Title: The cystine-glutamate exchanger (xCT, Slc7a11) is expressed in significant concentrations in a subpopulation of astrocytes in the mouse brain

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Main Points
Immunohistochemistry (validated using knockout mice) reveals a selective, but variable xCT expression in astrocytes and strong labeling in the leptomeninges, along blood vessels and in some tanycytes. The hippocampal xCT/EAA3 ratio is close to one.

**Keywords:** system xₐ; glutamate transporter; glia; astrocyte heterogeneity; tanycytes

**Abbreviations**

The abbreviations used are: BSA, bovine serum albumin; CAS, Chemical Abstracts Service Registry Number; CD98, cluster of differentiation 98 (4F2 heavy chain/ Slc3a2); cKO, conditional knockout; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; CNS, central nervous system; Cre, Cyclization recombinase; DTT, 1,4-dithiothreitol; EAATs, excitatory amino acid transporters; EAAT1, excitatory amino acid (glutamate) transporter 1 (Slc1a3); EAAT2, excitatory amino acid transporter 2 (Slc1a2); EAAT3, excitatory amino acid transporter 3 (Slc1a1); GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; NaPi, sodium phosphate buffer with pH 7.4; NCS, newborn calf serum; PBS, phosphate buffered saline (10 mM NaPi pH7.4 and 135 mM NaCl); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, tris-buffered saline (0.3M NaCl and 0.1 M Tris-HCl pH 7.4); TBST, TBS with 0.5 % (v/v) Triton X-100; WT, wild-type mice; xCT, cystine-glutamate exchanger (Slc7a11); xCT KO, xCT knockout mice (Sato et al., 2005).
Abstract
The cystine-glutamate exchanger (xCT) promotes glutathione synthesis by catalyzing cystine uptake and glutamate release. The released glutamate may modulate normal neural signaling and contribute to excitotoxicity in pathological situations. Uncertainty, however, remains as neither the expression levels nor the distribution of xCT have been unambiguously determined. In fact, xCT has been reported in astrocytes, neurons, oligodendrocytes and microglia, but most of the information derives from cell cultures. Here, we show by immunohistochemistry and by Western blotting that xCT is widely expressed in the CNS of both sexes. The labeling specificity was validated using tissue from xCT knockout mice as controls. Astrocytes were selectively labeled, but showed greatly varying labeling intensities. This astrogial heterogeneity resulted in an astrocyte domain-like labeling pattern. Strong xCT labeling was also found in the leptomeninges, along some blood vessels, in selected circumventricular organs and in a subpopulation of tanycytes residing the lateral walls of the ventral third ventricle. Neurons, oligodendrocytes and resting microglia, as well as reactive microglia induced by glutamine synthetase deficiency, were unlabeled. The concentration of xCT protein in hippocampus was compared to that of the EAAT3 glutamate transporter by immunoblotting using a chimeric xCT-EAAT3 protein to normalize xCT and EAAT3 labeling intensities. The immunoblots suggested an xCT/EAAT3 ratio close to one (0.75 ± 0.07; average ± SEM; n= 4) in adult C57BL6 mice. Conclusions: xCT is present in select blood/brain/CSF interface areas and in an astrocyte subpopulation, in sufficient
quantities to support the notion that system $x_c$ provides physiologically relevant transport activity.
**Introduction**

Glutamate is the major activator of excitatory neurotransmitter receptors in the mammalian CNS and the extracellular glutamate concentrations must be kept low (Zhou and Danbolt, 2014). While the mechanisms for glutamate removal have been identified (Danbolt, 2001), the release mechanisms are not yet fully characterized (Baker et al., 2002; Marx et al., 2015; Danbolt et al., 2016a). One of the latter is system x_c^-, which mediates glutamate release in exchange for cystine uptake (Bannai, 1986). Most, or all of, the system x_c^- activity in the brain is thought to be due to a heterodimer composed of a 4F2 heavy chain (CD98/Slc3a2) linked to the cystine-glutamate exchanger (xCT; Slc7a11) by a disulfide bridge (Sato et al., 1999). The substrate selectivity is controlled by xCT while the heavy chain is common to several heteromeric transporters (Palacin et al., 2005).

System x_c^- has received considerable attention both because cystine uptake is considered important for synthesis of glutathione (Sato et al., 2005; Jiang et al., 2015; Seib et al., 2011) and because the accompanying glutamate release may be significant (Baker et al., 2002; De Bundel et al., 2011; Massie et al., 2011) by modulating neurotransmission and possibly by aggravating neurological disorders (for review see: Bridges R et al., 2012; Lewerenz et al., 2013; Massie et al., 2015).

There is, however, still uncertainty as neither the localization nor the tissue concentrations of xCT have been determined. Firstly, fairly high xCT expression levels are required if the above functions indeed rely on xCT. Secondly, the physiological roles of xCT also depend on its cellular and regional distribution. *In situ* hybridization did not provide evidence for a wide distribution in brain parenchyma, but rather revealed xCT
mRNA in the leptomeninges, in some circumventricular organs and in select parts of the ventricular walls (Sato et al., 2002). Further immunohistochemistry has given inconsistent results partly because of poor antibody specificity and partly because most of the studies have been based on cell cultures rather than intact brains (e.g. Van Liefferinge et al., 2016; Massie et al., 2015). As a result, xCT protein is reported in neurons, astrocytes, microglia, oligodendrocytes and oligodendrocyte precursor cells (e.g. Soria et al., 2016; Burdo et al., 2006; La Bella et al., 2007). One of the most cited studies (Pow, 2001) on xCT localization, however, suggests that system x\textsubscript{c} is selective for astrocytes, but this study is based on antibodies to a substrate rather than the transporter itself and was performed before xCT knockout mice became available.

Here, we provide the first high-resolution immunohistochemical data validated with tissue from xCT knockout mice (xCT KO; Sato et al., 2005) as negative controls. We further provide quantitative data on the tissue content of xCT protein relative to excitatory amino acid transporter 3 (EAAT3) protein.

**Materials and Methods**

**Materials**

Sodium dodecyl sulfate (SDS) of high purity (>99 % C12 alkyl sulfate) was from Pierce (Rockford, IL, USA), and electrophoresis equipment were from Hoefer Scientific Instruments (San Francisco, CA, USA). N,N′-methylene-bisacrylamide, acrylamide, ammonium persulfate, TEMED and alkaline phosphatase substrates (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) were from Promega (Madison, WI, USA). Molecular mass markers for SDS-polyacrylamide gel electrophoresis and
nitrocellulose sheets (0.22 μm pores, 100 % nitrocellulose) were from Amersham (Buckinghamshire, UK). Prolong Gold AntiFade Mountant with DAPI (Cat. No. P-36935) was from Life Technologies (Carlsbad, CA, USA). Paraformaldehyde and glutaraldehyde EM grade were from TAAB (Reading, UK). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Animals

All animal experimentation was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80 23) revised 1996 and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Formal approval to conduct the experiments described was obtained from the animal subjects review board of our institutions. Care was taken to avoid suffering and minimize the number of animals used. Brain tissue for immunohistochemistry was obtained from young adult mice of both sexes that had been fixed by cardiac perfusion as described (Danbolt et al., 1998). The tissue used for estimating relative differences in regional xCT distribution by Western blotting was obtained from young adult female mice. The hippocampi used to compare xCT expression levels with those of EAAT3, and EAAT2, were from young adult mice of both sexes. The generation of mice lacking xCT and their genotyping, have been described in detail (Sato et al., 2005; Van Liefferinge et al., 2016; RRID:MGI:3608978). These mice have been backcrossed to C57BL/6J mice for at least 12 generations and kept in the animal facility of the Vrije Universiteit in Brussel. The Tie2-GFP (green) transgenic mice (Tg(TIE2GFP)287Sato/J; RRID:IMSR_JAX:003658) were obtained from Jackson
Laboratories. The mouse expressing tdTomato red fluorescent protein in astrocytes was generated by crossing a mGFAP-Cre driver (B6.Cg-Tg(Gfap-Cre)77.7Mvs/2J; RRID: IMSR_JAX:024098) with a STOP-flox tdTomato reporter Ai9 (B6;129S6-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/J; RRID: IMSR_JAX: 007905). To study whether xCT is expressed in reactive microglia, we examined brain sections from conditional glutamine synthetase knockouts which develop neurodegeneration accompanied by neuroinflammation (Danbolt et al., 2017). A total of three pairs of brains were obtained from a colony set up to produce conditional glutamine synthetase knockout and wildtype mice.

**Antibodies**

In-house developed anti-peptide antibodies against glutamate transporters were from the same batches as described previously. Because antibody batches may differ from each other (Danbolt et al., 2016b), we identify the antibody batches produced in our laboratory by the unique identification number (“Ab#”) they are given by our electronic laboratory information system (software provided by Science Linker AS, Oslo, Norway) as well as the Research Resource Identifiers (RRIDs) and a reference to the papers describing the production. The antibodies to xCT (Van Liefferinge et al., 2016) were raised in rabbits against peptides corresponding to residues 1-37 (Ab#618: MVRKPVVATISKGGYLQGNVSGRLPSVGDQEPPGHEK-amide; RRID:AB_2714118) and 471-502 (Ab#616: DKKPKWFRRLSDRITRTLQIILEVVPEDSKEL-free acid; RRID:AB_2714106) of rat xCT and were shown to be specific when tested both by immunoblotting and by immunohistochemistry on acetone fixed brain sections (Van
The antibodies to EAAT1 were the rabbit anti-A522 antibody (Ab#314; Holmseth et al., 2009; RRID:AB_2314561) and a commercial mouse monoclonal anti-GLAST antibody (NCL-EAAT1; lot. no.124104, Novocastra Laboratories, Newcastle, UK: Banner et al., 2002; RRID:AB_564088). Both of these antibodies recognized a peptide (PYQLIAQDNEPEKPVADSET-amide) representing the C-terminal residues 522-541 of rat EAAT1 (Storck et al., 1992). A third anti-EAAT1 antibody (Ab#286; Li et al., 2012; RRID:AB_2714072) was produced in sheep and directed to the N-terminal residues 1-25 (MTKSNGEEMPGRMFQFGVRKRC-amide) of rat EAAT1 (Storck et al., 1992). The sheep antibodies to EAAT2 (Ab#8; Li et al., 2012; RRID:AB_2714090) were directed to rat EAAT2 (Pines et al., 1992) residues 493-508 (YHLSKSELDTIDSQHR-amide), while the sheep antibodies to EAAT3, Ab#340 (Holmseth et al., 2005; RRID:AB_2714057) and Ab#565 (Holmseth et al., 2012a; RRID:AB_2714058) were directed to residues 510-524 (VDKSDTISFTQTSQF-free acid) of rat EAAT3 (Bjørås et al., 1996).

The other primary antibodies used were obtained commercially. The mouse monoclonal antibodies were: anti-glial fibrillary acid protein (anti-GFAP; cat. no. G3893, lot.no. 083M4785; RRID:AB_477010); anti-synaptophysin (cat. no. S5768; RRID:AB_477523); anti-CNPase (cat. no. C5922; RRID:AB_476854); anti-α-smooth-muscle-actin (α-SMA; cat. no. A5228, lot. no. 065M4762V; RRID:AB_262054) and anti-β-tubulin (cat. no. T4026; RRID:AB_477577), all from Sigma-Aldrich (St. Louis, MO, USA) and anti-glutamine synthetase (cat. no. MAB302, lot. no. 2571582; EMD Millipore, Billerica, Massachusetts, USA; RRID:AB_2110656). The polyclonal antibodies were: Rabbit-Anti-Iba-1 (rb-anti-Iba-1; cat. no. 019-19741; WAKO chemicals, GmbH, Germany;
RRID:AB_839504); Chicken-Anti-Iba-1 (ch-anti-Iba-1; cat. no. 234006, lot. no. 234006/1; Synaptic Systems, Germany; RRID:AB_2619949) and Rat-anti-mouse-F4/80 (anti-F4/80; cat.no. 14-4801; eBioscience, San Diego, USA; RRID:AB_467559).

The secondary antibodies and tertiary detection reagents were: Alexa Fluor 488-conjugated- Goat Anti-Chicken IgY (H+L) (cat. no. A11039); Goat Anti-Rat IgG (H+L) (cat. no. A11006, lot. no. 1605895); Donkey Anti-Rabbit IgG (H+L) (cat. no. A21206, lot. no. 1608521) and Goat Anti-Mouse IgG (cat. no. A11029, lot. no. 35220A); Alexa Fluor 555-conjugated- Goat Anti-Rabbit IgG (cat. no. A21429; lot. no. 34870A) and Donkey Anti-Sheep IgG (cat. no. A21436), all from Molecular Probes (Eugene, OR, USA); Alexa Fluor 680 AffiniPure Donkey Anti-Sheep IgG (H+L) (cat. no. 713-625-147, lot. no. 106399; Jackson ImmunoResearch, West Grove, PA, USA); IRDye 680RD Donkey Anti-Rabbit IgG (H+L) (cat. no. P/N 926-68073); 800CW Donkey Anti-Rabbit IgG (H+L) (cat. no. P/N 926-32213, lot. no. C50821-02), 800CW Donkey Anti-Mouse IgG (H+L) (Cat. No. P/N 926-32212), all from Licor Bioscience (Cambridge, UK); and Biotinylated Donkey Anti-Rabbit IgG (cat. no. RPN1004, lot. no. 399303) and Streptavidin-biotinylated horseradish peroxidase complex (cat. no. RPN1051, lot. no. 347752), both from Amersham Biosciences (Buckinghamshire, UK).

**Electrophoresis and immunoblotting**

Freshly frozen brain tissue was homogenized in 10-30 volumes of either water followed by centrifugation (Beckman JA20 rotor, 18000 rpm, 4°C, 10 min) to remove water soluble proteins prior to solubilization of the membrane proteins, or in 10 mM sodium phosphate buffer with pH 7.4 (NaPi) with SDS (10 mg/ml) as stated. The samples were
incubated (15 min) with or without reducing agent (30 mM DTT) as stated prior to SDS-polyacrylamide gel electrophoresis and immunoblotting (Holmseth et al., 2009). Briefly, the blots were first rinsed in phosphate buffered saline (PBS: 10 mM NaPi pH 7.4 and 135 mM NaCl) and then blocked (1 h) with casein (50 mg/ml) in PBS before incubation with primary antibodies (at concentrations as stated) diluted in bovine serum albumin (BSA, 30 mg/ml) in PBST (PBS with 1 ml/liter Tween 20 and 0.5 mg/ml NaN₃) over night at room temperature. Next, the membranes were rinsed (4x10 min) with PBST before incubation (1 h) in secondary antibody solution (1:10000 - 1:20000), then rinsed (3 x 5 min) with PBST, and finally with PBS (2 x 5 min) before scanning.

The blots were examined for immunofluorescence using an infrared scanner (Licor Odyssey system, LI-COR Biotechnology-UK Ltd, Cambridge, UK). Densitometric data were extracted from the resulting 16 bit monochrome images (one image for each channel) by means of the gel analyzer tool included in our electronic laboratory information system (software provided by Science Linker AS, Oslo, Norway). Analysis of the extracted data was performed using Microsoft Excel (Microsoft Corporation, WA, USA) and GraphPad Prism 4 (Graph-Pad Software Inc., San Diego, CA, USA).

Because water soluble proteins have been reported to interfere with the binding of transporter proteins to the blotting membranes (Danbolt et al., 2016b), we tested if water soluble proteins could interfere with detection of xCT. One mouse brain was homogenized in water, and divided in two equal aliquots. One aliquot was centrifuged (Beckman JA20 rotor, 18000 rpm, 4°C, 10 min) and the supernatant discarded. The ensuing pellet (referred to as membranes) and the untreated homogenate aliquot were brought up to the same volume by adding water, SDS (to 10 mg/ml) and NaPi (to 10
mM). The protein concentrations were estimated based on brain tissue mass assuming 100 mg total protein per gram wet weight and assuming that half of the protein is water soluble (Lowry, 1953; Lowry et al., 1954). Removal of the latter did not appear to influence the xCT immunoreactivity recorded on the blots (Figure 1a). This was repeated (Figure 1b) with tissue from another two mouse brains. Protein extracts were prepared and immunoblotted as above and the raw densitometric values (no background subtracted) were plotted against the total amount of membrane proteins loaded for each extract. Figure 1b shows the combined dataset (n=2) subjected to linear regression. When loading 1 - 25 µg of membrane proteins, the amount of xCT detected in the two types of extracts was a linear function of the amount of loaded membrane proteins and there was no statistically difference between the two (membranes: slope: 5.4 x 10^5, data fit: R^2=0.982; whole tissue: slope: 5.7 x 10^5, data fit: R^2=0.978).

Further, we found that xCT and CD98 were most efficiently separated when the disulfide link between them was reduced at room temperature rather than at 100°C (Figure 1c). By applying different amounts of protein in different lanes, we ensured that we were working within the linear range (Figure 1ab).

To calculate the relative level of xCT compared to EAAT3 protein the raw densitometric data were plotted and linear regression was used to define a linear equation. The y-intercept was set as background level and this background was equally extracted from all the raw densitometric values before calculating the relative xCT/EAAT3 level.

**Immunohistochemistry**
Mice were perfusion-fixed as described previously (Danbolt et al., 1998). Briefly, they were given a lethal dose of ZFR cocktail (mixture of Zolazepam (3.3 mg/ml; CAS 31352-82-6), Tiletamine (3.3 mg/ml; CAS 14176-49-9), Xylazine (0.5 mg/ml; CAS 7361-61-7) and Fentanyl (2.6 μg/ml; CAS 437-38-7); at least 0.1 ml per 10 g body weight). After cessation of all reflexes, the mice were perfused with 0.1 M NaPi to wash out blood, immediately followed by 4 % formaldehyde in 0.1 M NaPi for five minutes. The relevant tissues were post-fixed in fixative (2-3 h, room temperature). Sections (40 μm thick) were cut from the fixed unfrozen tissue using a Vibratome 1000 plus® (Vibratome, Bannockburn, UK). Alternatively, the tissue was cryoprotected by transferring it to a solution consisting of 0.1 M NaPi with 10 % (w/v) sucrose for 3-4 h (4°C), then in 0.1 M NaPi with 20 % (w/v) sucrose (over night, 4°C) and finally in 0.1 M NaPi with 30 % (w/v) sucrose and 0.2 mg/ml NaN₃ (over night, 4°C) before freezing on dry ice and storage at -80°C until cutting with a Cryotome Microm HM450 (ThermoFisher Scientific).

Diaminobenzidine-peroxidase labeling was performed as described (Lehre et al., 1995). Briefly, the sections were pre-incubated in 1 % hydrogen peroxide in 0.1 M NaPi, rinsed and treated (30 min, room temperature) in 1 M ethanolamine in 0.1 M NaPi with 0.5 % (v/v) Triton X-100 and rinsed (x 3) in PBS (0.135 M NaCl, 10 mM NaPi pH 7.4 with 0.5 % (v/v) Triton X-100) prior to incubation (1 h, room temperature) in blocking solution (10 % (v/v) newborn calf serum (NCS) in TBST (TBS with 0.5 % (v/v) Triton X-100), with 0.1 mg/mL NaN₃). Next, sections were incubated (over night, room temperature) with primary antibodies diluted (at concentrations as stated) in blocking solution. After rinsing (x 5) in TBST with 1 % (v/v) NCS, the sections were incubated (1 h, room temperature) with secondary antibodies diluted (1:300) in TBST with 1 % (v/v) NCS, and, after rinsed
as above, incubated (1 h, room temperature) with streptavidin-biotinylated horseradish peroxidase complex, diluted (1:300) in TBST with 1 % (v/v) NCS. The rinsing steps were repeated and sections were then rinsed (x3) in PBS prior to the color development step with diaminobenzidine-peroxidase.

Immunofluorescent labeling was done as previously described (Zhou et al., 2012). Briefly, the sections were rinsed (3 x 5 min) in TBST, treated with 1 M ethanolamine in 0.1 M NaPi pH 7.4 (30 min, room temperature), washed in TBST and blocked (1 h) in TBST containing 10 % NCS and 3 % BSA followed by incubation (over night, room temperature) with primary antibodies diluted in 3% NCS and 1 % BSA in TBST. Then sections were washed with TBST before incubation (1 h, room temperature) with secondary antibodies (1:1000). The sections were washed again with TBST and mounted with ProLong Gold antiFade mountant with DAPI.

**Preparation of xCT-EAAT3 chimeric protein**

**PCR:** 10 ng mouse xCT cDNA (Sato et al., 1999) was used as DNA template. A hybrid was synthesized by overlap extension PCR containing nucleotides 353–1858 of mouse xCT (GenBank accession number: NM_011990) and nucleotides 1601–1648 of mouse EAAT3 (Gen-Bank accession number: NM_009199). The primers used were forward primer xCT-Forward: 5’ CGGAATTCCATGGTCAGAAAGCCAGTTGTG 3’ containing the EcoRI restriction site and a reverse primer xCT-EAAT3 tag containing the XbaI restriction site and the 15 amino acid sequence tag of EAAT3 (amino acid 510-524, epitope for the EAAT3 antibody Ab#340, RRID:AB_2714057): 5’ GCTCGACTAGAACTGTGAGGTCTGAGTGAACGAGATGGTGTCAGATTTGTCTACTA
ATTCTTTAGAGTCTTCTGGTACAAC 3’. Briefly, the PCR reaction conditions were: denaturation of template at 94°C for 5 min, followed by 35 cycles of amplification (94°C for 30 s, 65°C for 30 s, 72°C for 1 min 45 s). Cloning: The PCR products and the pcDNA3.1+ vector were digested by EcoRI and Xbal restriction enzymes (Takara Bio USA). After gel purification (QIAaquick gel extraction kit, Hilden, Germany), the digested and purified PCR products were inserted into the EcoRI/Xbal sites of the pcDNA3.1+ vector by T4 DNA ligase (Thermo Fisher scientific). After transformation of ligation products and amplification in *E. coli*, plasmid DNA was extracted from over night cultures of single colonies by using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). The plasmid concentrations were determined by Nanodrop 2000. Transfection of mammalian cells: HEK293T cells were grown at 37°C in 5 % CO₂ in DMEM/F-12 (1:1) with GlutaMAX supplemented with 10% fetal bovine serum and 1% penicillin streptomycin antibiotics (Thermo Fisher Scientific). 24 h before transfection, the cells were passaged into a six well plate. Once they reached 85 % confluency they were transfected with plasmid DNA (2.5 µg/well) using the Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer’s instruction. The cells were harvested after 24-36 h and washed once with PBS. Ice-cold water containing 1 mM PMSF and 5 mM EDTA was added to each well and a cell scrape was used to lift the cells. The cell suspension was collected and centrifuged (13000 xg, 10 min, 4°C). The supernatant was discarded to remove water soluble proteins. The pellet was re-suspended in 1% (w/v) SDS (containing 1 mM PMSF and 5 mM EDTA) to a final protein concentration of about 5 mg/ml, sonicated and stored at -80 °C until it was used for immunoblotting as described above.
Microscopy

Overview pictures of immuno-peroxidase (Figure 2) and Nissl stained (Figure 6cd) sections were captured with Canon EOS 600D or Canon EOS 750D camera equipped with a Canon MP-E 65mm F1:2.8 1-5x Macro objective. Immunofluorescence labeled sections were scanned using Zeiss Axioplan 2 microscope equipped with a Zeiss LSM 510 meta confocal scanner head (Zeiss, Jena, Germany) except images in Figures 4g and 6b which were acquired with an ELYRA-S.1 microscope equipped with a Zeiss LSM 710 meta confocal scanner head. The objectives used were Plan-Apochromat 10x/0.45 and 20x/0.8 M27 and EC Plan-Neofluar 40x/1.30 and Plan-Apochromat 63x/1.40 oil DIC M27. The sizes of the scale bars in the figures correlate with the objectives: 100-200 µm, x10 objective; 50 µm, x20 objective; 20 µm, x40 objective; 2.5-10 µm, x63 objective; except when stated otherwise (Figure 3a). The secondary antibodies used were Alexa Fluor 488- and Alexa Fluor 555-conjugates. The laser lines used for excitation were of 405 (to visualize DAPI), 488 and 561 nm wavelength. For higher resolution image scans the original images were of an approximately size of (x63 objective) 133 x 133 µm or (x40 objective) 210 x 210 µm, and with a minimum of 1500x1500 pixels. The pinhole size was set to 1 airy unit as default, and adjusted to correspond to a similar optical slice thickness for all channels. For single optical scans with the x63 objective image acquisition was run with a pixel dwell of 3.2 µs (Figure 3e) or 17.5 µs (Figures 4a-f, 4h,6j, 7c and 8fst) and with an average acquisition line of 2 when using the Zeiss LSM 510 meta confocal scanner head. Images acquired with the LSM 710 confocal were scanned with a pixel dwell of 7.31 µs (Figure 4g) or 0.91 µs (Figure 6b) and with an
average line of 2. All images were scaled into appropriate sizes, cropped and assembled using the Adobe InDesign CS6 software, but not altered in any other way except when stated (Figure7a). Some figures (Figures 2b, 3a, 5a, 6ef, 7a and 8abgop) represent montages produced by merging multiple individual images using the Photomerge function in Adobe Photoshop CS6.

**Experimental Design and Statistical Analysis**

The validity of the present study depends on the specificity of the immunohistochemical procedures. As described in detail above, we have used antibodies both to the N-terminus and to the C-terminus of xCT with similar results. More importantly, we have used tissue from xCT knockout mice (Sato *et al.*, 2005) processed in parallel as negative controls. The images shown here demonstrate that labeling of wildtype mice is obtained when antibody concentrations and other parameters are set to minimize labeling of tissue from the knockout mice. As we have shown before, this is the single most important control (Holmseth *et al.*, 2012b). However, as outlined in our recently published strategy for immunohistochemical protein localization (Danbolt *et al.*, 2016b), it is also necessary to compare data obtained with different methods. These considerations, although important, do not readily lend themselves to statistical analysis. All experiments that were not statistically analyzed were independently repeated a minimum of three times to ensure consistency and reproducibility. The data is presented as average ±SEM. Statistically significant difference between group means were tested by one-way ANOVA, followed by Post hoc comparisons conducted with the use of
Tukey HSD test (Figure 2d). The statistical analysis was performed using IBM SPSS statistics 24 software.
Results

Specific visualization of xCT protein in formaldehyde fixed mouse tissue

We have recently generated specific xCT antibodies (Van Liefferinge et al., 2016). Here, an improved tissue processing protocol allowed detection of xCT in formaldehyde fixed tissue in which the tissue ultrastructure is well preserved. Both the antibody to the N-terminus (Ab#618) and the antibody to the C-terminus (Ab#616) of xCT, labeled tissue from wildtype mice without labeling tissue from xCT KO (Sato et al., 2005) processed in parallel (Figure 2a). Although the Ab#618 antibody gave stronger labeling than the Ab#616 antibody, the labeling patterns obtained with the two different antibodies were similar. Labeling with antibodies to EAAT1 was used as a positive control to check the quality of the xCT KO tissue.

As shown (Figure 2b), xCT expression was detected in all brain regions, but at somewhat higher levels in the cerebral cortex (neocortex and hippocampus), thalamus, hypothalamus and striatum than in the cerebellum and brain stem (Figure 2b). Particularly high labeling intensities were seen in the leptomeninges surrounding the brain and along some larger blood vessels (e.g. Figure 2c, arrows and arrow heads, respectively), but there was also widespread labeling of brain parenchyma, as shown for neocortex, hippocampus, striatum and cerebellum (Figure 2c1-4). In hippocampus the highest labeling intensities were observed in stratum lacunosum moleculare and in the molecular layer of the dentate gyrus (Figures 2c2). In the cerebellum, xCT was present in white matter and, with lower intensity, the granule cell layer, whereas no labeling was detected in the molecular layer (Figures 2c4 and 5a). The processes of Bergmann glia cells stretching radially through the molecular layer were visualized by
staining for GFAP, but were not labeled by antibodies to xCT (data not shown). The white matter tracts, including corpus callosum, commissura anterior, capsula interna, stria terminalis, fimbria of hippocampus and tractus opticus, were only sparsely labeled (Figure 2b and 2c\textsubscript{1-3}). Data from immunoblots (Figure 2d) were in good agreement with the immunohistochemistry in that xCT protein was detected in all brain regions with somewhat lower levels in the cerebellum.

**Within brain parenchyma xCT is expressed in astrocytes**

Both the C-terminal antibody and the N-terminal antibody to xCT gave rise to patchy labeling of brain parenchyma. This was particularly evident in the neocortex, in hippocampus and in both dorsal and ventral parts of striatum (Figure 2c\textsubscript{1-3} and 3a), as well as in the thalamus (Figure 3f) and in the hypothalamus (data not shown). The xCT-positive patches were seen in sections from wildtype mice (e.g. Figure 3abce) and not in sections from xCT KO (e.g. Figure 3d). The diameters of the patches were similar to those of astrocyte domains (Oberheim *et al.*, 2009). To test whether these patches actually represented astrocytes, immunofluorescence double labeling was performed by combining antibodies to xCT with various astroglial markers: EAAT1, glutamine synthetase (GS) and GFAP. The xCT-positive patches were positive also for EAAT1 (Figure 3ce). On the other hand, not all EAAT1 labeled astrocytes were xCT-positive. Double labeling for xCT and GS also suggested xCT expression localized to a subset of the cells positive for the astrocyte marker (data not shown). The single focal plane LSM scans were supplemented with z-stack imaging, which further indicated that the xCT labeling followed the three-dimensional projection of a subgroup of astrocyte domains.
In agreement, antibodies to GFAP labeled star-like structures (astrocyte cytoskeletons) located close to the center of each astrocyte domain, whether xCT-positive or not (Figure 3b). Consequently, the patchy labeling implied that neighboring astrocytes often had very different xCT contents. This was also observed when we tested the localization of xCT in astrocytes by performing xCT labeling of brain sections from a transgenic mouse expressing a red fluorescent protein variant (tdTomato) in astrocytes, controlled under the mouse GFAP promoter (Figure 3f1). We took advantage of the fact that this reporter line does not label all astrocytes, particularly in the subcortical region, and thereby nicely visualizes individual astrocyte domains. As shown (Figure 3f), xCT positive cells co-localized with tdTomato-expressing cells, and among the tdTomato-expressing cells, there were variations in the xCT labeling intensities. This astrocyte heterogeneity was consistently observed with both xCT antibodies (Figure 4) in all brain regions studied, including hippocampus (Figure 3abce), thalamus (Figure 3f and 4c) and neocortex (Figure 4a), as well as both the ventral and the dorsal striatum and the hypothalamus (data not shown).

**Co-localization of neuronal markers with xCT was not detected**

To examine whether xCT-positive astrocyte branches could mask an eventual presence of xCT protein in neurons, we did immunofluorescence double labeling by combining antibodies to xCT with different neuronal markers known to be present in cell bodies and dendrites (EAAT3; Holmseth et al., 2012a) and terminals (synaptophysin), respectively. Where xCT and neuronal markers were present within the same areas, high resolution examination of the xCT-positive patches did not reveal any co-
localization of xCT with markers for neuronal dendrites or somata (data not shown) or nerve terminals (Figure 4h). There was some labeling of neuronal nuclei, but as this was observed in the sections from both wildtype mice (e.g. Figure 3c) and xCT KO (e.g. Figure 3d), it was interpreted as cross-reactivity.

**Co-localization of oligodendrocyte marker with xCT was not detected**

The strongest xCT labeling in the cerebellar parenchyma was in white matter (Figures 2c, 4 and 5a). This raised the question of whether xCT could be expressed in oligodendrocytes. However, whereas double labeling for xCT and EAAT1 (using antibodies to both the N- and the C-termini of EAAT1) revealed co-localization of the two proteins (e.g. Figure 5b), no co-localization of xCT and CNPase (an oligodendrocyte marker) was observed (Figure 5c). Similarly, no co-localization of xCT and CNPase was detected in the corpus callosum, commissura anterior, capsula interna and chiasma opticum (data not shown). Thus, the observed weak xCT labeling in white matter tracts was due to expression in fibrous astrocytes. The present data did not provide evidence for xCT protein in oligodendrocytes.

**Co-localization of microglial markers with xCT was not detected**

To examine whether xCT is expressed in microglia, microglia marker Iba-1 (Ito et al., 1998) was used. At low magnification, Iba-1 positive cells appeared distributed independently of xCT positive cells (Figure 6a). Further, at sites where Iba-1 positive cells appeared located within an xCT positive patch, no co-localization was detected between Iba-1 and xCT proteins when examined at higher resolution (Figure 6bi). We
also examined xCT expression in one model of reactive microglia: mice lacking glutamine synthetase in the cerebral cortex. These mice show prominent neurodegeneration (e.g. CA1 atrophy with loss of pyramidal cells; compare Figure 6c to 6d), and thereby represent a good source of activated microglia (Figure 6h; Danbolt et al., 2017). Signs of activation of microglia comprise transformation to a bushy- or amoebae-like shape and upregulation of F4/80. Such microglial cells were observed in degenerating hippocampal tissue (compare Figure 6g to 6h), but despite these signs of activation there was still no co-localization between xCT and Iba-1 (Figure 6j). Thus, these results gave no indication of xCT expression, neither in resting microglia, nor in reactive microglia in this particular disease model. Interestingly, along with the neurodegeneration, we observed reduced xCT labeling in astrocytes (compare Figures 6e with 6f) with ensuing disappearance of the patchy labeling pattern described above (Figures 2bc, 3abce and 6ae), while intense xCT labeling related to leptomeninges and blood vessels was still present.

**xCT is highly expressed in relation to leptomeninges and larger blood vessels**

Intense xCT labeling was detected in the leptomeninges and along some blood vessels (e.g. Figures 2c, 3ac, 5a and 6ei). To examine whether endothelial cells express xCT, sections from Tie2-GFP transgenic mice were labeled for xCT. Confocal imaging, including both single section and Z-stacks scans, revealed that blood vessel related xCT was localized to cells clearly different from the endothelial cells (Figure 7a).

Noteworthy, the intense xCT labeling was mainly found associated with larger blood vessels, as several smaller vessels (capillaries) labeled in sections from the Tie2-
GFP mice did not show xCT labeling. Because pericytes closer to the arteriole end of the capillary bed surround larger blood vessels and have been found to express more smooth muscle α-actin (α-SMA; Attwell et al., 2016), we used antibodies against α-SMA to examine whether the xCT positive cells along blood vessels could be either vascular smooth muscle cells or α-SMA-expressing pericytes. Immunofluorescence double labeling for xCT and smooth muscle α-actin indicated that the xCT expressing cells along blood vessels were localized on the neuropil side of the α-smooth muscle actin (α-SMA) expressing cells in the blood vessel walls (Figure 7b). No further attempts were made to identify the xCT-positive cells associated with blood vessels.

**Examining xCT expression related to the ventricular system**

The lateral ventricles and third ventricle were examined in serial coronal sections. High xCT labeling intensities were found in a select area of the lateral walls of the ventral third ventricle, predominantly related to the medial hypothalamic nuclei and the caudal portions of the arcuate hypothalamic nuclei (Figure 8a-e). Labeling of xCT was not detected in the ventricle lining of the anterior third ventricle portion (Figure 8a), but started to appear in the ventricle lining in the middle of the tubular portion of the ventricle (Figure 8b). The highest labeling intensities were detected in the caudal parts of the tubular portion of the ventricle (Figure 8cd) and rostral to the mammillary recess (Figure 8e). In this area, intense xCT labeling was observed in cell structures facing the ventricle lumen (Figure 8f), and strongly labeled structures were observed projecting radially from parts of the lateral walls into the subjacent neuropil and seemingly towards the ventral meningeal surface (Figure 8c-e). The morphology and distribution of these
xCT-positive cells suggest that they are tanycytes (Berger and Hediger, 2001; Mullier et al., 2010). However, even within the ventrocaudal portion of the third ventricle the strong xCT labeling was found related only to a select part of the lateral walls of the ventricle. The ventricle floor, on the other hand, including the median eminence and its ependymal covering, was xCT-negative (Figure 8bc), suggesting that xCT is not expressed in all tanycytes. Highly intense xCT labeling was also detected in the circumventricular organs *area postrema* (Figure 8o) and subfornical organ (Figure 8q). The medial habenular nucleus was also strongly labeled (Figure 8i). Higher magnification revealed co-localization of xCT and the astrocyte marker EAAT1 also in these regions (e.g. Figures 4e and 8st). Epithelial ependymal cells in the ventricle lining of the dorsal parts of the ventral third ventricle (Figure 8h), the floor and lateral walls of the dorsal third ventricle (Figure 8i) and the lateral ventricles (Figure 8j), as well as ependymal cells in the choroid plexus (Figure 8k) were found to be xCT-negative based on observations from three different animals. The xCT antibodies however labeled some scattered non-ependymal cells in choroid plexus (Figure 8k; arrows). Further, the ependymal lining of the aqueduct, the fourth ventricle and the proximal part of the central canal also were xCT-negative (Figure 8l-n). Sections from xCT KO littermate tissue processed in parallel showed no xCT labeling related to ventricles and circumventricular organs (Figures 8g and 8p, r).

**The content of xCT protein relative to EAAT3 protein in mouse hippocampus**

Although the above immunohistochemistry and Western blotting indicated that xCT is both readily detectable with the antibodies used and widely expressed in the brain, none
of these experiments gave information on the total amount of xCT protein. The high sensitivity of the confocal image acquisition allows proteins to be visualized even when expressed at very low levels. As we have in the past quantified the glutamate transporters (Lehre and Danbolt, 1998; Holmseth et al., 2012a), we decided to compare the amount of xCT protein to the amounts of the glutamate transporters subtypes EAAT2 and EAAT3 by immunoblotting. However, labeling intensities obtained with two different antibodies to two different proteins cannot be compared without a common reference. Therefore, we created a chimeric xCT-EAAT3 protein (Figure 9a) and a chimeric xCT-EAAT2 protein (not shown). These chimeric proteins were run together with solubilized hippocampus tissue on immunoblots which were double labeled using antibodies to xCT and either EAAT3 (Figure 9b) or EAAT2 (not shown). The labeling intensities of the hippocampal extracts were divided by those of the chimeric protein. As the chimeric protein was the same, the number of xCT molecules relative to EAAT3, or EAAT2, molecules in the hippocampal samples could be calculated simply by dividing the xCT values (hippocampus protein/chimeric protein) by the EAAT3, or EAAT2, values (hippocampus protein/chimeric protein). This analysis revealed that the xCT/EAAT3 ratio equaled 0.75 ± 0.07 (average ± SEM; n= 4 immunoblots like that in Figure 9b). When the experiment was repeated with the chimeric xCT-EAAT2-protein, it was concluded that the EAAT2 levels are higher than the xCT levels by two orders of magnitude (data not shown) consistent with the EAAT2/EAAT3 ratio previously reported (Holmseth et al., 2012a).

Discussion
The aims of the present study were to map the distribution and to estimate the tissue content of xCT protein in mouse brain.

The regional distribution of xCT described here is in good agreement with in situ hybridization data (Sato et al., 2002) showing that the highest xCT expression levels in the adult mouse brain are in the area postrema, the subfornical organ, the medial habenular nucleus, and in parts of the lateral walls of the ventral third ventricle. The in situ hybridization also revealed scattered xCT expressing cells in the hypothalamus (Sato et al., 2002), but overall labeling of brain parenchyma was weak, raising the question whether xCT is expressed at physiologically relevant levels. Here, due to higher sensitivity, we were able to reveal widespread xCT protein expression throughout brain parenchyma. Quantitative analysis further confirmed that the in vivo xCT protein concentrations are considerable.

**Most xCT in normal brain parenchyma is concentrated in a subpopulation of astrocytes**

Within brain parenchyma, xCT was detected in a subpopulation of EAAT1-positive cells throughout the brain, with especially high levels in the medial habenular nucleus, the subfornical organ and the area postrema. Also a subpopulation of tanycytes in the lateral walls of the ventrocaudal third ventricle was highly xCT-positive. The selective expression of xCT in astrocytes is in agreement with transcriptome data (Zhang et al., 2014; Zeisel et al., 2015), and also with an elegant study of the distribution of system x_c^- activity, which was mapped with antibodies to a system x_c^- substrate (aminoadipate) in rat brain slices (Pow, 2001), before the generation of xCT knockout mice (Sato et al.,
In agreement with our data, aminoadipate was found accumulated in astrocytes, but not in neurons or oligodendrocytes. The distribution of accumulated aminoadipate, however, did not reveal astrocyte heterogeneity like the xCT data presented here, possibly because gap junctions allow aminoadipate to diffuse from one astrocyte to another. Interestingly, aminoadipate accumulated also in Bergmann glial cells which we found to be xCT-negative. In accordance with our findings, xCT mRNA was absent from the Purkinje cell layer (which contains cell bodies of both Bergmann glia and Purkinje cells), whereas mRNA encoding CD98 was present (Sato et al., 2002), possibly suggesting that Bergmann glia express another Slc7-type of transporter with affinity for aminoadipate.

The variability in xCT expression adds to the growing list of proteins contributing to astrocyte heterogeneity (Schitine et al., 2015, Hu et al., 2016). We have, however, no way of testing whether the observed variability in astrocytic xCT expression represents stable properties of the astrocytes or whether this changes with the functional state of the tissue. It is, however, interesting to note that a similar variability in xCT expression is found in gliomas (Robert et al., 2015).

**Expression of xCT in reactive microglia**

As induction of xCT has been reported in response to a number of different stimuli (for review see Bridges R J et al., 2012; Lewerenz et al., 2013; Dai et al., 2015; Massie et al., 2015), the possibility exists that other cell types than astrocytes, microglia in particular, may under certain conditions start expressing xCT. Most evidence for microglial xCT expression comes from *in vitro* studies, but there are some reports on
induced xCT expression in reactive microglia \textit{in vivo} (for references see: Bentea \textit{et al.}, 2017). Here, however, we did not detect xCT in reactive microglia from the glutamine synthetase-deficient mouse hippocampi, raising the question of whether the mode of activation determines if xCT expression is turned on.

\textbf{Role of xCT in the supply of cystine for glutathione synthesis}

Glutathione (a tripeptide consisting of glutamate, cysteine and glycine) is essential in many detoxification processes, and impaired glutathione metabolism is linked to human disease (Dringen \textit{et al.}, 2015). As cystine is reduced intracellularly to cysteine, xCT provides one of the components required for glutathione synthesis. However, lack of xCT in mice does not seem to cause reduced glutathione levels or oxidative stress \textit{in vivo} (De Bundel \textit{et al.}, 2011; Massie \textit{et al.}, 2011). In line with this, there is a redundancy in this system. Firstly, cysteine is not an essential amino acid and is produced in astrocytes via the transsulfuration pathway (McBean, 2012). Secondly, while system $x_c^-$ is probably the only transporter able to exchange cystine for glutamate, several transporters are capable of cystine transport (\textit{e.g.} Slc7a9, Slc7a13 and Slc7a15; Fotiadis \textit{et al.}, 2013; Fernandez \textit{et al.}, 2005; Nagamori \textit{et al.}, 2016), implying that the presence of other cystine transporters in brain remains a possibility. Further, there are several cysteine transporters in brain. Hence, if cystine is reduced to cysteine at the cell surface, cysteine can be taken up independently of system $x_c^-$. Transporters for cysteine comprise EAAT3 (Zerangue and Kavanaugh, 1996) which is selectively expressed in neurons (Holmseth \textit{et al.}, 2012a), and the alanine-serine-cysteine transporter (ASCT1, Slc1a4; Arriza \textit{et al.}, 1993) which
is expressed in astrocytes (Sakai et al., 2003), as well as NTT4 (Slc6a17), SNAT1 (Slc38a1), SNAT2 (Slc38a2) and possibly others (Mackenzie and Erickson, 2004; Zaia and Reimer, 2009; Bröer, 2008; Fotiadis et al., 2013, Bridges R et al., 2012)). In particular, EAAT3 may be as important as xCT in providing glutathione precursors considering that mice lacking EAAT3 have lower glutathione levels and age prematurely (Aoyama et al., 2006).

Concentrations of xCT protein and contributions to extracellular glutamate

The hypothesis that system x\textsubscript{c}\textsuperscript{\textendash} is an important mediator of glutamate release in normal tissue (Baker et al., 2002) was recently supported by the observation that absence of xCT induces a decrease in extracellular glutamate (De Bundel et al., 2011; Massie et al., 2011). However, for xCT to be able to release such amounts of glutamate two conditions must be met: (1) There must be enough external substrate available for exchange. (2) The concentrations of xCT protein must be sufficient to provide significant exchange capacity relative to the capacity of the removal mechanisms. The capacity of the latter is high (Otis and Kavanaugh, 2000, Bergles and Jahr, 1997, Lehre and Danbolt, 1998; Marx et al., 2015), but if the ambient glutamate levels are considerably lower than the K\textsubscript{m} of EAAT1-3 mediated uptake (Herman and Jahr, 2007; Herman et al., 2011), then even a modest xCT-mediated release might be functionally significant.

1. Substrate availability: Because system x\textsubscript{c}\textsuperscript{\textendash} exchanges internal and external substrates in a 1:1 relationship (Bannai, 1986), the rate of glutamate release depends, not only on the transporter proteins (density and kinetics), but also on the availability of extracellular substrates (Patel et al., 2004; Bridges R J et al., 2012). It has recently
become clear that cystine, which is only present at low concentrations, is not the only endogenous substrate. Also α-aminoadipate (Chang, 1982), homocysteate (Do et al., 1997) and cystathionine (Kobayashi et al., 2015) are able to exchange with intracellular glutamate via system $x_c^-$. The tissue content of cystathionine appears to be considerably higher than that of cystine (for references see: Kobayashi et al., 2015). The reported $K_m$-values are 0.081 mM for cystine and 0.16 mM for glutamate (McCormick and Tunnicliff, 1998), with the affinity for cystathionine being similar to that for glutamate (Kobayashi et al., 2015).

2. Exchange capacity: The exchange capacity of xCT depends both on the number of transporter molecules at the plasma membrane and on how fast they operate (the cycling time). The finding that mouse hippocampus contains similar amounts of xCT and EAAT3 proteins implies that xCT protein is robustly expressed (Holmseth et al., 2012a): the xCT levels are much higher than the forebrain levels of EAAT4 (Dehnes et al., 1998), but two orders of magnitude lower than EAAT2 (Lehre and Danbolt, 1998). However, as xCT is highly inducible (Massie et al., 2015), both the total xCT protein concentration and the percentage that is targeted to the plasma membrane, may differ depending on the situation.

EAAT3 is expressed in the cell bodies and dendrites of most, if not all neurons and is thereby distributed in a plasma membrane surface area of 1.5 $\mu m^2$ per $\mu m^3$ tissue (stratum radiatum, hippocampus CA1, young adult Wistar rats: Holmseth et al., 2012a). This plasma membrane surface area is quite similar to that of astrocytes (1.4 $\mu m^2$ per $\mu m^3$ tissue: data from the stratum radiatum, hippocampus CA1, young adult Wistar rats: Lehre and Danbolt, 1998). As xCT is more selectively distributed, but expressed at
comparable tissue concentrations, the xCT levels in highly expressing astrocytes must be higher than that of EAAT3 in neurons. Further, judging from labeling intensities, the expression levels in cells related to the leptomeninges, blood vessels, circumventricular organs and ventricular walls are probably even higher.

Unfortunately, the cycling time of xCT-mediated cystine-glutamate exchange is unknown. For comparison, the cycling times of EAAT2 and EAAT3 are around 30 glutamate molecules per second at $V_{\text{max}}$ (Otis and Jahr, 1998; Otis and Kavanaugh, 2000; Bergles et al., 2002; Grewer and Rauen, 2005) and the GABA transporters appear to operate at similar rates (Mager et al., 1993; Sacher et al., 2002; Karakossian et al., 2005; Gonzales et al., 2007). As the transport cycles of these groups of transporters (Zhou et al., 2014) are probably more complex than that of xCT, it is legitimate to hypothesize that system xc may be faster.

**Conclusions**

In mouse brain parenchyma, xCT is selectively expressed in scattered astrocytes throughout the brain. Further, especially strong xCT labeling is found in select blood/brain/CSF interface areas: in the leptomeninges, along larger blood vessels, in some circumventricular organs and in tanyocytes in parts of the walls of the ventral third ventricle. In the present study we did not find evidence for expression of xCT in microglia, oligodendrocytes and neurons in the normal adult mouse brain. Also reactive microglia in glutamine synthetase-deficient hippocampi were xCT-negative. This does not rule out low level expression, but as transporters are slow compared to ion channels, they must be expressed at fairly high numbers for their activity to be physiologically
relevant (for discussion see: Danbolt et al., 2016b). We estimated the total concentration of xCT in the hippocampus to be similar to the EAAT3 protein concentration within factor two. With the distribution of xCT being more limited than that of EAAT3, this implies high levels of xCT in some cells.
Acknowledgments

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Figure legends

Figure 1: Testing conditions for Western blotting. Panel a: Brain tissue was homogenized in water and either centrifuged to separate soluble proteins (s) from water insoluble (membrane) proteins (m), or processed as whole tissue (w). The samples were solubilized in SDS (Lanes 1-9 and 14-15) and brought up to the same volume, implying equal concentrations of membrane proteins in the two types of extracts (m and w), as indicated. The gels were immunoblotted with antibodies to xCT (Ab#618, 0.5 µg/mL). There was no detectable xCT in the water soluble fraction (s; Lane 9). Solubilization in 1 % (w/v) Triton X-100 (Lane 10) was less efficient than solubilization in SDS (compare with Lane 5). Reducing agent 1,4-dithiothreitol (DTT; 30 mM) or 5,5'-dithiobis-(2-nitrobenzoic acid) (Db) were added as indicated prior to incubation (15 min, room temperature) preceding electrophoresis. Reduction with DTT (Lanes 1-10) allowed xCT to migrate with apparent molecular mass of 35 kDa as reported previously (Van Liefferinge et al., 2016). Note higher apparent mass in the absence of reducing agent (Lanes 14-15). Lanes 11-13 are empty lanes separating DTT containing samples from samples not containing DTT. The whole blot is shown from start of gel to the dye front (DF). The amount (µg) of water soluble proteins (s; lane 9) or membrane proteins (all other lanes) in each lane is indicated. Panel b: Membrane protein extracts (m) and whole brain protein extracts (w) from two more different mouse brains were prepared and immunoblotted with antibodies to xCT as described above. Note the linear relationship between the amount of protein loaded and the immunoreactivity detected (as assessed by raw densitometric values), and also note that the immunoreactivity was not significantly affected by removal of water soluble proteins. Panel c: The best effect
of the reduction was seen when it was performed at room temperature (15 min; Lane 1) rather than at 100°C (2 min, boiling water bath; Lane 2). The two samples were loaded into neighboring lanes and this pair was repeated several times to produce several blot strips. The various strips were probed with antibodies to xCT (Ab#618, 0.5 µg/mL), EAAT2 (Ab#8, 0.3 µg/mL) and tubulin (Tub; anti-β-tubulin, 1:1000) as indicated. The two left strips represent the same physical membrane double labeled and therefore scanned twice (in different infrared channels) producing two images. The dye front (DF) is indicated.

**Figure 2: Regional distribution of xCT protein in mouse brain.** Panel a: Brain sections from wildtype (+/+) and xCT knockout (-/-) mice (littermates; 3 months old) were probed with antibodies to xCT (N-terminus: Ab#618, 1 µg/mL; C-terminus: Ab#616, 1 µg/mL) and developed with diaminobenzidine-peroxidase. Sections labeled with antibodies to EAAT1 (Ab#314, 0.1 µg/mL) were used as positive controls. Panel b shows a section from a wildtype mouse processed as above using the Ab#618 antibody to xCT (1 µg/mL), and imaged at higher magnification. Note that xCT labeling was obtained in all brain regions, but with somewhat lower intensities in the cerebellum (C) than in the neocortex (X), hippocampus (H), hypothalamus, thalamus (T) and striatum (S). Panel c: Excerpts from Panel b presented with higher magnification. Note the particularly intense labeling of the leptomeninges (arrows Panels c1, c4) and along some larger blood vessels (arrow heads Panels c1, c2, c4), but also that the brain parenchyma itself was labeled. Also note the patchy labeling, which was especially prominent in the neocortex (c1; X), hippocampus (c2), caudate putamen (c3; CPu) and
nucleus accumbens ($c_3$; AcbC, AcbSh). In hippocampus, the most intensely labeled regions were *stratum lacunosum moleculare* (LMol) and the molecular layer of dentate gyrus (Mol). In cerebellum ($c_4$) xCT was detected in the white matter (wm) and, with lower intensity, in the granule cell layer (gcl), whereas the cerebellar molecular layer (ml) was xCT-negative. Also the white matter tracts of *corpus callosum* (cc; including forceps minor, fmi), fimbria of hippocampus (fi), *commissura anterior* (ac), *stria terminalis* (st), *capsula interna* (ic) and *tractus opticus* (opt) were labeled, although weaker than most of the grey matter regions (*Panels b-c*). *Panel d*: The xCT protein levels in different brain regions were compared by immunoblotting (10 µg total protein per lane) with antibodies to both xCT (red; Ab#618, 0.5 µg/ml) and tubulin (green; anti-β-tubulin, 1:1000) as illustrated with an image of a representative immunoblot. The labeling intensities were determined and xCT data was normalized to tubulin data, and expressed as percent (average ± SEM; n = 3 mice) of the values from neocortex. There was a statistically significant difference between group means as determined by one-way ANOVA $F(6, 14) = 3.93$, $p = 0.016$. Post hoc comparisons conducted with the use of Tukey HSD test revealed significant pairwise difference between the mean scores of neocortex (mean: 1.30; SEM: 0.238; n=3) and cerebellum (mean: 0.440; SEM: 0.0897; n=3), difference between means: 0.861*, * $p=0.007$; 95 % KI: 0.210, 1.51). The remaining regions did not significantly differ from each other. Abbreviations: AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; B, olfactory bulb; C, cerebellum; H, hippocampus; S, striatum; T, thalamus and hypothalamus; X, neocortex. Scale bars, *Panel a*: 1 mm; *Panel b*: 0.5 mm; *Panel c*: 0.25 mm.
Figure 3: Astrocyte heterogeneity with respect to xCT expression.

Immunofluorescence labeling with antibodies to xCT (Ab#618, 1 μg/mL) reveals a patchy labeling pattern. **Panel a:** Montage of confocal micrographs from one section of a wildtype mouse hippocampus. **Panel b:** Single optical section scan from hippocampus CA1 from a section double labeled for xCT and GFAP (green; anti-GFAP, 1:200; astrocyte skeleton marker). Note that GFAP labels the astrocyte cytoskeleton and thereby reveals the centers of individual astrocytes domains. The approximate borders of two adjacent astrocyte domains are encircled to underline the variation in xCT labeling intensities between neighboring astrocytes. **Panel c:** Double labeling for xCT (red) and EAAT1 (green; NCL-EAAT1, 1:200) from hippocampus CA1 shows that the xCT-positive patches (*) are also positive for EAAT1 (green), which is a marker for astrocyte plasma membranes (Lehre et al., 1995). Note that the xCT antibody does not label sections from xCT knockout (-/-; **Panel d**) mice processed in parallel, with the exception for some unspecific binding to nuclei (e.g. in the pyramidal cell layer, P; upper part of panel c). **Panel e:** Higher resolution excerpts (x63/1.40 oil immersion objective) from the boxes in Panel c. **Panel f:** xCT labeling (green) in a brain section from a transgenic mouse expressing tdTomato (red) under the mouse GFAP promoter (mGFAP:tdTomato). Note that xCT co-localizes with tdTomato in this reporter line, but that the astrocyte domains visualized by tdTomato expression (Panel f, asterix) show quite a big variation in xCT labeling intensities. The image is from thalamus. DAPI (b, c3, f; blue) labels the nuclei. Abbreviations: O, stratum oriens; P, pyramidal cell layer; R, stratum radiatum; v, blood vessel. Scale bars, a: 200 μm (40x/1.30 oil immersion objective); b, c, d, f: 20 μm; e: 5 μm.
Figure 4: The xCT labeling co-localizes with astrocyte marker EAAT1, but not with neuronal markers. Panels a-f: The astrocyte domain-like labeling pattern of xCT in hippocampus (Figures 2c and 3) is also seen in other wildtype mouse brain regions (green; xCT), as here shown for neocortex (a), thalamus (b) and the medial habenular nucleus (c). In these regions, the xCT-positive cells are positive also for the astrocyte marker EAAT1 (red; Ab#286, 0.5 μg/mL). Note that xCT-antibodies targeted to the N-terminus (a, Ab#618, 1 μg/mL) and to the C-terminus (Panels c and e: Ab#616, 3 μg/mL) give rise to the same labeling pattern. Panels bdf show that, except for some unspecific nuclei staining, no xCT labeling is detected in the corresponding brain regions from xCT knockout (-/-) littermates imaged with the same confocal settings. Panel g: xCT (green; Ab#618, 3 μg/mL) is present in the fine astrocyte processes visualized by staining for EAAT1 (red; Ab#286, 1.5 μg/mL). In contrast, no co-localization could be observed with neuronal markers, as shown in Panel h with double labeling for xCT (red; Ab#618; 1 μg/mL) and the nerve terminal marker synaptophysin (green; anti-synaptophysin, 1:1000). Nuclei are stained blue with DAPI. Scale bars, a-h: 5 μm; inserts g, h: 2.5 μm.

Figure 5: Cellular localization of xCT in mouse cerebellum. Panel a (montage) shows one folium from a wildtype mouse cerebellum labeled with antibodies to xCT (red; Ab#618, 1 μg/mL) and to the C-terminus of EAAT1 (green; NCL-EAAT1, 1:200). The images are acquired in order to visualize the parenchymal xCT labeling implying that the stronger labeling related to the leptomeninges (indicated by arrows) and the
vasculature (v; indicated by arrow heads) is oversaturated. Note that xCT is expressed in white matter (wm) and, albeit with lower intensity, in the granule cell layer. No xCT labeling is detected in the molecular layer (ml), which is highly EAAT1 positive. Panel b shows a higher magnification image of double labeling for xCT (green; Ab#618, 1 μg/mL) and for the N-terminus of EAAT1 (red; Ab#286, 0.5 μg/mL). Note that the xCT labeling follows that of EAAT1 in white matter, suggesting that xCT is localized to fibrous astrocytes in the cerebellum. Similar to other brain regions, the xCT expression levels appear to vary greatly among astrocytes as xCT was not detected in all EAAT1-positive cells (e.g. arrows). Panel c: xCT (red; Ab#618, 1 μg/mL) labeling was not detected in cells positive for the oligodendrocyte marker CNPase (green; 1:200) in cerebellum white matter. The nuclei are stained with DAPI (b-c; blue). Scale bars, a: 200 μm; b, c: 20 μm.

Figure 6: xCT was not detected in Iba-1 (microglia marker) positive cells, neither in normal mouse brain nor in the glutamine synthetase-deficient mouse hippocampus. Panels a-b: Immunofluorescent double labeling for xCT (red; Ab#618, 1 μg/mL) and microglia marker Iba-1 (green; ch-anti-Iba1, 1:500) in young adult wildtype mouse brain. At lower magnification (Panel a) note that the distribution of xCT positive cells (red) is clearly different from the distribution of the Iba-1 labeled microglial cell population (green), as here shown for hippocampus CA1. Examination at higher resolution (Panel b; x63/1.40 oil immersion objective) reveals that where microglial cells are located within the same area as the xCT positive patches, xCT was not detected in Iba-1 positive structures, further indicating that the two proteins are localized in different cells. Panels c-j: Neurodegeneration and reactive microglia are observed in mice
lacking glutamine synthetase in the cerebral cortex (GS cKO: Zhou et al., 2017). As shown here with Nissl stained sections from a pair of littermates (15 weeks old), the pyramidal cells in the wildtype (WT) hippocampus CA1 (Panel c) have disappeared (arrow) in the GS cKO mice (Panel d). Immunofluorescence labeling for xCT (red; Ab#618, 1 μg/mL) in another pair of sections shows loss (Panel f, montage, GS cKO, 9 weeks) of the astrocyte domain-like xCT labeling pattern seen in brain parenchyma in wildtype mice (Panel e; montage, WT, 15 weeks). The labeling is, however, mostly preserved in the leptomeninges and along blood vessels. Immunofluorescent double labeling with marker proteins to macrophages (green; anti-F4/80, 1:500) and microglia (red; rb-anti-Iba-1, 1:500) in sections from the same 9 weeks old GS cKO mouse shows changed microglial morphology revealed by Iba-1 labeling and upregulation of F4/80 (Panel h) compared to the wildtype littermate (Panel g). Despite this strong microglial activation, no co-localization of xCT (red; Ab#618, 1 μg/mL) and Iba-1 (green; ch-anti-Iba1, 1:500) was observed (Panel j; hippocampus CA1). Thus, xCT was neither detected in resting microglia (Panels abi) nor in reactive microglia induced by glutamine synthetase deficiency (Panel j). DAPI (Panels a,b,e-j; blue) labels the nuclei.

Abbreviations: LMol, stratum lacunosum moleculare; O, stratum oriens; P, pyramidal cell layer; R, stratum radiatum; v, blood vessel. Scale bars, a: 100 μm; b: 5 μm; c-f: 100 μm; g-i: 20 μm; j: 10 μm.

**Figure 7:** Strong xCT labeling surrounding some large blood vessels. Panel a: A section from a Tie2-GFP transgenic mouse expressing green fluorescent protein in the endothelium was labeled with antibodies against xCT (red; Ab#618; 1 μg/mL). a1 shows
a montage of two microphotographs from the surface of neocortex with strongly labeled leptomeninges (arrows). An xCT-positive blood vessel is seen below the surface, but note that the smaller branch derived from this is xCT-negative (a1, arrow head). a2 and a3 are excerpts from a1, shown with higher magnification. Note that xCT (red) was not seen co-localized with the endothelium (green). Panel b: Double labeling for xCT (red; Ab#618, 1 μg/mL) and α-smooth-muscle-actin (green; anti-α-SMA, 1:500) revealed that xCT-expressing cells along the blood vessels are localized externally to the α-SMA expressing cells in the blood vessel wall, as shown here from thalamus. The parenchymal xCT labeling appears weak in this figure because the intensity and gain were set to avoid oversaturation of the labeling related to the leptomeninges and blood vessels. DAPI (b; blue) was used for nuclei staining. Scale bars, a1: 20 μm; a2, a3: 5 μm; b: 5 μm.

Figure 8: Strong xCT labeling in the subfornical organ, in area postrema and in parts of the lateral walls of the ventrocaudal third ventricle. Panels a-f show how xCT (red; Ab#618, 1 μg/mL) is distributed along a rostro-caudal axis related to the ventral part of the third ventricle. The xCT labeling in the ventricle lining were below detection limit in the rostral portion (a), but gradually appeared in more caudal portions (b, c, d and e). Panel f is an excerpt from Panel c. Tissue from xCT KO (-/-) did not show any xCT labeling related to the ventricular system when examined at corresponding Bregma levels (a representative image in Panel g). xCT could not be detected in ependymal cells lining the dorsal part of the ventral third ventricle (Panel h), the floor and lateral walls of the dorsal third ventricle (Panel i) or the lateral ventricles.
(Panel j, arrows) and was also not detected in ependymal cells of choroid plexus (Panel k: here shown for the lateral ventricle), but in a few, scattered non-ependymal cells of choroid plexus (arrows) probably associated with meningeal or vascular structures. Note that the medial habenular nucleus (i: MHb) is strongly labeled. Panels l-n: xCT (green; Ab#618, 1 μg/mL) was not found present in the ependymal lining of the aqueduct in midbrain (l), the fourth ventricle (m; here shown for the ventricle floor) or the proximal central canal (n). Panels o-r: The circumventricular organs area postrema (AP; O) and subfornical organ (Panel q; SFO) are highly positive for xCT (green; Ab#618, 1 μg/mL). No xCT labeling was detected in AP and SFO in sections from the xCT KO littermate (-/-; Panels p-r, respectively). Higher magnification (Panels s-t) revealed that xCT (green) is co-localized with EAAT1 (red; Ab#286, 0.5 μg/mL) also in these regions. The nuclei are labeled (blue) by DAPI. Abbreviations: 3V, third ventricle; 4V, fourth ventricle; AP, area postrema; Aq, aqueduct; Arc, arcuate hypothalamic nucleus; ArcMP, arcuate hypothalamic nucleus, medial posterior part; CC, central canal; CPu, caudate putamen; D3V, dorsal third ventricle; DM, dorsomedial hypothalamic nucleus; DTM, dorsal tuberomammillary nucleus; ME, median eminence; MHb, medial habenular nucleus; Pa, paraventricular hypothalamic nucleus; Pe, periventricular hypothalamic nucleus; PVP, paraventricular thalamic nucleus, posterior part; Sch, suprachiasmatic nucleus; SFO, subfornical organ; VMH, ventromedial hypothalamic nucleus. Where relevant, the distance from Bregma is given (in mm) in the upper right corner of each panel according to the mouse brain atlas (Paxinos and Franklin, 2001). Panels a, b, g, o and r are montages of two confocal microphotographs each. Scale bars, a-e: 100 μm; g-r: 50 μm; f, s, t: 10 μm.
**Figure 9**: Mouse hippocampus tissue contains similar amounts of xCT and EAAT3 protein. **Panel a**: A chimeric xCT-EAAT3 cDNA construct was made by adding the 45 nucleotides encoding the last 15 C-terminal amino acid residues (AA) of mouse EAAT3 (indicated in red) to the 3′-end of the mouse xCT sequence. The positions of the binding primers are underlined. Arrows point out the direction of primer extension. The part of the xCT protein sequence that corresponds to the synthetic peptide used to produce the Ab#618 xCT antibody is indicated in green. The chimeric xCT-EAAT3 cDNA construct was expressed in HEK293T cells, which were harvested in water, centrifuged to remove water-soluble proteins and solubilized in SDS (Chimera). Extracts from mock-transfected cells were produced as negative control (NC). **Panel b**: The above extracts were subjected to electrophoresis and immunoblotting together with extracts from the hippocampi of three different wild type mice (H1, H2 and H3; water soluble proteins removed). The amounts of protein loaded in each lane are indicated. The dye front (DF) and the lanes containing molecular marker proteins (M) are indicated. The blots were double labeled with antibodies to xCT (green; Ab#618, 0.5 µg/mL) and EAAT3 (red; Ab#340, 0.5 µg/mL) and scanned (LiCor Odessey infrared scanner) to obtain quantitative information. Both of the antibodies labeled the chimeric protein (yellow) and additionally labeled the different respective target proteins in the hippocampus extracts as they should. The mobilities of the labeled molecules in the latter extracts are as previously reported: about 35 kDa for xCT (Van Liefferinge et al., 2016) and close to 70 kDa for EAAT3 (Holmseth et al., 2012a). When using the chimeric protein to normalize the labeling intensities obtained with the two antibodies, the number of xCT protein
molecules were found to be similar to that of EAAT3 protein molecules: 0.75 ± 0.07
(average ± SEM; n= 4 immunoblots) times lower.
Figure 2
Figure 5
Hybrid protein sequence:

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Figure 9