Imaging Taurine in the Central Nervous System Using Chemically Specific X-ray Fluorescence Imaging at the Sulfur K-Edge

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Abstract

A method to image taurine distributions within the central nervous system and other organs has long been sought. Since taurine is small and mobile, it cannot be chemically “tagged” and imaged using conventional immunohistochemistry methods. Combining numerous indirect measurements, taurine is known to play critical roles in brain function during health and disease and is proposed to act as a neuro-osmolyte, neuro-modulator, and possibly a neuro-transmitter. Elucidation of taurine’s neurochemical roles and importance would be substantially enhanced by a direct method to visualize alterations, due to physiological and pathological events in the brain, in the local concentration of taurine at or near cellular spatial resolution *in vivo* or *in situ* in tissue sections. We thus have developed chemically specific X-ray fluorescence imaging (XFI) at the sulfur K-edge to image the sulfonate group in taurine *in situ* in *ex vivo* tissue sections. To our knowledge, this represents the first undistorted imaging of taurine distribution in brain at 20 μm resolution. We report quantitative technique validation by imaging taurine in the cerebellum and hippocampus regions of the rat brain. Further, we apply the technique to image taurine loss from the vulnerable CA1 (cornu ammonis 1) sector of the rat hippocampus following global brain ischemia. The location-specific loss of taurine from CA1 but not CA3 neurons following ischemia reveals osmotic stress may be a key factor in delayed neurodegeneration after a cerebral ischemic insult

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.6b02298. Tables showing intensity with energy for standards (Table S1) and fit results for bulk XAS (Tables S2 and S3); figures showing a comparison of chemically specific sulfur XFI and Fourier transform infrared imaging (FTIRI) (Figure S1), slope of the pre-edge region (Figure S2), and effects of energy calibration (Figure S3) (PDF)

and highlights the significant potential of chemically specific XFI to study the role of taurine in brain disease.

Graphical Abstract

The ability to observe changes in the distribution and local concentration of biological molecules is critical to understanding the mechanistic pathways of brain diseases. Without this knowledge, development of targeted patient therapies is severely hindered. Although many innovative and advanced techniques have been developed to image the brain, a method to image cerebral taurine to date has remained elusive.

Taurine (2-aminoethanesulfonic acid) is a small mobile organic acid found in millimolar concentrations in the mammalian brain. Taurine is critical for healthy brain function, with deficiency during development producing neurological deficits.^{1–3} Although most mammals can synthesize taurine *de novo*, some such as cats rely exclusively on their diet for taurine. Taurine deficiency in such animals at any stage of life (neonatal through to adulthood), or disruption to the taurine transport protein genome in animals capable of taurine synthesis *de novo*, produces cellular degeneration within the central nervous system (CNS) with retina photoreceptor cells particularly vulnerable.^{4,5} A likely rationale for the importance of taurine in the brain is its function as an osmolyte, allowing neurons to control cell volume and water content during events such as membrane depolarization and ion influx or efflux that occurs during neuro-transmission.^{2,6–8} Consistent with this role, analysis of tissue slice fluid or microdialysis of extra-cellular fluid *in vivo* reveals that neurons release large quantities (millimolar levels) of taurine into the extracellular space during hypo-osmotic extracellular conditions or in response to overexcitation of neurons.^{8–11} Release of taurine from neurons during excitation and in response to other sources of increased intracellular osmotic pressure or decreased extracellular osmotic pressure prevents swelling and loss of cell viability.^{7,8,12} In addition to a role as a neuro-osmolyte, taurine has been proposed to act as a neuro-transmitter or neuro-modulator for several classes of interneurons.^{13,14} However, without a method to image endogenous brain taurine, the extent to which taurine acts as an osmolyte or a neuro-transmitter, and its exact functions in healthy and diseased brain, remain undetermined.

Thus, a method to image endogenous taurine within the central nervous system has been long sought.¹⁵ Nuclear magnetic resonance spectroscopy (NMR) has revealed a wealth of information about neurotransmitters (glutamate) and metabolites (organic phosphates, lactate). Indeed, endogenous taurine may be detected *in vivo* with ¹H or ³³S NMR; however,

the relatively low spatial resolution of NMR imaging typically limits analysis to the whole brain or large regions of an organ.^{16–18} Further, weak signals and complex baselines often confound spectral analysis.^{16,17} Antisera have been developed specific to various taurine conjugates in chemically fixed tissue; however, contradictory results were often obtained, attributed to the small mobile nature of taurine whose distribution is altered during perfusion and chemical fixation.^{19,20} For example, although biochemical assays demonstrate taurine to be abundant in the cerebellum,²¹ immunohistochemical studies do not reliably stain cerebellar taurine.^{19,20} Exogenously administered radiolabeled taurine has informed on the physiology and location of taurine uptake within tissue^{22,23} but limits studies to biological processes with a time frame governed by the radionuclide decay.

Here we demonstrate that X-ray absorption spectroscopy (XAS) at the sulfur K-edge provides a long sought after technique to image endogenous taurine at the near-cellular level (20 μm in this study). XAS is well suited to provide a “fingerprint” of sulfur speciation in biological samples due to the wide variability in energy of the sulfur K-edge (14 eV). The exquisite sensitivity of XAS to the oxidation state and molecular geometry of sulfur has been used by ourselves and others to characterize sulfur speciation in biological samples such as cell cultures, blood, bacteria, and brain tissue.^{24–30} Previously we used XAS to study the bulk concentration of taurine *in situ* within *ex vivo* brain tissue sections and in living mammalian cells.^{24–26} Herein, we advance from our previous work to describe the development and validation of a novel imaging technique, chemically specific X-ray fluorescence imaging (XFI) at the sulfur K-edge. We use this technique to provide the first undistorted images of taurine distribution in the cerebellum and hippocampus. We show that taurine is abundant in CNS tissue regions containing neuron soma (gray matter) or dendrites (molecular layers), but is not abundant in myelinated axons (white matter).

Further, we apply our novel imaging method to study alterations in taurine distribution following global brain ischemia, which can occur during cardiac arrest and results in delayed neurodegeneration of CA1 (cornu ammonis 1) pyramidal neurons but generally not of CA3 pyramidal neurons or granule neurons of the dentate gyrus. Using this unique ability to image taurine at the near-cellular level, we show for the first time the loss of taurine from tissue layers susceptible to brain ischemia. Moreover, we demonstrate that taurine is lost from tissue layers containing both CA1 pyramidal neuron soma and dendrites. These results provide strong support that overexcitation of neurons and ensuing osmotic stress is a driving mechanism for delayed cell death after ischemic insult to the brain. We also show how our novel imaging method can image additional sulfur biomarkers, including sulfate-esters, an important component of the myelin sheath, and the thiol–disulfide ratio, a marker of cellular redox. This study thus comprises a breakthrough in direct biochemical imaging of sulfur species, provides new insight into mechanisms of delayed neurodegeneration occurring after global ischemia, and demonstrates how the imaging approach can enable the study of a range of important sulfur species within the brain.

EXPERIMENTAL SECTION

Animal Model of Global Brain Ischemia and Tissue Collection

Male, Sprague–Dawley rats (8-week old) were randomized to sham surgery or global brain ischemia (2-vessel occlusion), as previously described for our laboratory.³¹ Brain tissue was obtained from the rats 5 days after sham surgery or brain ischemia ($n = 5$ for sham surgery and $n = 5$ for ischemia). All rats were housed with a 12 h light/12 h dark cycle with ad libitum access to chow and water. To prevent introduction of chemical artifacts that can occur during the preparation of biological samples,^{25,26} all rats were anaesthetized with isoflurane and humanely killed through decapitation; the head was immediately frozen by immersion in liquid nitrogen.²⁵ This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. The frozen brain was chiselled out from the head on dry ice.

Tissue Sectioning for Spectroscopic Analyses

Duplicate 10 μm -thick coronal sections of cerebellum (a subset of 4 animals from sham surgery) or hippocampus (10 animals total, 5 ischemic animals, 5 sham animals) were cut on a cryomicrotome at $-16\text{ }^{\circ}\text{C}$ and melted onto either glass microscope slides for routine histology or Thermanox plastic coverslips for sulfur K-edge XAS analysis. Hippocampal sections were cut at coordinates between -2.7 and -3.3 mm relative to Bregma. Tissue sections were maintained at dry ice temperature for 2–5 days prior to being air-dried and analyzed within 48 h for sulfur K-edge XAS analysis (bulk-XAS, micro-XAS, or chemically specific XFI). An additional subset of three cerebellum tissue sections were cut as described above and mounted on Si_3N_4 membranes to enable Fourier transform infrared spectroscopic imaging prior to X-ray analysis (Figure S1).

Standard Compounds

Standard compounds (Sigma-Aldrich) representative of thiols (reduced glutathione), thioethers (methionine), disulfides (oxidized glutathione), sulf-oxides (methionine sulfoxide), sulfinic acids (hypotaurine), sulfonic acids (taurine), O-linked sulfate esters (dextran sulfate), N-linked sulfate esters, and inorganic sulfates (Na_2SO_4) functional groups were analyzed as 30–100 mM solutions in PBS at pH 7.4 (to minimize the self-absorption artifacts previously reported).^{27,28} The reduced glutathione solution was also analyzed at pH 13 (thiolate). Solutions were analyzed in custom sulfur free polycarbonate cells with polypropylene windows.

Bulk Sulfur K-Edge XAS

XAS data were collected using beamline 4-3 of the Stanford Synchrotron Radiation Light-source (SSRL) or the soft X-ray microcharacterization beamline (SXRMB) of the Canadian Light Source (CLS). Both beamlines employed a Si(111) double crystal monochromator. The incident beam was reduced to $2\text{ mm} \times 6\text{ mm}$ (4-3) or $2\text{ mm} \times 4\text{ mm}$ (SXRMB) by vertical and horizontal slits, with incident intensity measured using a helium gas filled ion chamber. Samples (tissue sections or model compound solutions) were mounted at 45° to the

incident beam, and X-ray fluorescence was collected either with a Stern-Heald-Lytle detector filled with nitrogen gas (4-3) or with a 4 element Si drift detector (SXRMB). Prior to spectra collection, the sample chamber was purged with He until its relative O₂ content was less than 0.5% to eliminate X-ray attenuation from air and maintain essentially zero humidity to preserve sample integrity. Spectra, collected at ambient room temperature, were calibrated against the spectrum of a Na₂S₂O₃·5H₂O powder solid standard, with the lowest energy peak set to 2469.2 eV, as described previously.²⁶ Data acquisition was controlled with XAS-Collect (4-3)³² or Aquaman software (SXRMB), with spectra collected in the 2450–2515 eV range taking approximately 5 min per sweep.

Chemically Specific XFI and Micro-XAS

Sulfur K-edge chemically specific XFI and micro-XAS data were collected at SSRL beamline 14–3, at SXRMB at the CLS, and at beamline 2-ID-B at the Advanced Photon Source (APS). A monochromatized incident beam was generated from a Si(111) double crystal (14-3, SXRMB) or multilayered spherical grating monochromator (2-ID-B). A small spot was produced either from a Kirkpatrick-Baez (KB) mirror pair (5 $\mu\text{m} \times 7 \mu\text{m}$ on 14-3; 10 $\mu\text{m} \times 11 \mu\text{m}$ on SXRMB) or a 40 μm pinhole aperture (2-ID-B). Incident intensity was measured upstream of the optic with a helium filled ion chamber. Samples were mounted at 45° to the incident beam, in a sample box purged with He (vacuum box for SXRMB). The Si drift fluorescence detector, either a single element Vortex (14-3, 2-ID-B) or a Bruker 4 element (SXRMB), was mounted at 90° to the incident beam. At 14-3, the detector readout was synchronized to the stage movement speed, and data collected continuously, such that the full emission spectrum was collected every 500 ms, for an average stage movement of 20 μm (i.e., “rapid” or “fly” scanning). At SXRMB and 2-ID-B images were collected in step scan mode, with a 20 μm step and 500 ms dwell time.

All beamlines used the bulk XAS energy calibration strategy. The full emission spectrum was recorded at each pixel, in addition to single channels for P, S, and Compton scatter. Chemically specific XFI of taurine used sulfur X-ray fluorescence images at incident energies of 2475, 2478.2, 2479.8, and 2510 eV. An experiment at 14-3 additionally mapped for sulfur reduced forms using incident energies of 2465, 2469.85, 2470.55, 2470.8, 2473.5, 2478.2, 2479.8, and 2510 eV.

Micro-XAS spectra were collected on the chemically specific XFI apparatus using bulk XAS spectral acquisition parameters. Three replicate spectra were collected at each position to monitor for beam damage.

X-ray Data Processing

Bulk and micro-XAS spectra were processed using the EXAFSPAK program suite.³³ Using the DATFIT program, spectra collected from tissue sections were fitted to linear combinations of reference spectra. Standards were excluded from the fits unless they contributed at least 0.5% of the total spectra, at a value greater than three times their standard deviation of measurement (calculated from the diagonal elements of the variance-covariance matrix). All spectra from brain tissue could be fitted to a linear sum of thiol, thio-ether, disulfide, sulfoxide, sulfonate, and O-linked sulfate ester functional groups. Sulfinic

acids, N-linked sulfate esters and inorganic “free” sulfate were not detected, in agreement with our previous studies.^{25,26}

Chemically specific images were generated using Sam’s Micro-Analysis Tool Kit (<http://smak.sams-xrays.com/>) and ImageJ JV1.36 (<http://rsb.info.nih.gov/ij/download.html>). We have previously published the approach used to generate the chemically specific images.³⁴ In brief, to visualize n chemical forms of sulfur, $n + 2$ fluorescence maps of sulfur are collected. A map is collected with incident energy tuned to the energy of the strongest peak of each of the n chemical forms of sulfur, plus two additional maps below and above the sulfur K-edge, respectively, equivalent to a background, nonsulfur fluorescence map and a total sulfur map. The background fluorescence map is subtracted from each of the remaining $n + 1$ fluorescence maps. The background image was recorded at 2475 eV for the taurine four energy imaging approach or at 2465 eV for the 8 energy approach to reveal thiols, thioethers, disulfides, sulfoxides, taurine, and sulfate ester. Normalized fluorescence intensities were determined from model compound spectra of the n sulfur chemical forms (Table S1). With each pixel intensity expressed as a linear combination of the normalized intensity of components multiplied by their abundance at that pixel, simultaneous equations are solved by matrix inversion to yield maps for the n sulfur chemical forms. Dividing by the total sulfur map (2510 eV) expresses the sulfur in each chemical form as percentage of total sulfur.

Average taurine and sulfate ester content was calculated from regions of interest drawn around anatomical structures using bright field microscope images of the unstained section and histology of the adjacent tissue section as a guide.

Routine Histology

Hematoxylin and eosin (H&E) histology on tissue sections following X-ray analysis, and on adjacent sections mounted on glass slides, was described previously.³⁵

Statistical Analysis

All statistical analysis was performed using animal replicates as the replicate data values to calculate averages, standard-deviations, and tests for statistical significance. For imaging data, the multiple pixels within the regions of interest were averaged to give a single data value per region of interest, per animal. For micro-XAS statistical analysis, 5 micro-XAS spectra were collected per region of interest to minimize the effect of the small sampling area interrogated by the $5\ \mu\text{m} \times 7\ \mu\text{m}$ beam and were averaged to give a single value per region of interest, per animal. One tissue section per animal was analyzed for both imaging and micro-XAS.

Statistical analysis was performed to test for a significant difference in the sulfur speciation between the inner layer (white matter), granular layer (gray matter), and molecular layers of the cerebellum. A paired t -test was applied to test if the sulfur speciation within the granular layer and molecular layer was significantly different from the inner layer. An additional paired t -test was applied to test for a significant difference between the granular layer and molecular layer. All tests were two-tailed and used the 95% confidence limit. As this statistical approach used multiple t -tests, the p values obtained for each test were multiplied

by the number of individual *t*-tests performed (i.e., multiplied by 3, Bonferoni Correction) as a conservative approach to minimize cumulative random error.

A two-tailed paired *t*-test using the 95% confidence limit was used to test for a significant difference in the sulfur speciation between the vulnerable CA1 layer of the hippocampus following global ischemia in the rat and the less vulnerable CA3 layer of the hippocampus. In addition, a two-tailed student's *t*-test using the 95% confidence limit was employed to test for a significant difference in sulfur speciation in the vulnerable CA1 sector of the hippocampus following global ischemic in the rat compared to the CA1 sector in sham animals.

RESULTS AND DISCUSSION

XAS Quantifies Relative Taurine Concentration within Biological Samples

Sulfur K-edge XAS is sensitive to oxidation state and molecular geometry, providing a spectroscopic “fingerprint” of the sulfur functional groups within the sample. This property enables the determination of the relative concentration of a specific sulfur chemical form within a complex mixture.^{28,29} We previously used this capability to quantify relative bulk taurine concentrations within different regions of brain tissue.^{25,26} More correctly, XAS quantifies sulfonates rather than taurine; however, molecular specificity is implied for brain tissue, as nearly all of the brain sulfonate pool exists as taurine.^{19–21,26,36} Spectra of a suite of model compounds reflecting the sulfur species contained within brain tissue show the wide spectral variation that correlates with oxidation state and molecular geometry (Figure 1A). A close inspection of the 2475–2485 eV range highlights that variations in local atomic environment result in spectral differences between O-linked sulfate esters, N-linked sulfate esters, and inorganic sulfate, which all contain S^{VI} (Figure 1B). The sensitivity of the sulfur K-edge to both oxidation state and molecular geometry enables taurine (sulfonate, S^{IV}) to be differentiated from these S^{VI} species (Figure 1B), as described previously.^{25,26}

Previous bulk biochemical assays on microdissected tissue studies showed taurine to be abundant in the granular layer (gray matter) and molecular layers and less abundant in the inner white matter layer.^{18,21,36} Here we demonstrate that sulfur K-edge micro-XAS of the cerebellum can provide identical scientific conclusions from *in situ* analysis of tissue sections, without the need for tissue microdissection or homogenization (Figure 2, Table S2). Our results reveal that the inner white matter (Figure 2A) contains lower taurine content relative to both the granular and molecular layers (Figure 2B,C).

The relative taurine content was 15.1 ± 1.2 and $15.2 \pm 1.2\%$ within the granular and molecular layer, respectively, compared to $11.6 \pm 1.8\%$ within the inner layer (Table S2). These results support that taurine is more abundant in both neuron soma of the granular layer and neuron dendrites that extend into the molecular layer but not in myelinated axons of the inner white matter layer. As the molecular layer is rich in glial cells (i.e., Bergman glia), these results raise the possibility that glia are rich in taurine and play a crucial role in storing or supplying taurine to neurons to help regulate osmotic pressure, consistent with mounting indirect evidence.^{7,22,23} In addition to quantifying the relative taurine content, the micro-XAS spectra show spectroscopic differences characteristic of the underlying

biochemistry associated with each tissue layer. Specifically, micro-XAS of the inner layer of the cerebellum displays strong absorbance across the energy range 2478–2480 eV, while the granular and molecular layer display strong absorbance around 2478 eV but weaker absorbance near 2480 eV. The results of linear fitting highlight that these features arise from a significantly larger contribution of O-linked sulfate esters to the total sulfur pool in the inner layer of the cerebellum ($25.1 \pm 1.9\%$) relative to the granular (0.9 ± 0.5) and molecular ($1.2 \pm 1.6\%$) layers (Table S2). The high concentration of sulfatide lipids within the myelin sheath of white matter tracts can explain the high level of sulfate esters in this tissue region, consistent with mass spectroscopic imaging of the cerebellum and white matter tracts of the brain.³⁷ The fitting results also revealed additional spectroscopic differences, such as lower thio-ether content of the inner ($26 \pm 3\%$) relative to the granular ($43 \pm 1\%$) and molecular layers ($45 \pm 1\%$). Although the reason for the differences in thio-ether content are not yet known, they may be accounted for by variations in relative abundance of cysteine and methionine residues within proteins and in glutathione content within white and gray matter. Thiolates, sulfinic acid, N-linked sulfate ester and inorganic sulfate were not detected in any tissue type, agreeing with our previous studies.^{25,26}

Development of Chemically Specific XFI to Image Taurine in Brain Tissue Sections

Linear fitting of spectra of model compounds to the spectrum of a mixed sample, such as brain tissue, is a sensitive and robust method to determine the relative content of specific sulfur species (as demonstrated in Figures 1 and 2). However, to generate an image with this approach requires an entire XAS spectrum per pixel, which gives rise to impractical data collection times (e.g., at 5 min per spectrum, a $1000 \mu\text{m} \times 1000 \mu\text{m}$ region imaged with a $20 \mu\text{m}$ step or pixel size would take 8.5 days). In chemically specific XFI, instead of collecting an entire spectrum, a series of sulfur fluorescence images are recorded at n select energies across the sulfur K-edge, with each energy corresponding to the highest intensity peak (white line) position of the n chemical forms of sulfur. An additional image is collected below the sulfur edge for background subtraction, and above the edge for a measurement of total sulfur, giving a total of $n + 2$ images.

Building on the above strategy, we developed a four energy imaging approach to rapidly image brain taurine in a much shorter time frame (<5 h per sample). Sulfur fluorescence images are collected at 2475, 2478.2, 2479.8, and 2510 eV (Figure 3B, top line), corresponding to a background image of nonsulfur and low oxidation state sulfur (2475 eV), images at the spectral peaks of sulfonate and O-linked sulfate ester (2478.2 and 2479.8 eV) and a total sulfur image (2520 eV). After subtracting the background (2475 eV) from the 2478.2 and 2479.8 eV signals, normalized intensities of sulfonate and O-linked sulfate ester standards are used to solve by matrix inversion for relative contributions of sulfonates and O-linked sulfate esters to the total fluorescence intensities at 2478.2 and 2479.8 eV. These values are then divided by the total sulfur fluorescence signal (2520 eV) to image the relative content of sulfonate (taurine) and sulfate ester (Figure 3). An image of the total P distribution is measured simultaneously with each of the sulfur maps (Figure 3), since the incident energy is above the phosphorus K-edge in all cases.

Our imaging approach uses several assumptions. (1) The background signal (scatter, other fluorescence) that is not the primary sulfur fluorescence is assumed to be invariant with energy. Supporting this, a linear baseline with negligible slope is seen below the sulfur K-edge in unprocessed micro-XAS spectra (Figure S2). (2) The sulfur fluorescence signal from lower oxidation state species is assumed to be constant across the postedge region where sulfonates and sulfate esters absorb (2475–2480 eV). This approximation has been used by others,³⁰ and we recently showed that large variations in proportions of low oxidation state species (i.e., thiols, disulfides, thio-ethers, sulfoxides) produces only minor variation in signal across the range 2475–2480 eV.³⁴ (3) It is assumed that sulfonate and O-linked sulfate esters are the only chemical forms of sulfur with intense features at 2475–2480 eV, which is supported by our linear fitting results (Figure 1 and 2). However, if additional chemical forms of sulfur are detected for other tissue types, imaging at one additional energy for each additional chemical component will be required. (4) The method requires accurate energy calibration which is stable over the duration of the entire data set. A miscalibration of just 0.5 eV can lead to markedly changed conclusions (Figure S3).

In this study we validate that the four energy imaging method yields the same scientific conclusions as linear fitting of spectra with quantitative results within 10% of those from linear fitting (Figure 4B). Several previous studies used imaging at the sulfur K-edge to study sulfur chemical forms in onion tissue, malignant rat brain tissue and bovine cornea.^{38–40} However, the latter two studies did not account for variations in edge intensity that occur between different sulfur compounds (e.g., 1 mM taurine and 1 mM sulfate have different edge intensities. Further, none accounted for the spectral variation between compounds of the same oxidation state but different molecular geometry, namely, O-linked sulfate esters, N-linked sulfate esters, and inorganic sulfate. Failure to consider these factors would result in large errors when determining the amounts of sulfonates and sulfates in brain tissue. Therefore, this study has substantially extended beyond previous work to develop and optimize a robust, accurate, and rapid method to reveal the distribution of taurine in brain tissue.

Imaging Taurine in the Rat Cerebellum

We applied our imaging approach to yield the first undistorted image of taurine in the rat cerebellum at 20 μm spatial resolution (Figure 4). The results are in excellent agreement with those obtained from micro-XAS linear fitting analysis, yielding average taurine concentrations of 11.9 ± 0.2 and $12.5 \pm 0.4\%$ within the granular and molecular layer, respectively, and a lower taurine content of $8.4 \pm 0.5\%$ within the inner layer. Standard deviations are smaller for values from chemically specific XFI, which averages taurine concentration from a larger tissue region, compared to fitting micro-XAS data, which uses a limited sampling area. Further, we confirm that taurine distributions in cerebellum imaged at three synchrotron facilities yield the same scientific conclusions (Figure 4B). The abundance of taurine in tissue layers containing neuron soma and dendrites (i.e., granular and molecular layers) indicates that taurine may be critical for healthy function of these structures, consistent with a role as a neuro-osmolyte. In our proof of principle study, taurine was imaged at 20 μm spatial resolution. However, future imaging of taurine or other sulfur species at low- or submicrometer resolution (cellular level) should be possible using

currently available optics. Such future high-resolution studies have potential to colocalize taurine to specific cell types, which may help solve longstanding questions of whether taurine is a neuro-transmitter for certain classes of interneurons. Complementary imaging techniques are expected to be critical in the future use of our chemically specific sulfur XFI method; we demonstrate how the imaging method can be combined with Fourier transform infrared imaging and routine H&E histology of the same section of cerebellum tissue (Figure S1).

Taurine Depletion within the Hippocampus CA1 Sector Following Global Brain Ischemia in the Rat

Specific brain regions are highly vulnerable to ischemia, such as the CA1 sector of the hippocampus. Delayed neuron death within this region is observed clinically in humans and accounts for working memory deficits observed in victims of global brain ischemia (survivors of cardiac arrest). Rodent models of global ischemia are routinely used to study the biochemical pathways of delayed cell death within the CA1 sector of the hippocampus, resulting in a large volume of literature on this topic.^{41–43} It is widely accepted that overexcitation of CA1 pyramidal neurons, or neuro-excitotoxicity, is a driving mechanism of cell death within this region.^{42,43} Despite extensive studies on the mechanisms of cell death after global ischemia, the effect of global ischemia on the content and distribution of taurine within the hippocampus has not been shown previously. Here we have applied our novel imaging method to reveal alterations in the distribution and relative content of taurine within the rat hippocampus following ischemic insult. Not unexpectedly, a substantial loss of taurine was observed within the CA1 sector on day 5 after ischemic insult, corresponding to the time of CA1 neuron death (Figure 5). Taurine content fell from $12 \pm 1\%$ in the CA1 sector of sham animals to $6.1 \pm 1.4\%$ in ischemic animals. A similar decrease was not observed in the less vulnerable CA3 sector in ischemic animals ($12 \pm 2\%$). These results are supported by linear fitting of micro-XAS spectra, which revealed a taurine content of $9.3 \pm 1.4\%$ in the CA1 sector of ischemic animals, significantly lower than that of the less vulnerable CA3 sector of ischemic animals ($17.1 \pm 2.9\%$) and of the CA1 sector of sham animals ($19.0 \pm 1.4\%$) (Figure 5, Table S3). Interestingly, taurine loss was not confined to the pyramidal layer that contains neuron soma but occurred in dendritic layers also (oriens, lucidum, and radiatum layers). This result is consistent with a role of taurine as a neuro-osmolyte within both neuron soma and dendrites. We speculate that taurine loss from these tissue layers acts to maintain cell volume and prevent osmotic shock during early stages of neuro-excitotoxicity after ischemic insult. However, prolonged overexcitation of neurons will eventually deplete intraneuronal taurine reserves, giving rise to osmotic stress and potentially contributing to neuron death. Whether this is a primary driving mechanism of cell death, a contributing factor or a concurrent event is not yet known but may now be investigated using the imaging method reported in this study.

In addition to revealing relative taurine contents, linear fitting micro-XAS spectra from the CA1 sector of the hippocampus showed an increase in disulfides and a decrease in thiols concomitant with neuron death (Table S2). Although this study focused on developing a new method to image taurine, we also show how our imaging method can be adapted to image a wider range of sulfur chemical forms in the brain. An 8 energy imaging approach was

employed to map thiols, thio-ethers, disulfides, sulfoxides, sulfonates, and O-linked sulfate esters in the vulnerable CA1 sector and the less vulnerable CA3 sector of the hippocampus after global ischemia (Figure 6) and in a representative sham animal (Figure 7). The results clearly show decreased thiols and increased disulfides within the CA1 sector, concomitant with taurine loss and neuron death following ischemia. This was not observed in the CA3 sector that is less vulnerable to ischemic insult or in the sham animal. These results highlight the immense potential for future studies to use this imaging method to study not only brain taurine but also the thiol–disulfide ratio, an important marker of oxidative stress, which is not easily imaged by other techniques.

CONCLUSIONS

This study has demonstrated a novel technique to image taurine in the CNS. To our knowledge this technique is unique in imaging an undistorted distribution of endogenous taurine at cellular or near-cellular resolution. Our results reveal that taurine is abundant in the granular and molecular layers of the cerebellum, consistent with the location of neuron soma and dendrites and supporting a neuro-osmolyte role for taurine. This study showed a significant loss of taurine from vulnerable CA1 pyramidal neurons following global ischemia, suggesting osmotic stress may contribute to the mechanisms of delayed cell death that occur in this region. We also demonstrate how chemically specific XFI can image other sulfur species such as sulfate esters which are abundant in white matter and thiols and disulfides, important markers of oxidative stress and thiol redox. We expect that this unprecedented ability for direct *in situ* imaging of sulfur speciation afforded by this technique will find widespread use in neuroscience.

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Figure 1.

(A) X-ray absorption spectra at the sulfur K-edge of a suite of model compounds that reflect the different chemical forms of sulfur likely to be found in brain tissue. Some spectra have been scaled by a factor of 1/2 to simplify comparison between the edge position of all spectra.

Figure 2.

Micro-XAS analysis of sulfur speciation in different tissue layers of the cerebellum. (A) Representative spectra from the inner layer (white matter), granular layer (gray matter), and molecular layer. (B–D) Representative of linear fitting of spectra of the (B) inner layer, (C) granular layer, and (D) molecular layer, showing data (circles), fit (red line), residual (line offset below), and the components (black lines), scaled according to their contributions to the fit (Table S2).

Figure 3.

Chemically specific imaging methodology applied to image taurine (sulfonic acid, sulfonate at physiological pH) and sulfate ester levels in the cerebellum. (A) Images of sulfur fluorescence are collected at select incident energies that correspond to a background position to account for nonsulfur fluorescence and sulfur fluorescence from low oxidation state species (2475 eV), white line of sulfonic acids (2478.2 eV), white line of sulfate esters (2479.8 eV), and total sulfur image (2510 eV). The position of these energies on a representative micro-XAS spectrum are shown by red lines. (B) Representative images of sulfur fluorescence collected from the cerebellum with incident energies set at 2475, 2478.2, 2479.8, and 2510 eV. Imaging at the 4 energies allows the distribution of taurine (sulfonate) and sulfate esters to be determined, along with the distribution of total P and total S. Comparison with hematoxylin and eosin (H&E) histology reveals that sulfate esters are abundant in the inner layer white matter (1), while taurine is abundant in the granular layer gray matter (2) and molecular layers (3) of the cerebellum. Scale bar = 100 μm . Data collected with a step size of 20 μm , beam spot size of 5 $\mu\text{m} \times 7 \mu\text{m}$.

Figure 4.

Chemically specific imaging of the distribution of taurine (sulfonate) and sulfate esters in the cerebellum. (A) Chemically specific images of the cerebellum collected at three different synchrotron facilities, the Stanford Synchrotron Radiation Lightsource (SSRL), the Canadian Light Source (CLS), and the Advanced Photon Source (APS). Sulfate esters were always observed to be highest in the white matter of the inner layer of the cerebellum (1) and lowest in the granular (2) and molecular (3) layers. In contrast, taurine was highest in the granular (2) and molecular (3) layers. Scale bar = 100 μm . (B) The results from quantification of chemically specific images (CSI) and fitting micro-XAS spectra (fitting) revealed the same scientific conclusions. A significant difference in mean ($\pm$ standard deviation) % composition values of gray matter and molecular layer relative to white matter was found for both taurine and sulfate esters (Student's *t* test, $*p < 0.05$, $\dagger p < 0.01$, $\delta p < 0.001$, $n = 4$). Using the same approach, no significant differences in the speciation of sulfur were found between gray matter and molecular layer. Data collected at 14-3 with a step size of 20 μm , beam spot size of 5 $\mu\text{m} \times 7 \mu\text{m}$, at CLS with a step size of 20 μm , and beam spot size of 10 $\mu\text{m} \times 11 \mu\text{m}$, and at APS with a step size of 20 μm , beam spot size of 40 $\mu\text{m} \times 40 \mu\text{m}$.

Figure 5.

Taurine is depleted from the CA1 subsector of the hippocampus at 5 days after global brain ischemia in the rat. (A) Routine H&E histology and chemically specific images of the distribution of taurine (sulfonate) and sulfate ester in the hippocampus of representative sham and ischemic animals. Eosinophilic (bright pink) neurons, a marker of dying or dead cells are evident within the CA1 subsector of the hippocampus in the ischemic animals, along with depleted taurine levels. Scale bar = 100 μm in all images except for higher resolution H&E images collected at 20 $\times$ magnification, where the scale bar is 50 μm . (B–D) Representative micro-XAS analysis of the speciation of sulfur within CA1 subsector of the hippocampus in sham (B, C) and ischemic (B, D) animals. Note: For clarity, spectra from the less vulnerable CA3 subsector of the hippocampus are not shown; however, spectra from the CA1 subsector of the hippocampus in sham animals closely resemble spectra collected from the CA3 subsector in both ischemic and sham animals. (E) Quantification of the relative taurine content (mean $\pm$ standard deviation) within subsectors of the hippocampus in sham and ischemic animals ($n = 5$, Student's t test, $*p < 0.05$ comparison between CA1 ischemic and CA3 ischemic, $**p < 0.01$ comparison between CA1 ischemic and CA3 ischemic $\dagger p < 0.05$ comparison between CA1 ischemic and CA1 sham). Both chemically specific imaging and linear fitting of micro-XAS spectra yielded similar results and identical scientific conclusions. Data collected with a step size of 20 μm , beam spot size of 5 $\mu\text{m} \times 7 \mu\text{m}$.

Figure 6.

Chemically specific sulfur imaging with eight energies is used to reveal the distribution of thiol, thio-ether, disulfide, sulfoxide, sulfonate (taurine), and sulfate ester functional groups within the hippocampus exposed to ischemia. H&E histology reveals cell loss within the vulnerable CA1 sector that is characterized by decreased thiols, increased disulfides and decreased sulfonate, relative to the less vulnerable CA3 sector. Scale bar = 100 μm . Data collected with a step size of 20 μm , beam spot size of 5 $\mu\text{m} \times 7 \mu\text{m}$.

Figure 7.

Chemically specific sulfur imaging with 8 energies is used to reveal the distribution of thiol, thio-ether, disulfide, sulfoxide, sulfonate (taurine), and sulfate ester functional groups within the hippocampus of a sham animal. H&E histology reveals no cell loss or eosinophilic cells within the vulnerable CA1 sector. There is a homogeneous distribution of thiols and sulfonate across the CA1 and CA3 sectors and low levels of disulfides across both these regions. This is in contrast to the ischemic hippocampus presented in Figure 6 that showed reduced thiols and sulfonate and increased disulfide levels in the CA1 sector. Scale bar = 100 μm . Data collected with a step size of 20 μm , beam spot size of 5 $\mu\text{m} \times 7 \mu\text{m}$.