at room temperature for 20 min prior to analysis. CE-LIF was performed on a custom in-house-built instrument using a 420-nm diode laser, 490-nm bandpass filter, and PMT for LIF detection. Additional instrument details have been described elsewhere (Kirschner et al., 2007). Samples were injected onto a bare fused silica capillary of dimensions 48 cm $\times$ 25 μm (45 cm to detection window) for 1 sec at 380 mbar vacuum and separated by using negative polarity (-21 kV). Separation buffer optimized for analysis of D-ser and L-glu is a 25 mM phosphate background electrolyte (BGE) adjusted to pH 2.15 using equal molar additions of phosphoric acid and NaH_2PO_4 . BGE additionally contained 20 g/liter sulfated β -cyclodextrin as chiral selector. PeakFit software (SeaSolve Software Inc., Framingham, MA) was used to process raw data and quantify peak areas in all experiments based on a Gaussian peak shape for each analyte. Linear calibration curves were constructed for analytes as a function of concentration verses the peak area ratio (analyte area/internal standard area). Efflux of analytes is expressed as percent of baseline, where baseline is defined as the average value of 10 samples collected prior to onset of treatment, a period of sampling in which analyte levels were stable.

Monitoring Treatment Profile

Monitoring actual treatment exposure is critical for precise timing of transmitter efflux. The actual treatment exposure time in perfusion experiments is influenced by multiple factors including flow rate, chamber dimensions, lag time between pump and chamber, and lag time between chamber and sample collection and properties of flow within the chamber. Flow profile was monitored using different concentrations of D-glutamate (D-glu) as a marker of aCSF and OGD solutions. D-glu was quantified with endogenous amino acids in our chiral CE-LIF preparation. D-glu does not have any known biological roles in the CNS, and is reportedly found in only trace levels in a few brain regions (Quan and Liu, 2003; Mangas et al., 2007). In our preparations, we detected no D-glu in hippocampus slice perfusates. We therefore used 0.5 μM D-glu in aCSF solutions and 3.0 μM D-glu in treatment solutions.

Statistical Analysis

Data was analyzed using SAS v9.1 software (SAS Institute Inc., Cary, NC) by two-way ANOVA with treatment analyzed as a between subjects variable and time analyzed as a repeated measures variable. Tukey post-hoc comparisons were used to compare OGD and control groups at specific time points. Significance was indicated by $P < 0.05$. Data are shown as mean $\pm$ SEM.

Results

An optimized narrow pH range of 2.15–2.20 for the CE-LIF run buffer allowed for baseline separation of D-ser, L-glu, L-aspartate, and L-glutamine CBI-amino acids. Exogenously applied D-glu partially overlapped with L-thr. The partial overlap of L-thr and D-glu was resolved using Gaussian peak-fitting software (PeakFit software; SeaSolve Software Inc.).

Calibration curves using a weighted least squares linear regression for CBI-amino acids were obtained, and the equations, R^2 values, linear dynamic range investigated, and estimated concentration limits of detection (CLOD) for each analyte are given in Table I. Larger dynamic ranges were calibrated for L-glu, L-aspartate, and L-glutamine compared with other CBI-amino acids, because preliminary results suggested that these transmitters could fluctuate markedly during treatment with OGD.

A stepwise change in D-glu produced a sixfold higher concentration of D-glu in treatment compared with aCSF. The resultant profile was used to establish the timing of OGD treatment (Fig. 2). D-glu profiles were conveniently quantified by CE-LIF, which chirally resolves exogenous D-glu from endogenous L-glu in perfusate samples.

OGD induced a significant increase in all amino acids investigated except for L-glutamine, as demonstrated by a comparison of CE electropherograms of basal and OGD perfusates (Fig. 3). Average basal concentrations of analytes in slice perfusates, including both control ($n = 5$) and OGD ($n = 8$) slices, are as follows: D-ser ($0.34 \pm 0.03 \mu\text{M}$ OGD, $0.34 \pm 0.03 \mu\text{M}$ CTL), L-glu ($0.93 \pm 0.19 \mu\text{M}$ OGD, $1.07 \pm 0.18 \mu\text{M}$ CTL), L-aspartate ($0.66 \pm 0.20 \mu\text{M}$ OGD, $0.80 \pm 0.15 \mu\text{M}$ CTL), L-glutamine ($10.68 \pm 1.20 \mu\text{M}$ OGD, $9.83 \pm 0.74 \mu\text{M}$ CTL), and L-thr ($1.64 \pm 0.14 \mu\text{M}$ OGD, $2.00 \pm 0.21 \mu\text{M}$ CTL).

Within a few minutes after the onset of OGD treatment, there is a significant, rapid increase in all amino acids except for L-glutamine (Fig. 4a–e). Stable, pretreatment D-ser efflux rapidly increases during OGD and is significantly different from CTL values within 14 min of onset of OGD ($P < 0.05$; Tukey post hoc comparison). D-ser efflux increases to $254\% \pm 24\%$ of baseline levels, then decreases within the first few minutes of reperfusion (reintroduction of oxygenated aCSF), returning to near pretreatment values within 12 min. D-ser remains at pretreatment levels throughout the remainder of the reperfusion phase (Fig. 4a).

Likewise, efflux of L-glu is rapid, and the timing of efflux is similar to that of D-ser. Stable, pretreatment L-glu efflux increases and is statistically different from CTL levels within 16 min of onset of OGD ($P < 0.05$; Tukey post hoc comparison) and reaches a maximum of $1,675\% \pm 259\%$ of baseline 24 min after onset of OGD. Within the first 2–4 min of reperfusion, L-glu begins to decline slowly, and this decline continues over the course of the 50-min reperfusion. The magnitude of L-glu efflux is larger than that of D-ser, and, unlike D-ser, L-glu remains significantly higher ($244\% \pm 56\%$ of baseline values) for up to 46 min after onset of reperfusion ($P < 0.05$; Fig. 4b).

Stable, baseline L-asp efflux increases rapidly and is statistically different from CTL levels within 18 min of onset of OGD ($P < 0.05$; Tukey post hoc comparison). L-asp levels increase significantly to $519\% \pm 128\%$ of baseline shortly after OGD, with maximal efflux corresponding to that of L-glu at 24 min from onset of OGD. L-asp returns to basal values within 10–12 min of reperfusion (Fig. 4c). Stable, pretreatment L-thr efflux initially increases from CTL levels modestly and is statistically significant by 4 min after onset of OGD ($P < 0.05$; Tukey post hoc comparison). After 12–14-min treatment, L-thr efflux rises more rapidly, with maximal efflux at 24 min from onset, with a magnitude of $313\% \pm 33\%$ of basal values. L-thr returns to near baseline within 12 min of reperfusion (Fig. 4d).

Interestingly, the effect of OGD on L-gln efflux is markedly different from other amino acids investigated here (Fig. 4e). First, in control slices, L-gln efflux slowly decreases throughout the entire experiment ($46.3\% \pm 4.6\%$ basal values after 94 min of perfusion). Initially it appears that L-gln efflux increases briefly early during OGD treatment; however, statistical analysis of these early events does not support this observation ($P > 0.05$; Tukey post hoc comparison, time periods 6, 8, and 10 min). OGD treatment then produces, compared with control, a further and significant drop in L-gln efflux that reaches statistical significance compared with CTL at 24 min from onset ($P < 0.05$; Tukey post hoc comparison). Ultimately, L-gln efflux reaches a minimum of $30.7\% \pm 6.0\%$ baseline after 4 min of reperfusion (vs. $62.3\% \pm 5.8\%$ at the same time point in control slices). L-gln does not significantly recover to baseline levels over the next 30 min of reperfusion.

Discussion

D-Ser Efflux From Hippocampal Slices During OGD

We demonstrate that D-ser, the endogenous coagonist of NMDAR, increases 2.5-fold during 24 min of in vitro modeled ischemia in hippocampus and, for the first time with 2-min temporal resolution, show that the timing of the rise and fall of D-ser precedes or parallels that of L-glu. These results therefore demonstrate that both coagonists are available to activate NMDAR and contribute to excitotoxicity. Endogenous D-ser is essential for NMDA-induced excitotoxicity, whereas endogenous levels of glycine are not sufficient to support excitotoxicity (Shleper et al., 2005). D-ser release is therefore expected to enhance excitotoxicity, and, indeed, antagonists of the D-ser (“gly”) binding site are neuroprotective in animal models of stroke (Wasterlain et al., 1996; Ohtani et al., 2000, 2003).

To our knowledge, there is only one other report of a temporal response of D-ser to model ischemia. Lo and colleagues (1998) using in vivo microdialysis with HPLC and fluorescence detection investigated efflux of D-ser with 10-min temporal resolution in response to 2-hr focal ischemia in rabbit cortex (Lo et al., 1998). As supported by our study, they found that D-ser efflux was significantly elevated throughout the ischemic period but did not have sufficient time resolution to address whether D-ser and L-glu release occurred within the same functional time window. Additionally, this is the first report of D-ser efflux from hippocampus during modeled ischemia. Hippocampus is highly susceptible to global cerebral ischemia experienced during cardiac arrest (Schmidt-Kastner and Freund, 1991; Kirino, 2000), and hippocampal

tissue has been shown to have particularly high concentrations of both D-ser and NMDAR (Hashimoto and Oka, 1997).

Validity of Microperfusion Chamber

Similarities between results reported here in acute hippocampal slices and results during cerebral ischemia in vivo support the validity of the microperfusion technique. We demonstrate that L-glu increases ~17-fold from baseline during 24 min of in vitro ischemia (Fig. 4b). In support of our data, Mitani and Tanaka (2003) have shown that, during 20 min of bilateral occlusion of the common carotid artery in mouse hippocampus, glu levels, sampled by microdialysis from probes placed in the CA1 region, rose ~14-fold and did not return to basal values after 40 min of reperfusion. With the microperfusion chamber used in the present study, L-asp increases approximately sixfold during 24-min OGD and returns to basal values soon after reperfusion. Similarly, L-asp increases in response to ischemia in vivo (Benveniste et al., 1984; Dawson et al., 2000).

Three points of evidence in our current data support slice viability despite low flow rates. First, we demonstrate stable basal levels of glutamate and other transmitters over the course of 2 hr in the microchamber. Second, treatment of stable basal slices with OGD results in robust efflux of glutamate, which is compelling evidence that slices are viable prior to switching to treatment, insofar as it demonstrates that cells maintain Na⁺ gradients and that these are reversed during OGD. The capacity for slices to recover basal levels of glu that persist at a slightly higher level than basal levels as demonstrated following in vivo ischemia further demonstrates that slices are able to recover ion homeostasis post-OGD. Furthermore, the magnitude of maximal evoked glutamate release and levels during recovery demonstrated here are similar to reported magnitudes for hippocampus in vivo during cerebral ischemia. Taken together, these results suggest that slices are viable in this microperfusion preparation. This validates the microperfusion device for hippocampal slice OGD experiments.

With our 24-min OGD exposure, several factors likely contribute to the delayed glu response seen in these hippocampal slices. First, several minutes are likely required for significant depletion of ATP from the slice preparation. Mitani et al. (1994) demonstrated that over 3 min was required for ~80% ATP depletion in acute hippocampal slices exposed to OGD in a flow-through chamber at 33°C. Second, the temporal response of our slice chamber is about 4.5 min for a complete turnover. This may have the effect of further delaying total ATP depletion and initial chemical response, because it takes longer for tissue to be exposed to the full 100% OGD solution. Third, we expect that chemical diffusion from synaptic regions in the tissue matrix to the bulk medium may limit overall temporal response in 400-μm-thick slices during perfusion experiments.

Significance of L-Thr Efflux

L-thr increases significantly in response to OGD in a manner similar to that of D-ser, with the exception that we note a small but significant elevation of L-thr earlier on in the OGD time course compared with D-ser. The chemical similarity between L-thr and D-ser suggests that these two amino acids use similar transport mechanisms. The sodium-dependent neutral amino acid transporter alanine serine cysteine transporter-2 (ASCT-2) has indeed been demonstrated to show high micromolar affinity for both L-thr and D-ser (Ribeiro et al., 2002; Rutter et al., 2007). ASCT-2 is therefore an attractive candidate for efflux of these amino acids during OGD when transmembrane sodium ion gradients are reversed. Unfortunately, selective inhibitors of ASCT-2 have not yet been developed, so the relative contribution of this Na⁺-dependent mechanism to L-thr and D-ser efflux during OGD remains to be determined.

Significance of L-Gln Efflux

L-gln response to 24-min OGD treatment is different from that of the other amino acids investigated here. The basal level of L-gln steadily decreases in control slices. This result is similar to that of Kapetanovic and colleagues (1993), who demonstrated a dramatic, time-dependent loss of L-gln in mouse and rat acute hippocampal slices under static (no flow) and superfused (3 ml/min, constant flow) conditions. Several studies have demonstrated a similar drop in L-gln during cerebral ischemia in vivo, although never with the same temporal resolution as shown here during OGD (Benveniste et al., 1984; Silverstein et al., 1991; Uchiyama-Tsuyuki et al., 1994; Van Hemelrijck et al., 2005). Many authors have postulated that the decrease of L-gln during ischemia may be due to loss as L-glu via the Gln-Glu cycle (Silverstein et al., 1991; Uchiyama-Tsuyuki et al., 1994; Huang and Hertz, 1995a,b; Phillis et al., 2001; Shen et al., 2008). The particular role of L-gln in excitotoxicity has not been investigated; however, L-gln may enhance excitotoxicity by serving as a source for L-glu.

Advantages of Microperfusion/CE-LIF Technique

The importance of D-ser in the mammalian brain and its relationship to glutamate via NMDA receptor coactivation has led to the need for robust and sensitive approaches for quantifying this transmitter with L-glu in various brain preparations. Chiral HPLC assays have been extensively utilized for D-ser quantification from brain homogenates and in vivo microdialysis samples (Hashimoto et al., 1995; Fukushima et al., 2004; Grant et al., 2006). HPLC assays typically suffer from long migration times (40–60 min) and expensive chiral columns and column switching protocols. Recently, Bowser and colleagues (Ciriacks and Bowser, 2006; Klinker and Bowser, 2007) developed powerful online capillary electrophoresis (CE) microdialysis assays for monitoring D-ser, L-glu, and other amines in vivo and in vitro (O'Brien et al., 2004; O'Brien and Bowser, 2006). The latter studies involved assaying D-ser and L-glu in single excised retina and acute cortical tissue using a microliter-sized retinal perfusion chamber coupled online to microdialysis CE-LIF. To our knowledge, these were the first reports of monitoring rapid dynamic release of endogenous D-ser from an in vitro preparation. Their unique system was capable of monitoring efflux of D-ser and L-glu every 15 sec, although temporal resolution (time required to detect an instantaneous stepwise change in analyte concentration) of the transmitter response was limited by the turnover rate of the chamber (turnover every 4 min), not by sampling rate. Furthermore, the practical limitation of this online CE-LIF approach for routine studies in vitro relates to the fact that multiple CE-LIF platforms would be required for each chamber run in parallel, thus limiting throughput. In addition, the prerequisite for microdialysis sampling of the perfusion medium as it exits the chamber adds unwanted complexity and costs for in vitro studies and compromises quantitative recovery of analyte. Overall, we demonstrate that the microperfusion/CE-LIF technique offers several inherent advantages, including 1) the ability to simultaneously sample from multiple brain slices from a single animal; 2) complete recovery of neurotransmitter, because microdialysis is not required; 3) high temporal in vitro response of neurotransmitter efflux; and 4) ability to monitor simultaneously multiple analytes by CE-LIF with high sensitivity.

Temporal response with the microperfusion approach could be increased further than reported here in future work by increasing perfusion flow rate and sampling frequency. Indeed, one of the advantages of the microperfusion approach in combination with sensitive CE-LIF is the potential to realize these improvements. Our CE-LIF technique utilizes minute volumes of perfusion media and, based on S/N shown in Figure 3 for CBI-amino acids, would likely allow for doubling of flow rate without loss in capacity to detect the amino acids of interest.

In summary, we show that OGD induces D-ser and L-glu efflux from acute slices with similar timing of release, which is important when considering that both are required for NMDAR excitotoxicity. In addition, our data represent the first temporal monitoring of both excitotoxic

chemicals D-ser and L-glu in hippocampus. We describe a new microperfusion technique that is coupled to offline chiral CE-LIF to investigate ischemia-evoked efflux of neurochemicals. The use of our previously reported CE-LIF approach allowed for monitoring efflux profiles of L-asp, L-thr, and L-gln in addition to the targeted excitotoxic chemicals D-ser and L-glu with 2-min temporal resolution. We speculate that this new approach will be valuable for future studies investigating mechanisms of neurochemical regulation.

Acknowledgments

We thank Eric Johansen and Ned Manning from the UAF Engineering Department machine shop for aid in construction of microchambers. We thank Dr. Brian Rasley for his useful comments.

Contract grant sponsor: U.S. Army Research Office; Contract grant number: W911NF-05-1-0280; Contract grant sponsor: U.S. Army Medical Research and Materiel Command; Contract grant number: 05178001; Contract grant sponsor: National Institute of Neurological Disorders and Stroke; Contract grant sponsor: National Institute of Mental Health; Contract grant number: NS041069-06.

References

- Arundine M, Tymianski M. Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. *Cell Mol Life Sci* 2004;61:657–668. [PubMed: 15052409]
- Benveniste H, Drejer J, Schousboe A, Diemer NH. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J Neurochem* 1984;43:1369–1374. [PubMed: 6149259]
- Ciriacks CM, Bowser MT. Measuring the effect of glutamate receptor agonists on extracellular D-serine concentrations in the rat striatum using online microdialysis-capillary electrophoresis. *Neurosci Lett* 2006;393:200–205. [PubMed: 16266784]
- Curras MC, Pallotta BS. Single-channel evidence for glycine and NMDA requirement in NMDA receptor activation. *Brain Res* 1996;740:27–40. [PubMed: 8973795]
- Dawson LA, Djali S, Gonzales C, Vinegra MA, Zaleska MM. Characterization of transient focal ischemia-induced increases in extracellular glutamate and aspartate in spontaneously hypertensive rats. *Brain Res Bull* 2000;53:767–776. [PubMed: 11179841]
- Fukushima T, Kawai J, Imai K, Toyo'oka T. Simultaneous determination of D- and L-serine in rat brain microdialysis sample using a column-switching HPLC with fluorimetric detection. *Biomed Chromatogr* 2004;18:813–819. [PubMed: 15386574]
- Grant SL, Shulman Y, Tibbo P, Hampson DR, Baker GB. Determination of d-serine and related neuroactive amino acids in human plasma by high-performance liquid chromatography with fluorimetric detection. *J Chromatogr* 2006;844:278–282.
- Hashimoto A, Oka T. Free D-aspartate and D-serine in the mammalian brain and periphery. *Prog Neurobiol* 1997;52:325–353. [PubMed: 9247969]
- Hashimoto A, Oka T, Nishikawa T. Anatomical distribution and postnatal changes in endogenous free D-aspartate and D-serine in rat brain and periphery. *Eur J Neurosci* 1995;7:1657–1663. [PubMed: 7582120]
- Huang R, Hertz L. Neuroprotective effect of phenylsuccinate, an inhibitor of cytosolic glutamate formation from glutamine, under anoxic conditions but not during exposure to exogenous glutamate. *Neurosci Lett* 1995a;183:22–26. [PubMed: 7746478]
- Huang R, Hertz L. Potentiation by K⁺ of anoxic release of newly synthesized neuronal glutamate. *Neuroreport* 1995b;6:2404–2408. [PubMed: 8747163]
- Kapetanovic IM, Yonekawa WD, Kupferberg HJ. Time-related loss of glutamine from hippocampal slices and concomitant changes in neurotransmitter amino acids. *J Neurochem* 1993;61:865–872. [PubMed: 8103084]
- Kartvelishvily E, Shleper M, Balan L, Dumin E, Wolosker H. Neuron-derived D-serine release provides a novel means to activate N-methyl-D-aspartate receptors. *J Biol Chem* 2006;281:14151–14162. [PubMed: 16551623]

- Katsuki H, Nonaka M, Shirakawa H, Kume T, Akaike A. Endogenous D-serine is involved in induction of neuronal death by N-methyl-D-aspartate and simulated ischemia in rat cerebrocortical slices. *J Pharmacol Exp Ther* 2004;311:836–844. [PubMed: 15240826]
- Kirino T. Delayed neuronal death. *Neuropathology* 2000;20(Suppl):S95–S97. [PubMed: 11037198]
- Kirschner DL, Jaramillo M, Green TK. Enantioseparation and stacking of Cyanobenz[f]isoindole-amino acids by reverse polarity capillary electrophoresis and sulfated beta-cyclodextrin. *Anal Chem* 2007;79:736–743. [PubMed: 17222044]
- Kleckner NW, Dingledine R. Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. *Science* 1988;241:835–837. [PubMed: 2841759]
- Klinker CC, Bowser MT. 4-Fluoro-7-nitro-2,1,3-benzoxadiazole as a fluorogenic labeling reagent for the in vivo analysis of amino acid neurotransmitters using online microdialysis-capillary electrophoresis. *Anal Chem* 2007;79:8747–8754. [PubMed: 17929877]
- Lipton P. Ischemic cell death in brain neurons. *Physiol Rev* 1999;79:1431–1568. [PubMed: 10508238]
- Lo EH, Pierce AR, Matsumoto K, Kano T, Evans CJ, Newcomb R. Alterations in K⁺ evoked profiles of neurotransmitter and neuromodulator amino acids after focal ischemia-reperfusion. *Neuroscience* 1998;83:449–458. [PubMed: 9460753]
- Mangas A, Covenas R, Bodet D, Geffard M, Aguilar LA, Yajeya J. Immunocytochemical visualization of D-glutamate in the rat brain. *Neuroscience* 2007;144:654–664. [PubMed: 17084987]
- Mitani A, Tanaka K. Functional changes of glial glutamate transporter GLT-1 during ischemia: an in vivo study in the hippocampal CA1 of normal mice and mutant mice lacking GLT-1. *J Neurosci* 2003;23:7176–7182. [PubMed: 12904478]
- Neumar RW, Nolan JP, Adrie C, Aibiki M, Berg RA, Bottiger BW, Callaway C, Clark RS, Geocadin RG, Jauch EC, Kern KB, Laurent I, Longstreth WT Jr, Merchant RM, Morley P, Morrison LJ, Nadkarni V, Peberdy MA, Rivers EP, Rodriguez-Nunez A, Sellke FW, Spaulding C, Sunde K, Vanden Hoek T. Post-cardiac arrest syndrome Epidemiology, pathophysiology, treatment, and prognostication A Consensus Statement From the International Liaison Committee on Resuscitation (American Heart Association Australian and New Zealand Council on Resuscitation, European Resuscitation Council, Heart and Stroke Foundation of Canada, InterAmerican Heart Foundation, Resuscitation Council of Asia, and the Resuscitation Council of Southern Africa); the American Heart Association Emergency Cardiovascular Care Committee; the Council on Cardiovascular Surgery and Anesthesia; the Council on Cardiopulmonary, Perioperative, and Critical Care; the Council on Clinical Cardiology; and the Stroke Council. *Circulation*. 2008in press
- O'Brien KB, Bowser MT. Measuring D-serine efflux from mouse cortical brain slices using online microdialysis-capillary electrophoresis. *Electrophoresis* 2006;27:1949–1956. [PubMed: 16703626]
- O'Brien KB, Esguerra M, Miller RF, Bowser MT. Monitoring neurotransmitter release from isolated retinas using online microdialysis-capillary electrophoresis. *Anal Chem* 2004;76:5069–5074. [PubMed: 15373444]
- Ohtani K, Tanaka H, Yasuda H, Maruoka Y, Kawabe A, Nakamura M. Blocking the glycine-binding site of NMDA receptors prevents the progression of ischemic pathology induced by bilateral carotid artery occlusion in spontaneously hypertensive rats. *Brain Res* 2000;871:311–318. [PubMed: 10899297]
- Ohtani K, Tanaka H, Ohno Y. SM-31900, a novel NMDA receptor glycine-binding site antagonist, reduces infarct volume induced by permanent middle cerebral artery occlusion in spontaneously hypertensive rats. *Neurochem Int* 2003;42:375–384. [PubMed: 12510020]
- Oliet SH, Mothet JP. Molecular determinants of D-serine-mediated gliotransmission: from release to function. *Glia* 2006;54:726–737. [PubMed: 17006901]
- Phillis JW, Ren J, O'Regan MH. Studies on the effects of lactate transport inhibition, pyruvate, glucose and glutamine on amino acid, lactate and glucose release from the ischemic rat cerebral cortex. *J Neurochem* 2001;76:247–257. [PubMed: 11145998]
- Quan Z, Liu YM. Capillary electrophoretic separation of glutamate enantiomers in neural samples. *Electrophoresis* 2003;24:1092–1096. [PubMed: 12658700]
- Ribeiro CS, Reis M, Panizzutti R, de Miranda J, Wolosker H. Glial transport of the neuromodulator D-serine. *Brain Res* 2002;929:202–209. [PubMed: 11864625]

- Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell C, Roger V, Sorlie P, Steinberger J, Thom T, Wilson M, Hong Y. Heart disease and stroke statistics—2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2008;117:e25–e146. [PubMed: 18086926]
- Rutter AR, Fradley RL, Garrett EM, Chapman KL, Lawrence JM, Rosahl TW, Patel S. Evidence from gene knockout studies implicates Asc-1 as the primary transporter mediating d-serine reuptake in the mouse CNS. *Eur J Neurosci* 2007;25:1757–1766. [PubMed: 17432963]
- Schmidt-Kastner R, Freund TF. Selective vulnerability of the hippocampus in brain ischemia. *Neuroscience* 1991;40:599–636. [PubMed: 1676492]
- Shen J, Rothman DL, Behar KL, Xu S. Determination of the glutamate-glutamine cycling flux using two-compartment dynamic metabolic modeling is sensitive to astroglial dilution. *J Cereb Blood Flow Metab.* 2008in press
- Shleper M, Kartvelishvily E, Wolosker H. D-serine is the dominant endogenous coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices. *J Neurosci* 2005;25:9413–9417. [PubMed: 16221850]
- Silverstein FS, Naik B, Simpson J. Hypoxia-ischemia stimulates hippocampal glutamate efflux in perinatal rat brain: an in vivo microdialysis study. *Pediatric Res* 1991;30:587–590.
- Uchiyama-Tsuyuki Y, Araki H, Yae T, Otomo S. Changes in the extracellular concentrations of amino acids in the rat striatum during transient focal cerebral ischemia. *J Neurochem* 1994;62:1074–1078. [PubMed: 8113794]
- Van Hemelrijck A, Sarre S, Smolders I, Michotte Y. Determination of amino acids associated with cerebral ischaemia in rat brain microdialysates using narrowbore liquid chromatography and fluorescence detection. *J Neurosci Methods* 2005;144:63–71. [PubMed: 15848240]
- Wasterlain CG, Adams LM, Wichmann JK, Sofia RD. Felbamate protects CA1 neurons from apoptosis in a gerbil model of global ischemia. *Stroke* 1996;27:1236–1240. [PubMed: 8685935]

Fig. 1. Custom microperfusion chamber for single acute brain slices showing polycarbonate microperfusion chamber (a), schematic diagram of major components of microchamber (b; actual chamber dimensions $L \times W \times H \sim 9 \times 5 \times 0.7$ mm, with 0.3-mm deep microchannel support), magnification of microchamber well illustrating microchannel support designed to increase fluid flow around slice (c), and schematic of microperfusion setup from syringe pump to fraction collection (d).

Fig. 2. OGD treatment profile determined by using trace D-glu perfusion across slices. A stepwise change in D-glu levels (0.5 μ M switched to 3.0 μ M) occurs in $\sim$ 4.5 min, which establishes the turnover rate of the microchamber and allows for precise monitoring of the treatment profile.

Fig. 3.

Comparison of typical electropherograms from basal (**left**) and OGD (**right**) perfusate samples demonstrating changes in analyte concentrations relative to internal standard (IS). Basal sample shown is from perfusate collected 10 min prior to OGD treatment, whereas OGD sample shown was collected 24 min into OGD treatment. Both basal and OGD samples were obtained from the same acute slice experiment.

Fig. 4. Efflux of D-ser occurs in parallel with efflux of L-glu during OGD in acute hippocampal slices. Percentage change in endogenous amino acid concentrations relative to average of last 10 pretreatment samples are shown: D-ser (a), L-glu (b), L-asp (c), L-thr (d), and L-gln (e). Sampling and analysis were carried out using the newly developed microperfusion/CE-LIF strategy. Temperature was maintained at 36°C throughout perfusion. In control experiments, perfusate treatment was administered by switching to standard oxygenated aCSF medium. Data points represent average $\pm$ SEM of n slices as indicated. * $P < 0.05$, OGD vs. CTL (Tukey post hoc comparison).

TABLE I
Weighted Least Squares Linear Regression Results for Concentration Determinations for Each Analyte Demonstrating Linearity of the CE-LIF Method[†]

Analyte	Equation	R ²	Calibrated range, μ M	CLOD [★] , nM
L-glu	$y = 0.388x - 0.032$	0.997	0–63.0	14.7 ± 3.8
L-asn	$y = 0.308x + 0.002$	0.999	0–25.2	16.0 ± 2.1
D-ser	$y = 0.260x - 0.001$	0.9961	0–1.80	19.3 ± 4.4
L-thr	$y = 0.247x - 0.005$	0.9995	0–7.20	26.2 ± 8.8
L-gln	$y = 0.357x - 0.031$	0.9968	0–36.0	12.5 ± 1.5

[†]Weighted standard curve equations ($y = mx + b$; CBI-AA/internal standard = $m[\text{CBI-AA}] + b$), square of correlation coefficient (R^2), calibrated range, and estimated CLOD. Curves were generated from weighted linear regression of ≥ 6 standard solutions, with $n = 5$ for each standard.

[★]CLOD calculated from response of a low-concentration standard ($n = 5$) assuming $S/N = 3$.