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Differential regulation of cell proliferation in neurogenic zones in mice lacking cystine transport by xCT

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Abstract

The cystine/glutamate exchanger (xCT) supplies intracellular cyst(e)ine for the production of glutathione, a major cellular anti-oxidant. xCT is enriched in brain regions associated with neurogenesis. Previous studies have shown that the malfunction of this protein greatly attenuates cell proliferation *in vitro* and is associated with brain atrophy *in vivo*. Using mice that are homozygous for a function-blocking deletion in xCT (Sut mice), we examined *in vivo* the role of xCT in cell proliferation in neurogenic regions of the subventricular zone (SVZ) and denate gyrus (DG) in the adult brain. Our results indicate that a high level of cellular proliferation in the adult brain persists even in the absence of functional xCT. Furthermore, in both young adult and middle-aged mice (3 and 11 months old), rates of SVZ cell proliferation were comparable between Sut and wild-type controls, although there was trend towards reduced proliferation in Sut mice (12% and 9% reduction, respectively). To our surprise, rates of cell proliferation in the DG were elevated in both 3- and 11-month-old Sut mice relative to controls (22% and 28% increase, respectively). These results demonstrate that xCT expression plays a role in regulating cellular proliferation in the DG, but not the SVZ of adult mice. Furthermore, unlike previous *in vitro* studies, our *in vivo* observations clearly indicate that xCT is not essential for ongoing cellular proliferation.
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In mammals, neurogenesis persists throughout adulthood in two regions: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampal formation [1] and the subventricular zone (SVZ) of the lateral ventricles [2]. In both neurogenic regions, these cells arise from stem cell precursors, migrate towards their respective targets, differentiate and become integrated into the existing circuitry [3,4]. Although it is not entirely certain what functional role these new neurons play, growing evidence suggest that these cells participate in normal processes such as spatial learning, memory storage, and olfactory discrimination [5–7]. In addition, alterations in the rate of cellular proliferation have also been implicated in the etiology of psychiatric and neuropathological conditions such as depression [8] and epilepsy [9]. Considering the potential importance of neurogenesis in multiple aspects of normal and abnormal brain functioning, it is essential that we understand what molecular mechanisms regulate the ongoing proliferation of these cells.

Anti-oxidant proteins play an essential role in the survival of brain cells exposed to various metabolic and oxidative challenges [10], therefore it would seem likely that they could also influence the production of new cells [11]. Indeed, studies have shown that certain conditions known to promote oxidative stress such as traumatic brain injury [12] and ischemia [13], greatly impact rates of adult neurogenesis. Glutathione (GSH) is a major cellular anti-oxidant that plays a pivotal role in a cell's defence against oxidative stress [14]. A decrease in cellular GSH levels increases the

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brain's susceptibility to oxidative injuries [15,16]. The Na^+ independent cystine–glutamate exchange antiporter (xCT) uptakes cystine which subsequently participates in the maintenance of intracellular cyst(e)ine which is essential for GSH production [17–19]. xCT can be detected in astrocytes and developing neuronal preparations, although its levels are a factor of 10 higher in the meninges and the ependymal cells of periventricular regions [19,20]. Consistently, *in vivo* imaging of fluorescent indicators for GSH show that it is highly enriched in the ependymal cells of the lateral ventricle, and the subgranular cell layer of the hippocampus [21]. Therefore, the abundance of GSH in the developing brain and its enrichment in neurogenic regions of the adult suggest that factors involved in the regulation of GSH, such as xCT, may be important for cell proliferation [19].

The subtle gray pigmentation mutant phenotype (Sut) is a natural truncation mutation in the gene Slc7a11 (gene coding for the light chain of xCT transporter), leading to non-functional xCT protein [22]. *In vitro* work suggests that xCT is critical for cell proliferation given that melanocytes [22], fibroblasts [18], astrocytes and meningeal [19] cells do not proliferate without the addition of the anti-oxidant β-mercaptoethanol (β-ME) which reduces extracellular cystine to cysteine allowing it to bypass xCT transport. Here, we utilized Sut mice to determine what role, if any the xCT exchanger protein plays in cellular proliferation in neurogenic regions of the adult brain *in vivo*.

Materials and methods

Animals. All experiments were approved by the University of British Columbia Animal Care Committee and were conducted in strict accordance with guidelines set by the Canadian Council on Animal Care. Sut mice breeding pairs were obtained from Dr. R. Swank (Roswell Park Cancer Institute, Buffalo, NY) [22]. The C3H/HeSnJ control background strain was obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained at the University of British Columbia Animal Care Facility in a 12 h light/dark cycle with food and water ad libitum.

Injections and tissue processing. Labelling of new cells in the adult brain was accomplished by administering two intraperitoneal injections (2 h apart, see Fig. 1A) of the thymidine analog bromodeoxyuridine (BrdU, 100 mg/kg, Sigma–Aldrich). BrdU was dissolved at a concentration of 10 mg/ml in 0.9% NaCl (w/v).

To examine rates of cellular proliferation, all mice were sacrificed 24 h after the first BrdU injection. Mice were deeply anaesthetized using pentobarbital (100 mg/kg) and perfused intracardially through the left ventricle with 10 ml of phosphate buffered saline (PBS, 0.9% NaCl in 0.1 M phosphate buffer, pH 7.4) followed by 10 ml of phosphate buffered 4% paraformaldehyde (PFA). Brains were removed and post-fixed for 2 days in PFA at 4 °C, then immersed in 30% sucrose solution for another 2 days. Brains were cut frozen, at 40 μm in the coronal plane on a sliding microtome and collected into a series of six wells containing PBS with 0.02% sodium azide.

BrdU immunohistochemisty. Free-floating sections were denatured by incubation in a solution containing 50% deionized formamide (Sigma–Aldrich) and 50% 2× saline citrate buffer (SSC, 0.9% NaCl in 0.03 M saline citrate buffer, pH 7.6) at 65 °C for 2 h. After a wash in SSC buffer, sections were immersed in 2 N HCl at 37 °C for 30 min. To eliminate endogenous peroxidase activity, sections were treated with 0.3% H_2O_2 in dH2O for 10 min. For BrdU immunolabeling, sections were incubated in primary antibodies raised against BrdU (mouse anti-BrdU, 1:1000 dilution, Sigma–Aldrich) with 2% normal horse serum (Vectastain) in PBS containing 0.3% Triton X-100 (PBS+), overnight at room temperature. Sections were then immersed in secondary antisera (biotinylated horse–anti-mouse IgG, 1:1000 dilution, Vectastain ABC kit) in PBS+ at room temperature, followed by 1 h incubation in avidin–horseradish peroxidase complex (1:1000 dilution, Vectastain ABC kit) at room temperature. BrdU labelled nuclei were visualized by incubating sections in chromagen solution containing 0.02% diaminobenzadine, 0.08% nickel chloride and 0.009% H_2O_2 in 0.1 M Tris buffered saline for approximately 1–2 min at room temperature. Sections were then washed three times in PBS at room temperature, mounted, dehydrated and coverslipped using Permount (Fisher Scientific).

Data analysis. To assess the role of xCT in cellular proliferation, an observer blind to experimental condition would count BrdU positive nuclei in the SVZ and DG using a Zeiss Axiophot microscope in bright field mode using a 40× objective (NA = 0.75). Cells in the uppermost focal plane were excluded to reduce double counting of split nuclei. For the SVZ, labelled nuclei were counted along the lateral walls of the lateral ventricles for a total of five sections per mouse, beginning at 1.18 mm anterior of bregma (every sixth section was counted). For the DG, all BrdU labelled cells within two cell diameters from the inner edge of the granule cell layer (GCL) of the DG were included in the analysis. Due to systematic differences in brain size between genotypes, quantification of cell proliferation rates were expressed as the total number of BrdU positive nuclei per unit area. Measurements of brain area and morphology were performed using NIH Image J software (v1.35) from images taken using 4× objective lens on a Zeiss Axiphot microscope equipped with a 12-bit digital camera (Retiga EXi, Q imaging).

Results are presented as means ± SEM. Statistical analysis of raw data was performed with Microsoft Excel. Comparisons between groups were done using two-tailed Students *t*-tests. Probability values less than 0.05 were deemed statistically significant.

Results

Subventricular zone and dentate gyrus structure

The Sut mice have previously been reported to have alterations in brain morphology [21]. Therefore morphological measurements of the SVZ and DG GCL in 3- and 11-month-old mice were first performed. There were no differences between mice in SVZ thickness, however, SVZ length and area were reduced in Sut mice relative to the wild-type C3H control (Table 1, 3 month $n = 8$, 11 month $n = 5$). In the DG, Sut mice showed a reduction in GCL length, an increase in thickness, but were comparable in area when relative to C3H mice (Table 2, 3 month $n = 8$, 11 month C3H $n = 5$, Sut $n = 4$). We also measured cortical thickness, as well as hemispheric, hippocampal, and lateral ventricular area. Our results are in agreement with previous findings showing a reduction in the size of the Sut mouse brain compared to its background strain control C3H [19] (data not shown).

Cellular proliferation in the subventricular zone and dentate gyrus

To examine the effect of xCT disruption on cellular proliferation, BrdU labelling was examined in the SVZ 24 h after two systemic injections of BrdU (Fig. 1A). BrdU is a thymidine analogue that becomes incorporated into the DNA of cells undergoing S phase of mitotic division [23]. Consistent with previous reports [2], BrdU labelled nuclei

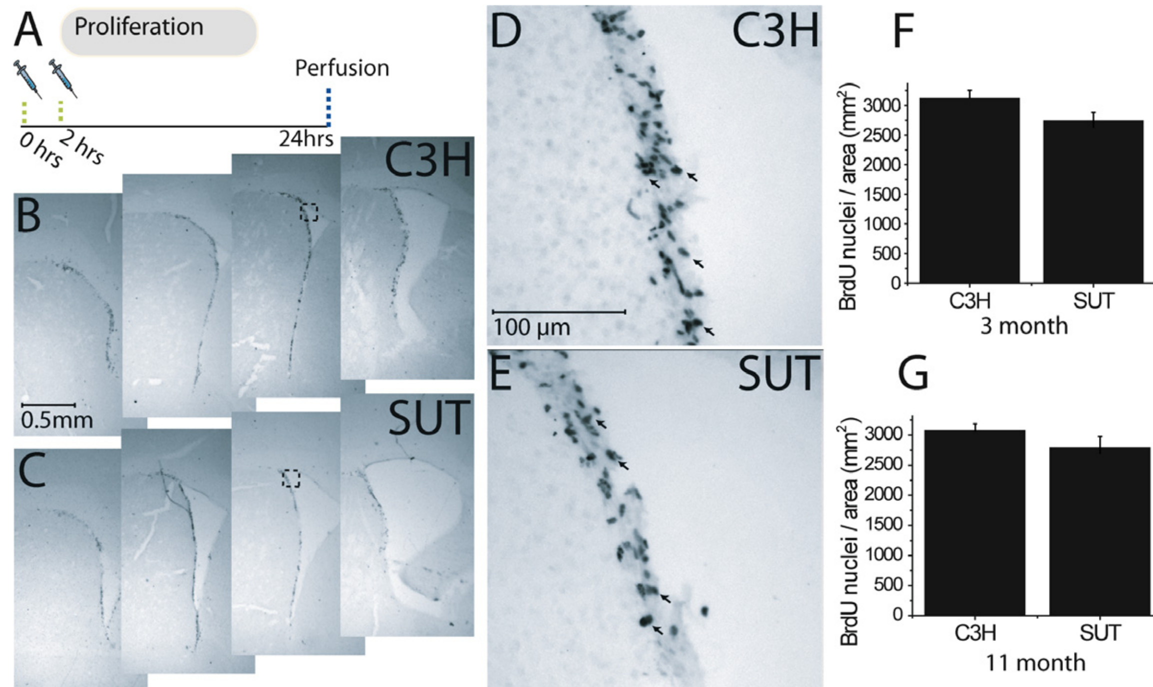

Fig. 1. BrdU labelling of proliferating cells in the SVZ 24 h after injection. (A) Experimental design for proliferation studies. All mice received two injections of BrdU (i.p. 100 mg/kg) and were sacrificed 24 h from the time of the first injection. (B,C) Low-magnification bright field images showing anterior–posterior profile of BrdU labelling in the SVZ of wild-type C3H and Sut mice (each section is approximately 480 μm apart). (D,E) Higher-magnification images (20× objective) of boxed regions in B and C. (F,G) Quantitative analysis of BrdU positive nuclei in the SVZ of 3- and 11-month-old mice.

Table 1
Subventricular zone area, length and thickness in 3 ($n = 8$) and 11 month ($n = 5$) old mice

		SVZ area (mm^2)	SVZ length (mm)	SVZ thickness (mm)
3 month	C3H	0.0838 ± 0.0016	1.85 ± 0.029	0.0453 ± 0.00086
	SUT	0.0765 ± 0.0017	1.70 ± 0.050	0.0451 ± 0.00051
	P value	0.0082	0.00088	0.86
11 month	C3H	0.0785 ± 0.0024	1.85 ± 0.049	0.0425 ± 0.0013
	SUT	0.0750 ± 0.0026	1.72 ± 0.026	0.0437 ± 0.0016
	P value	0.343	0.106	0.562

Table 2
Dentate gyrus granule cell layer area, length and thickness in 3 ($n = 8$) and 11 month old mice (C3H $n = 5$, Sut $n = 4$)

		SVZ area (mm^2)	SVZ length (mm)	SVZ thickness (mm)
3 month	C3H	0.819 ± 0.015	4.16 ± 0.055	0.197 ± 0.0032
	SUT	0.774 ± 0.016	3.74 ± 0.065	0.207 ± 0.0025
	P value	0.0552	0.0002	0.0412
11 month	C3H	0.778 ± 0.029	4.31 ± 0.105	0.180 ± 0.0035
	SUT	0.738 ± 0.042	3.86 ± 0.075	0.191 ± 0.0079
	P value	0.45	0.0137	0.20

were found throughout the lateral portions of the SVZ (Fig. 1B–E). Quantitative analysis of BrdU labelling in the SVZ did not reveal a significant effect of genotype on cell proliferation in either 3-month-old ($t(14) = 1.983$, $P = 0.067$, $n = 8$) or 11-month-old ($t(8) = 1.336$, $P = 0.218$, $n = 5$) mice (Fig. 1F and G). However, in both 3- and 11-month-old Sut mice, there was a trend towards fewer BrdU labelled nuclei in the SVZ relative to wild-type C3H mice (12.1% and 9.4% reduction relative to controls in 3 and 11 month olds, respectively). The fact that proliferation rates did not differ significantly between genotypes (when normalized to area) suggests that xCT mediated cystine uptake is not an absolute requirement for cell proliferation in the SVZ *in vivo*.

Twenty-four hours after BrdU injection, immunopositive nuclei were also found along the subgranular zone of the dentate gyrus (Fig. 2A–D), a second major zone of neurogenesis in adult rodents. In sharp contrast to our results in the SVZ, labelling of proliferating cells in the SGZ was significantly increased in Sut mice at 3 months of age (Fig. 2E, 21.6% increase relative to controls, $t(14) = 2.978$, $P = 0.01$, $n = 8$). At 11 months, there was a trend towards more labelled nuclei in the SGZ of Sut mice, although this did not reach statistical significance (Fig. 2F, $t(7) = 2.232$, $P = 0.061$, C3H $n = 5$ Sut $n = 4$).

Discussion

Morphological and cell proliferation alterations

It is evident from *in vitro* studies that xCT plays a pivotal role in cellular proliferation [18,19,22]. The present study has examined the *in vivo* involvement of xCT in cell proliferation in neurogenic zones of the adult mouse brain. Despite an effect *in vitro*, we report relatively modest changes in the rates of cell proliferation measured *in vivo* using BrdU labelling after a 24 h period. A simple explanation for these findings is that compensatory mechanisms may support cell proliferation despite a lack of xCT mediated cystine transport.

Past research has shown that Sut mice display signs of atrophy such as enlarged ventricles and reduced overall brain size [19]. We suspected that the morphology of neurogenic zones was also altered. The SVZ of Sut mice has the same thickness, but showed a reduction in length and consequently a reduction in overall area. In the DG, the GCL of Sut mice showed a reduction in length, an increase in thickness and a trend toward a reduction in overall area. Thus, these results parallel previous findings of brain atrophy observed in the Sut mice [19]. In the SVZ, the absolute BrdU cell count is reduced in the Sut mice; however, changes in cell density are less prominent due to the reduced area of the Sut mice SVZ. On the contrary, in the DG Sut mice shown an increase in absolute BrdU cell count and a similar increase was seen in their overall GCL BrdU density.

Regional specific expression of xCT

xCT deficient Sut mice show a modest reduction in SVZ cell proliferation, but a significant increase in DG neurogenesis. These differential effects on proliferation in the two neurogenic regions studied may be accounted for by the specific distribution of xCT in the brain as well as the region specific differences in exposure levels to oxidative stress. Using in situ hybridization, RT-PCR and immunoblotting, xCT has been detected in both ependymal cells of the lateral ventricles, as well as in the hippocampus [19,20,24,25]. Interestingly, in regions bordering the CSF-brain barrer (ependymal cells), xCT expression levels were more than 10-fold higher than elsewhere in the brain possibly due to a requirement for local GSH production [19,20,24,25]. Thus, xCT expression levels near the SVZ are much greater than that in the SGZ of the DG. Therefore, functional loss of xCT could have a larger impact on SVZ cell proliferation than SGZ cell proliferation as we observed in Sut mice.

Evidence supporting compensatory mechanism for oxidative stress

The observation that cultured Sut mouse astrocytes, meninges [19] and fibroblasts [18] do not initially proliferate *in vitro* suggests that compensatory mechanisms are

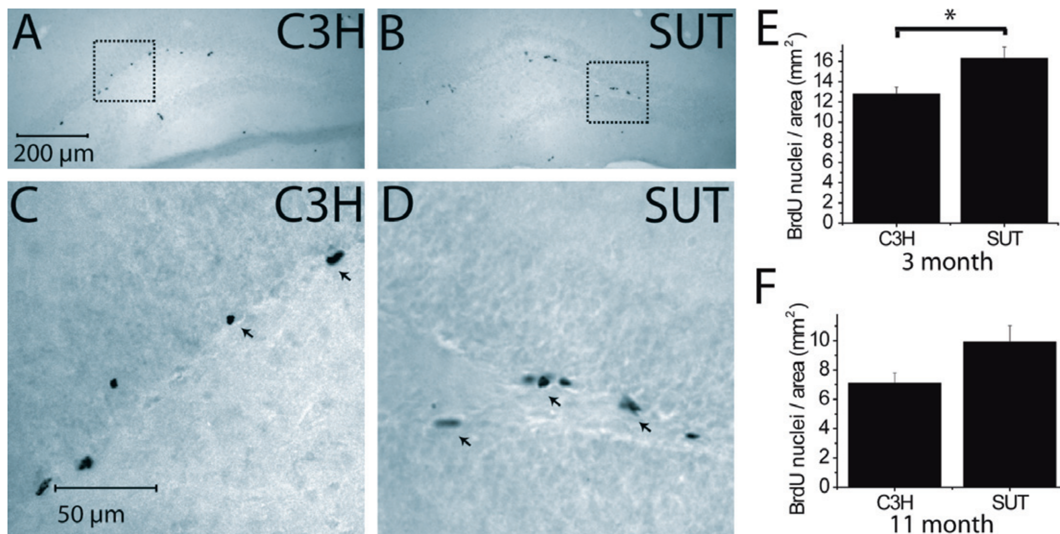

Fig. 2. Role of xCT transporter on cellular proliferation in the DG. (A–D) Bright field images showing BrdU immunopositive nuclei decorating the inner lining of the DG in both wild-type C3H and Sut mice. (E,F) Histograms showing the significant increase in cell proliferation in the DG of Sut mice at 3 months, but not at 11 months of age $^{*}P < 0.01$.

effective *in vivo* that allow xCT to be dispensable. Interestingly, with Sut astrocytes and meninges grown *in vitro*, compensatory mechanisms become effective after 1 week in culture, and the cells can survive independently of the reductant β-ME [19]. Additionally, cultured xCT deficient mouse cells also showed a low velocity cystine transport via a Na^{+}-dependent mechanisms resistant to inhibition by glutamate an xCT blocker [18]. Lastly, it is evident that xCT deficient mice have lower plasma [18] and cellular [19] GSH. This suggests that Sut mice are more likely to be in a state of oxidative stress that could induce anti-oxidant pathways such as Nrf2 activation. Nrf2 is a transcription factor, which upon activation will trigger anti-oxidant pathways [26,27]. Thus, the loss of xCT function could trigger a global compensatory mechanism, which would explain the elevation of cell proliferation in the DG SGZ. The elevation in oxidative stress in xCT deficient mice may also be associated with increased cell death. This may consequently trigger a regeneration mechanism as reflected by the elevation in cell proliferation in the DG of Sut mice. A similar phenomenon has been reported in ischemic stroke, where oxidative stress is elevated and DG neurogenesis is increased [28–30].

In conclusion, we have shown that the natural loss of functional xCT transport activity in Sut mice was accompanied by relatively normal rates of cellular proliferation in the SVZ, and an enhancement of cell production in the DG. These results implicate xCT in the regulation of hippocampal neurogenesis and demonstrate that, unlike previous *in vitro* studies, the absence of cystine transport via xCT is not an essential component of ongoing cell production in the adult brain.

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