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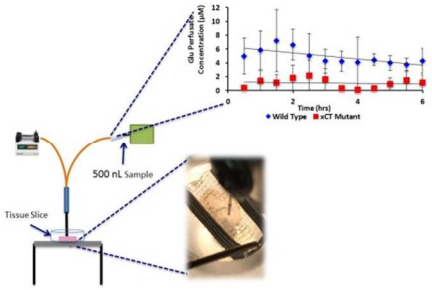

Low flow push-pull perfusion is used to measure extracellular glutamate levels from mouse brain tissue slices.

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ARTICLE

Sample Collection and Amino Acids Analysis of Extracellular Fluid of Mouse Brain Slices with Low Flow Push-Pull Perfusion.

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Brain tissue slices are a common neuroscience model that allows relatively sophisticated analysis of neuronal networks in a simplified preparation. Most experimental methodology utilizes electrophysiological tools to probe these model systems. The work here demonstrates the adaptation of low-flow push-pull perfusion sampling (LFPS) to a brain slice system. LFPS is used to sample from the hippocampus of mouse brain slices. Perfusate amino acid levels are quantified following sampling with capillary electrophoresis. Glutamate was measured from the CA1 region of the hippocampus in slices taken from a cystine-glutamate transporter deletion mutant, *xCT*$^{-/-}$, and the background strain C57BL/6J. Sampling is performed over up to 6.5 hr with standard tissue slice preparation and experimentation methods. Four amino acids were quantified to demonstrate the ability to perform LFPS and show good agreement with published literature. Perfusate glutamate levels are found to be significantly lower with *xCT*$^{-/-}$ slices (1.9 (± 0.5) μM) relative to controls (4.90 (± 1.1) μM). But, experiments with control slices show a significant decrease in glutamate over the 6 hr sampling period that are not seen with *xCT*$^{-/-}$ slices. Increasing the LFPS sample collection rate during the first 90 min of sampling did not show a sampling artifact in perfusate glutamate content. Sampling immediately following slicing did not show an early increasing glutamate level that would be indicative of a significant contribution from blood or tissue damage. The data presented here show a complementarity to electrophysiological studies of tissue slices. The ability to characterize extracellular fluid chemical content with LFPS in these slices provides an alternative data stream for probing neurochemical signaling networks in brain tissue slices.

Introduction

Sampling the extracellular space has provided important insights into the patterns of chemical signaling in the central nervous system (CNS).[1] *In vivo* approaches such as microdialysis,[2–4] electrochemical measurements,[5,6] direct sampling,[7] and push-pull perfusion[8–12] have been important tools to monitor molecules in the brain. While *in vivo* sampling provides unique information for understanding neurochemical signaling in the brain, a brain slice model system offers advantages of ease of access to individual cells and networks and manipulation of the slice environment. Brain slices are a common experimental model for electrophysiological studies.[13] Unfortunately, the thin tissue geometry presents a challenge for most *in vivo* sampling approaches for obtaining chemical content information from thin tissue slices. In this work the *in vivo* sampling approach low-flow push-pull perfusion sampling (LFPS) is explored as a means to collect and monitor chemical content of the extracellular fluid of brain slices.

Low flow push-pull perfusion (LFPS) is an *in vivo* sampling technique to monitor neurotransmitters in the brain. LFPS has been previously used to monitor the amino acid content in extracellular fluid *in vivo* with rodents.[8–12,14] With the low flow rates ~50 nL/min commonly used, LPFS has *in vitro* recoveries as high as 80% and has not shown visible tissue damage at the sampling probe tip common to earlier push-pull perfusion studies.[10] The small dimensions of the probe and the low flow rates likely contribute to this reduction in probe damage. Because sampling proceeds at a capillary tip, the perfusion tissue volume is limited and provides highly localized sampling *in vivo* that may be applicable to sampling from tissue slices.[10,14,15]

Although only a 200-300 micron thick slice of the brain, many *in vivo* features such as brain architecture and local synaptic circuitry are preserved allowing experimental control of tissue environment and access to the individual cells in their native networks.[16] Most studies are electrophysiological which do not provide direct chemical information.[17] While slice-based

systems provide fitting experimental access to tissue, the thin tissue geometry is typically not well suited to typical tissue volumes studied with *in vivo* sampling approaches, and it remains unclear whether the extracellular chemical composition in slices remains similar to that in intact brains. The unknown extracellular chemical composition with tissue slices is compounded by high flow rate superfusion bath that is used with well-established methodology to keep the tissue alive and functioning.

There has been a number of efforts to obtain extracellular content information from tissue slices. A couple of early efforts utilized the 1-2 mm active length of a microdialysis probe lying on top of a mouse brain slice for serotonin.[18,19] There is also a report of an in-house constructed, 0.22 mm-long microdialysis probe that was used to monitor glutamate in the nucleus acumens and prefrontal cortex of rats brain slices.[20] Another approach is use applied electric fields across tissue slice bath and the tip of a collection capillary.[21] In order to improve the spatial resolution of the sample collection method an electric field was applied between a "push" and "pull" capillary placed within a tissue slice.[22] These electrogenerated flow approaches have mostly been used in conjunction with substrate delivery to study tissue ectoenzyme activities[21,23] but, there is also a report of the measurement of endogenous aminothiols.[24]

Here, we focus on glutamate. Glutamate is the major excitatory neurotransmitter in the mammalian CNS and understanding the endogenous levels is key to modeling individual transporters and receptors as well as characterizing neuronal networks. Reports of the concentration of glutamate found endogenously in the nervous system vary by more than three orders of magnitude (nM to μM)[14,25] Glutamate can be released from cells in the CNS via swelling activated anion channels, gap junction hemi channels, purinergic (P2X) receptors, synaptic vesicles and cysteine-glutamate exchangers.[26] The contribution to clearance and recycling mechanisms needs to be better defined and may be a result of tissue anatomy.[26] Determination of glutamate in the CNS is essential to understand neurochemical control. The cysteine-glutamate transporter (xCT) is a plasma membrane protein that exchanges intracellular glutamate for extracellular cystine.[25] xCT has been hypothesized to regulate the extracellular glutamate levels *in vivo*.[14,26–28] The glutamate levels in *xCT*$^{-/-}$ mice have been measured through microdialysis[20] and LFPS[6] to understand the role of xCT in maintaining extracellular glutamate levels.

In this work, we demonstrate the feasibility of LFPS as a means for *ex vivo* monitoring of glutamate and other neurotransmitters in thin brain slices. LFPS collection followed by capillary electrophoresis separation and quantitation was used to obtain perfusate levels of extracellular amino acid concentrations. Extracellular glutamate content was monitored for up to a 6.5-hr period in order to define fluctuations in perfusate levels. Sampling procedures and tissue preparation conditions were studied relative to observed glutamate levels. As an application of this slice-LFPS *ex vivo* sampling approach, basal perfusate levels of glutamate were determined from mutant mice (*xCT*$^{-/-}$) with a knocked-out cystine-glutamate transporter as well as C57BL/6J mice with the same genetic background but an intact cystine-glutamate transporter gene.

Materials and Methods:

Chemical and Reagents:

Chemicals were purchased from Fisher Scientific (Itasca, IL) unless otherwise noted. L-glutamic acid was obtained from Sigma-Aldrich (St. Louis, MO.) The composition of the artificial cerebrospinal fluid (aCSF) was 2 mM CaCl, 124 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 1.25 NaH_2PO_4, 1 mM $MgSO_4$, and 10 mM glucose, pH~7.3. The aCSF had an osmolarity of 300-305 mΩsm and was used after bubbling with 95% O_2, 5% CO_2 for an hour. All solutions, unless stated otherwise, were prepared in deionized water, from US Filter Purelab Plus water purification system (Lowell, MA) and filtered with 0.22-μm Millex GP filters purchased from Millipore Corp. (Bedford, MA). L-glutamate stock solutions were prepared in aCSF. Epoxy-based sealant (Quick setting Epoxy Adhesive) was purchased from Home Depot (Chicago, IL). CO_2 and O_2 gas tanks were obtain from Linde gas (Chicago, IL).

Animals:

All procedures were reviewed and approved by the University of Illinois at Chicago Animal Care and Use committee. xCT knockout (xCT$^{-/-}$) mice (Slc7a11^{tm1Hsat}; RRID: MGI_MGI :3608978), were generously provided by Hideyo Sato, Yamagata University, Japan.[29] The xCT knockout mutation was generated in the C57BL/6J background and outcrossed with C57BL/6J more than 10 times. xCT expression is completely eliminated in these *xCT*$^{-/-}$ mice. Homozygous C57BL/6J mice were used as controls.

Low Flow Push-Pull Probe:

The push-pull probe construction procedure was described previously.[10] Briefly, the sampling probe is a concentric construction of fused-silica capillaries and stainless steel tubing (Polymicro Technologies, Phoenix, AZ) that permits direct infusion of a physiological saline and withdrawal of perfusate to the tip of the sampling probe. A Harvard PhD 2000 syringe pump (Harvard Apparatus, Holliston, MA) is connected to an outer infusion capillary (360/75 μm o.d./i.d) that feeds a 355/180 μm o.d/i.d. stainless steel probe tip. Simultaneous perfusate sample collection via vacuum (Thermo Scientific, Barrington, IL) is through an withdrawal capillary (150/75 μm o.d./i.d) inserted into the stainless steel tip and connected to a 4-cm, ~270 μm i.d. tygon tubing that is replaced with the collection of individual samples.

Slice Preparation

To obtain brain tissue, male P14-22 mice were anesthetized with isoflurane and decapitated. Whole brains were then immediately removed, and the hippocampus isolated. The

hippocampus was isolated by a method described as the "Magic cut".[30] A vibrating microtome was used to prepare 300 μm thick hippocampal slices in an ice-cold cutting and storage solution (87 mM NaCl, 2.5 mM KCl, 7 mM $MgCl_2$, 75 mM sucrose, 25 mM glucose, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 2.0 mM $CaCl_2$). Slices were then immersed in a freshly prepared aCSF at 35°C for 30-45 minutes, after which they were allowed to incubate in aCSF at room temperature.

Sampling from Brain Slices

The LFPS probe was mounted on a three-axis high-resolution micromanipulator for control and placement while sampling. Prior to placing the brain slice into the sampling 2.0 mL reservoir, the position of the bottom of the reservoir was recorded and used to place the probe tip 30 µm within the tissue. This is similar to patch pipet placement in our previous study.[31] For placement of the aCSF-filled probe the flow rate of the infusion capillary was increased to 500 nL/min to prevent clogging of the probe tip while the withdrawal remained 50 nL/min. Immediately after placement, the infusion flow rate was returned to 50 nL/min. All LFPS samples were taken from the CA1 region of the hippocampus. The sampling time is referenced to LFPS probe placement (time = 0). At time = 0, a 10 min sample was collected. Subsequent 10 min samples were collected every 30 min for a 6 hr period. There was continuous perfusion during the 20 min between sample collections but this perfusate was discarded. All collected samples were analyzed for amino acid content by CE. In order to increase the temporal resolution in some experiments, samples were collected every 10 min. A total of 5 trials performed for the C57BL/6J . Only 3 trials were performed with the $xCT^{-/-}$ due to an infanticide phenotype behavior exhibited by the $xCT^{-/-}$ mutant.

In order to study the effect of slice preparation methods, the LFPS probe was inserted into the tissue immediately after slicing and placement in the sampling reservoir. To match tissue processing from other experiments the temperature of the reservoir aCSF was maintained at 35 ˚C by flowing aCSF at this temperature for 30 min. After this period, the bath superfusion was changed to room temperature (20 ˚C) aCSF, to mimic procedures typically used for electrophysiological experiments

Electrophoretic Assay:

The conditions for the detection of the amino acids in small volume samples were previously optimized in our laboratory.[6] Samples were derivatized with fluorescamine in a 1:1 volume ratio (sample:fluorescamine). Electrophoretic analysis was performed on a laboratory-built CE system with a commercial high voltage power supply (Spellman, NY) and a ZETALIF detector (Picometrics, Paris, France) modified in-house with a LED 365 nm (Prizmatix, Modiin-Ilite, Israel). All separations were achieved in 360/50µm o.d./i.d. fused-silica capillary (Polymicro Technologies) with a total length of 50 cm (35 cm effective) at 27 kV applied potential. The CE capillary, buffer vials, and electrode assembly were isolated with a Plexiglas box to separate the components and protect the operator. Before use, the capillary was conditioned with 1.0 M NaOH, deionized water, and the separation buffer at intervals of 45 to 60 sec each. Samples were injected gravimetrically at 19 cm for 15 s allowing ~7.6 nL to be injected into the capillary. Consecutive injections were performed from the same vial.

Data Analysis:

Fluorescence data from the electrophoretic run was exported from a custom LabVIEW data acquisition program to Microsoft Excel in order to plot electropherograms. Peak heights were measured from electropherograms by subtracting the baseline, which was considered to be the average signal 1 s before the first peak of the electropherogram, from the peak maximum. The noise was calculated as the standard deviation of the same baseline section of the electropherogram. The signal-to-noise ratio (S/N) was calculated from the peak height with baseline subtracted divided by the standard deviation of the baseline. Concentrations were derived from calibration curves generated with standards. Microsoft Excel was used to perform statistical tests.

Results and Discussion:

LFPS with mouse brain slices:

LFPS has been reported for *in vivo* sampling within the brain[10,14] and has been demonstrated to be used at the vitreoretinal interface of the rat.[11,12,32] The sampling geometry is such that infused saline is mixed and withdrawn at the 100-200 µm diameter probe tip. Here, LPFS is placed 30 µm below the surface of the tissue slice within the CA1 of mouse hippocampal slices. This localized, nanoliter *ex vivo* sampling of extracellular fluid for content analysis was performed using tissue from homozygous $xCT^{-/-}$ mutant mice as well as the background strain C57BL/6J. Probe placement was performed by visualization of CA1 synapse region similar to previously published work.[31]

In Figure 1, example electropherograms are plotted showing a number of amino acid peaks that were identified via spiking with standard amino acids. Figure 1A is a representative electropherogram of a sample collected from a control mouse hippocampal slice. Qualitatively, the amino acid profile appears very similar to results that we have previously obtained from intact anesthetized animals.[14] Importantly, glutamate was easily identified and found to be present at a level for quantitation. Three other amino acids were also identified from perfusate samples and average concentrations observed are listed in Table 1. The presence of GABA, another important neurotransmitter expected to be found, could not be confirmed by standard spiking in all samples. The observation of amino acids from this preparation is significant because the thin slice of tissue is placed in a superfusion bath with a 2-3 mL/min flow of aCSF as is typically for most brain tissue slice preparations. The diffusion of amino acids from the tissue into the bath combined with the relatively high turnover of the superfusion fluid might be expected to contribute to significant loss of amino acids.

The ability to collect extracellular fluid chemical content from a tissue slice provides a means to study tissue slice models. These simplified tissue preparations are often used to probe neuronal networks that may not be possible or as facile in an *in vivo* model. The LFPS format with sample collection at a capillary tip is well suited to the thin tissue slice and avoids the need for a custom microdialysis probe[20] or collection on the surface of a tissue preparation.[19] The pressure driven format with LFPS may also afford a simple alternative to electroosmotic sampling approaches.[22]

Figure 1B shows an example electropherogram of a sample collected from an *xCT*$^{-/-}$ hippocampal slice. Similar to the electropherogram in Figure 1A, several amino acids were identified in common between the *xCT*$^{-/-}$ and control, including glutamate, glycine, aspartate and glutamine. These electropherograms are also similar to those obtained after *in vivo* LPFS from the striatum of a different xCT mutant allele (C3H/HeSnJ-*Slc7a11*^{sut}).[14] There is an observed difference in migration time between the electropherograms in Figure 1. However, this difference can be attributed to the extent of protein absorption to the capillary wall which results in reductions in electroosmotic flow (EOF).

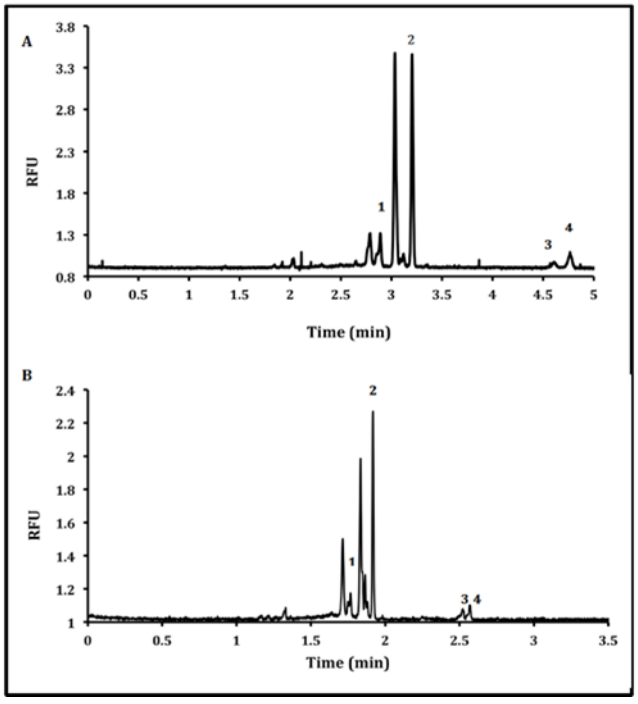

Figure 1. Representative electropherograms of 500 nL samples of extracellular fluid collected with LFPS from the CA1 region of hippocampal slices of A) wild type, B) xCT-/-mutant mice. Samples were labelled with the amine-reactive fluorophore fluorescamine for LED-induced detection during separation with CE. Separation conditions: 50/360 µm (id/od) fused-silica capillary, 35/50 cm effective length/total length, 540 V/cm field strength and run buffer 20 mM borate. Peaks identified by spiking with standards: 1-Gln,2-Gly,3-Glu,4-Asp.

A major difference seen between the previous *in vivo*[14] and *ex vivo* experiments here was the presence of an arginine peak with *in vivo* samples. The loss of arginine *ex vivo* might be due to diffusional losses of the analyte to the bath without replacement by a compensatory mechanism or an actual difference in tissue composition between these different strains and brain regions. More experiments will be needed to explore these possibilities considering the standard high flow rate superfusion bath could be contributing an extracellular composition bias. The overall qualitative similarity in the electropherogram peak profiles obtained from these mouse models collected both from a tissue slice and *in vivo* support the validity of the data obtained with this slice LFPS approach.

The average measured concentrations for the four identified amino acids from both control and *xCT*$^{-/-}$ slices can be seen in Table 1. The only significant difference observed was a 61% decrease in glutamate concentration from the *xCT*$^{-/-}$ slices relative to C57BL/6J controls ($p<0.0001$). This is comparable to the decrease observed in the hippocampus as measured by *in vivo* microdialysis.[25,33] This data, in contrast to the arginine data above, suggests that alterations in extracellular glutamate can be maintained in *in vitro* slice preparation with the superfusion and the difference compositions can be detected by the LPFS.

Table 1. Average LFPS perfusate concentration of amino acids from mouse hippocampal slices

Amino Acid	Control (µM)	XCT$^{-/-}$ (µM)
Glutamate	4.9±1.1***	1.9±0.5***
Aspartate	1.1±0.4	1.2±0.5
Glutamine	8.2±2.4	6.1±1.6
Glycine	3.1±1.6	2.6±0.9

***Significant difference control (N=5) vs. *xCT*$^{-/-}$ (N=3) t-test ($p<0.0001$).

The xCT amino acid transporter is a trans-membrane protein that is hypothesized to exchange intracellular glutamate with extracellular cystine in a 1:1 ratio. There are also other reports in the literature that showed similar glutamate concentrations *in vivo* with microdialysis in the striatum in the same strain of mice.[28,33] A comparison of C57BL/6J perfusate glutamate levels here to our previous report of *in vivo* striatum glutamate levels measured in C3H/HeSnJ mice show slice perfusate levels to be higher.[14] In contrast, the mutant perfusate glutamate both here with hippocampal *xCT*$^{-/-}$ and with striatal C3H/HeSnJ-*Slc7a11*^{sut} are nearly identical.[14] This suggests that there may be substantial variation in glutamate between different 'control' mouse strains, which are nonetheless similar in the absence of xCT.

Moreover, aspartate levels seen here are quite similar between the C57BL/6J and the *xCT*$^{-/-}$ slice preparations. This is somewhat surprising due previously observed significant differences found in the levels of aspartate in the C3H/HeSnJ-*Slc7a11*^{sut} mice *in vivo*[14]. This again suggests interesting previously undescribed differences between mouse strains or

the different brain regions studied, or possibly differences between *in vivo* and *ex vivo* sampling. While not a significant decrease, the 16% lower glycine perfusate concentration for the *xCT*$^{-/-}$ compared to the C57BL/6J should be further explored as possibly related to the lower endogenous glutamate levels.

Monitoring Glutamate levels through time:

One of the features of a sampling approach is the capability to monitor neurotransmitter concentrations through time. In particular, the availability of chemical content information would complement electrophysiological studies if available from tissue during slice model preparation and storage as well as during experimentation.

In Figure 2 the average perfusate glutamate concentrations for 10 minute samples are plotted every 30 min for 6 hr. In both plots for control and mutants the traces show indistinguishable average relative fluctuations between individual time points. The observed glutamate levels at each time point show a 22.8%RSD for control and 28.5%RSD for *xCT*$^{-/-}$. Moreover, these levels of fluctuation are entirely in line with *in vivo* data.[14] Consistent with the average glutamate concentrations over the entire sampling period, glutamate levels are consistently lower from *xCT*$^{-/-}$ slice preparations compared to those of C57BL/6J. Further differences are observed when regression lines are calculated over the 6 hr sampling experiment for mutants and control slices. The linear regression for the control, y=-0.44x+6.3, shows a nearly order-of-magnitude steeper slope (Δ[Glu] μM/Δhr) compared to the regression for *xCT*$^{-/-}$, y=-0.043x+2.1. The *xCT*$^{-/-}$ mutant regression appears nearly flat. Although there is a large difference in the average slope, a regression analysis of these slopes shows that there is not a significant difference (P-value=0.06). While the average [Glu] concentration in C57BL/6J control tissue is consistent with *in vivo* values over the entire sampling period, the values measured from slices also appear to decrease the longer that the tissue is bathed, suggesting that glutamate is diffusing out of the slice. This difference between control and *xCT*$^{-/-}$mutant mice is significant as both tissues are subject to the same brain slice preparation and superfusion maintenance procedures. However, the first few measurements are not very different from those after 6 hours. Thus, an alternative possibility is that there is a rise in the glutamate concentration that occurs during the first 90 min of sampling for the control brain slices, and the apparent reduction thereafter is simply a return to 'normal'. In this case, it is notable that peak concentrations around 90 minutes were not seen for the *xCT*$^{-/-}$mutant mice.

To measure any possible increase in the glutamate levels over the first 90 min of sampling of C57BL/6J control slices, the sample collection rate was increased to continuous sample collection every 10 min. In Figure 3 average perfusate glutamate concentrations are plotted for the first 90 min of

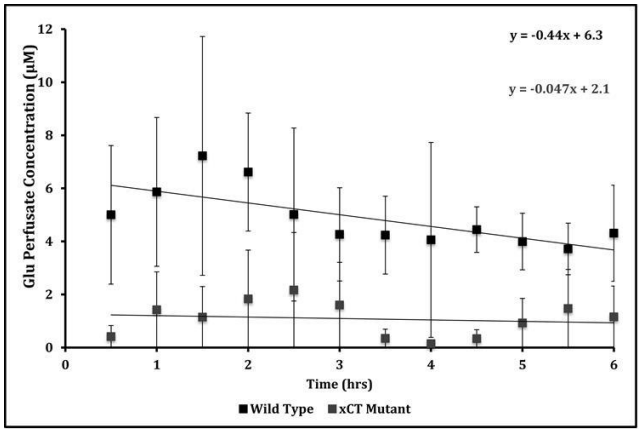

Figure 2. Comparison of LFPS perfusate glutamate levels obtained from control and *xCT*$^{-/-}$ hippocampal mouse brain slices at 20°C over 6 hr. Linear regressions of data are over entire sampling period. Wild type control N=5, *xCT*$^{-/-}$ mutant N=3.

sampling with samples collected every 10 min, and for comparison, the first 90 min of control data from Figure 2 is replotted. The first 90 min of sample collection at either sampling frequency does show a positive slope with a regression of the measured glutamate. However, a one-way ANOVA of the data showed there was not a significant change in glutamate concentration over the 90 min period (P>0.7). This suggests that there is not an increase in glutamate during the first 90 min of sampling that is only present in control slices and contributes to the decrease in glutamate levels over sampling time.

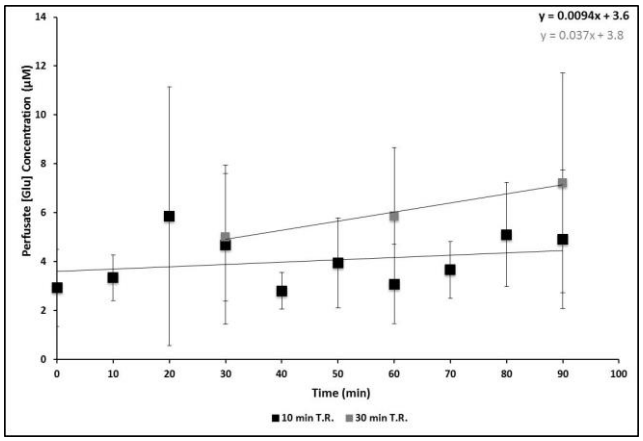

Figure 3. Comparison of 10 min/500 nL LFPS perfusate glutamate levels obtained control mouse hippocampal brain slices collected every 10 (black squares) or 30 min (gray squares). The linear regression equations include all time points up to the first 90 min of sampling at 30 min (N=5) and 10 min (N=3) sampling rates.

Slice preparation procedure and glutamate levels:

Typically in brain slice experiments, harvested brain tissue is sliced and stored for periods extending several hours prior to study. The brain tissue is sliced at temperatures near 0˚C, then allowed to recover for almost an hour in a quiescent 35˚C bath before being stored at room temperature (~20˚C) in a superfused bath prior to experiments. The slices are maintained in artificial cerebrospinal fluid that is bubbled with O_2 and CO_2

but not proteins, amino acids or other small molecules typically found in cerebrospinal fluid.[34] To mimic the tissue preparation and storage effects on glutamate in the tissue, LFPS was performed both during tissue incubation in the warmer 35˚C bath for 30 min immediately following slicing and for 6 hr at the room temperature (20 ˚C) in a superfused bath.

The glutamate levels observed for both the high temperature and subsequent room temperature baths are plotted in Figure 4. The levels of glutamate in the higher temperature bath do trend toward a decrease over the first 30 min of sampling as a one-way ANOVA (P>0.0507) suggests. However, the absolute level of glutamate in the first sample is not significantly higher over samples collected during the first 90 minutes of sampling. The disruption cause by slicing tissue might be expected to dramatically increase tissue glutamate levels due to the 10-fold higher blood concentration or cell damage,[35] at least initially. The observation that glutamate levels are not significantly elevated may be explained by the fact that sampling is being performed 30 µm within the tissue away from slicing-induced damage. It is possible that any chemical contribution from tissue damage is likely drawn away from the tissue down the steeper concentration gradient into the superfusion bath rather than into the tissue where the tip of the sampling probe is positioned.

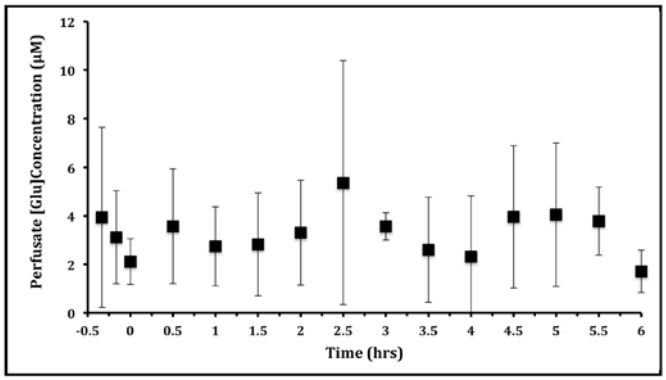

Figure 4. Average of 10 min LFPS perfusate glutamate levels collected from hippocampal mouse brain slices of wild type animals upon placement of slice into a 35°C aCSF bath after slicing for 30 min and for 6 hr after transfer to the 20°C bath. N=3.

Also seen in Figure 4 are the glutamate concentrations during the subsequent 6 hr sampling period in the superfused, room temperature bath. These data are similar to those in Figure 2 with the exception that the probe had been positioned 30 min earlier. A Students' *t*-test of the average glutamate concentrations for the 6 hours following removal from the warm bath are not significantly different from glutamate levels from controls displayed in Figure 2. Nor is there a difference seen via F-test in the average 30.3% (%RSD) fluctuations in glutamate levels between these data sets. Probe placement is expected to lead to damage that leads to higher observed levels of glutamate in samples that typically require 30-120 min to stabilize *in vivo*. These data with tissue slices suggest that any stabilization may be much more rapid. Alternatively, there may be ongoing probe damage after probe placement that leads to a damage-induced elevation of glutamate levels that precludes observation of a difference between an extra 30 min of sampling. Histological experiments similar to others[36–38] would assist in determining the potential for damage-induced elevation of glutamate.

Complementary of LFPS and electrophysiological data:
Electrophysiology is the primary means to study brain slices but the use of data from sampling can be complementary because it is a direct measure of chemical composition. In the work here LFPS has shown a decrease in extracellular glutatmate in hippocampal slices from *xCT*$^{-/-}$ mutants compared to C57BL/6J controls. This is consistent with the hypothesis that loss of xCT function leads to lower extracellular glutamate. The lower extracellular glutamate is then hypothesized to lead to an increase in the number of AMPA glutamate receptors.[26] Consistent with this hypothesis a recent report shows both electrophysiological and immunohistochemical data that there is an increase in AMPA receptors with *xCT*$^{-/-}$ mutants compared to controls.[31] Further, C57BL/6J control tissue shows increased response from AMPA receptors over time with bath superfusion treatment of glutamate-free aCSF. After 3-6 hours of aCSF treatment control tissue slices electrophysiologically phenocopied *xCT*$^{-/-}$ mutants.[31] This control tissue increase in AMPA receptor response with time is not seen when glutamate-containing aCSF is used. In Figure 5, the average level of extracellular glutamate measured via LFPS is shown both for the first and last 3 hours of superfusion with glutamate-free aCSF. A statistical analysis shows a significant decrease in the glutamate levels for the C57BL/6J controls during the first 3 hours of sampling when compared with the last 3 hours of sampling ($p<0.05$).

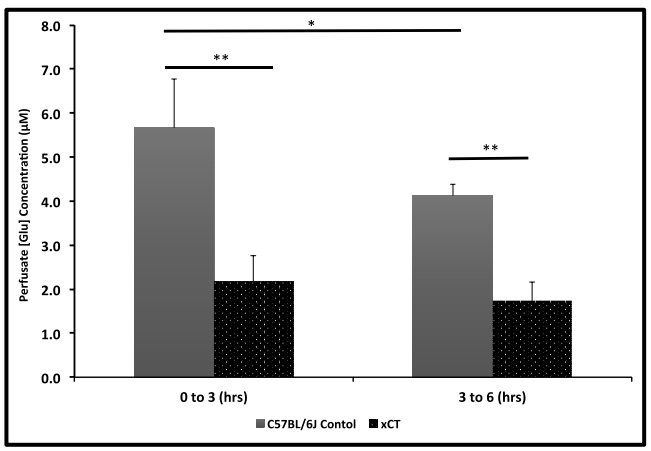

Figure 5. Average glutamate levels during the first three hours of sampling in comparison with the last three hours of sampling. *Significant difference via *t*-test of C57BL/6J samples between first and last 3 hours of sampling ($p<0.05$). **Significant difference via *t*-test between glutamate levels for C57BL/6J and the *xCT*$^{-/-}$ during the first or final 3 hours of sampling ($p<0.0001$). C57BL/6J N=5, *xCT*$^{-/-}$ N=3

No decrease is observed over time with the same glutamate-free aCSF superfusion treatment of *xCT*$^{-/-}$ mutants. Although

glutamate is significantly higher in controls versus mutants at both time points, the significant decrease in glutamate level over the 3 hour treatment of control tissue is consistent with electrophysiological data[31] and the extracellular glutamate-driven mechanism for electrophysiological and immunohistochemical differences seen between *xCT*$^{-/-}$ mutants and C57BL/6J controls. While there may be other possible mechanisms that ought to be experimentally studied to explain this observation, the addition of extracellular chemical composition data from LFPS provides a unique line of evidence that is complementary to that generated by electrophysiology.

Conclusions

In this work we have demonstrated a capability of LFPS sampling for thin brain slices. Amino acid content was quantified in extracellular fluid perfusates as seen by capillary electrophoresis. Glutamate, a major neurotransmitter, was identified and quantified in control and a *xCT*$^{-/-}$ mouse mutant where the cysteine-glutamate transporter is not expressed. A 61% difference in observed extracellular glutamate was found between control and *xCT*$^{-/-}$ similar to that seen by others measuring from intact brains in living mice. These data support the theory of the involvement of this transporter in regulating the extracellular glutamate levels. Collecting samples at a higher sampling frequency does not appear to influence observed glutamate levels. The observed glutamate levels also cannot be attributed to tissue damage due to probe placement, which was the same in both genotypes. Finally LFPS was used to complement electrophysiology data to enhance possible interpretations. In future the combination of electrophysiological data collection with LFPS would provide a more full picture of state of tissue in brain slice experiments.

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Notes and references

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