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Abstract

Rationale

Gaps in our understanding of glutamatergic signaling may be key obstacles in accurately modeling complex CNS diseases. System x_c^- is an example of a poorly understood component of glutamate homeostasis that has the potential to contribute to CNS diseases.

Objectives

This study aims to determine whether system x_c^- contributes to behaviors used to model features of CNS disease states.

Methods

In situ hybridization was used to map mRNA expression of xCT throughout the brain. Microdialysis in the prefrontal cortex was used to sample extracellular glutamate levels; HPLC was used to measure extracellular glutamate and tissue glutathione concentrations. Acute administration of sulfasalazine (8–16 mg/kg, IP) was used to decrease system x_c^- activity. Behavior was measured using attentional set shifting, elevated plus maze, open-field maze, Porsolt swim test, and social interaction paradigm.

Results

The expression of xCT mRNA was detected throughout the brain, with high expression in several structures including the basolateral amygdala and prefrontal cortex. Doses of sulfasalazine that produced a reduction in extracellular glutamate levels were identified and subsequently used in the behavioral experiments. Sulfasalazine impaired performance in attentional set shifting and reduced the amount of time spent in an open arm of an elevated plus maze and the center of an open-field maze without altering behavior in a Porsolt swim test, total distance moved in an open-field maze, or social interaction.

Conclusions

The widespread distribution of system x_c^- and involvement in a growing list of behaviors suggests that this form of nonvesicular glutamate release is a key component of excitatory signaling.

Introduction

Glutamate is often described as the primary excitatory neurotransmitter in the brain (Coyle and Puttfarcken 1993; Franks et al. 2002; Greenamyre et al. 1988; Javitt et al. 2011; Marino et al. 2001; Tapia et al. 1999), and as such, changes in its activity is primarily or secondarily involved in most, if not all, disease of the brain. However, there are fundamental gaps in our understanding of glutamatergic signaling that may represent critical obstacles in accurately modeling complex brain disease states.

Excitatory signaling is typically depicted as the release of glutamate from a presynaptic terminal, diffusion throughout the synaptic cleft resulting in activation of post- and presynaptic glutamate receptors, subsequent

overflow from the cleft, and finally, clearance by sodium-dependent glutamate transporters primarily expressed on astrocytes (Franks et al. 2002; Rusakov et al. 2011). However, it is now becoming clear that excitatory signaling is achieved by a more elaborate network involving reuptake and release mechanisms expressed by both neurons and astrocytes regulating the activation of glutamate receptors located in and outside the synaptic cleft (Moran et al. 2005; Pettit and Augustine 2000; Rodriguez et al. 2013; Wu et al. 2012). The need to better understand the cellular mechanisms regulating glutamate homeostasis is evident from studies demonstrating that nonvesicular glutamate release has been implicated in diverse CNS processes ranging from activation of NMDA receptors to neurodevelopment (Behar et al. 1999; Cavelier and Attwell 2005; Han et al. 2013; Hirai et al. 1999; Jabaudon et al. 1999; Kihara et al. 2002; Kupchik et al. 2012; Moran et al. 2005; Moussawi et al. 2009, 2011; Navarrete and Araque 2010; Pirttimaki et al. 2011; Simonian and Herbison 2001).

System x_c^- is an example of a poorly understood source of nonvesicular glutamate release. Originally identified over 30 years ago as a sodium-independent glutamate transporter (Bannai and Kitamura 1980), system x_c^- functions as a cystine–glutamate antiporter that couples the uptake of one molecule of cystine to the release of one molecule of glutamate (Bannai 1986; Bridges et al. 2012b; Lo et al. 2008; Piani and Fontana 1994). Thought to be expressed by both neurons and astrocytes, system x_c^- is comprised of two proteins: xCT which is a light chain that is unique to system x_c^- and 4F2hc which is a heavy chain incorporated into several amino acid transporter systems (Bridges et al. 2001; Sato et al. 1999). The relative concentration gradient of each of the substrates strongly favors the uptake of cystine and the release of glutamate. Glutamate released from cystine–glutamate exchange by system x_c^- has been shown to exert broad effects on synaptic transmission, likely through the activation of high-affinity glutamate receptors (Baker et al. 2002; Kupchik et al. 2012; Moran et al. 2005; Moussawi et al. 2009, 2011).

The purpose of the present study was to explore the involvement of system x_c^- in an array of behaviors used to study diverse CNS disease states. First, we examined the distribution of xCT in structures known to be critical in regulating a diverse range of behaviors, including the prefrontal cortex, amygdala, hippocampus, and bed nucleus of the stria terminalis. Next, we examined the impact of inhibiting system x_c^- on behaviors measured using an elevated plus maze (Pellow et al. 1985; Tan et al. 2012), open-field maze (Britton and Britton 1981; Grivas et al. 2013; Joffe et al. 1973), Porsolt swim test (Porsolt et al. 1977; Rada et al. 2003; Wolak et al. 2013), social interaction paradigm (Baker et al. 2008; File and Hyde 1978; Sams-Dodd 1995), and attentional set shifting which can be used to reflect multiple aspects of cognition (Berg 1948; Floresco et al. 2006; Gilmour et al. 2013; Owen et al. 1991).

Materials and methods

Animals and surgeries

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 300–400 g were individually housed in a temperature-controlled room with a 12-h light/dark cycle with food and water ad libitum. The housing conditions and care of the rats were in accordance with the Animal Welfare Act, and all procedures were approved by the Marquette University IACUC Committee. Rats used in the microdialysis study were anesthetized using pentobarbital (50 mg/kg, IP) with atropine sulfate (1 mg/kg, IP) pretreatment to limit tracheobronchial secretions. Bilateral guide cannula (20 gauge, 14 mm; Plastics One, Roanoke, VA) were implanted using coordinates +3.1 mm anterior, ±1.0 mm mediolateral to bregma, and −0.75 mm ventral from the surface of the skull at a 6° angle from vertical (Paxinos and Watson 1986). Bilateral cannula was used to limit attrition resulting from the potential obstruction of a single cannula. Rats were given at least 6 days to recover from surgery prior to testing. Rats used for behavioral testing were experimentally naïve and used in only a single behavioral paradigm (i.e., attentional set shifting, elevated plus maze, or open-field maze including assessment of social interaction).

Drug treatments

Sulfasalazine (0–16 mg/kg, IP; Sigma Chemical Co., St Louis, MO), an inhibitor of system x_c^- (Bernabucci et al. 2012; Gout et al. 2001; Sontheimer and Bridges 2012), was dissolved in isotonic saline and brought to a pH between 6.0 and 8.0 using NaOH. Testing commenced 2 h following acute sulfasalazine treatment as described below. In the data depicted in Figs. 4 and 5, rats also received vehicle or N-acetyl cysteine (60 mg/kg, IP), which was dissolved in saline, brought to a pH of 7.0 using NaOH, and administered 1 h prior to testing.

In situ hybridization

Brains were serially sectioned coronally at 12 μm using a cryostat, thaw-mounted onto electrostatically clean slides, and stored at −80 °C until post-fixed. Prior to hybridization, sections were post-fixed in 4 % paraformaldehyde, rinsed in 0.1 M PBS (pH 7.4), equilibrated in 0.1 M triethanolamine (pH 8.0), and acetylated in triethanolamine containing 0.25 % acetic anhydride. Standard in vitro transcription methods were used to generate both sense and antisense riboprobes recognizing xCT transcripts (Choi; Milwaukee, WI), which were subsequently diluted in hybridization cocktail (Amresco; Solon, OH) with tRNA. Sections were hybridized overnight at 55 °C with fluorescein (FITC)-labeled riboprobes. After hybridization, slides were treated with RNase A and stringently washed with 0.5× SSC at 65 °C for 30 min. Slides were then incubated with an antibody against FITC (Jackson Immunoresearch; West Grove, PA) overnight at 4 °C. Following washes, slides were incubated at room temperature with a biotinylated secondary antibody for 1 h, washed again, and signal amplification was performed using the peroxidase-based Vectastain Elte ABC kit (Vector; Burlingame, CA). Finally, riboprobe signal was further enhanced using the TSA-plus fluorophore system with FITC (PerkinElmer; Waltham, MA). Image capture was performed using fluorescent microscopy (Axioskop-2, Zeiss; Thornwood, NY) and Axiovision image analysis software (Zeiss; Thornwood, NY).

Quantification of extracellular glutamate levels

Microdialysis experiments were conducted as described previously (Baker et al. 2002). Briefly, removable probes extending 2 mm past the guide cannula were inserted through the cannulae into the prefrontal cortex and microdialysis buffer (5 mM glucose, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.15 % phosphate-buffered saline, pH 7.4) was pumped through the probes at 1 μl/min for at least 3 h prior to collecting baseline samples. Afterwards, four 20-min samples were collected to obtain measures of basal glutamate. Rats were then injected with sulfasalazine (0–16 mg/kg, IP) and additional microdialysis samples were collected for 4 h. Samples were frozen at −80 °C until analyzed for glutamate content. Glutamate content was measured by precolumn derivatization with *o*-phthalaldehyde using a Shimadzu LC10AD VP autosampler. The mobile phase consisted of 100 mM Na_2HPO_4, 0.1 mM EDTA, pH 5.90, and 13 % acetonitrile. Chromatographic separation of glutamate was obtained using a reverse-phase column (Synergi Hydro-RP 80 A, 4 μm, 150 × 4.6 mm; Phenomenex, Torrance, CA), and detected using a Shimadzu 10RF-AXL fluorescence detector with an excitation and emission wavelength of 320 and 400 nm, respectively. Tissue slices obtained from rats included in the microdialysis studies were stained with cresyl violet to verify probe placements. Rats determined to have misplaced guide cannula were excluded from all analyses. Data is expressed as percent baseline, normalized to the three baseline samples prior to the sulfasalazine injection or as area under the curve following sulfasalazine injection.

Quantification of tissue cysteine and glutathione levels

Brains were extracted following rapid decapitation and 1 mm punches were taken from each hemisphere targeting the prefrontal cortex. The tissue punches were immediately submerged in 100 μl of a 4:1 mixture of dialysis buffer/thiol mobile phase solution. The samples were homogenized with a probe sonicator and analyzed for protein and glutathione content using the BCA method and HPLC coupled to EC detection, respectively. Once the protein content had been determined, the homogenized samples were spun through a centrifugal filter (PES, 3K MWCO; VWR, Radnor, PA) and the resulting protein free sample was injected onto a Shimadzu HPLC system

(SCL-10AVP controller, LC-10ADVP pump, SIL-10ADVP autosampler) coupled with an electrochemical detector (Decade II, Magic Diamond working electrode, +1.80 V, 200 nA range, 0.5 Hz filter, Antec Leyden, Netherlands). Separation was obtained with a reverse-phase column (Kinetex XB-C18 100 A, 2.6 µm, 150 × 4.6 mm; Phenomenex, Torrance, CA) and an ion-pairing mobile phase (50 mM citric acid, 10 mM octane sulfonic acid, pH 2.80, 1 % acetonitrile). Resulting cysteine and glutathione concentrations were normalized by the protein content.

Activity in an open-field maze

The open-field apparatus measured 150 × 100 × 40 cm. The session lasted 15 min and began by placing the rat into the chamber facing a wall. The amount of time animals spent in the center zone, defined as 15 cm from each wall, and total distance traveled were recorded using video-tracking software (EthoVision; Leesburg, VA).

Elevated plus maze

The maze consisted of two open arms (50.8 cm × 10.2 cm) connected to two enclosed arms (50.8 cm × 10.2 cm × 30 cm) by an open square (12.7 cm × 12.7 cm), all of which was elevated at a height of 55 cm from the floor. Each rat was placed in the maze facing an open arm. Entries into each arm, defined as placement of 4 feet into an arm, and time spent in each open arm, was recorded by an individual blind to drug treatment.

Attentional set shifting

Prior to testing, rats were food deprived to 85 % of their free-feeding weight, introduced to sucrose pellets in their home cage, and habituated to all four arms of the test maze platform. The maze platform consisted of four arms (60 × 20 × 12 cm) in the shape of a plus sign (+). Dividers at the end of each arm were used to conceal the presence of sugar pellets used to reinforce behavior.

For each of the test sessions, rats were run in blocks of 12 trials. For each trial, only three of the four arms were made accessible by blocking the fourth arm, thereby creating a maze resembling a T. In this configuration, one arm was used as a starting chamber, the other two were used as choice arms one of which was baited with a sucrose pellet. The configuration of the T-maze (i.e., the exact arms used as the starting and choice arms) was identical for each of the 12 trials in a given block. Following completion of the 12 trials, a new T-maze configuration was achieved by blocking a new arm. Thus, the orientation of the T-maze essentially rotated around four-armed platform (+) for each block of trials. The rotation of the T-maze diminished the utility of spatial cues in the room, thereby refining effective strategies to the reinforcement criteria in place for each test session.

A distinct strategy used to determine which arm would be baited arm on the three test days. On day 1 (visual cue learning), rats were reinforced with a sugar pellet when entering an arm containing a visual cue, which was a laminated sheet of paper with black and white stripes. The order of placement of the visual cue in the two choice arms was designed to be unpredictable (e.g., semi-random) while ensuring roughly equivalent placement in each of the arms. On day 2 (attentional set shifting), the reinforcement criteria switched from a visual to a spatial cue in that rats were reinforced when turning in a fixed direction (e.g., always right or always left). Finally, on day 3 (reversal learning), rats were reinforced when turning in a direction opposite to what was reinforced on day 2.

For each of the above sessions, a trial was deemed correct when the rat entered the baited choice arm. If the animal entered the incorrect arm, he was returned to his home cage to await the beginning of the next trial, which commenced once the maze was appropriately staged with the placement of a visual cue and sucrose pellet. Each test day continued until rats successfully completed ten consecutive trials plus one additional trial after rotating the three arms used for testing.

Social interaction

Individual rats were habituated to the open-field apparatus described above during two daily sessions lasting 15 min each. On the test day, rats that had received identical sulfasalazine treatments were placed in opposite ends of the open-field apparatus and allowed to explore the maze for 10 min. Social interaction was defined as the amount of time rats were within 20 cm of each other and was measured using video-tracking software (EthoVision; Leesburg,VA).

Porsolt swim test

Rats underwent habituation and test sessions during which rats were placed for 5 min into a cylindrical-shaped container (diameter of 34 cm and height of 53 cm) filled to a depth of 42 cm with water at room temperature. A video-tracking system (EthoVision; Leesburg, VA) mounted directly above the cylinder recorded the time each rat spent climbing (upward movement of paws out of the water), swimming, or immobile. Videos were then scored by individuals blind to drug treatment. To do this, the individual determined whether the subject predominately exhibited climbing behavior, horizontal activity, or was immobile during 5-s intervals. After each session, rats were removed from the cylinder, dried, and placed on a heating pad.

Statistics

The SPSS statistics package (version 21) was used to perform the statistical analyses. Behavioral data were analyzed using analysis of variance (ANOVA) with drug treatment as the between subject factor. Microdialysis data were analyzed with drug treatment as a between subjects factor and time (20-min samples) as a repeated measure. Comparison of basal glutamate levels included the four samples that preceded the injection of sulfasalazine. Comparison of post-sulfasalazine levels included the 13 samples that followed the injection. Dunnett's *T* was used for post hoc comparisons with significance set at $p < .05$. Each comparison was two-sided except for the analyses of the microdialysis data depicted as area under the curve given the a priori prediction that sulfasalazine would decrease extracellular glutamate levels.

Results

Figure 1a depicts expression patterns of mRNA for xCT, the active subunit for system x_c^- in the prefrontal cortex, basolateral amygdala, hippocampus, and bed nucleus of the stria terminalis. Interestingly, xCT mRNA exhibits a discrete pattern of distribution in the prefrontal cortex and basolateral amygdala, and a diffuse pattern in the hippocampus and bed nucleus of the stria terminalis. In each of these structures, the xCT sense probe did not produce a signal indicating the selective nature of the antisense probe. Figure 1b illustrates co-labeling of the antisense probe and DAPI confirming the cellular expression of the antisense signal.

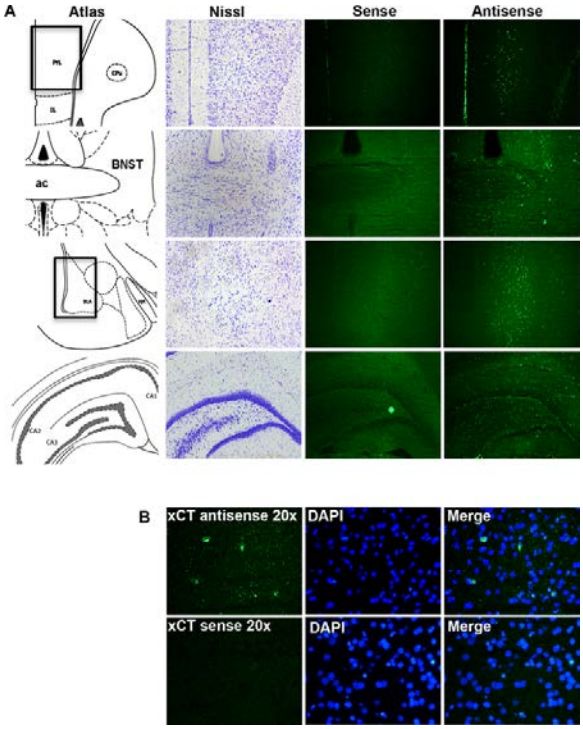

Fig. 1 In situ hybridization demonstrating xCT mRNA expression. **a** Staining for FITC-labeled xCT antisense probe (*far right column*) in the prelimbic cortex (*PrL*), bed nucleus of the stria terminalis (*BNST*), basolateral amygdala (*BLA*), and the hippocampus as reflected by the schematic images adapted from *The Rat Brain in Stereotaxic Coordinates* (*far left*; Paxinos and Watson 1998). Adjacent sections were stained with cresyl violet (*middle left*) or processed with the sense probe (*middle right*). **b** In situ hybridization of both sense and antisense probes for xCT at ×20 magnification (*left column*), distribution of DAPI labeling (*middle column*), merged image of both labeling patterns (*right column*). *Cpu* = caudate putamen, *IL* = infralimbic cortex, *ac* = anterior commissure, *opt* = optic tract, *CA1*, *CA2*, *CA3* = hippocampal cell fields

Figure 2 illustrates extracellular glutamate levels in the medial prefrontal cortex before and after an acute injection of sulfasalazine, which was done in order to identify an effective dose range of sulfasalazine when administered intraperitoneally. The average (±SEM) concentration of glutamate (1.13 ± 0.26 µM) in the baseline samples ranged between 0.71 ± 0.2 and 2.1 ± 0.88 µM, but did not differ across groups (one-way ANOVA, $p > .05$). As a result, we used a mixed ANOVA with time (microdialysis sample) as a within-subjects factor and sulfasalazine treatment as a between-subjects factor to compare extracellular glutamate levels depicted as percent baseline before and after a systemic injection of sulfasalazine. Prior to sulfasalazine, the main effect of treatment, time (sample), and the interaction between these variables failed to reach significance ($p > .05$). However, following the injection of sulfasalazine, we obtained a main effect of sulfasalazine [$F_{3,26} = 2.99$, $p < .05$] and time [$F_{12,312} = 6.09$, $p < .001$] in the absence of an interaction between these variables ($p > .05$). Post hoc analyses revealed that glutamate levels were significantly lower in rats treated with sulfasalazine at a dose of 8 or 16 mg/kg when compared to saline controls (Dunnett's *T*, $p < .05$).

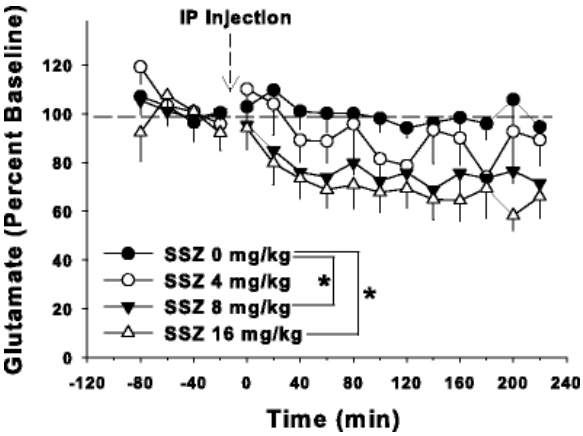

Fig. 2 Systemic administration of the system x_c^- inhibitor sulfasalazine dose-dependently reduces extracellular glutamate levels in the prefrontal cortex. This graph depicts extracellular glutamate levels in the prefrontal cortex before and after an injection of vehicle (1 ml/kg, IP; *N* = 7) or sulfasalazine (4–16 mg/kg, IP; *N* = 7–8/group). *$p < .05$ indicates a significant reduction relative to vehicle-treated subjects in post-injection extracellular glutamate levels in the prefrontal cortex (one-sided Dunnett's *T*)

The uptake of cystine and subsequent conversion to its reduced form, cysteine, contributes to glutathione synthesis, which has been proposed to be altered in several disease states. Because of this, we measured the effect of acute sulfasalazine treatment on tissue levels of glutathione and cysteine. Mean ± SEM levels of glutathione in tissue punches obtained from the prefrontal cortex in rats treated with vehicle, 8 mg/kg sulfasalazine, or 16 mg/kg sulfasalazine were 5.90 ± 0.22, 6.1 ± 0.23, and 5.94 ± 0.26 μg/mg protein, respectively. Mean ± SEM levels of cysteine in tissue punches obtained from the prefrontal cortex in these same rats were 49.7 ± 8.73, 48.2 ± 2.04, and 48.5 ± 5.19 ng/mg protein. An ANOVA with sulfasalazine as a between-subjects factor did not yield a significant main effect of sulfasalazine treatment on either cysteine or glutathione levels ($p > .05$).

The impact of acute blockade of system x_c^- on behavior in an open field maze is depicted in Fig. 3. An ANOVA used to compare total distance traveled in the maze did not indicate a significant main effect of sulfasalazine treatment. However, there was a significant main effect of sulfasalazine treatment on time spent in the center of the maze [$F_{2,32} = 4.38$, $p < .05$], which is depicted in Fig. 3b. Post hoc analyses revealed a significant reduction in time spent in the center of the maze by rats treated with 8 or 16 mg/kg sulfasalazine (Dunnett's *T*, $p < .05$).

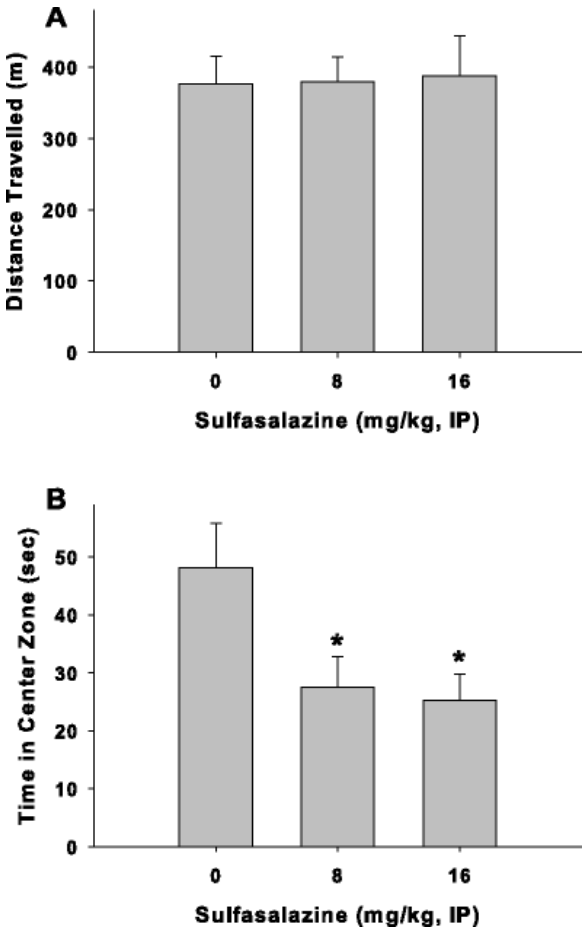

Fig. 3 Acute administration of sulfasalazine reduces time spent in the center of an open-field maze without altering overall levels of locomotor activity. **a** This graph depicts the distance traveled by rats injected with vehicle (1 ml/kg, IP; *N* = 10) or sulfasalazine (8–16 mg/kg, IP; *N* = 12–13/group). **b** This panel illustrates the amount of time rats pretreated with vehicle or sulfasalazine (8–16 mg/kg, IP) spent in the center of the open field. *$p < .05$ indicates a significant reduction relative to vehicle-treated subjects (two-sided Dunnett's *T*)

Figure 4 illustrates the impact of acute sulfasalazine treatment on the amount of time spent in the open arm of an elevated plus maze. This was done to further evaluate the possibility that acute blockade of system x_c^- activity may be producing an anxiogenic state, as indicated by the above reduction in time spent in the center of an open-field maze. An ANOVA yielded a significant main effect of sulfasalazine treatment [$F_{2,22} = 5.26$, $p < .05$]. Subsequent post hoc comparisons revealed that subjects receiving either dose of sulfasalazine spent significantly less time in the open arm of the maze than subjects receiving vehicle (Dunnett's *T*, $p < .05$). To confirm that sulfasalazine produced an anxiogenic state by inhibiting system x_c^-, we examined the impact of co-administration of the cysteine prodrug N-acetyl cysteine on sulfasalazine-induced changes in open arm time (Fig. 4b). An ANOVA with drug treatment as a between-subjects variable yielded a significant main effect [$F_{3,23} = 5.15$, $p < .01$]. Subsequent post hoc analyses revealed that sulfasalazine (8 mg/kg, IP) did not alter time spent in the open arm when co-administered with N-acetyl cysteine (Dunnett's *T*, $p < .05$).

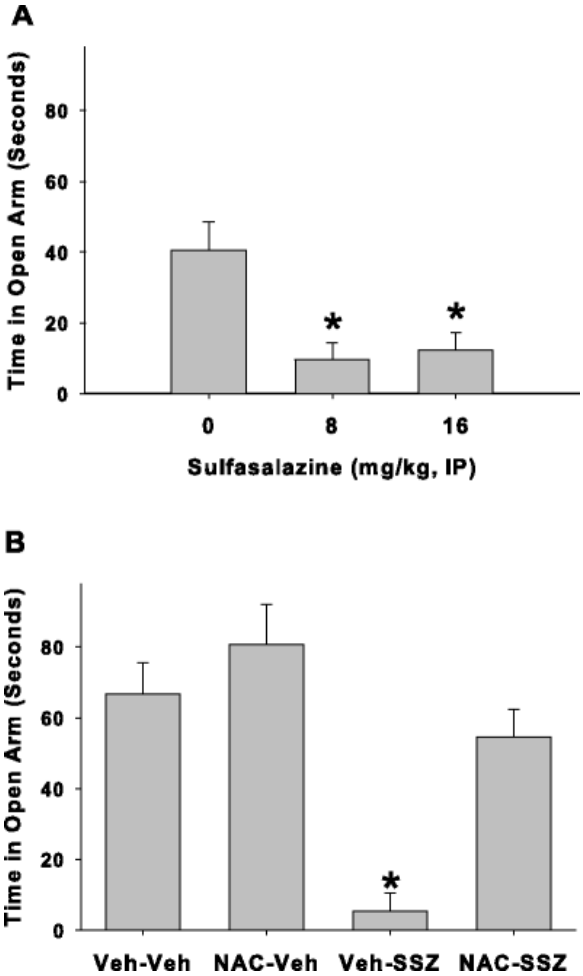

Fig. 4 An anxiogenic effect produced by acute sulfasalazine administration is reversed by co-administration of the cysteine prodrug N-acetyl cysteine. **a** This figure illustrates the amount of time spent in the open arm of an elevated plus maze by rats pretreated with vehicle ($N = 9$) or sulfasalazine (8–16 mg/kg, IP; $N = 5$–11/group) 2 h prior to testing. *$p < .05$ indicates a significant reduction relative to vehicle-treated subjects (two-sided Dunnett's *T*). **b** This panel presents the amount of time spent in the open arm of an elevated plus maze by rats pretreated with vehicle ($N = 4$), sulfasalazine (8 mg/kg, IP; $N = 5$–9/group; administered 2 h prior to testing), and/or N-acetyl cysteine (100 mg/kg, IP; administered one hour prior to testing). *$p < .05$ indicates a significant difference relative to vehicle-treated subjects (two-sided Dunnett's *T*)

An ANOVA was used to compare the impact of drug treatment on the number of trials needed to reach criterion on each of the three distinct test days in the attentional set shifting model. We obtained a main effect of treatment on days 2 and 3 only [day 1, $p > .05$; day 2: $F_{3,29} = 3.59$, $p < .05$; day 3: $F_{3,29} = 3.99$, $p < .05$]. The mean ± SEM number of trials required for each treatment group on day 1 were 44.4 ± 5.81 (saline only), 33.9 ± 5.8 (N-acetyl cysteine only), 53.8 ± 5 (sulfasalazine only), and 53.5 ± 9.1 (N-acetyl cysteine + sulfasalazine); values for each group obtained on test days 2 (attentional set shifting) and 3 (reversal learning) are depicted in Fig. 5. Further analyses of the main effect on day 2 revealed only a trend towards an increase in the number of trials required by rats treated with sulfasalazine ($p = .08$). On day 3, however, rats receiving sulfasalazine alone required significantly more trials to complete the test session (Dunnett's *T*, $p < .05$), an effect that was blocked by co-administration of N-acetyl cysteine with sulfasalazine.

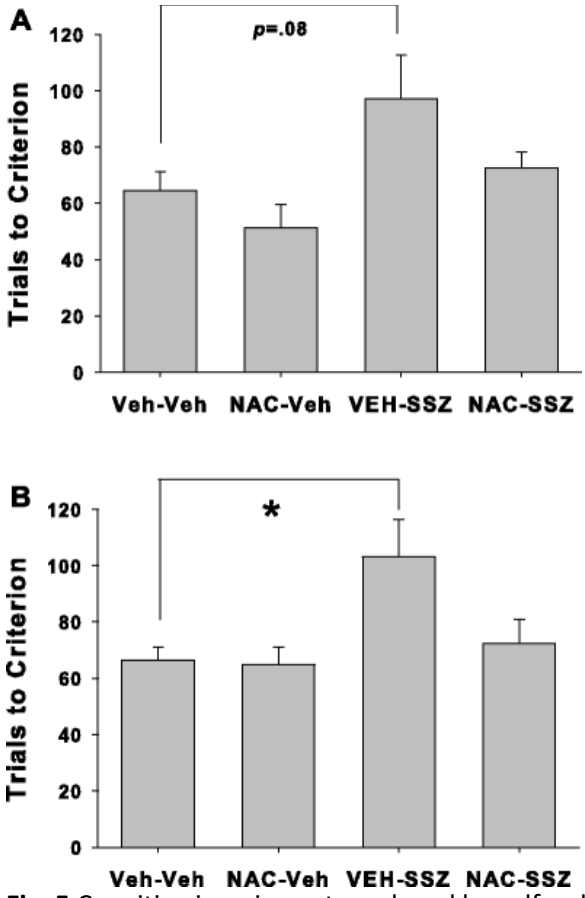

Fig. 5 Cognitive impairment produced by sulfasalazine administration is reversed by co-administration of the cysteine prodrug N-acetyl cysteine. Rats were pretreated with vehicle (*Veh*; *N* = 8), N-acetyl cysteine alone (*NAc*; 60 mg/kg, IP; *N* = 8), sulfasalazine alone (*SSZ*; 8 mg/kg, IP; *N* = 9), or NAC + SSZ (*N* = 8) 2 h prior to testing. **a** This figure illustrates the number of trials needed to reach criterion on a task reinforced when rats, previously reinforced to enter an arm cued by a visual stimulus, adopted a strategy guided by spatial cues (i.e., turning in a direction opposite to their previously determined turn bias). **b** Data represent the number of trials needed to reach criterion when the reinforcement strategy was opposite to the previous day (i.e., turning in the direction of their previously established turning bias). **p* < .05 indicates a significant increase relative to vehicle-treated subjects (two-sided Dunnett's *T*)

Sulfasalazine did not produce a significant reduction in several behaviors, including social interaction, or behavior measured during a Porsolt swim test including climbing, horizontal activity, or immobility. In each case, we failed to obtain a main effect of sulfasalazine ($p > .05$).

Discussion

System x_c^- is an intriguing component of excitatory signaling, the study of which may yield insights into the pathology and treatment of CNS diseases. Originally described as a sodium-independent glutamate transporter (Bannai and Kitamura 1980), system x_c^- is capable of nonvesicular glutamate release by coupling the uptake of one molecule of cystine to the release of one molecule of glutamate (Bannai 1986; Bridges et al. 2012b; Lo et al. 2008; Piani and Fontana 1994). The importance of this mechanism has been demonstrated by studies revealing that system x_c^- regulates excitatory signaling and/or synaptic plasticity in several structures (Baker et al. 2002; Buckingham et al. 2011; Carmeli et al. 2012; Kupchik et al. 2012; Li et al. 2012; Moran et al. 2005; Moussawi et al. 2009, 2011). Key findings from the present study include our observations that mRNA for xCT, the catalytic subunit for system x_c^-, is distributed throughout the brain and that acute inhibition of system

x_c^- impairs cognition and produces an apparent anxiogenic state. These data are consistent with the conclusion that complex CNS activity is a product of excitatory signaling achieved by an intricate network of release and reuptake mechanisms, many of which are poorly understood, in part, because they are unique to glutamate.

As an initial step in evaluating the possibility that system x_c^- may be an important component of excitatory signaling throughout the brain, we examined xCT mRNA distribution. A few structures, including the basolateral amygdala and prefrontal cortex, displayed high levels of xCT mRNA distributed in a defined pattern. In contrast, many of the examined regions, including the hippocampus and bed nucleus of the stria terminalis, exhibited a diffuse, even distribution pattern of xCT mRNA that is similar to what has previously been observed for proteins primarily expressed by astrocytes, such as GLT-1 (Berger et al. 2005). It will be important to determine if these distinct patterns reflect differential expression of xCT by neurons or astrocytes. Regardless, these data clearly illustrate that xCT mRNA is widely distributed throughout the brain, and thus, may be involved in a wide array of behaviors.

In order to acutely inhibit system x_c^- function throughout the brain, we opted to systemically administer the inhibitor sulfasalazine. Although there are valid concerns with the use of this drug, in part because it can be metabolized by bacteria in the colon (Azadkhan et al. 1982), there are several studies demonstrating altered CNS activity following IP-administered sulfasalazine (Buckingham et al. 2011; Chung and Sontheimer 2009). Consistent with these reports, we found that sulfasalazine produced a dose-dependent reduction in extracellular glutamate in the brain, specifically in the prefrontal cortex. In addition, we observed behavioral deficits that were reversed by N-acetyl cysteine, which is thought to function by supplying the system x_c^- substrate cystine (Baker et al. 2008; Bridges et al. 2012a; Kau et al. 2008; Moran et al. 2005). Interestingly, we did not observe reduced tissue levels of cysteine or glutathione in the prefrontal cortex. To the extent that this finding can be extrapolated to other brain regions, it implies that our observed behavioral changes may be more likely to be due to disrupted glutamate release from system x_c^- rather than reduced synthesis of thiols, for which there are potentially redundant cysteine/cystine transport systems, for review see (Bridges et al. 2012a). It is important to note that systemic sulfasalazine would be expected to reduce system x_c^- activity throughout the brain, and thus, behavioral changes observed in this study are not necessarily the result of impaired glutamatergic signaling in the prefrontal cortex.

In apparent contrast with our findings using systemic administration of sulfasalazine, a previous study found that local delivery of S-4-carboxyphenylglycine into the prefrontal cortex did not significantly alter extracellular glutamate levels (Melendez et al. 2005). The discrepancy between these studies may be due to either the route of administration used in each study and/or the specific inhibitor used to decrease system x_c^- activity. Regardless, it is important to note that Melendez et al. (2005) observed that S-4-carboxyphenylglycine produced a significant decrease in extracellular glutamate when co-administered with an inhibitor of glutamate uptake. Thus, at least under certain conditions, both inhibitors/routes of administration have been shown to reduce extracellular glutamate levels in the prefrontal cortex.

We next conducted a series of behavioral studies to determine the consequence of acute system x_c^- blockade. Using an attentional set-shifting paradigm, we found that acute sulfasalazine produced a significant deficit in reversal learning. Co-administration of the cysteine prodrug N-acetyl cysteine normalized behavior in this paradigm, which is consistent with sulfasalazine inhibition of system x_c^- (Baker et al. 2008; Bridges et al. 2012a; Kau et al. 2008; Moran et al. 2005). Interestingly, impaired reversal learning has been observed in several diseases that are altered by manipulating system x_c^- activity, including drug addiction and schizophrenia (Amen et al. 2011; Baker et al. 2003; Berk et al. 2008; Calu et al. 2007; Izquierdo and Jentsch 2012; Knackstedt et al. 2009; Murray et al. 2008; Owen et al. 1991; Pantelis et al. 1999; Stalnaker et al. 2009), for review see Bridges et al. (2012a). However, the potential for altered system x_c^- to contribute to other CNS diseases involving impaired cognition is largely unexplored.

Sulfasalazine treatment altered several anxiety-related behaviors, including elevated plus maze and time spent in the center of an open-field maze. Similar to impaired cognition, the anxiogenic state produced by sulfasalazine was reversed by the cysteine prodrug N-acetyl cysteine. Surprisingly, acute sulfasalazine treatment failed to alter social interaction, a measure influenced by anxiety levels (File and Hyde 1978; File and Lister 1984). While most studies report that anxiogenic or anxiolytic substances produce parallel changes in elevated-plus maze and social interaction, there have been discrepant outcomes (Baldwin et al. 1989; Johnston et al. 1988; Koss et al. 2004; Leblanc-Duchin and Taukulis 2004). For instance, yohimbine has been shown to reduce open-arm time without altering social interaction (Johnston et al. 1988). Several groups have proposed that each assay may underlie distinct aspects of anxiety (File 1995; Fuchs and Fliugge 2006; Green et al. 2013; Morley and McGregor 2000). If this is the case, then there may be unique mechanisms underlying elevated plus maze and social interaction.

The inability of acute sulfasalazine treatment to alter behavior in a Porsolt swim test, locomotor activity in an open-field maze, or social interaction may reflect a lack of involvement of system x_c^- activity in these phenomena, although it is possible that long-term decreases in system x_c^- activity may be necessary to produce deficits in these paradigms. Alternatively, these behaviors may be more sensitive to increased system x_c^- activity, in part because the utility of these screens primarily involves therapeutic predictive validity rather than construct or face validity (Fuchs and Fliugge 2006; McGonigle 2013; Razafsha et al. 2013). In this regard, it is important to note that the cysteine prodrug N-acetyl cysteine has been shown to attenuate depression in man and rodent behaviors in the Porsolt swim test which is used as a preclinical screen for depression (Costa-Campos et al. 2013; Smaga et al. 2012). Similarly, N-acetyl cysteine reduces the severity of negative symptoms of schizophrenia and phencyclidine-induced social withdrawal, which is used as a preclinical screen for schizophrenia (Baker et al. 2008; Berk et al. 2008).

Excitatory signaling is typically depicted as the release of glutamate from a presynaptic terminal, diffusion throughout the synaptic cleft resulting in activation of post- and presynaptic glutamate receptors, subsequent overflow from the cleft, and finally, clearance by sodium-dependent glutamate transporters primarily expressed by astrocytes (Franks et al. 2002; Rusakov et al. 2011). However, the widespread distribution of system x_c^- and involvement in a growing list of behaviors suggest that this form of nonvesicular glutamate release is another key component of excitatory signaling. The potential importance of nonvesicular glutamate release mechanisms, such as system x_c^-, highlight the degree to which we need to better understand how a complex network of transporters and release mechanisms function to regulate the primary excitatory neurotransmitter in the brain. By better understanding the cellular and molecular regulation of glutamate, we will be better able to model complex disease states, and in doing so, may continue to identify novel therapeutic targets for the treatment of CNS disorders.

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Conflict of interest

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