



# The Cystine/Glutamate Antiporter, System $x_c^-$ , Contributes to Cortical Infarction After Moderate but Not Severe Focal Cerebral Ischemia in Mice

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Understanding the mechanisms underlying ischemic brain injury is of importance to the goal of devising novel therapeutics for protection and/or recovery. Previous work in our laboratory and in others has shown that activation of cystine/glutamate antiporter, system  $x_c^-$  ( $Sx_c^-$ ), facilitates neuronal injury in several *in vitro* models of energy deprivation. However, studies on the contribution of this antiporter to ischemic brain damage *in vivo* are more limited. Since embolic or thrombotic transient or permanent occlusion of a cerebral blood vessel eventually leads to brain infarction in most stroke cases, we evaluated the contribution of  $Sx_c^-$  to cerebral ischemic damage by comparing brain infarction between mice naturally null for SLC7a11 (SLC7a11<sup>sut/sut</sup> mice) – the gene that encodes for the substrate specific light chain for system  $x_c^-$  – with their wild type (SLC7a11<sup>+/+</sup>) littermates following photothrombotic ischemic stroke of the middle cerebral artery (PTI) and permanent middle cerebral artery occlusion (pMCAo) rendered by cauterization. In the PTI model, we found a time-dependent reduction in cerebral blood flow that reached 50% from baseline in both genotypes 47–48 h post-illumination. Despite this, a remarkable reduction in incidence and total infarct volume of SLC7a11<sup>sut/sut</sup> mice was revealed 48 h following PTI as compared to SLC7a11<sup>+/+</sup> mice. No difference in injury markers and/or infarct volume was measured between genotypes when occlusion of the MCA was permanent, however. Present data demonstrate a model-dependent differential role for  $Sx_c^-$  in focal cerebral ischemic damage, further highlighting that ischemic severity activates heterogeneous biochemical events that lead to damage engendered by stroke.

**Keywords:** mouse, cystine/glutamate exchanger, photothrombosis, system  $x_c^-$ , permanent ischemia, transient ischemia

## INTRODUCTION

It is well established that neuronal cell death in the acute phase of cerebral ischemia is caused by extracellular accumulation of the excitatory amino acid glutamate and consequent over-stimulation of postsynaptic glutamate receptors (Meldrum et al., 1985; Choi, 1988; Benveniste, 1991; Lipton and Rosenberg, 1994; Dirnagl et al., 1999). Surrounding this ischemic core is a region of reduced blood

flow (10–50%) known as the penumbra. Evidence from both animal and human studies indicates that injury in this area continues to progress for hours and perhaps days following disruption of blood flow to the core (Heiss et al., 1992; Garcia et al., 1993; Du et al., 1996; Marchal et al., 1996; Baird et al., 1997; Schwamm et al., 1998). The mechanisms contributing to progression of injury in the penumbra are incompletely understood, although studies suggest that this may ensue from hypoxic spreading depression-like depolarizations that promote ongoing excitotoxicity (Somjen, 2001; Pietrobon and Moskowitz, 2014). Using a mixed cortical cell culture system, we previously found that astrocytic system  $x_c^-$  ( $Sx_c^-$ )—a cystine/glutamate antiporter that exports Glu when importing cystine—contributed to glutamate-mediated excitotoxic neuronal death under simulated ischemic penumbral conditions (Fogal et al., 2007; 2010; 2012). Others demonstrated that pharmacological block of  $Sx_c^-$  reduced oxygen-glucose deprivation-induced neuronal currents (i.e., anoxic depolarizations) and cell death in slice and slice cultures, respectively (Soria et al., 2014), as well as in cortical cells (Hsieh et al., 2017). *In vivo*, a rapid increase in  $Sx_c^-$  activity in rat brain after focal experimental cerebral ischemia induced by transient middle cerebral artery occlusion (tMCAo) was found (Soria et al., 2014) along with increased xCT protein levels localized to microglia/macrophages, neurons and astrocytes (Domercq et al., 2016; Hsieh et al., 2017). All together, these data support the idea that  $Sx_c^-$  may play a deleterious role in brain damage that follows cerebral ischemia. Thus, in this study we determined the contribution of  $Sx_c^-$  to neocortical infarction engendered by moderate or permanent focal ischemia, by comparing brain damage of mice wild type ( $Slc7a11^{+/+}$ ) or null ( $Slc7a11^{sut/sut}$ ) for  $Sx_c^-$  following photothrombotic ischemia (PTI) or permanent middle cerebral artery occlusion (pMCAo), respectively.

## MATERIALS AND METHODS

### Animals and Animal Husbandry

This study was conducted in accordance with the National Institute of Health guidelines for the use and care of experimental animals as approved by the Institutional Animal Care and Use Committee. We utilized male mice on the C3H/HeSnJ background that have a naturally occurring deletion mutation in *Slc7a11*, the gene that encodes for the substrate-specific light-chain for  $Sx_c^-$  (xCT) (Chintala et al., 2005). Experimental littermate mice—wild-type ( $Slc7a11^{+/+}$ ) or lacking system  $x_c^-$  ( $Slc7a11^{sut/sut}$ )—were derived from F1 heterozygous ( $Slc7a11^{+/sut}$ ) breeding units created by crossing  $Slc7a11^{sut/sut}$  C3H/HeSnJ male mice [Jackson Laboratories (JAX) Stock #001310] with  $Slc7a11^{+/+}$  C3H/HeSnJ female mice (JAX, Stock #000661). Mice were only used up to the F3 generation.

At weaning, genotyping was performed *via* PCR analysis of tail genomic DNA samples as described (Sears et al., 2019), after which mice were housed three to five per cage such that at least one mouse of each genotype was represented (pseudo-randomized design). Genotype was reconfirmed *via* PCR upon sacrifice. Mice were maintained in a controlled temperature

environment operating on a 12 h light/dark cycle with standard mouse chow and water provided *ad libitum*. These breeding and housing strategies were utilized to control for environmental differences, genetic background influences, and genetic drift (Wolfer and Lipp, 2000; Wolfer et al., 2002).

### Ischemic Stroke Models

On each of the 5 days prior to a study, mice (23–30 g; 9–14 weeks) were physically held so that they would become accustomed to being touched. On the surgical day, mice were brought into the procedure room, weighed, and allowed to acclimatize for at least 1 h. Investigator was blind to mouse's genotype at time of experimentation (i.e., surgery) and during all subsequent analyses. All mice were sacrificed 48 h after the induction of cerebral ischemia as described below. **Supplementary Figure 1** provides a diagrammatic schematic depicting the timeline of each protocol. In both models, ischemic damage is largely restricted to the neocortex.

### Photothrombosis-Induced Ischemia

Cerebral ischemic damage was induced *via* photothrombosis *via* laser irradiation of the photosensitive compound Rose bengal (Boquillon et al., 1992; Ding et al., 2009). Mice, fully anesthetized with avertin (0.4 g/kg), were placed in a stereotaxic frame using tooth and ear bars. Using aseptic technique, the skull was exposed by making a 1.5–2 cm incision above the sagittal suture. A 5.5 mm × 5.0 mm rectangle metal sheet with a 1.5 mm diameter hole in the middle was gently attached to the right frontal-parietal area with glue (3M Vetbond®). Freshly made Rose bengal (Abcam, Cambridge, United Kingdom) was then injected retro-orbitally (0.03 g/kg in saline) using a 28-gauge, 1/2 inch, 0.5 ml insulin needle and syringe. Three minutes after injection, the frame containing the mouse was mounted on an upright microscope platform and the exposed but otherwise intact skull was illuminated through the 1.5 mm diameter hole for 2 min using a 10x objective with a green light of bandwidth 540–580 nm by means of an X-cite 120Q light source to activate the dye to induce a thrombus. A heating pad was used to maintain mouse's body temperature (36.5–37°C) during and following the procedure. Mortality with this procedure is very low with only one mouse of each genotype lost during surgery.

Ischemic damage was determined 48 h after surgery. Mice were perfused with cold PBS followed by 4% (v/v) paraformaldehyde (PFA). After perfusion, brains were removed and post-fixed in 4% PFA/PBS for 12 h and then placed in 20% (w/v) sucrose for an additional 12–24 h (4°C). Tissue was then embedded in Tissue Tec OCT and solidified in liquid nitrogen. Brain sections (20  $\mu$ m) cut serially through the rostro-caudal extent of each brain (–2.0 to –3.6 relative to bregma) (Microm HM-550 cryostat, Thermo Fisher Scientific) were stained with 0.5% thionin as described in detail (Claycomb et al., 2011). Images were acquired by a DP73 digital color camera (Digital Video Camera Co.) mounted on an Olympus IX50 inverted microscope controlled by CellSens Standard software (Olympus, Center Valley, PA). Infarct area (A) was directly measured using the free hand tool of ImageJ by tracing the area of the ipsilateral cortex that lacked thionin stain. The lesion area, identified by

absence of thionin staining, was quantified using NIH Image J at four levels spanning from  $-2.0$  to  $-3.6$  from bregma by two individuals blind to genotype. Area measurements were converted to volume using Cavalieri's principle:  $V = A_i \times D + A_{ii} \times D + A_{iii} \times D + A_{iv}$  with  $V =$  total infarct volume ( $\text{mm}^3$ ) and  $A_i =$  mean infarct area of each section derived from two measurements and  $D$  represents the distance between 2 sections (Shih et al., 2003).

Laser Speckle Contrast Analysis was used to measure microvascular cerebral blood flow in the right MCA territory where the Rose Bengal was activated [PSI HR real time laser speckle perfusion imager; Pericam (Las Vegas, NV)] in a separate cohort of mice (see **Supplementary Figure 2** for representative video). Briefly, after making the incision and exposing the skull, mouse was moved to the imager to access the baseline of blood flow for 30 sec per manufacturer's instruction. Then a 5.5 mm  $\times$  5.0 mm rectangle metal sheet with a 1.5 mm diameter hole in the middle was gently attached to the right frontal-parietal area with glue (3M Vetbond®), followed by the retro-orbital injection and laser activation of Rose Bengal as described above. After the activation, the metal sheet was gently removed, and mouse was immediately moved to the imager to access the real time blood flow for 30 sec (time zero). The incision was sealed lightly by glue, after which the mouse was put back into their cage containing a heating-pad and allowed to recover. Mice were re-anesthetized, the incision gently re-opened and additional blood flow measurements taken over the same region at 4–6, 24, and 47–48 h post-illumination. Data at each time point was normalized to baseline for each mouse and expressed as mean percentage  $\pm$  S.D.

## Permanent Middle Cerebral Artery Occlusion

Permanent cerebral cortical ischemia was induced in avertin-anesthetized male mice by ligating the right common carotid artery (CCA) and cauterizing the right middle cerebral artery (MCA) distal to the striatal branch as described in Piao et al. (2009) and Cui et al. (2015). The mouse's body temperature was maintained at  $36.5$ – $37^\circ\text{C}$  during the surgery and during recovery *via* heat pad. With respect to mortality, we lost one  $\text{Slc7a11}^{+/+}$  and two  $\text{Slc7a11}^{\text{sut/sut}}$  mice 1 day after surgery. Those surviving were sacrificed 48hr later and brains directly sectioned into 1 mm coronal sections. The 4th, 6th, 8th, and 10th sections were stained with 2% 2,3,5-triphenyl-tetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO) for 30 min at  $37^\circ\text{C}$  in the dark. Slices were then carefully transferred into 10% formalin for 24 h. Images (300 dpi) were captured by scanning (Epson Perfection 3170). The non-injury areas of both contralateral and ipsilateral hemispheres—denoted by red staining—were measured using NIH ImageJ for all sections by two experimenters blind to the genotype of the mice and infarct area and volume determined. Infarct area (A) was determined by subtracting the non-injured ipsilateral area from the area of the contralateral hemisphere. Total infarct volume ( $\text{mm}^3$ ) was calculated using the following equation:  $V = A_i \times 2 + A_{ii} \times 2 + A_{iii} \times 2 + A_{iv}$  with  $V =$  total infarct volume and  $A_i =$  infarct area of each

section with 2 representing the distance between slices (i.e., 2 mm) (Shih et al., 2003).

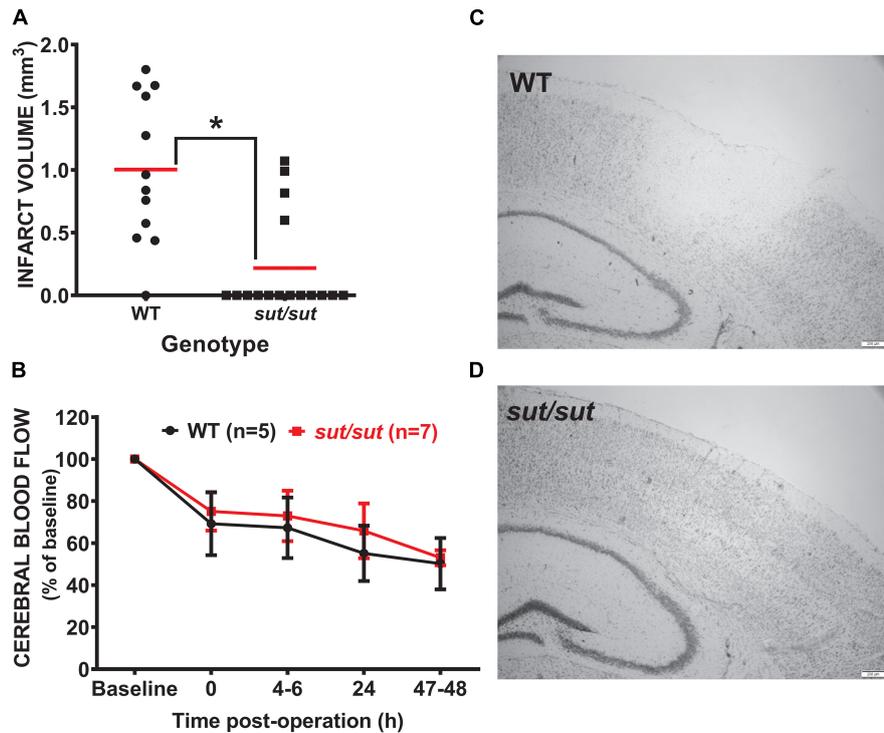
To measure spectrin breakdown products, shown previously to correlate nicely with final infarct volume (Davoli et al., 2002), contralateral and ipsilateral sides of the 5th, 7th, and 9th sections were separately pooled and homogenized in RIPA buffer containing: 0.5% Sodium deoxycholate, 25 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 5 mM iodoacetamide, 5 mM EDTA, 5 mM EGTA, and 1 X Complete Protease Inhibitor (Roche). Cellular debris was removed by centrifugation (12,000 g; 20 min;  $4^\circ\text{C}$ ). Supernatants were collected and 15  $\mu\text{g}$  (BCA assay; Pierce; Rockford, IL) of protein was separated by 10% SDS-PAGE under reducing conditions, followed by electrophoretic transfer to PVDF membrane (Bio-Rad; Hercules, CA). Membranes were blocked (Odyssey® blocking buffer; at  $25^\circ\text{C}$  for 1 h) and then probed ( $4^\circ$ , overnight) with an anti- $\alpha$  Fodrin monoclonal antibody (AA6, 100 ng/ml; Enzo) and a mouse monoclonal antibody directed against  $\beta$ -actin to correct for protein loading (650 ng/ml; Sigma). Species-specific secondary antibodies labeled with spectrally distinct IRDye® fluorescent dyes (LI-COR Biosciences; Lincoln, NE) were used to detect primary antibodies (1 h at  $25^\circ\text{C}$ ). Results were recorded on LI-COR ODYSSEY® Fc Imaging system (LI-COR Biosciences) and protein levels quantified using Image Studio 3.1 (LI-COR Biosciences; Lincoln, NE). Results were obtained by normalizing fodrin protein levels to their respective  $\beta$ -actin levels and expressed as mean fold change over the corresponding contralateral hemisphere (set to 1).

## Statistical Analysis

All statistical analyses and graphics were compiled using GraphPad Prism (Version 6.0.3, GraphPad Software, Inc. or higher; Graphpad Software, Inc., La Jolla, CA). Cerebral blood flow (CBF) were analyzed using a mixed effects model with the Geisser-Greenhouse correction for normality followed by Šidák's multiple comparisons test.  $\alpha$ II-spectrin breakdown products (SBDPs) where analyzed using two-way ANOVA of log transformed data [ $y = \log(y)$ ] followed by Šidák's multiple comparisons test. Infarct volumes were analyzed *via* two-tailed Mann Whitney  $U$ -test. Exact or adjusted  $p$ -values are included in text, in a table and/or described in figure legends as appropriate.

## RESULTS

Anatomical damage due to PTI was greatly reduced in mice lacking  $\text{Sx}_c^-$  with overall infarct volume being significantly smaller (two-tailed Mann Whitney  $U$ -test;  $p = 0.0007$ ) in  $\text{SLC7a11}^{\text{sut/sut}}$  as compared to  $\text{SLC7a11}^{+/+}$  littermate controls (**Figures 1A,C,D**). This change was driven mostly by a reduction in the overall incidence of damage evidenced by the fact that 92% of  $\text{SLC7a11}^{+/+}$  mice (12/13) had quantifiable cortical infarction as compared to just 25% (4/16) of  $\text{SLC7a11}^{\text{sut/sut}}$  mice (**Figure 1A**; Fishers exact test,  $p = 0.005$ ). Differing amounts of occlusion could not account for the dissimilarity in PTI-mediated damage as similar decreases in rCBF starting



**FIGURE 1** | Comparison of infarct incidence and size between WT and  $Sx_c^-$  null mice following photothrombotic-induced ischemia. **(A)** Forty-eight h after PTI, brains of *sut/sut* ( $n = 16$ ) mice and their WT ( $n = 13$ ) littermates were removed. Coronal sections ( $20 \mu\text{m}$ ) were cut along the rostral-caudal axis ( $-2.0$  to  $-3.6$  relative to bregma) and stained with thionin, with areas lacking thionin staining represent ischemic damage. Infarct volume was determined as described in Methods. Each data point (black dot: WT; black square: *sut/sut*) represents total infarct volume in  $\text{mm}^3$  from an individual mouse, whereas the horizontal red line represents the mean **(B)** Depicts rCBF as determined *via* laser speckle contrast imaging represented as mean fold change  $\pm$  SD from baseline (set to 100) taken immediately prior to illumination and at intervals following illumination as described in methods. **(C)** Representative thionin staining of brain tissue of *Slc7a11*<sup>+/+</sup> (WT) and **(D)** *Slc7a11*<sup>sut/sut</sup> (*sut/sut*) mice. A significant difference in infarct volume ( $*p = 0.0007$ ) was determined by two-tailed Mann Whitney *U*-test. Infarct incidence—defined as the number of mice with any size infarct divided by the total number of mice—also differed significantly between genotypes as assessed by Fisher's exact test ( $p = 0.005$ ).

at  $\approx 25\text{--}30\%$  ( $69.2 \pm 14.9$  vs.  $75.1 \pm 9.1$  of baseline levels for *SLC7a11*<sup>+/+</sup> vs. *SLC7a11*<sup>sut/sut</sup>, respectively) measured directly after laser illumination progressing to  $\approx 50\%$  reduction of pre-illumination levels ( $50.2 \pm 12.2$  vs.  $53.0 \pm 3.6$  for *SLC7a11*<sup>+/+</sup> vs. *SLC7a11*<sup>sut/sut</sup>, respectively) by the time of sacrifice occurred irrespective of the mouse's genotype (**Figure 1B**). Mixed effect model analysis shows a significant effect of time ( $p < 0.0001$ ) with *post hoc* analyses revealing a significant reduction from baseline in *SLC7a11*<sup>+/+</sup> mice occurring at 24 and 48 h, respectively. In contrast, significant changes from baseline occurred at all-time points post-illumination in *SLC7a11*<sup>sut/sut</sup> mice. Pertinently, no significant between genotype differences was found at any time post-illumination (**Table 1**).

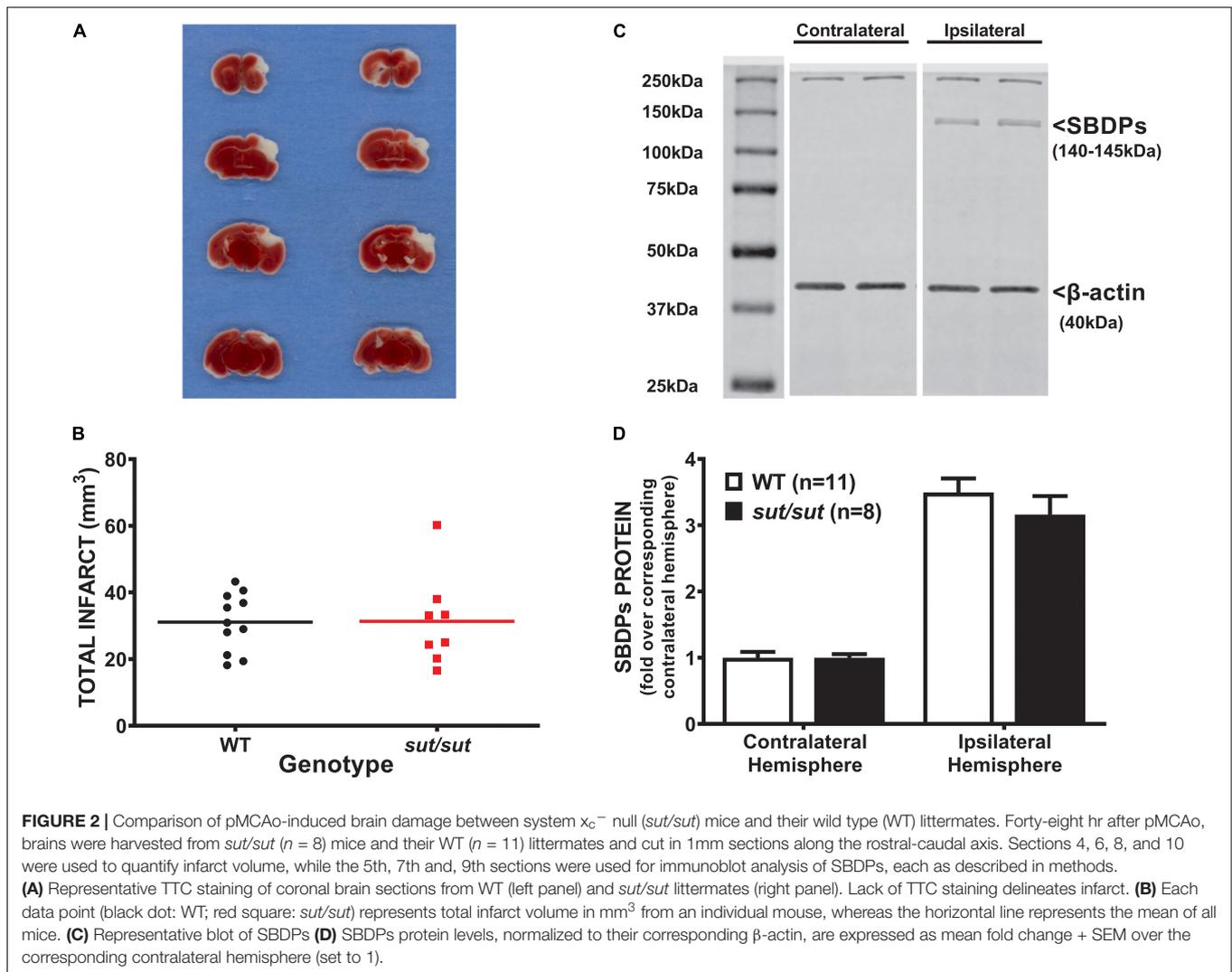
In contrast to what we found with PTI, a well demarcated infarct was visible 48 hr following pMCAo in all mice regardless of genotype (**Figure 2A**). Analysis of infarct volume showed no significant difference in infarct size between *SLC7a11*<sup>+/+</sup> and *SLC7a11*<sup>sut/sut</sup> mice (two tailed Mann Whitney *U*-test,  $p > 0.999$ ) (**Figure 2B**). The levels of  $\alpha$ II spectrin breakdown products, measured as an additional index of ischemic injury, also did not differ between genotypes (**Figures 2C,D**; two-way ANOVA;  $p = 0.925$ ).

## DISCUSSION

Occlusion of a major blood vessel, most notably the middle cerebral artery (Olsen et al., 1985), leads to cerebral ischemia in the vast majority of stroke cases. The pace of brain circuitry loss in humans from a typical large vessel occlusion is estimated to be a staggering 120 million neurons and 830 billion synapses per hour (Saver, 2006). Given that  $> 750,000$  persons in the US will have a stroke this year, it is imperative that more therapies be made available, which necessitates our need to understand more fully

**TABLE 1** | Statistical analysis of rCBF following PTI (**Figure 1B**).

Blood flow	<i>SLC7a11</i> <sup>+/+</sup>	<i>SLC7a11</i> <sup>sut/sut</sup>	+/+ vs. sut/sut
Post-illumination time (h)	<i>P</i> -value (change from baseline)	<i>p</i> -value (change from baseline)	<i>p</i> -value (between group difference)
0	0.096	0.004	0.956
4–6	0.069	0.023	0.970
24	0.016	0.005	0.666
47–48	0.008	$<0.0001$	0.994



the mechanisms underlying ischemic brain damage. Previous PET imaging studies demonstrated increased  $Sx_c^-$  activity in rat brain *in vivo* following transient focal ischemia produced *via* intraluminal occlusion of MCA followed by reperfusion (Soria et al., 2014; Domercq et al., 2016). The models chosen in this study to assess the role of  $Sx_c^-$  to ischemic damage directly mimic different clinical conditions. Photosensitization of intravascular Rose Bengal by laser illumination (Watson et al., 1985; Pevsner et al., 2001) at the territory of the MCA induces platelet aggregation, vascular thrombosis and, as we demonstrated herein, incomplete blood flow reduction, while pMCAo *via* direct electrocoagulation mimics severe ischemic stroke without vessel recanalization (Sommer, 2017). Present data demonstrate that activity of  $Sx_c^-$  promotes cortical infarction after moderate (PTI) but not severe (pMCAo) focal cerebral ischemia in mice.

The mouse utilized in this study was first identified by its subtle gray coat pigmentation that sequencing and subsequent experimentation revealed resulted from a large deletion mutation in Exon 12 of the *Slc7a11* gene (Chintala et al., 2005), which encodes the substrate specific light chain (xCT) for the

cystine/glutamate exchanger, system  $x_c^-$ . Importantly, we and others (Swank et al., 1996) have found that on a non-agouti background the reduction in pigmentation arising from the *sut* mutation becomes nearly imperceptible when the mice are adults making homozygotes difficult to distinguish from wild-type littermate controls; hence our ability to remain blind to genotype during experimentation. Additionally, despite the identification of an alternative transcript *via* 3'RACE, northern blot analysis reveals no xCT mRNA in brain of *Slc7a11<sup>sut/sut</sup>* mice (Chintala et al., 2005). This is likely due to the fact that loss of the 3'UTR *via* this deletion leads to absence of polyadenylation, which is necessary for mRNA stability and translation (Muhlemann and Jensen, 2012; Lykke-Andersen and Bennett, 2014). Additionally, no xCT protein in brain was found *via* Western Blot analysis (McCullagh and Featherstone, 2014).

We initially considered that changes in clotting—either initial aggregation or more pronounced lysis—might explain the results in the PTI model given a reduction in collagen-mediated platelet aggregation *ex vivo* has been reported in *SLC7a11<sup>sut/sut</sup>* mice (Swank et al., 1996). However, our data

showing a sustained reduction in CBF over the 48 hr period suggests this is not the case. This may not be too surprising given the complexity of factors/mechanisms known to facilitate platelet aggregation *in vivo* (Rumbaut and Thiagarajan, 2010). Given this, we conclude that the neocortical tissue of mice is remarkably less vulnerable to death when  $Sx_c^-$  function is absent under conditions where blood flow is moderately disrupted (**Figure 1**).

Our PTI results are somewhat in keeping with those of Hsieh and colleagues, who demonstrated a reduction in infarct volume in genetically manipulated mice lacking  $Sx_c^-$  ( $xCT^{-/-}$ ) when subjected to cerebral ischemia followed by reperfusion (Hsieh et al., 2017). Both of these findings might seem surprising given the importance of  $Sx_c^-$  to the production of GSH, at least, *in vitro*, where growth of  $xCT$ -deficient cells is dependent on the addition of a reducing agent (Bannai and Tateishi, 1986; Chintala et al., 2005; Shih et al., 2006; Jackman et al., 2010). However,  $xCT^{-/-}$  and  $Slc7a11^{sut/sut}$  mice have normal brain GSH levels (De Bundel et al., 2011; Sears et al., 2019), at least under basal conditions, suggesting that other cyste(i)ne transporter systems compensate for loss *in vivo* (Sosnoski et al., 2020). We cannot discount that there might be GSH dysregulation following ischemic stress. However, if so, the impact would appear minimal as evidenced by a reduction in damage following PTI and a lack of injury enhancement after pMCAo.

Interestingly, following tMCAo,  $xCT^{-/-}$  mice had appreciably less extracellular glutamate levels in the ischemic cortex at early (2–5 hr) and late time points (1–3 days), indicating a role for  $Sx_c^-$  as a source of extracellular glutamate post-ischemia (Hsieh et al., 2017). Relevant to this, we demonstrated increased astrocytic system  $x_c^-$  levels and activity contributes to enhanced extracellular Glu levels, which precipitates excitotoxic neuronal cell death in an *in vitro* model of the ischemic penumbra (Fogal et al., 2007; Jackman et al., 2010). Together, these results help explain the intriguing observation made by Obrenovitch that neither vesicle exocytosis or reversed Glu uptake could account for all the cerebral ischemic extracellular Glu levels measured *in vivo* (Obrenovitch, 1996). Together, these results extend the current, perhaps oversimplified, concept of excitotoxicity that has heretofore dominated current thinking to include  $Sx_c^-$ . Given the lack of specificity of the commercial antibodies directed against  $xCT$  (Van Liefveringe et al., 2016), we did not explore its cell-type expression following ischemia in our models. However, others have demonstrated increased immunofluorescence for  $xCT$  in microglia/macrophages at 3 and 7 days post-ischemia and in astrocytes up to 28 days post-ischemia (Domercq et al., 2016) while Hsieh and colleagues report a post-ischemic increase in expression in both astrocytes and neurons (Hsieh et al., 2017).

Despite the evidence demonstrating the contribution of  $Sx_c^-$  to ischemic damage when blood flow is moderately (this study) or transiently (Hsieh et al., 2017) reduced, a very different picture emerged when the MCA was permanently occluded. Finding no difference in infarct volume or other markers of injury (**Figure 2**), we can only conclude that other biochemical mechanisms clearly predominate when blood flow disruption is severe. This is perhaps not surprising as many potential therapeutics have been described in the literature that show potential promise in

transient but not permanent ischemia models and the idea has been put forth that salvageable tissue exits only in the ischemic penumbra (for review see Moskowitz et al., 2010).

Studies in genetically modified mice are useful for potential target identification. However, in the interest of therapeutic development, it is important to note that in keeping with the observations described above, pharmacological inhibition of  $Sx_c^-$  reduced ischemia-induced inflammation (Domercq et al., 2016) and infarct volume (Hsieh et al., 2017) subsequent to transient ischemia in rats, but not following permanent ischemia in mouse (our unpublished observations). While it is clear that additional studies are needed to determine the relevance of our and other findings to human stroke, it is intriguing to speculate that should strategies to mitigate  $Sx_c^-$  activity ever be employed in a clinical setting, it may only be beneficial to patients whose vessel recanalizes leading to revascularization or in patients with penumbra where blood flow is mildly to moderately interrupted (10–50% of baseline).

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Syracuse University's Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

YH and SH: conceptualization, methodology, funding acquisition, writing of original draft, reviewing and editing, and statistical analysis. YH: investigation. SH: resources and supervision. Both authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fncel.2022.821036/full#supplementary-material>

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