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REVIEW ARTICLE

Regulation of xCT expression and system x_c^- function in neuronal cells

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Abstract The glutamate/cystine antiporter system x_c^- transports cystine into cells in exchange for glutamate at a ratio of 1:1. It is composed of a specific light chain, xCT, and a heavy chain, 4F2, linked by a disulfide bridge. Intracellularly, cystine is reduced into cysteine, the rate-limiting precursor of glutathione (GSH), an important small molecule antioxidant. Several lines of evidence suggest that the expression of xCT and thereby the presence system x_c^- activity plays an important role in the brain. First, it regulates extracellular glutamate concentrations. Second, as brain is prone to oxidative stress due to its high oxygen consumption and lipid content, system x_c^-, by favoring GSH synthesis, may prevent oxidative damage. Thus, to understand how xCT expression and system x_c^- activity are regulated in the central nervous system is of utmost importance. In this review, we will summarize the current knowledge about the molecular basis by which xCT expression and system x_c^- activity are regulated in neuronal cell lines, especially the hippocampal cell line, HT22. In addition, we will relate these pathways to findings in other cell types, especially those found in the central nervous system. We will focus on the signaling pathways that modulate the transcription of the xCT gene. Furthermore, we describe possible pathways that modify system x_c^- activity beyond the level of xCT transcription, including regulation on the level of membrane trafficking and substrate availability, especially the regulation by glutamate transport through excitatory amino acid transporters.

Keywords Cystine · Glutamate · xCT · Oxidative stress · Neurons

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Introduction

The cystine/glutamate antiporter system x_c^- is an obligate sodium-independent amino acid antiporter (Makowske and Christensen 1982), which mediates the uptake of the non-essential sulfur-containing amino acid cystine into cells in exchange with the excitatory amino acid L-glutamate (Glu) in a 1:1 ratio (Bannai 1986). The driving force for cystine import into cells via system x_c^- is the gradient of high-intracellular and low-extracellular Glu concentrations along which Glu is counter transported (Bannai and Kitamura 1980). The activity of system x_c^- depends on the presence of chloride (Patel et al. 2004) and can be inhibited by extracellular Glu, which is the only proven physiologically relevant inhibitor and alternative substrate identified so far (Bannai 1986; Patel et al. 2004). After uptake by system x_c^-, cystine is rapidly reduced to cysteine, which is the rate-limiting amino acid in the synthesis of GSH. GSH is the most important antioxidant in the brain (Dringen and Hirrlinger 2003). System x_c^- therefore has two roles, one in the control of extracellular Glu (Baker et al. 2002a) and one in the defense against oxidative stress (Tan et al. 2001).

System x_c^- consists of a specific light chain, SLC7A11 or xCT, which confers specificity, and a heavy chain,

4F2hc or CD98 (Sato et al. 2000). Replacing 4F2hc by the alternative heavy chain, rBAT, was reported to still lead to a fully functional transporter by some (Wang et al. 2003) but not by others (Bassi et al. 2001). The subunits are linked by a disulfide bridge. This structure is shared by other members of the heterodimeric amino acid transporters, LAT1, LAT2, y^+LAT1, y^+LAT, and $b^{0,+}$AT, which correspond to the functionally defined transporter systems L (LAT1 and LAT2), system y^+L (y^+LAT1, y^+LAT) and b0,+ ($b^{0,+}$AT) (Verrey et al. 2004).

In 1999, Sato and co-workers identified mouse xCT by functional expression cloning (Sato et al. 1999). Human xCT consists of 501 amino acids with 89% sequence identity compared to mouse xCT (Sato et al. 1999). A membrane topology was proposed that contains 12 transmembrane domains with the N- and C-termini located inside the cell and a re-entrant loop within intracellular loops 2 and 3 (Gasol et al. 2004). In this review, we will summarize the current knowledge of the pathways that regulate the expression of xCT, the specific subunit of system x_c^-, mainly in neuronal cells. In addition, we will describe possible pathways, which might modify system x_c^- activity beyond alterations in the levels of xCT expression including translation, membrane trafficking and finally regulation of system x_c^- activity by the concentration of substrate amino acids across the membrane. Conrad and Sato (2011) provide further details on system x_c^- cloning, regulation and functions, mainly on non-neural cells, that could also be relevant for glial cells (Had-Aissouni 2011).

xCT expression in brain cells

Northern blot analysis revealed that human xCT mRNA is most prominently expressed in the brain (Sato et al. 1999). xCT mRNA was detected at low levels in all mouse brain areas, while very high levels were demonstrated in meninges by Northern blotting and RT-PCR (Sato et al. 2002). However, non-radioactive in situ hybridization only detected xCT mRNA in very restricted areas of the brain parenchyma, the area postrema, the subfornical organ, the habenular nucleus, and the hypothalamus, all areas with significant contact to the cerebrospinal fluid, in addition to strong expression in ependymal cells of the lateral wall of the third ventricle and the meninges (Sato et al. 2002). Immunohistochemically, xCT was localized in both neurons and astrocytes in the mouse and human brain in addition to the border areas between the brain and the blood or cerebrospinal fluid including vascular endothelial cells, ependymal cells, choroid plexus, and leptomeninges (Burdo et al. 2006). In contrast, a different approach to localize system x_c^- at the cellular level in the brain using immunohistochemistry with an antibody against α-aminoadipate to detect its intracellular accumulation in acute rat brain slices after exposure to this substrate inhibitor of system x_c^- in vitro showed α-aminoadipate accumulation in astrocytes but not in neurons (Pow 2001). However, control experiments demonstrating the specificity of this method for system x_c^- were only performed in retina but not in brain slices (Pow 2001), a fact that limits the impact of this interesting work. Immunoblotting showed that xCT expression in brain and spinal cord increases during development, reaching the highest expression in adulthood (La Bella et al. 2007). Using an independent antiserum and immunoblotting, the presence of xCT protein was also demonstrated in the cortex, hippocampus, striatum, and to a lesser degree, in the cerebellum (Shih et al. 2006). This study replicated the increase of xCT protein expression in adult brain compared to embryonic brain at day 18 as well as strong expression of xCT protein in meninges (Shih et al. 2006). Functionally, robust system x_c^- activity was demonstrated in freshly dissociated brain cells from rat E17 embryos (Sagara et al. 1993). In contrast, system x_c^- activity was found to be almost absent in freshly prepared hepatocytes and macrophages (Takada and Bannai 1984; Watanabe and Bannai 1987). A sodium-independent, chloride-dependent Glu uptake activity, which resembles the pharmacological profile of system x_c^-, was reported in adult rat synaptosomes but not in peripheral tissues (Zaczek et al. 1987). Moreover, in adult rats system x_c^- activity was detected in striatal tissue as uptake of radiolabeled cystine sensitive to Glu and homocysteate in acute brain slices (Baker et al. 2002b) and using microdialysis in multiple brain areas (Baker et al. 2002b, 2003; Moran et al. 2005). The difference regarding the widespread detection of xCT protein (Burdo et al. 2006; La Bella et al. 2007; Shih et al. 2006) and the demonstration of system x_c^- activity (Baker et al. 2002b, 2003; Moran et al. 2005) in the brain parenchyma while xCT mRNA was not detected (Sato et al. 2002) might result from insufficient sensitivity of the non-radioactive technique used for in situ hybridization.

In cell culture, either primary astrocytes or neurons or neuronal or glial cell lines might even more rely on system x_c^- for GSH synthesis because the more pro-oxidant conditions of cell culture favor the oxidation of extracellular cysteine to cystine. Here, inhibition of system x_c^-, either by high concentrations of Glu or Glu analogs like homocysteate or quisqualate, induces GSH depletion, oxidative stress and finally cell death (Chen et al. 2000; Maher and Davis 1996; Murphy et al. 1989, 1990; Oka et al. 1993; Schubert and Piasecki 2001), a process called oxidative Glu toxicity or oxytosis (Albrecht et al. 2010; Tan et al. 2001).

Properties of the murine and human xCT mRNA

In mouse macrophages as well as in the murine neuronal cell line HT22, xCT mRNA is present in multiple variants, of which three of approximately 2.5, 3.5, and 12 kb predominate (Lewerenz et al. 2006; Sato et al. 1999), although two additional ones with sizes between 3.5 and 12 kb can be detected (Sato et al. 2001). The 12 kb variant was also detected in mouse (Sato et al. 1999) and rat cortex and astrocytes (Gochenauer and Robinson 2001). 5′ RACE was applied to identify the transcriptional start site (Sasaki et al. 2002). Using this start site, the length of the mouse 5′ UTR is 329 bp. The longest mouse xCT cDNA (NM_011990.2) published online is 9181 bp with a 7351 bp long 3′ UTR without the poly-A tail (http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=nucleotide&dopt=GenBank&RID=TU0CM91C016&log%24=nucltop&blast_rank=1&list_uids=80861466). Human xCT cDNA was cloned from a human cDNA library and the transcriptional start site identified in W126Va$_4$ cells (Sato et al. 2000), from the human retinal pigment epithelial cell line ARPRE-19 (Bridges et al. 2001) and the human teratoma cell line NT2 (Bassi et al. 2001). Putative transcripts of 1885 and 6568 bp (Sato et al. 2000), 2482 bp (Bridges et al. 2001), and 3144 bp (Bassi et al. 2001) were identified, which share a 231 bp 5′ UTR and a 1506 bp open reading frame (ORF) including the stop codon but divergent 3′ UTRs. The protein encoded by these cDNAs has 501 amino acids and shows 89% identity and 96% similarity with mouse xCT (Bridges et al. 2001; Sato et al. 2000). The reference cDNA (NM_014331.3) published online (http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=nucleotide&dopt=GenBank&RID=TU4E6EH2014&log%24=nucltop&blast_rank=1&list_uids=80861465) is 9648 bp with a 321 bp 5′ UTR and 7962 bp 3′ UTR.

In human fibroblasts, using the probes containing either the ORF or the several kb long 3′ UTR as probes in combination with Northern blotting a 12 kb mRNA was detected (Sato et al. 2000). The 12 kb mRNA was also shown to predominate in human brain, which is the tissue with the highest xCT expression among the organs studied (Bassi et al. 2001). In contrast, Kim et al. (2001) reported an 8.5 kb mRNA expressed in human spinal cord, brain, and pancreas and both a 8.5 and 2.8 kb mRNA expressed in human U87 glioma cells using a probe containing the ORF. This group also identified a splice variant (hxCTb), which encodes a 495 amino acid protein in which the 20 C-terminal amino acids of the 501 amino acid long variant (hxCTa) are replaced by 13 divergent amino acids (Kim et al. 2001). Using RT-PCR, Kim et al. (2001) showed that the mRNA encoding the new splice variant is expressed at high levels in U87 glioma cells while the variant identical with the mouse homolog predominates in brain, spinal cord, and pancreatic tissue. Negligible expression of hxCTb compared to hxCTa was reported in an independent human glioma cell line, SNB-19 (Patel et al. 2004). In oocytes, cRNA of both human xCT variants induce system x_c^- activity when co-injected with 4F2hc cRNA (Bassi et al. 2001; Kim et al. 2001; Sato et al. 2000). Until present, the precise molecular identity of the 12 kb variant that predominates in murine and human tissues and cell lines in most studies is unknown. Most likely a variant with an even longer 3′ UTR exists as shown for the 9.2 kb variant published online.

Transcriptional regulation of xCT expression

The cloning of the mouse and human xCT cDNA allowed the localization and analysis of the murine and human xCT gene including the 5′ flanking region. The 5′ flanking region of the mouse and human xCT gene contains multiple putative AP-1 binding sites (Sato et al. 2000, 2001). A putative binding site for NFκB was identified in the murine xCT gene 5′ flanking region which might be responsible for the known induction of xCT mRNA and system x_c^- activity by lipopolysaccharides in macrophages (Sato et al. 2001) although direct evidence that NFκB binds to the xCT promoter and thereby activates transcription of the xCT gene has not been presented. Recently, NFκB has been reported to mediate the upregulation of EAAT2 and thereby the neuroprotective properties of the β-lactam antibiotic ceftriaxone in human astrocytes (Lee et al. 2008). We reported that ceftriaxone upregulates xCT expression in multiple cell types including astrocytes and stem cell-derived motor neurons. However, induction of xCT expression by ceftriaxone was most probably mediated by Nrf2 (Lewerenz et al. 2009). The proximal 5′ flanking region of the mouse xCT gene contains four putative antioxidant response elements, of which the most 5′ mediates induction of xCT promoter activity by the transcription factor Nrf2 and oxidative stress (Sasaki et al. 2002) and is fully conserved in the human xCT 5′ flanking region (Sato et al. 2000). Upregulation of Nrf2 protein levels by treatment with the Nrf2 inducer *tert*-butylhydroquinone (*t*BHQ) strongly increases xCT protein and system x_c^- activity in the hippocampal cell line HT22 (Fig. 1a, b). Similar findings were observed in rat astrocytes cultures, where retroviral over-expression of Nrf2 led to a robust induction of xCT protein levels detected by Western blotting (Shih et al. 2006).

In addition, a tandem of amino acid response elements (AAREs) has been identified in the mouse xCT gene, the more 5′ of which binds the transcription factor ATF4 and both AAREs cooperatively mediate the activation of the xCT promoter by amino acid starvation (Sato et al. 2004).

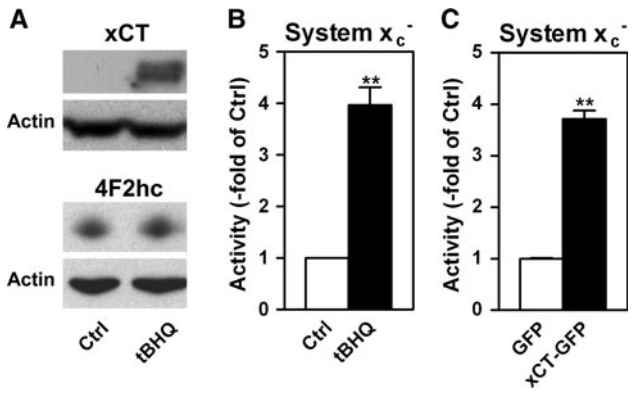

Fig. 1 In the hippocampal cell line, HT22, system x_c^- activity is regulated by xCT expression (**a**) Western blots of HT22 cells treated with 20 μM *t*BHQ for 24 h. Membrane proteins and Western blots were performed as previously described (Lewerenz and Maher 2009). The xCT antibody was a kind gift from Dr. Sylvia Smith, Medical College of Georgia, Augusta, GA, the 4F2hc antibody was used as described previously (Burdo et al. 2006). Actin served as a loading control. **b** HT22 cells were treated similarly as in (**a**) and system x_c^- activity was measured as described previously as glutamate-sensitive uptake of 35-S cystine and normalized to cellular protein (Lewerenz and Maher 2009). **c** As a control experiment, HT22 cells were transfected with a vector expressing GFP or a xCT construct with a c-terminal GFP-tag (xCT-GFP), which was a kind gift from Dr. Andy Y. Shih, University of San Diego, La Jolla, CA as described previously (Lewerenz and Maher 2009). System x_c^- function was measured as in (**a**). (**a**) and (**c**) were at least reproduced once (**b**) represents four independent experiments, $^{**}P < 0.01$, one sample *t* test against 1 (**b**) or two-tailed *t* test (**c**)

The two AAREs oriented in opposite direction are completely conserved among mouse, rat, bovine, and human xCT 5′ flanking regions (Lewerenz and Maher 2009). We recently showed that phosphorylation of translation initiation factors eIF2α at serine 51 is a critical regulator of ATF4 protein levels in HT22 cells as well as mouse embryonic fibroblasts and the rat pheochromocytoma cell line PC12 (Lewerenz and Maher 2009). ATF4 in turn upregulates the transcription of the xCT mRNA, xCT protein levels and system x_c^- activity (Lewerenz and Maher 2009). Interestingly, PC12 cells selected for resistance against oxidative damage by the amyloid-β peptide, which is involved in the pathogenesis of Alzheimer's disease (AD), exhibit a strong activation of the phospho-eIF2α/ATF4/xCT signaling module (Lewerenz and Maher 2009). Moreover, we showed that phospho-eIF2α and ATF4 are also upregulated in AD brains (Lewerenz and Maher 2009). Thus, this pathway might represent an adaptive response to oxidative stress in AD.

Post-transcriptional regulation of xCT expression

Amino acid limitation induces the phosphorylation of the translation initiation factor eIF2α. Phosphorylated eIF2α inhibits cap-dependent translation. Interestingly, some proteins that are preferentially induced by amino acid limitation contain short upstream ORFs (uORFs) and/or internal ribosomal entry sites (IRES) in the 5′ leader sequence of the mRNA some of which overlap with the ORF of the protein induced by amino acid limitation (Wek et al. 2006), e.g., the transcription factor ATF4, which contains two uORFs in the mRNA region 5′ of the ATF4 ORF (Vattem and Wek 2004). Similar, but not identical mechanisms have been described in other proteins induced by ATF4 and amino acid limitation. These include the ATF4-induced nuclear factor CHOP (Chen et al. 2010), the eIF2α phosphatase GADD34 (Lee et al. 2009), the arginine/lysine transporter cat-1 (Fernandez et al. 2002), and the neutral amino acid transporter SNAT2 (Gaccioli et al. 2006). When the transcriptional start site identified by Sasaki et al. (2002) is applied, the mouse xCT mRNA has a 324 bp 5′ leader sequence. System x_c^- activity is robustly induced by amino acid starvation (Sato et al. 2004). Moreover, induction of eIF2α phosphorylation by treatment with the eIF2α phosphatase inhibitor, salubrinal, which reduces cap-dependent translation in favor of cap-independent mechanisms and robustly induces ATF4 protein, increases xCT protein expression and system x_c^- activity in PC12 cells (Lewerenz and Maher 2009). The 5′ UTR of the mouse xCT mRNA contains an upstream uORF starting with an ATG from 13 to 108 bp, encoding a 36 amino acid long hypothetical peptide. Whether this uORF ensures efficient protein translation under conditions of impaired cap-dependent translation remains to be elucidated. However, when the start site identified by Sato et al. (2000) is applied, uORFs are absent in the human xCT mRNA 5′ UTR (unpublished results).

In the cat-1 mRNA, the 3′ UTR participates in mRNA stabilization during nutritional stress (Yaman et al. 2002). Whether the different long 3′ UTRs of the xCT mRNA are involved in mRNA stabilization and thereby the regulation of xCT protein expression is not known.

Regulation of system x_c^- activity by protein modification of xCT

The 4F2hc heavy chain of the transporter complex is glycosylated. Mouse xCT lacks a potential site for *N*-linked glycosylation, whereas the human xCT protein shows a putative *N*-linked glycosylation site at Asn-314 (Bridges et al. 2001). In human xCT, a potential cAMP-dependent phosphorylation site at Thr-45 and a potential protein kinase C (PKC)-dependent phosphorylation sites at Thr-103 were predicted and both have an intracellular localization (Kim et al. 2001). Of note, findings by Tang and Kalivas (2003) in rat cortical astrocytes suggest that

activation of protein kinase A (PKA) and PKC inhibits system x_c^- activity. However, the fact that the authors did not present the incubation times they used weakens the significance of their findings as long-term activation of PKA was reported to be associated with the induction of system x_c^- in astrocytes and in HT22 cells rather than with inhibition (Gochenauer and Robinson 2001; Lewerenz et al. 2003). Moreover, phosphorylation of the xCT protein itself remains to be demonstrated.

Regulation of system x_c^- activity by protein trafficking, interaction with other proteins and cell surface expression

A heterodimer consisting of the xCT protein and the 4F2hc subunit represent the functional unit of system x_c^-. Cysteine-158 in mouse and human xCT represents the conserved cysteine involved in the formation of the disulfide bond with the heavy chain characteristic for heterodimeric amino acid transporters (Bassi et al. 2001; Sato et al. 1999). Transfection studies in oocytes showed that the 4F2hc subunit is required both for the transport of xCT to the cell surface and system x_c^- activity (Bassi et al. 2001; Sato et al. 1999). However, 4F2hc is known to form heterodimers with at least five other specific amino acid transporter subunits (Verrey et al. 2004). Thus, as xCT might compete with the other transporter subunits for interaction with 4F2hc, the expression of 4F2hc might theoretically regulate the expression of system x_c^- at the cell surface. However, in NIH3T3 cells transfected with xCT cDNA alone, the activity of system x_c^- was significantly increased (Wang et al. 2003). In HEK293 cells, transient over-expression of xCT substantially increased system x_c^- function more than 10-fold, whereas simultaneous over-expression of 4F2hc reduced system x_c^- by $\sim$40% (Shih and Murphy 2001). In the retinal pigment epithelial cell line, ARPE-19, the NO-induced increase in system x_c^- activity was associated with an increase in xCT but not 4F2hc mRNA (Bridges et al. 2001). In neuronal cells, we found a tight correlation of xCT mRNA and system x_c^- activity when comparing wild-type hippocampal HT22 cells with HT22 cells selected for Glu resistance with both xCT mRNA and system x_c^- activity being induced robustly in the Glu-resistant cells (Lewerenz et al. 2006). Moreover, treatment of HT22 cells with the Nrf2-activating compound *t*BHQ, which induces ARE-mediated xCT mRNA expression (Sasaki et al. 2002), is associated with a robust induction of xCT protein and system x_c^- activity in HT22 cells, while 4F2hc remains unaffected (Fig. 1a, b). Transient over-expression of xCT in HT22 cells robustly induces system x_c^- activity (Fig. 1c). In rat astrocytes, overexpression of xCT by adenoviral vectors also effectively induced system x_c^- activity more than 5-fold (Shih et al. 2006).

Together, these observations favor the view that xCT expression is the main determinant for system x_c^- activity in cells of central nervous system origin as well as other cell types studied. Thus, we conclude that 4F2hc is expressed in excess in these cell types. However, 4F2hc expression is highly regulated in some cells, part by microRNAs targeting the 3′ UTR of the 4F2hc mRNA (Nguyen et al. 2010). Thus, competition for association with 4F2hc among different amino acid transporter light chains might exist in other cell types which have not been examined.

The observation that xCT mRNA levels correlate well with system x_c^- activity speaks against the hypothesis that post-transcriptional mechanisms play an important role in regulating xCT protein expression and system x_c^- activity in neuronal cells. However, this conclusion is only valid if xCT mRNA levels actually reflect xCT gene transcription rather than xCT mRNA stability. At least in hamster BHK21 cells and mouse NIH3T3 fibroblasts this appears to be the case as the induction of system x_c^- activity by cystine starvation or diethyl maleate treatment, respectively, was reflected in increased activity of the xCT promoter (Sasaki et al. 2002; Sato et al. 2004). However, the relative level of cell surface localization of xCT protein was reported to be decreased by increased cell culture density and increased by treatment with oxidants in neuroblastoma cells (Chase et al. 2009), indicating regulation of system x_c^- activity by membrane trafficking at least in some cells.

Regulation of system x_c^- activity by substrate availability and endogenous inhibitors

For cystine uptake, system x_c^- is an obligate exchanger of cystine and Glu in a 1:1 ratio and depletion of intracellular Glu inhibits cystine uptake (Bannai 1986). However, under experimental conditions where intracellular reduction of cystine to cysteine is inhibited, system x_c^- can also mediate Glu uptake coupled to cystine release (Bannai 1986). These findings demonstrate that a bi-directional transport of both amino acids is possible. Moreover, the fact that high-intracellular concentrations of Glu stimulate both cystine and Glu uptake via system x_c^- (Bannai 1986) suggests that system x_c^- can also act as a Glu/Glu exchanger. Due to the efficient reduction of imported cystine to cysteine, the intracellular cystine concentration is very low. Thus, it is very likely that when system x_c^- is measured in vitro using uptake of radiolabeled Glu (Gochenauer and Robinson 2001; Lewerenz et al. 2010), Glu/Glu exchange is the primary mechanism being looked at. As the Glu

concentration of approximately 2–3 μM is considerably higher than the cystine concentration in the brain, which has been reported to be as low as 200 nM (Baker et al. 2003) Glu/Glu exchange might predominate in vivo. However, other molecules might be transported in vivo by system x_c^- and thereby modify cystine uptake. These include the system x_c^- inhibitor α-aminoadipate, which is present in the brain as a metabolite of lysine metabolism (Chang 1978, 1982).

As the extra- and intracellular concentrations of Glu are critical for cystine uptake via system x_c^-, pathways that modify the distribution of this amino acid might be important regulators of system x_c^- activity. In the brain, the most important systems that modulate Glu uptake are sodium-dependent excitatory amino acid transporters (EAATs). Astrocytic membranes in the vicinity of synapses express a high density of EAAT, especially GLT-1 (EAAT2, SLC1A2) and GLAST (EAAT1, SLC1A3), which remove Glu released into the synaptic cleft during action potentials within milliseconds (Rothstein et al. 1996). After uptake into astrocytes, Glu is converted to glutamine by glutamine synthetase and then transported back to neurons, where it is deaminated to Glu by glutaminase (Broer and Brookes 2001). EAAC1 (EAAT3, SLC1A1) is the major neuronal Glu transporter and is mainly expressed in the extrasynaptic membranes of neurons (Rothstein et al. 1994). The mean cerebral Glu concentration in brain lysates is 10 mM (Kvamme et al. 1985). However, intracellular Glu concentrations in astrocytes and non-glutamatergic neurons have been reported to be 4- to 5-fold lower than in glutamatergic nerve terminals (Ottersen et al. 1992). Thus, EAATs may have an important role in the regulation of system x_c^- activity by decreasing extracellular Glu and increasing intracellular Glu, preferentially in glial cells and non-glutamatergic neurons in the brain where intracellular Glu concentrations tend to be relatively low. Indeed, a similar role has been assigned to EAATs in macrophages where Glu taken up by EAATs has been shown to increase intracellular GSH by transactivating cystine uptake via system x_c^- (Rimaniol et al. 2001). Analysis of Glu release by system x_c^- by glioma cells showed that a high percentage of the Glu released is instantly taken up again by a sodium-dependent mechanism (Patel et al. 2004), possibly EAATs. To test the hypothesis that EAAT support system x_c^- acting by redistributing Glu, we used the paradigm of oxidative Glu toxicity in the HT22 hippocampal cell line, which expresses both EAAT1-3 and system x_c^- (Lewerenz et al. 2006). We showed that over-expression of xCT and EAAT3 cooperatively protected HT22 against oxidative Glu toxicity by increasing intracellular GSH. Moreover, pharmacological inhibition of EAATs exacerbated oxidative Glu toxicity by decreasing intracellular Glu and GSH (Lewerenz et al. 2006). These data supported our hypothesis that EAATs are neuroprotective by supporting system x_c^- function. To extend this idea, we analyzed the effect of the β-lactam antibiotic ceftriaxone, which was identified as an EAAT-inducing compound (Rothstein et al. 2005), on oxidative Glu toxicity in the HT22 cells (Lewerenz et al. 2009). Ceftriaxone was previously shown to be protective in experimental models of amyotrophic lateral sclerosis, ischemic stroke, HIV-dementia, Huntington's disease, and multiple sclerosis (Lipski et al. 2007; Melzer et al. 2008; Miller et al. 2008; Rothstein et al. 2005; Rumbaugh et al. 2007), all diseases in which oxidative stress is thought to play a role (Browne and Beal 2006; Ilieva et al. 2007; Steiner et al. 2006; van Horssen et al. 2008; Wong and Crack 2008). The protective effect of ceftriaxsone was mainly attributed to the upregulation of EAAT2 and the subsequent reduction of excitotoxic neuronal cell death due to a reduction of extracellular Glu. However, we showed that system x_c^- plays a major role in ceftriaxone-mediated protection from oxidative stress. Ceftriaxone increased system x_c^- activity and GSH levels independently of its effect on EAATs by induction of the transcription factor Nrf2, a known inducer of the specific x_c^- subunit, xCT. Similarly, ceftriaxone-stimulated changes in Nrf2, system x_c^- activity and GSH were observed in rat cortical and spinal astrocytes, and ceftriaxone-induced xCT mRNA expression in stem cell-derived human motor neurons (Lewerenz et al. 2009). We think that dual activators of xCT and EAATs might be especially powerful tools against neurodegeneration as EAATs can neutralize the potentially detrimental Glu release by system x_c^-

How the concentration of the other substrate of system x_c^-, cystine, is regulated in the brain is not known. The extracellular cystine concentration in the brain is very low, about 200 nM. There are several possible explanations for this. First, GSH release from astrocytes and a subsequent disulfide exchange reaction with cystine generating cysteine-GSH disulfide and cysteine. Second, the ectoenzyme γ-glutamyltransferase can deplete cystine by transferring the γ-glutamyl residue of GSH to cystine thereby generating γ-glutamylcystine. Furthermore, the source of extracellular cystine in the brain is not known. Presumably, it is generated by the oxidation of cysteine released from cells or it is generated from released GSH or it could be imported from plasma (see Aoyama et al. 2011 and references therein) or synthesized from methionine (see McBean 2011 and references therein). In addition to its concentration, another mechanism regulates cystine availability for uptake by system x_c^-. The pK values of the two NH_3^+ groups of cystine are 7.48 and 9.02, respectively (Meister 1963). This means that the amino group with the

lower p*K* is increasingly protonated with decreasing pH within the range that occurs in physiological and pathophysiological states of the brain. It was shown that the transport of cystine via system x_c^- is inhibited by extracellular acidosis of ~pH 6.5 by ~60% whereas Glu transport via system x_c^- is affected much less when analyzed in human fibroblasts (Bannai and Kitamura 1981) or when human xCT and 4F2hc were over-expressed in oocytes (Bassi et al. 2001). We obtained similar findings in the hippocampal cell line HT22 (Lewerenz et al. 2010). These observations are compatible with the hypothesis that only the anionic form of cystine is transported by system x_c^- and thus modulation of extracellular pH regulates the substrate availability for system x_c^- by protonation of cystine.

Acidosis in the brain, e.g., due to cerebral ischemia, is often associated with substantial accumulation of lactate (Folbergrova et al. 1995; Wagner et al. 1992). One report demonstrated the inhibition of system x_c^- by lactate in rat cortical astrocytes (Koyama et al. 2000). However, in the hippocampal cell line HT22, system x_c^- is resistant to extracellular lactate in concentrations up to 20 mM (Lewerenz et al. 2010). The reason for this discrepancy is not known.

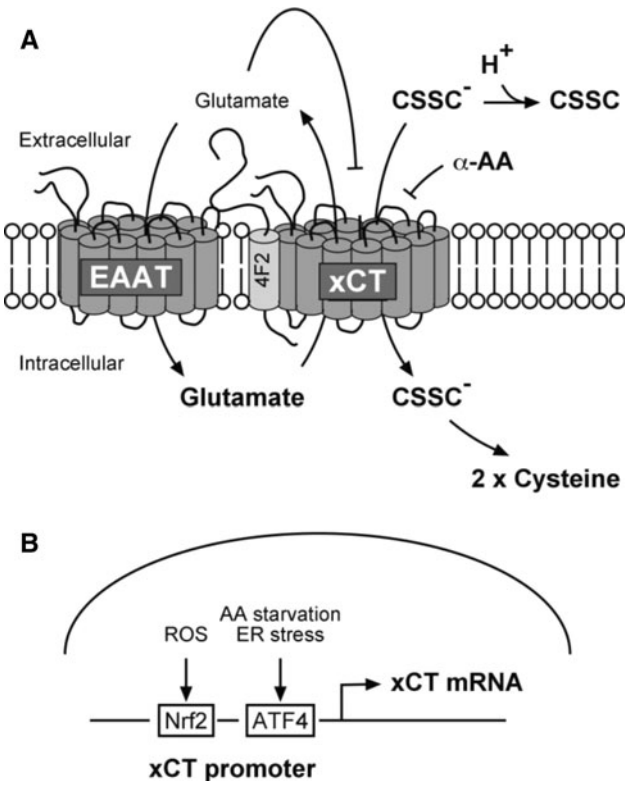

Fig. 2 Scheme of the major pathways of modulation system x_c^- activity (**a**) and the regulation of xCT expression (**b**) in neuronal cells. *AA* amino acids, *α-AA* α-aminoadipate, *CSSC* cystine, *CSSC*$^-$ cystine anion, *Cys* cysteine, and *ROS* reactive oxygen species

Summary

In neuronal and glial cells, the activity of the cystine/Glu antiporter system x_c^- is regulated on multiple levels (Fig. 2a, b). The transcription of the specific subunit of system x_c^-, xCT, is regulated by the transcription factors Nrf2 and ATF4, leading to the induction of xCT by oxidative stress and amino acid starvation. The heavy subunit, 4F2hc, seems to be expressed in excess and thus does not regulate system x_c^- activity in cells. Whether the modulation of mRNA stability or translation contributes to the regulation of xCT expression and thereby the activity of system x_c^- in neuronal and glial cells, remains to be explored. There is preliminary evidence that membrane trafficking regulates surface expression of the system x_c^- antiporter at least in some cell types. How much cystine is imported by system x_c^- is not only dependent on the extracellular cystine concentration, but also on the extra- and intracellular concentration of the counter-transported substrate and substrate inhibitor Glu. Therefore, pathways and transporters such as EAATs that regulate the extra- and intracellular Glu concentration are important modulators of system x_c^- activity in neuronal and glial cells. The role of other small molecules present in the brain such as lactate or α-aminoadipate in modulating system x_c^- activity remains controversial or to be explored, respectively.

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