Interleukin 1β Regulation of the System x_c^- Substrate-specific Subunit, xCT, in Primary Mouse Astrocytes Involves the RNA-binding Protein HuR*

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System x_c^- is a heteromeric amino acid cystine/glutamate antiporter that is constitutively expressed by cells of the CNS, where it functions in the maintenance of intracellular glutathione and extracellular glutamate levels. We recently determined that the cytokine, IL-1β, increases the activity of system x_c^- in CNS astrocytes secondary to an up-regulation of its substrate-specific light chain, xCT, and that this occurs, in part, at the level of transcription. However, an in silico analysis of the murine xCT 3′-UTR identified numerous copies of adenine- and uridine-rich elements, raising the possibility that undefined trans-acting factors governing mRNA stability and translation may also contribute to xCT expression. Here we show that IL-1β increases the level of mRNA encoding xCT in primary cultures of astrocytes isolated from mouse cortex in association with an increase in xCT mRNA half-life. Additionally, IL-1β induces HuR translocation from the nucleus to the cytoplasm. RNA immunoprecipitation analysis reveals that HuR binds directly to the 3′-UTR of xCT in an IL-1β-dependent manner. Knockdown of endogenous HuR protein abrogates the IL-1β-mediated increase in xCT mRNA half-life, whereas overexpression of HuR in unstimulated primary mouse astrocytes doubles the half-life of constitutive xCT mRNA. This latter effect is accompanied by an increase in xCT protein levels, as well as a functional increase in system x_c^- activity. Altogether, these data support a critical role for HuR in mediating the IL-1β-induced stabilization of astrocyte xCT mRNA.

System x_c^- (Sx_c^-)^2 is a heteromeric plasma membrane amino acid transporter consisting of two subunits: a lower molecular weight light chain and a higher molecular weight heavy chain, referred to as xCT and 4F2hc, respectively (1, 2). xCT is encoded by the Scl7a11 gene and confers substrate specificity, whereas 4F2hc is common to several amino acid transporters and allows plasma membrane expression of the functional Sx_c^- (3–6). Sx_c^- exports glutamate to the extracellular space and concomitantly imports cystine into the cytosol of cells, where it is rapidly reduced to cysteine to be incorporated into various catabolic processes, including protein synthesis and synthesis of the antioxidant GSH (7, 8). Apart from this crucial role in production of GSH (8–13), overwhelming evidence suggests that Sx_c^- regulates the ambient extracellular glutamate levels in the CNS (i.e. calcium-independent, tetrodotoxin-insensitive nonvesicular release) (14–22). At the cellular level, this serves an important physiological role in controlling synaptic strength in glutamatergic and dopaminergic neurotransmission (14, 16, 19, 20, 23). It also influences courtship behavior in drosophila (24) and drug seeking, anxiety, and depression-like behaviors in rodents (15, 23, 25, 26). The cellular source of this glutamate is likely the astrocyte (14, 19, 24). Thus, although astrocytes are known to play a key role in removal of glutamate from the extracellular space at glutamatergic synapses (27), they are capable of releasing glutamate as well (for review see Ref. 28).

Recently, our laboratory determined that IL-1β facilitates neuronal injury via a glutamate-mediated mechanism in the setting of energy deprivation (i.e. removal of oxygen or glucose) via enhancement of Sx_c^- expression and function in CNS astrocytes (29–31). Conversely, we demonstrated that IL-1β could also protect astrocytes against oxidant-induced injury via up-regulation of GSH synthesis (32). Thus, the ability of IL-1β via regulation of astrocyte Sx_c^- to promote, halt, or repair injury appears to depend on the context of the particular insult. For these reasons, understanding the molecular regulation of Sx_c^- by IL-1β in astrocytes is necessary so that we may use this information to devise strategies to harness the beneficial effects while mitigating the probability of excitotoxic injury under various pathological conditions.

There is ample evidence to suggest that Sx_c^- activity is dynamically regulated. In this regard, the 5′-flanking region of the murine xCT gene contains multiple putative transcription factor binding sites that contribute to the stimulus and/or cell type-dependent regulation of xCT gene transcription (3, 7, 13, 33–36). In addition to transcriptional regulation, the mRNA of xCT contains a long 3′-UTR, ~7366 nucleotides, raising the possibility for post-transcriptional regulation as well.

Consistent with this notion, we identified numerous adenosine- and uridine-rich elements in the murine xCT 3′-UTR, which are known to bind trans-acting factors that influence gene expression at the post-transcriptional level (37). Several trans-acting ELAV-like proteins are expressed in the CNS, including HuR (HuA), Hel-N1 (HuB), HuC, and HuD. HuR is ubiquitously expressed, whereas HuB, HuC, and HuD are neuron-specific (38, 39). Given its known ability to regulate genes induced by IL-1β (40–42), we hypothesized that HuR was an

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2 The abbreviations used are: Sx_c^-, system x_c^-; HuR, human antigen R; qPCR, quantitative PCR; MOI, multiplicity of infection; ActD, actinomycin D.

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Experimental Procedures

Experimental Media—Cell culture medium compositions were as follows: media stock containing l-glutamine-free modified Eagle’s medium (Earl’s salt; Corning, Manassas, VA) supplemented with l-glutamine, glucose, and sodium bicarbonate to final concentrations of 2.0, 25.7, and 28.2 mM, respectively; glial plating medium was media stock containing 10% heat-inactivated FBS (HyClone, Logan, UT) and 10% heat-inactivated calf serum (HyClone), 10 ng/ml epidermal growth factor (Invitrogen), and 50 IU penicillin/50 μg/ml streptomycin (Gibco); and maintenance medium was media stock containing 10% calf serum and 50 IU penicillin/50 μg/ml streptomycin.

Primary Cell Culture—Primary astrocytes were cultured from the pooled cortices of day 1–3 postnatal CD1 (Charles River Laboratories), C3H/HeSnJ (JAX, Bar Harbor, ME; stock no. 000661) or xCT-deficient mice (43) (sut/sut; JAX; stock no. 001310) mouse pups as described (31, 44). Dissociated cells were plated in multwell plates (Falcon Primaria; BD Biosciences) or tissue culture dishes (BD Biosciences) in glial plating medium (see above). Once cells achieved confluence and hence were contact-inhibited, 8 μM β-d-arabinofuranoside (Sigma) was added once for 6 days to reduce the number of microglia. Cells were then placed in maintenance media (see above), which was changed once per week until experimentation. In most cases, cultures highly enriched for astrocytes were generated by removing residual microglia by treatment with 50 mM l-leucine methyl ester for 30 min, 1 day prior to experimentation (45). Experiments were performed on cultures 21–35 days in vitro.

RNA Immunoprecipitation—Astrocytes were treated with recombinant murine IL-1β (3–5 ng/ml; R&D Systems, Minneapolis, MN) in an incubation/vehicle buffer of media stock (see above) containing 0.1% fatty-acid free BSA (Sigma) for 4 h. The medium was aspirated, 6 ml of ice-cold PBS was added, and the cells irradiated to 150 ml/cm² (Stratagene; UV Stratalinker 1800) (46, 47). Cells harvested by scraping were collected and spun (600 × g, 5 min, 4 °C). The resulting pellet was resuspended in 2 ml of polysyme lysis buffer containing 10 mM HEPES (pH 7.0), 100 mM KCl, 5 mM MgCl₂, 0.5% Nonidet P-40, and 1 mM DTT with 1× Complete Protease Inhibitor (Roche), 20 units of RNase inhibitor (New England Biolabs, Inc.), and 400 μM vanadyl ribonucleoside complex (New England Biolabs, Inc.). RNA in the immunoprecipitate was isolated with TRIzol LS (Life Technologies), and following first strand cDNA synthesis, RT-PCR was used to detect the presence of xCT (target sample), COX-2 (positive control), and β-actin (positive control) cDNA.

Reverse Transcriptase-Polymerase Chain Reaction—Total RNA was isolated, and first strand cDNA was synthesized as previously described (48). cDNA samples (1 μl) were amplified for 40 cycles (94 °C for 45 s, 52 °C for 30 s, and 72 °C for 60 s) using Taq DNA polymerase (Invitrogen) and target-specific primers in a total volume of 25 μl. Amplifiers for analysis of xCT mRNA were 5′-CATCTGCCCAGGATTGAGAT-3′ (forward) and 5′-CTGAGGTCCTGCTACTGTG-3′ (reverse). PCR amplifiers for assessment of COX-2 mRNA were 5′-CCGTTTCTCGTGTACTTCT-3′ (forward) and 5′-CTCTGGAGGTTTCTCG-3′ (reverse). Amplifiers for β actin were 5′-GTGCGCGGTAGGCAATGCA-3′ (forward) and 5′-TTTGGATGCACGGACTT-3′ (reverse). COX-2 and β-actin mRNAs were assessed as positive controls for HuR immunoprecipitation in vehicle and IL-1β-treated cells because both are targets of HuR (49, 50). PCR products, separated in either a 1% (β-actin) or 2% agarose (xCT and COX-2) gel containing ethidium bromide (200 μg/ml), were visualized with the LI-COR Odyssey Fc infrared imaging system. RT-qPCR was performed using mouse-specific primer pairs (TaqMan Gene Expression Assays, Applied Biosystems) for system xCT light chain, xCT (Mm00442530_m1), and β-actin (Mm01205647_g1). The reactions were run in the Eppendorf Realplex² real time PCR system. System and relative quantification performed using the comparative cycle threshold method (ΔΔCT), where Ct values of xCT were normalized to β-actin Ct values from the same sample and then compared with a calibrator (i.e. control) Ct value to determine the relative fold change in mRNA levels. β-Actin Ct values were unaffected by IL-1β treatment.

Adenovirus Infection—Adenoviruses expressing either green fluorescent protein (AdGFP) or HuR (AdHuR) (a generous gift from Dr. Myriam Gorospe, NIH, Bethesda, MD) were amplified and titered in HEK 293A cells using procedures essentially as described (51). Infection of astrocyte cultures was carried out for 16 h at MOI 5, which according to the GFP signal, resulted in nearly 100% infection efficiency. Adenovirus expressing shRNA against HuR or scrambled shRNA, both also containing GFP, were purchased from Vigenese Biosciences Inc. (Rockville, MD). Infection of astrocytes cultures was carried out for 16 h at MOI 5. Depending on the subsequent experimental protocol, the medium was then aspirated and replaced with either maintenance media or media stock.

mRNA Stability—To determine whether IL-1β altered xCT mRNA stability, astrocytes were treated with vehicle or recombinant murine IL-1β (5 ng/ml; R&D Systems) for 4 h, after which the medium was exchanged, and actinomycin D (ActD; 10 μg/ml) was added to halt transcription. To determine whether HuR overexpression could stabilize xCT mRNA,
astrocyte cultures were infected with AdGFP or AdHuR (see above). Forty hours after infection (16 h of infection + 24 h of recovery), ActD (10 μg/ml) was added to the cell culture medium. To determine whether HuR regulates the enhanced xCT mRNA stability that follows IL-1β exposure, astrocyte cultures were infected with Adscramble-shRNA (control) or AdHuR-shRNA (knockdown). Forty hours after following infection (16 h of infection + 24 h of recovery), the cells were treated with recombinant murine IL-1β (5 ng/ml; R&D Systems) for 4 h, after which the medium was exchanged, and ActD (10 μg/ml) was added to halt transcription. At various times following the addition of ActD, RNA was isolated, and xCT mRNA levels were determined using RT-qPCR (see above). To calculate mRNA half-life, curves were analyzed using one-phase exponential decay using GraphPad Prism (version 6.0.2; GraphPad Software, Inc.).

**Immunoblotting**—For cytosol fractionation, astrocytes cultured in 100-mm dishes were treated with IL-1β or vehicle for various times. The cells were harvested by gentle scraping and spun (600 × g for 5 min at 4 °C). The resulting pellets were washed once with cold PBS and resuspended by gentle trituration in a cytosol extraction buffer consisting of 10 mM HEPES (pH 7.4), 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.075% Nonidet P-40. After 3 min of incubation on ice, tubes were spun (210 × g for 5 min at 4 °C), and the supernatants were transferred to new tubes. These were spun again (16,500 × g for 10 min at 4 °C), and the resulting supernatants were collected as the cytosol fraction samples. For nuclear fractionation, the pellet resulting from the 210 × g spin above was washed once with cytosol extraction buffer without Nonidet P-40 and respun (210 × g for 5 min at 4 °C). These pellets were suspended in a nuclear extraction buffer (1× Complete protease inhibitor, 20 mM Tris HCl, pH 8.0, 420 mM NaCl, 1.5 mM MgCl₂, 25% glycerol, 0.2 mM EDTA, and 0.1% Nonidet P-40) and incubated on ice for 10 min; every 1–2 min, tubes were vigorously vortexed. Cellular debris was removed by centrifugation (16,500 × g for 10 min at 4 °C), and the resulting supernatants were collected as the nuclear fraction samples. For total cell lysis, astrocytes cultured in 6-well plates were washed once with ice-cold PBS, harvested by gentle scraping into 1 ml of ice-cold PBS, and then pooled (two wells from two separate plates). Cells were spun (600 × g for 5 min at 4 °C), and the resulting pellet was suspended in lysis buffer A (see above) followed by incubation on ice (30 min). Cellular debris was removed by centrifugation (12,000 × g for 20 min at 4 °C). For gel electrophoresis and protein detection, 30–50 μg of protein (BCA assay; Pierce) was separated by 12% SDS-PAGE under reducing conditions and electrophoretically transferred to a PVDF membrane (Bio-Rad). The membranes were blocked in Odyssey blocking buffer (LI-COR) for 1 h before overnight incubation with primary antibodies at 4 °C (xCT: 1 μg/ml, rabbit polyclonal, Novus Biologicals; β-actin: 0.3 μg/ml, mouse monoclonal, Sigma; HuR: 1 μg/ml, rabbit polyclonal, Millipore; GAPDH: 0.5 μg/ml, mouse monoclonal, Roche). Proteins of interest were detected using species-specific secondary antibodies labeled with spectrally distinct IRDye® fluorescent dyes and visualized using the LI-COR ODYSSEY® Fc imaging system (LI-COR). Analysis was performed using Image Studio 3.1 (LI-COR), and protein levels were normalized to the respective β-actin levels of each experiment. Anti-histone 3 antibody was used to check for nuclear contamination of the cytosolic fractions, and no signal was detected (data not shown). GAPDH was used to check for cytosolic contamination of nuclear fractions, and no signal was detected (data not shown).

**Immunocytochemistry**—HuR and GFAP protein were detected by indirect immunofluorescence. Astrocyte cultures were treated with IL-1β (3 ng/ml) or vehicle. The cells were fixed with 4% paraformaldehyde for 30 min and then incubated for 7 min with PBS containing 0.25% Triton X-100. Cells were washed with PBS, and nonspecific binding sites were blocked with 10% normal goat serum in PBS (25 °C, 30 min). To localize HuR in astrocytes, a rabbit polyclonal HuR antibody (3 μg/ml; Millipore) and a rat monoclonal GFAP antibody (1 μg/ml; Zymed Laboratories Inc.) were added in PBS containing 2% normal goat serum. HuR and GFAP were visualized with goat anti-rabbit Cy3 (7.5 μg/ml; Jackson ImmunoResearch) and goat anti-rat FITC (7.5 μg/ml; Jackson ImmunoResearch), respectively. DAPI (0.2 μg/ml) was used to visualize nuclei. Fluorescent images (40× magnification) were acquired using a CRX digital camera (Digital Video Camera Co, Austin, TX) mounted on an Olympus IX50 inverted microscope outfitted with epifluorescence and processed identically using Adobe Photoshop software.

**Measurement of GSH**—Total extracellular glutathione GSHₓ (GSH + GSSG) concentrations were determined using the GSH-Glo glutathione assay (Promega, Madison, WI) per the manufacturer’s instruction. The medium was collected for analysis of the extracellular GSH ([GSHₓ]), and GSSG within the samples was converted to GSH with the reducing agent TCEP-HCl (final concentration = 1 mM; 10 min; 25 °C; Thermo Scientific; Waltham, MA). Luciferase activity was measured using a Synergy 2 microplate reader (BioTek, Winooski, VT). Total extracellular GSH were normalized to standards prepared in GSH-Glo reaction buffer and media stock containing 0.1% fatty acid free BSA, respectively. Standards were linear over the range of 0–5 μM.

**Statistical Analysis**—All statistical analyses were performed using GraphPad Prism (version 6.0.2; GraphPad Software, Inc.) as described in each figure legend. Prior to parametric analyses, qPCR data were retransformed to the geometric means, whereas a log transformation was performed on the Western blot data. In all experiments, the data are expressed as the means ± S.E. Significance was assessed at *p* < 0.05, although the precise *p* values are indicated in each figure legend.

**Results**

**IL-1β Increases Steady State xCT mRNA Levels in Primary Astrocytes**—In extension of our previous study (31), we demonstrate that IL-1β increased the level of steady state xCT mRNA in primary cultures of mouse cortical astrocytes in a concentration-dependent manner measured 4 h post-exposure. Maximal increase occurred between 1 and 10 ng/ml IL-1β (Fig. 1A). As such, 3–5 ng/ml IL-1β was used for all subsequent studies.

**IL-1β Increases Stability of mRNA Encoding xCT in Primary Astrocytes**—To determine the contribution of mRNA stabilization to steady state xCT mRNA expression (Fig. 1A), the half-
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**FIGURE 1. IL-1β Increases steady state xCT mRNA levels in primary mouse astrocytes in a concentration-dependent manner via enhancement of mRNA stability.** A, astrocyte cultures were treated with vehicle or increasing concentrations of IL-1β. 4 h later, cells were harvested for RNA isolation, and relative mRNA expression for xCT was assessed via qPCR. The data are expressed as means ± S.E. fold change in xCT mRNA compared with untreated cells. An asterisk indicates values that are significantly different from control as determined using the Kruskal-Wallis test followed by Dunn’s test for comparison to control (n = 4–5 from four separate dissections; p < 0.04). B, astrocyte cultures were treated with vehicle or IL-1β (5 ng/ml) for 4 h. Cultures were washed free of IL-1β, and actinomycin D (10 μg/ml) was added. At the times indicated, samples were harvested for RNA isolation, and relative mRNA expression was assessed via qPCR. The data are expressed as the means ± S.E. scaled to zero time (set to 100%). The mRNA half-life (t1/2) was calculated using nonlinear regression analysis, specifically one phase exponential decay (n = 5 from five separate dissections).

Life of xCT transcript in control and IL-1β-treated astrocytes was compared. As shown in Fig. 1B, the xCT mRNA half-life was 3-fold greater in IL-1β-treated cultures as compared with vehicle-treated cells (t1/2 of 13 h versus 4 h, respectively).

**IL-1β Induces HuR Translocation from Nucleus to Cytoplasm in Primary Astrocytes—**Next, we examined the effect of IL-1β on the subcellular localization of HuR. Using indirect immunofluorescence microscopy, we found that HuR was largely restricted to the nuclear compartment in untreated astrocytes (Fig. 2A). However, within 15 min of treatment with IL-1β, HuR translocated to the cytosolic compartment, as indicated by a reduction in nuclear magenta staining and an increase in diffuse red staining throughout cytoplasm. Translocation was essentially complete by 60–120 min (Fig. 2A). Consistent with the immunocytochemistry results, Western blot analysis revealed a time-dependent increase in cytosolic HuR after IL-1β exposure with statistically significantly elevations occurring after 60 and 120 min (Fig. 2B, upper panel). Despite this, we did not detect a concomitant decrease in HuR in the nuclear fraction (Fig. 2B, lower panel). It is possible that the decrease was not sufficient enough to be detected by Western blot analysis. Importantly, the total amount of HuR protein in cell lysates was not changed by IL-1β exposure, suggesting that an increase in HuR production was unlikely to have accounted for this effect (data not shown).

**IL-1β Treatment Enhances HuR Binding to xCT mRNA—**Because the xCT mRNA 3’-UTR possesses putative HuR binding elements, it seemed possible that the stabilization of xCT mRNA by IL-1β was mediated by HuR. If true, HuR should directly bind to xCT 3’-UTR in an IL-1β-dependent manner. To test this, HuR protein was immunoprecipitated from astrocyte polysome extracts 2 h after treatment with IL-1β or its vehicle, and the presence of xCT mRNA was assessed using RT-PCR. HuR was successfully immunoprecipitated from both IL-1β and vehicle-treated samples (Fig. 3A). However, the 3’-UTR of xCT was only detected in IL-1β treated extracts (Fig. 3B). Immunoprecipitation of HuR protein also pulled down COX-2 and β-actin mRNAs, both of which are known targets of HuR (49, 50).

**HuR Knockdown Decreases IL-1β-induced xCT mRNA Stability—**Having confirmed binding of HuR to the xCT 3’-UTR, we next set out to determine whether it contributed to the enhanced mRNA stability that followed IL-1β treatment (Fig. 1B). To answer this question, we performed HuR knockdown in astrocyte using adenovirus shRNA (MOI 5). Cells infected with AdHuR shRNA expressed nearly 50% less HuR when compared with control Adscurble-shRNA infected cells (Fig. 4A). In keeping with a role for HuR in xCT stabilization, the IL-1β-induced increase in xCT mRNA half-life was reduced from 18.9 h in Adscurble-shRNA cells to 12.9 h in those infected with AdHuR-shRNA (Fig. 4B).

**HuR Overexpression Increases the Stability of xCT mRNA and xCT Protein Levels—**To confirm a role for HuR in xCT mRNA stabilization, we augmented HuR protein levels in cells using a recombinant HuR viral expression vector (AdHuR). The results were compared with cells infected with a control expression vector expressing GFP (AdGFP). Infection with AdHuR (MOI 5) resulted in a marked overexpression in HuR that was accompanied by an enhancement in xCT and COX-2 (positive control) protein levels as compared with AdGFP infected control cultures (Fig. 5A). Interestingly, AdGFP infection alone increased xCT protein levels relative to uninfected cells (control; Fig. 5A). Saliently, overexpression of HuR increased the xCT mRNA half-life 2-fold over that measured in AdGFP-infected cells (Fig. 5B), which itself was enhanced over that found in uninfected cells (compare Fig. 5B with Fig. 1B).

**Change in xCT Expression Is Associated with an Increase in Functional Sx− Activity—**Treatment of primary astrocytes with IL-1β enhances their synthesis and export of GSH, a process mechanistically linked to Sx− activity, as shown by the fact that the enhanced GSH export that follows IL-1β treatment is abolished in astrocytes derived from xCT deficient (sut/sut) mice (43) (Fig. 6A). The increase in xCT protein expression that followed HuR overexpression was also associated with an increase in GSH exported into the extracellular medium by astrocytes when compared with AdGFP-infected cells (Fig. 6B). Hence, the HuR-mediated increase in xCT mRNA stability and the resultant increase in cellular protein level ultimately led to a change in astrocyte Sx− functional activity.
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Discussion

By virtue of its role in cellular glutathione synthesis and its influence on ambient tissue glutamate levels, Sx$_c^-$ (cystine/glutamate antiporter) contributes importantly to CNS homeostasis. However, evidence also indicates that under particular circumstances, Sx$_c^-$ can contribute to certain brain pathologies (for comprehensive review see Refs. 52–54). The cytokine IL-1$\beta$ also produces dichotomous results within the CNS (reviewed in Ref. 55). Our laboratory recently determined that the effects of these two molecules are not mutually exclusive. For example, we found that under conditions of hypoxia when glutamate uptake was compromised (29), induction of astrocytic system x$^{-}$ activity by IL-1$\beta$ contributed to enhanced extracellular glutamate levels, which precipitated excitotoxic neuronal cell death (29, 31). However, we also found that under normoxic conditions, treatment with IL-1$\beta$ increased astrocyte glutathione production and protected the cells from oxidative stress (32). Thus, we posit that IL-1$\beta$-mediated up-regulation of astrocyte Sx$_c^-$ represents a protective mechanism that could, under certain circumstances, become maladaptive. This underscores the importance of understanding the mechanisms by which IL-1$\beta$ controls system x$^{-}$ expression in CNS astrocytes.

It is well known that Sx$_c^-$ activity is dynamically controlled and that changes in xCT expression have a major impact on its activity in numerous cellular systems. Transcriptional mechanisms undeniably play an important role in xCT expression under normal and pathophysiological conditions (52, 56). Several transcriptional regulatory elements have been identified in the 5$'$ enhancer-promoter region of the Slc7a11 gene (34, 35, 57), and the transcriptional regulatory proteins that bind to these cis-elements have been characterized under different circumstances. For example, ATF4 contributes to basal transcription from the Slc7a11 gene via a mechanism involving eIF2$\alpha$ and the amino acid response element (34, 58). ATF4 has also been implicated in the up-regulation of Slc7a11 gene transcription by excessive excitatory neuronal activity in cortical neurons (59). Moreover, ATF4 contributes to the induction of xCT mRNA transcription in response to amino acid deprivation or proteasome inhibition, in the latter case together with Nrf2 (34, 57). Electrophiles and oxidative stress in general also up-regulate xCT expression in an Nrf2-dependent manner in various cells, including cancer and neural cells (13, 35, 36). In these cases, Nrf2 regulation likely occurs via an antioxidant/electrophile response element in the Slc7a11 gene (35). Interestingly, the transcriptional repressors, KEAP1 and Oct-1, negatively control transcription from the Slc7a11 gene (36, 60). Finally, our previous results demonstrated that IL-1$\beta$ strongly up-regulated Slc7a11 gene expression in astrocytes and that this effect was due to transcriptional mechanisms, possibly through the action of the transcription factor, NF-$\kappa$B (31, 32).

Whether post-transcriptional regulation of xCT occurs in any cell type has yet to be fully characterized. The Slc7a11 gene
transcript possesses a long 3′-UTR of >7.3 kb (nucleotides 1861–9227 of the transcript; see NCBI Reference Sequence: NM_011990.2). The 3′-UTR region of many gene transcripts serves as an important post-transcriptional control point in gene expression (61). For example, mRNA stability and/or translation can be influenced by cis-acting regulatory elements located in the mRNA 3′-UTR (62). Evidence from at least two studies suggests that such mechanistic control of xCT expression might be relevant to cancer cell survival. One study demonstrated that cancer cell survival was supported by an elevation in Slc7a11 gene expression, which correlated with an increase in Slc7a11 mRNA stability (63). Results from another study demonstrated that binding of microRNA, miR26b, to conserved elements contained within the 3′-UTR region of the mRNA suppressed Slc7a11 mRNA expression, thus predisposing these breast cancer cells to apoptosis (64).

Interestingly, we found that the mouse Slc7a11 gene mRNA contains 11 adenine- and uridine-rich elements widely dispersed throughout the 3′-UTR region. These classic pentameric AUUUA sequences (65, 66) influence gene expression by serving as binding sites for regulatory RNA-binding proteins (37). Primary among these RNA-binding proteins are the ELAV (embryonic lethal abnormal vision)-like proteins. Of these, HuR (HuA; Elav4), Hel-N1 (HuB; Elav2), HuC (Elav3), and HuD (Elav4) are expressed in the CNS; however, the latter three are neuron-specific (38, 39). Given the abundance of putative HuR binding sites in xCT 3′-UTR and its known contribution to the stabilization of other genes induced by IL-1β (40–42), we examined whether stabilization of xCT mRNA by HuR contributes to IL-1β-induced up-regulation of xCT mRNA expression in astrocytes (Fig. 1A). First, we found that IL-1β increased Slc7a11 mRNA stability in astrocytes (Fig. 1B), suggesting that post-transcriptional mechanisms might indeed contribute. Next, we found that IL-1β induced the translocation of HuR from the nucleus to the cytoplasm in astrocytes (Fig. 2). This would be expected because, in addition to its role in mRNA stabilization, HuR is necessary for the export of adenine- and uridine-rich element-containing mRNAs from the nucleus to the cytosol (67, 68). Our finding that HuR was bound directly to xCT adenine- and uridine-rich element-containing mRNAs from the nucleus to the cytoplasm in astrocytes (Fig. 2). This strengthens this conclusion (Fig. 3). Finally, shRNA-mediated knockdown of HuR abrogated the ability of IL-1β to increase astrocyte xCT mRNA stability (Fig. 4), demonstrating a direct contribution of HuR to this enhancement.

Overexpression of HuR alone has been demonstrated to increase the stability of adenine- and uridine-rich element-containing mRNAs (69). When we overexpressed HuR in astrocytes, xCT mRNA half-life was doubled when compared with

**FIGURE 3.** IL-1β enhances HuR binding to xCT adenine- and uridine-rich elements. Astrocyte cultures were treated with vehicle (control, Ctrl) or IL-1β (5 ng/ml) for 2 h. Thereafter, cells were harvested and lysed with polysome lysis buffer. RNA immunoprecipitation was performed using a HuR monoclonal antibody or a nonimmune mouse IgG. A, efficiency of IP as determined by Western blot; representative of five blots. B, assessment of xCT mRNA bound to HuR as determined by RT-PCR. Evaluations of COX-2 and β-actin mRNA bound to HuR are included as positive controls (representative of five gels).

**FIGURE 4.** HuR knockdown decreases the IL-1β-mediated increase in xCT mRNA stability. A, infection of astrocyte cultures with adenovirus expressing shRNA against HuR (AdHuR-shRNA) or scrambled shRNA (Adscramble) was carried out at MOI 5 for 16 h. Cells were harvested 40 h postinfection, and Western blot analysis was performed using antibodies directed against HuR and β-actin (loading control) to determine the effectiveness of the shRNA knockdown. HuR protein levels were normalized to their respective β-actin levels and expressed as percentages of scramble (set to 100). B, astrocyte cultures were infected with Adscramble or AdHuR-shRNA (MOI 5) for 16 h. After a 24-h recovery period, cells were treated with IL-1β (5 ng/ml) for 4 h. Cultures were washed, and actinomycin D (10 μg/ml) was added. 1, 3, 5, 8, and 11 h later, samples were harvested for RNA isolation, and relative mRNA expression was assessed via qPCR. The data are expressed as the means ± S.E. scaled to 0 time (set to 100%). The mRNA half-life (t1/2) was calculated using nonlinear regression analysis, specifically one phase exponential decay (n = 4).
values obtained in GFP-infected cells (Fig. 5B). Interestingly, overexpression of GFP itself markedly lengthened the half-life of xCT mRNA over that found in noninfected cells (compare with Fig. 1B). Although numerous studies demonstrate that the xCT steady state mRNA can be induced by stress factors, this enhancement has generally been considered to be transcrip-
tional in nature (10, 11). Our results suggest there may be additional HuR-independent post-transcriptional control as well.

Finally, the HuR-mediated enhancement in changes in xCT mRNA stability was paralleled by increases in xCT protein expression (Fig. 5A) and Sx_{c}^- functional activity (Fig. 5B). These results are consistent with several studies, in neural and non-neural systems—including astrocytes as shown herein (Fig. 6A)—that demonstrate an association between xCT expression and Sx_{c}^- activity (9, 33, 34, 73–75).

In summary, these data demonstrate that HuR binds to xCT 3′-UTR mRNA in an IL-1β-dependent manner to facilitate xCT mRNA stability, indicating that post-transcriptional mechanisms do indeed contribute to the effect of IL-1β on Slc7a11 mRNA expression in astrocytes. This change is associated with increased protein expression and system x_{c}^- functional activity. More broadly, our results herein raise the possibility that post-transcriptional control of Slc7a11 gene expression may contribute to the control of system x_{c}^- function in the brain under neuroinflammatory conditions.

Author Contributions—J. S. performed and analyzed all experiments, save for that in Fig. 6A. Y. H. designed, performed, and analyzed the experiment shown in Fig. 6A and contributed to the preparation of the figures. S. J. H. and J. A. H. conceived and coordinated the study. J. S., S. J. H., and J. A. H. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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