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Slc7a11 (xCT) protein expression is not altered in the depressed brain and system xc- deficiency does not affect depression-associated behaviour in the corticosterone mouse model

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ABSTRACT

Objectives

The cystine/glutamate antiporter (system xc-) is believed to contribute to nonvesicular glutamate release from glial cells in various brain areas. Although recent investigations implicate system xc- in mood disorders, unambiguous evidence has not yet been established. Therefore, we evaluated the possible role of system xc- in the depressive state.

Methods

We conducted a protein expression analysis of the specific subunit of system xc- (xCT) in brain regions of the corticosterone mouse model, Flinders Sensitive Line rat model and *post-mortem* tissue of depressed patients. We next subjected system xc- deficient mice to the corticosterone model and analysed their behaviour in several tests. Lastly, we subjected additional cohorts of xCT deficient and wildtype mice to N-acetylcysteine treatment to unveil whether the previously reported antidepressant-like effects are dependent upon system xc-.

Results

We did not detect any changes in xCT expression levels in the animal models or patients compared to proper controls. Furthermore, loss of system xc- had no effect on depression- and anxiety-like behaviour. Finally, the antidepressant-like effects of N-acetylcysteine are not mediated via system xc-.

Conclusions

xCT protein expression is not altered in the depressed brain and system xc- deficiency does not affect depression-associated behaviour in the corticosterone mouse model.

KEYWORDS

Depression; glutamate; slc7a11; system xc-; N-acetylcysteine

1. INTRODUCTION

The monoamine hypothesis was the main driver for pathophysiological and pharmacological research on mood disorders for half a century. Recently, however, perturbations in the glutamatergic system are getting more attention. The glutamate hypothesis dates back to the early 1990’s, with the observation that N-methyl-D-aspartate receptor (NMDAR) antagonists exert antidepressant-like effects in animal models (Trullas and Skolnick 1990). Since this discovery, numerous research papers have been published on this matter. The most important clinical findings are based on imaging and monitoring studies demonstrating alterations in glutamate levels in plasma and brain tissue of patients (Yuksel and Ongur 2010), as well as pharmacological studies showing antidepressant effects of ionotropic glutamate receptor (iGluR) antagonists (Zarate et al. 2006). Recent comprehensive reviews of the most recent developments in pharmacological research are published by (Machado-Vieira et al. 2015) and (Park et al. 2015). In the pathophysiology of major depressive disorder (MDD), chronic stress (and associated elevation of glucocorticoid levels) seems to have a substantial influence on different aspects of synaptic glutamate neurotransmission, i.e. vesicular glutamate release in the prefrontal cortex (Moghaddam 2002), glutamate receptor signalling and trafficking, as well as glutamate removal from the extrasynaptic space by glial excitatory amino acid transporters (EAATs).

The cystine/glutamate antiporter system xc- (specific subunit xCT; slc7a11) exchanges extracellular cystine for intracellular glutamate (Sato et al. 1999) and is thereby of high importance for controlling extracellular glutamate levels (De Bundel et al. 2011, Massie et al. 2011) and generation of the antioxidant glutathione (GSH) (Massie et al. 2015). Glutamate that is released via system xc- can activate mGluRs and as such modulate synaptic neurotransmission (Moran et al. 2005) or extrasynaptic iGluRs and induce excitotoxicity, explaining its reported involvement in several neurological disorders (for review see (Massie, Boillee, Hewett, Knackstedt and Lewerenz 2015)).

We recently demonstrated that naive "non-stressed mice", lacking functional system xc- (xCT-/-), have lower depression- and anxiety-like behaviour in a battery of behavioural paradigms compared to their wildtype littermates (xCT+/+) (Bentea et al. 2015). On the other hand, Lutgen and colleagues observed an anxiogenic effect, when administering the non-specific system xc- inhibitor sulfasalazine in "non-stressed rats" (Lutgen et al. 2014). Furthermore the antioxidant N-acetylcysteine (NAC), an agent known to modulate also the function of system xc-, has recently gained attention as a potential treatment for various psychiatric disorders (Bauzo et al. 2012, Deepmala et al. 2015). In the bulbectomized rat model for depression, chronic NAC treatment leads to a significant decrease in forced swim test (FST) immobility (Smaga et al. 2012), while a recent clinical trial provided limited evidence for NAC treatment in depressed patients (Berk et al. 2014). When we take these few research reports into consideration, the involvement of system xc- in the pathophysiology of MDD and the stressed glutamate synapse, seems contradictory. Consequently, unravelling the role of system xc- in the depressive state seems elemental to better understand the bigger picture of aberrant glutamate neurotransmission in MDD. To this end, we first analysed xCT protein levels in two different animal models of depression and in *post-mortem* tissue of depressed patients. The first model we used is the in-house validated corticosterone (CORT) mouse model (Demuyser et al. 2015), in which the chronic excessive stressor of combined chronic CORT administration and social isolation induces robust depression- and anxiety-like behaviour. Besides this inducible mouse model, we documented xCT expression levels in a validated model of depression, the Flinders Sensitive Line (FSL) rat model. The FSL rats were genetically selected from Sprague-Dawley (SD) rats and show different features of construct, face and predictive validity of the depressive illness, such as decreased appetite, reduced general activity and changes in sleep patterns (Overstreet and Wegener 2013). Finally, we acquired *post-mortem* tissue of patients diagnosed with a major depressive illness, which allowed us to verify xCT expression in the clinically most relevant tissue of human patients. We analysed varying brain regions related to depression, i.e. the hippocampus as the memory centre of the brain and key region for emotional regulation, cingulate cortex as an integrative centre of motivation and emotional behaviour, nucleus accumbens as the pleasure centre and amygdala as the most important region for anxiety and fear regulation.

Apart from these observational experiments attesting possible alterations in xCT expression levels in depressed tissues, we modulated system xc- in a valid preclinical model for depression, namely the chronic CORT model. As we have previously described that under physiological conditions, naive "non-stressed mice" deficient of system xc-, show clear antidepressant- and anxiolytic-like behaviour (Bentea, Demuyser, Van Liefferinge, Albertini, Deneyer, Nys, Merckx, Michotte, Sato, Arckens, Massie and Smolders 2015), we now investigated how "mild- and excessive chronically stressed" xCT-/- and xCT+/+ mice behave in the same battery of paradigms. Finally we reproduced recent data, showing an antidepressant effect of chronic NAC treatment in rats in the same mouse model of depression (Smaga, Pomierny, Krzyzanowska, Pomierny-Chamiolo, Miszkiel, Niedzielska, Ogorka and Filip 2012). Since we used xCT-/- mice and their xCT+/+ littermates, we were able to differentiate system xc- specific effects of NAC from other off-target effects.

2. MATERIALS AND METHODS

2.1.Depressed patients and controls

Frozen brain tissue of depressed patients and non-depressed, non-demented controls was obtained from The Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. All tissues have been collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by the Netherlands Brain Bank. Frozen tissue was stored at -80 °C until use.

Age at death, gender and the delay between decease and autopsy of the depressed patients and controls, are outlined in Table 1.

2.2.Animals

All animals in this study were housed and maintained according to national guidelines on animal experimentation and all experiments were approved by the Ethical Committee for Animal Experimentation of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel. To the best of our abilities the results are described in accordance to the ARRIVE guidelines for reports in animal research (Kilkenny et al. 2010).

Male C57BL/6J mice (Charles River Laboratories, France) weighing 25-30 g and male xCT-/- mice and xCT+/+ littermates weighing 28-33 g were used to induce the CORT mouse model for depression and anxiety, as described before (Demuyser, Deneyer, Bentea, Albertini, Van Liefferinge, Merckx, De Prins, De Bundel, Massie and Smolders 2015). The mutant mice used in this study are high-generation descendants of the strain previously described (Sato et al. 2005), and were bred in the animal facilities of the Vrije Universiteit Brussel. The xCT null mutants were generated by targeted disruption of the START codon in exon 1 of the Slc7a11 gene, and were backcrossed for more than 12 generations on a C57BL/6J background. Genotypes were confirmed by PCR amplification of ear DNA using REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, USA) and the following primers: 5′-GATGCCCTTCAGCTCGATGCGGTTCACCAG-3′ (GFPR3); 5′-CAGAGCAGCCCTAAGGCACTTTCC-3′ [mxCT5′flankF6]; 5′-CCGATGACGCTGCCGATGATGATGG-3′ [mxCT(Dr4)R8]. All mice were housed in a 14/10 h light/dark cycle, at a constant temperature of 25 °C and with free access to food and tap water.

Male SD rats (Harlan, The Netherlands) and FSL rats (bred in-house) were housed under standard laboratory conditions in the Karolinska Institutet in Stockholm, Sweden (12 h light/dark cycle with controlled room temperature of 22 °C).

2.3.Induction of the corticosterone mouse model and N-acetylcysteine treatment

C57BL/6J mice as well as xCT-/- mice and their xCT+/+ littermates were randomly assigned to two different groups, receiving either CORT or SHAM treatment through subcutaneous (s.c.) injections, and were respectively single or group housed (2-4 mice per cage). S.c. SHAM and CORT injections in the neck region were given once daily, for 21 consecutive days between 9 and 11 AM. Corticosterone 21-acetate (Sigma Aldrich, Germany) (equivalent to 20 mg/kg/day CORT) was suspended in physiological saline (0.90% w/v of NaCl, Baxter, USA), containing 1% polyoxyethylene glycol sorbitan monooleate (Tween-80, Fagron, Belgium). A SHAM suspension consisted of 1% Tween-80 in physiological saline.

An additional cohort of xCT-/- and xCT+/+ mice was assigned to four different groups, with groups one and three receiving 21 days of s.c. SHAM treatment (group housed) and groups two and four treated with s.c. CORT injections (single housed). Furthermore the last 10 days were combined with an additional intraperitoneal (i.p.) NAC injection in groups three and four (100 mg/kg/day of NAC in dissolved in saline, Sigma Aldrich), while groups one and two received additional i.p. SHAM (physiological saline) treatment.

2.4.Western blot analysis

For quantification of xCT expression, C57BL/6J mice were sacrificed by cervical dislocation, after three weeks of SHAM/CORT treatment and subsequent two weeks of behavioural assessment (brain tissue originates from mice that were subjected to behavioural paradigms in (Demuyser, Deneyer, Bentea, Albertini, Van Liefferinge, Merckx, De Prins, De Bundel, Massie and Smolders 2015)). All rats were slightly sedated with isoflurane before decapitation. Total brain tissue was rapidly dissected and frozen on dry ice.

Cryosections (300 µm) were prepared from mouse, rat and human brain tissue. Slices were stored at -20 °C until desired brain regions were dissected with biopsy punch needles (Palkovits 1983). From all brain samples, hippocampus and cingulate cortex were dissected (with additional nucleus accumbens and amygdala dissections from the mouse and rat brains, brain regions identified according to Paxinos and Franklin atlas).

The dissected brain structures were homogenized in extraction buffer (2% sodium dodecyl sulfate polyacrylamide, 60 mM Tris base (pH 7.5), 100 mM dithiothreitol, 1% (v/v) phosphatase inhibitor cocktail 3 (Cantharidin(-)-p-Bromotetramisole and Calyculin A; Sigma Aldrich) and 2 tablets/100 ml protease inhibitor (4-(2-aminoethyl)benzenesulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin, and EDTA; Sigma Aldrich)). After incubation for 30 min at 37 °C, homogenates were centrifuged at 9500 g (4 °C) during 20 min. Supernatants were stored at −20 °C. Total protein concentrations were measured by means of the Qubit fluorometer (Invitrogen, Groningen, the Netherlands). SDS-polyacrylamide gel electrophoresis (4–12% Bis-Tris gel, Biorad Laboratories, Nazareth, Belgium), under reducing conditions, was used for the separation of the proteins. After separation, proteins were transferred to a polyvinylidene fluoride membrane (Biorad) using the Criterion Blotter (Biorad). The protein transfer was followed by a blocking step of non-specific binding in 5% membrane blocking agent (bovine serum albumin in Tris-buffered saline, GE Healthcare, Roosendaal, the Netherlands). Thereafter, membranes were incubated overnight with anti-xCT primary antibodies (specificity of the antibodies was verified with xCT-/- tissue). Different antibodies, raised in rabbits, were used for analysis of rodent brain tissue (Ab to mouse xCT GQTHHFKDAFSGRD-(amide) peptide sequence, 0.206 µg/ml in membrane blocking agent at 4 °C (Massie et al. 2008)) and for human samples (Ab #622 to rat xCT MVRKPVVATISKGGYLQGNVSGRLPSVGDQEPPGHEK-(amide) peptide sequence, 0.1 µg/ml in membrane blocking agent at 4 °C) (Van Liefferinge et al. 2015). The following day, membranes were incubated during 30 min with secondary antibody (horseradish-peroxidase-conjugated anti-rabbit 1/15000; Product No. P045001; Dako, Glostrup, Denmark). After rinsing, immunoreactive signals were visualized by means of enhanced chemiluminiscence (ECL Select, GE Healthcare) using an ImageQuant LAS 4000 detector (GE Healthcare). Next, membranes were rinsed overnight with Tris-buffered saline and Serva Purple staining (Serva Electrophoresis, Heidelberg, Germany) was applied to visualize the total protein content on the membrane. Therefore membranes were stripped by incubation in a stripping buffer (20 mg/ml SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris base (pH 6.7)). After thorough rinsing, blots were incubated for 30 min in a Serva Purple solution at room temperature. Next, fluorescent proteins were visualized using the LAS detector (GE Healthcare). For all comparisons that are made, i.e. SHAM- versus CORT-treated mice; SD versus FSL rats; non-depressed controls versus depressed patients, samples were loaded on the same membrane and experiments were repeated at least three times for each brain region. ImageJ software (National Institute of Health, USA) was used for densitometric analysis of immunoreactive and fluorescent bands. Optical densities (OD) of xCT immunoreactive bands were normalized to OD of the fluorescent protein bands of the entire lane detected on the same membrane. Expression levels are calculated relative to a pool of the control samples. xCT protein expression levels are reported as the percentage of the pool.

2.5.Behavioural assessment of xCT-/- and xCT+/+ mice

After three weeks of CORT treatment and single housing or SHAM treatment and group housing, xCT-/- mice and their xCT+/+ littermates were subjected to a series of tests for depression- and anxiety-like behaviour (graphical schedule see Fig. 1). Mice were acclimatized to the testing room at least 12 h prior to assessment. All experiments were videotaped and analysed by a scientist blinded to the treatment and genotype. All experiments were carried out in the light phase of the light/dark cycle.

In mice that received both CORT and NAC treatment, the last NAC injection was administered half an hour before the behavioural assessment.

2.5.1. Forced swim test (FST)

A modified version of the FST, originally described by Porsolt et al. (1977) was used to assess depression-like behaviour (Porsolt et al. 1977). Mice were placed in a glass tank cylinder (30 cm diameter) filled with 30 cm of water (25 ± 1 °C) and videotaped during 5 min. The light levels in the room created an illuminance of 400 lux. The inescapability of the set-up induces a state of helplessness. Three types of behaviour were distinguished. Climbing behaviour consisted of upward directed movements of the forepaws along the side of the swim tank (also called thrashing). Swimming behaviour was defined as mostly horizontal movement across the swim tank. Immobility was assigned when no additional activity was observed other than that required to keep the mouse’s head above the water surface. Cryan et al. (2000) previously described a time sampling technique, whereby the predominant behaviour in each 5 seconds period was noted. As the trial time was 300 seconds, a total number of 60 counts per mouse were recorded, which were divided between climbing, swimming, and immobility (Cryan and Lucki 2000).

2.5.2. Mouse-tail suspension test (MTS)

Apart from the FST, the MTS is one of the most widely used tests for examining depression-like behaviour in mice. The set-up was similar to the one described by Steru and co-workers (Steru et al. 1985). Mice are suspended by the tip of their tail for 5 min to induce an inescapable, short-term stress situation. The time of immobility is measured as a parameter for depression-like behaviour. Mice that climbed their tail were excluded from the experiment. The light levels in the room created an illuminance of 400 lux.

2.5.3. Novelty-suppressed feeding test (NSF)

The NSF is a conflict test that evokes competing motivations: the drive to eat and the fear to enter the centre of a brightly lit box. In this way hyponeophagia can be a parameter for depression- and anxiety-like behaviour. The procedure was slightly adapted from Mineur et al. (2007) (Mineur et al. 2007). Mice were deprived from food for 24 h before the start of the experiment, water remaining available *ad libitum*. Each subject was placed in a corner of an open box (60 cm x 60 cm; height 60 cm) (Plexiglas), with black opaque walls that prevent observation of visual cues outside the arena. The base of the arena was covered with 1 cm layer of bedding, one food pellet was placed in the centre and an illuminance of 150 lux was created in the centre of the arena. The time to the first feeding episode was recorded manually.

2.5.4. Light/dark paradigm (LDP)

The LDP investigates the spontaneous exploratory behaviour of rodents in response to mild stress, in this case a novel and well-illuminated environment. The test apparatus consists of an open box (60 cm x 60 cm; height 60 cm / 700 lux) manufactured in clear Plexiglas, with black opaque walls that prevent observation of visual cues outside the arena. A small dark square-shaped compartment (one fourth of the total area / 0.5 lux), manufactured in black high-pressure laminate (Volkern-Trespa), is positioned in one of the corners of the open field arena. Mice have an innate aversion to brightly illuminated areas; therefore they preferably stay in the small dark compartment. The test takes 5 min in total and mice are placed in the dark zone at the start. Thirty minutes prior to the start, mice are placed in a dark testing room. The trial is videotaped and timed manually. Anxiety-like behaviour is characterized by the time the subject spends outside the shelter and the latency time before exiting the first time (Pogorelov et al. 2007).

2.5.5. Open field test (OFT)

The OFT investigates the exploratory behaviour and general activity of rodents. The open field is a square box (60 cm x 60 cm; height 60 cm) with surrounding walls that prevent escape, manufactured in clear Plexiglas, with black opaque walls that prevent observation of visual cues outside the arena. The centre of the arena (150 lux) was defined as the central 40 cm × 40 cm zone. Total movement and distance travelled are calculated as a measure of exploratory and locomotor behaviour, while the time spent and distance travelled in the centre zone and number of rearings are measures for anxiolytic-like behaviour. The experiment is recorded by a video tracking system (Ethovision software, Noldus, The Netherlands). Both 5 min (for acute behaviour) and 30 min time bins (for longer term exploration and acclimatization) are integrated from the same trial.

2.6.Statistical analysis

Data of the relative xCT protein expression levels are expressed as mean ± standard error of the mean (SEM) and are analysed applying a two-sided Mann-Whitney U-test. All behavioural outcomes are expressed as mean + SEM and are analysed by a two-way ANOVA followed by Tukey's post hoc tests. The α value was set at 0.05. Statistical analyses were performed using GraphPad Prism 6.01 software.

3. RESULTS

3.1.xCT protein levels in depression related brain areas

We investigated the protein levels of xCT in brain samples of different animal models for depression and in *post-mortem* tissue of depressed patients (Fig. 2). In the CORT mouse model, xCT expression did not differ between SHAM and CORT treated C57BL/6J mice in any of the investigated brain regions, i.e. hippocampus (P = 0.7768, Fig. 2A), cingulate cortex (P = 0.9305, Fig. 2B), nucleus accumbens (P = 0.5231, Fig. 2C) or amygdala (P = 0.8211, Fig. 2D). Also in the FSL rat model, no significant differences were observed compared to control SD rats: hippocampus (P = 0.3814, Fig. 2E), cingulate cortex (P = 0.9305, Fig. 2F), nucleus accumbens (P = 0.3754, Fig. 2G) and amygdala (P = 0.3663, Fig. 2H). Finally the comparison of xCT protein expression in *post-mortem* tissue of depressed patients versus non-depressed controls showed no significant difference in both the hippocampus (P = 0.6753, Fig. 2I) and cingulate cortex (P = 0.5152, Fig. 2J). All individual relative expression levels of the human *post-mortem* samples are also summarized in Table 1.

3.2.Behavioural analysis of xCT-/- and xCT+/+ mice after chronic CORT treatment and social isolation

Depression-like behaviour was assessed in two different behavioural despair paradigms, the FST and MTS. In the immobility mean counts of the FST, no significant difference was observed between SHAM and CORT treatment [treatment factor: F1,95 = 0.05383, P = 0.817; Fig. 3A], but an overall trend towards decreased immobility was observed in xCT-/- mice [genotype factor: F1,95 = 3.425, P = 0.0673]. CORT treatment induced a significant overall difference in the swimming mean counts [treatment factor: F1,95 = 5.795, P = 0.018; Fig. 3B] and climbing mean counts [treatment factor: F1,95 = 23.63, P < 0.0001; Fig. 3C]. No difference is observed when comparing both genotypes in swimming [genotype factor: F1,95 = 1.589, P = 0.2106] or climbing behaviour [genotype factor: F1,95 = 0.8496, P = 0.359]. In the MTS, both a significant treatment [treatment factor: F1,92 = 31.8, P < 0.0001; Fig. 3D] and genotype effect [genotype factor: F1,92 = 5.719, P = 0.0188] was observed; Tukey's multiple comparisons post hoc test revealed only a significantly decreased immobility in SHAM treated xCT-/- versus xCT+/+ mice (P = 0.0199). It is interesting to mention that the increase in immobility time from the "SHAM treated mice" to the "CORT treated mice" is higher in xCT-/- mice, compared to xCT+/+ mice, as the relative increase in immobility time is significantly different between both groups (P = 0.0263, as calculated by means of a t-test).

The NSF is a paradigm to assess both depression- and anxiety-like behaviour. Here the latency time to feeding was significantly increased with CORT treatment [treatment factor: F1,96 = 12.93, P = 0.0005; Fig. 3E], but not with genotype [genotype factor: F1,96 = 0.4319, P = 0.5126]. The LDP showed a clear increase in anxiety-like behaviour (time outside shelter and latency time to exit shelter) with CORT treatment [treatment factor: F1,96 = 21.8, P <

0.0001; Fig. 3F], [treatment factor: F1,94 = 10.65, P = 0.0015; Fig. 3G]. On the other hand, both parameters were not significantly altered when comparing xCT-/- and xCT+/ mice [time outside, genotype factor: F1,96 = 0.4812, P = 0.4895] and [latency to exit, genotype factor: F1,95 = 3.002, P = 0.0865] respectively. A final measure for anxiety-like behaviour is the time in the centre in the OFT. Both in the 5 min trial and 30 min trial no significant difference was observed with treatment [5 min, treatment factor: F1,96 = 2.225, P = 0.1391; Fig. 3H], [30 min, treatment factor: F1,96 = 1.052, P = 0.3076; Fig. 3J]. xCT deletion had only a significant effect in the 30 min trial, with increased time in the centre in xCT-/- mice [genotype factor: F1,96 = 7.002, P = 0.0095] but not in the first 5 min of the test [genotype factor: F1,96 = 0.06025, P = 0.8066].

Finally, neither treatment nor genotype had an effect on locomotor activity (as measured with distance travelled) in the first 5 min or total 30 min trial of the OFT [5 min, treatment factor: F1,96 = 0.6178, P = 0.4338 / genotype factor: F1,96 = 0.2371, P = 0.6274; Fig. 3I], [30 min, treatment factor: F1,94 = 0.2844, P = 0.5951 / genotype factor: F1,96 = 0.3406, P = 0.5608; Fig. 3K].

3.3. Behavioural analysis of CORT administered xCT-/- and xCT+/+ mice after treatment with NAC

An additional cohort of SHAM and CORT exposed xCT+/+ and xCT-/- mice was treated with NAC. In the MTS, NAC treatment had no effect on immobility time in SHAM exposed mice [treatment factor: F1,18 = 0.04205, P = 0.8398 and genotype factor: F1,18 = 2.686, P = 0.1186; Fig. 4A], but it induced a significant decrease in immobility time in CORT exposed mice [treatment factor: F1,26 = 4.422, P = 0.0453], independent of genotype [genotype factor: F1,26 = 0.6999, P = 0.4104; Fig. 4B]. Apart from an antidepressive-like effect in the

MTS, NAC treatment did not significantly affect anxiety-like behaviour in SHAM or CORT treated xCT+/+ and xCT-/- mice (data not shown).

4. DISCUSSION

Presently the glutamate hypothesis of depression receives a lot of attention as a downregulation of EAAT expression levels has been uncovered both in animal models of depression (Chen, Yao, Xu, Qian, Wang, Liu, Wang and Wang 2014, Zink et al. 2010) and in human *post-mortem* studies (Choudary et al. 2005). ~~F~~urthermore several pharmacological agents, such as mGluR2/3 antagonists, have proven to exert antidepressant-like effects (Ago et al. 2013) and extrasynaptic NMDARs seem to be especially important for excitotoxicity (Parsons and Raymond 2014). Another key regulator of extracellular glutamate is system xc-, which is a major contributor to extracellular glutamate in hippocampus and striatum (De Bundel, Schallier, Loyens, Fernando, Miyashita, Van Liefferinge, Vermoesen, Bannai, Sato, Michotte, Smolders and Massie 2011, Massie, Schallier, Kim, Fernando, Kobayashi, Beck, De Bundel, Vermoesen, Bannai, Smolders, Conrad, Plesnila, Sato and Michotte 2011). Because this system xc- - dependent glutamate release can activate extrasynaptic receptors, changes in functioning of system xc- might impact the physiological functioning of the glutamatergic synapse (Bridges et al. 2012).

In the current research paper we provide evidence for unaffected expression levels of the specific subunit of system xc- (xCT) in various relevant preclinical and clinical tissue samples of depression. First of all, we observed unaffected xCT protein expression in different depression- and anxiety-related brain areas, i.e. hippocampus, cingulate cortex, nucleus accumbens and amygdala, in both the CORT mouse model (Demuyser, Deneyer, Bentea, Albertini, Van Liefferinge, Merckx, De Prins, De Bundel, Massie and Smolders 2015) and the widely used genetic FSL rat model (Femenia et al. 2015), compared to proper controls. Next, we observed unaltered xCT protein expression levels in *post-mortem* hippocampal and cingulate cortex tissue of depressed patients compared to non-depressed controls, in accordance to our observations in the animal models. Yet, an important side note should be made regarding the age of the depressed patients and matched control subjects included in this study. Since age-related changes in protein expression can be complex and variable, late-life depression can entail a more heterogeneous set of neurochemical changes, different from adult depression (McCullumsmith et al. 2014). In our previous study, we observed age-related interactions between loss of system xc- and depressive-like behaviour in mice, with a stronger antidepressant-like behaviour in the FST in aged xCT-/- mice compared to age-matched xCT+/+ counterparts (Bentea, Demuyser, Van Liefferinge, Albertini, Deneyer, Nys, Merckx, Michotte, Sato, Arckens, Massie and Smolders 2015). Although the latter data suggest an alternative involvement of system xc- in late-life depression, we did not observe any differences in xCT expression in our aged patient population. Another remark is warranted with regard to the statistical rigor in this section. Since only a limited sample set of human tissues of depressed patients and matched control subjects could be obtained to compare xCT protein expression levels, the analysis of hippocampal and cingulate cortex tissue is lacking power. The probability that subtle changes are not detected is rather high, with a consequent important chance of retaining false negative data. Therefore we should be careful drawing conclusions with regard to the xCT expression levels in the *post-mortem* human tissue.

Lack of changes in protein expression levels is not sufficient to exclude possible beneficial effects of targeting a certain protein with pharmacological intervention. Therefore we opted to further unravel the role of system xc-, using mice lacking functional system xc- and their wildtype controls. In our previous report, we described a clear reduction in depression- and anxiety-like behaviour of naive xCT-/- mice (below further referred to as "non-stressed mice") compared to wildtype littermates, in several acute-stress based behavioural paradigms (Bentea, Demuyser, Van Liefferinge, Albertini, Deneyer, Nys, Merckx, Michotte, Sato, Arckens, Massie and Smolders 2015). In the present study we took a next step towards clinical translation and subjected xCT-/- and xCT+/+ mice to a chronic excessive stressor of three weeks daily CORT injections and social isolation (depicted below as "excessive chronically stressed mice"), and compared them to matched group-housed mice receiving SHAM injections (denoted as "mild chronically stressed mice" since they receive a daily painful injection during 21 days). A clear overall increase in depression- and anxiety-like behaviour independent of genotype was observed in the "excessive chronically stressed mice", as described previously in the same background strain (Demuyser, Deneyer, Bentea, Albertini, Van Liefferinge, Merckx, De Prins, De Bundel, Massie and Smolders 2015). In addition to our previous observations, we see a clear overall increase in swimming behaviour and decrease in climbing behaviour in the excessive chronically stressed mice, compared to controls and independent of genotype. This shift from climbing to swimming behaviour could possibly be related to changes in monoaminergic neurotransmission (Slattery and Cryan 2012). Furthermore, we observe a significant decrease in depression-like behaviour in the MTS with absence of functional system xc- and an overall non-significant trend in reduced immobility mean counts in the FST. The findings in the MTS seem mostly related to a difference in the "mild chronically stressed mice", since a subanalysis of the "excessive chronically stressed mice" does not show any differences between both genotypes. In the

MTS, absence of functional system xc- seems protective in “non-stressed mice” (Bentea, Demuyser, Van Liefferinge, Albertini, Deneyer, Nys, Merckx, Michotte, Sato, Arckens, Massie and Smolders 2015) and in “mild chronically stressed mice”. After subjecting these mice to a chronic excessive stressor, that is CORT injections and social isolation, the antidepressive-like effect of system xc- deletion disappears. It is important to mention that the increase in immobility time from the “mild chronically stressed mice” to the “excessive chronically stressed mice” is higher in xCT-/- mice compared to xCT+/+ mice. This difference could point at a suppressive effect of system xc- activity on chronic excessive stress-mediated changes in depressive-like behaviour or to the possibility that the protective effect of absence of functional system xc- is only present in “non-stressed mice” and “mild chronically stressed mice”, not in “excessive chronically stressed mice”. Similar effects are depicted in the tests for anxiety-like behaviour, since the anxiolytic-like effects that were observed in “non-stressed mice” lacking functional system xc-, seem to disappear in “mild chronically stressed” and “excessive chronically stressed mice”. Indeed in our previous report we showed a clear increase in time outside shelter in the LDP and decrease in latency time to feeding in the NSF in “non-stressed xCT-/- mice”, compared to their wildtype littermates (Bentea, Demuyser, Van Liefferinge, Albertini, Deneyer, Nys, Merckx, Michotte, Sato, Arckens, Massie and Smolders 2015). After “mild chronic stress” or “excessive chronic stress”, no differential responses in genotype are observed in LDP or NSF. These findings seem to point at a diminishing protective effect of absence of functional system xc-, with increased chronic stress. Since chronic stress causes dynamic changes in receptor composition and trafficking, the downstream effects of extrasynaptic glutamate release through system xc- might be different in “non-stressed” versus “chronically stressed” mice (Popoli, Yan, McEwen and Sanacora 2012). A sole non-depression related observation that is consistent in all (“non-stressed” to “excessive chronically stressed”) xCT-/- mice, is the increased time in centre zone in the 30 min OFT trials. These data, therefore, suggest that xCT-/- mice habituate faster to a novel environment compared to xCT+/+ littermates, even after chronic excessive stress.

To elaborate on the involvement of system xc- in the previously reported antidepressant effects of NAC in a rat model of depression, we treated "chronically stressed" xCT-/- and xCT+/+ mice with a similar dose and treatment duration (Smaga, Pomierny, Krzyzanowska, Pomierny-Chamiolo, Miszkiel, Niedzielska, Ogorka and Filip 2012). Smaga and colleagues described a significant antidepressant-like effect of NAC treatment (100 mg/kg/day, during 10 days) in bulbectomized rats, but they did not observe any effect of NAC in the SHAM operated animals. We do not observe any effect of NAC treatment in the "mild chronically stressed mice", but we do see a clear decrease in immobility time with NAC treatment in the "excessive chronically stressed mice", both in xCT-/- and xCT+/+ mice. These effects are consequently not related to system xc-. Possibly the antidepressant-like effects of NAC are related to the antioxidative actions of cysteine and GSH, without the involvement of system xc-. Indeed NAC is the precursor of cysteine, an antioxidative molecule that can have an important effect on the redox balance without its conversion to cystine and import through system xc-. Also NAC itself, without being a precursor of cysteine, can directly act as an antioxidant without the need to first produce GSH (Kerksick and Willoughby 2005). Since MDD is associated with increased oxidative stress (Reus et al. 2015), a shift in the redox balance might be protective in the current animal model. Indeed NAC has been shown to decrease oxidative parameters in the depressive state (Arent et al. 2012, Smaga, Pomierny, Krzyzanowska, Pomierny-Chamiolo, Miszkiel, Niedzielska, Ogorka and Filip 2012) and increase circulating GSH in depressed patients (Berk et al. 2008).

In conclusion, xCT protein expression is not altered in several brain regions of the corticosterone mouse model, the Flinders sensitive rat model and depressed patients. xCT deletion does not affect depression-associated behaviours in the corticosterone mouse model. Finally, antidepressant-like effects exerted by N-acetylcysteine seem not dependent on a functional system xc-.

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DECLARATION OF INTEREST

The authors report no conflicts of interest

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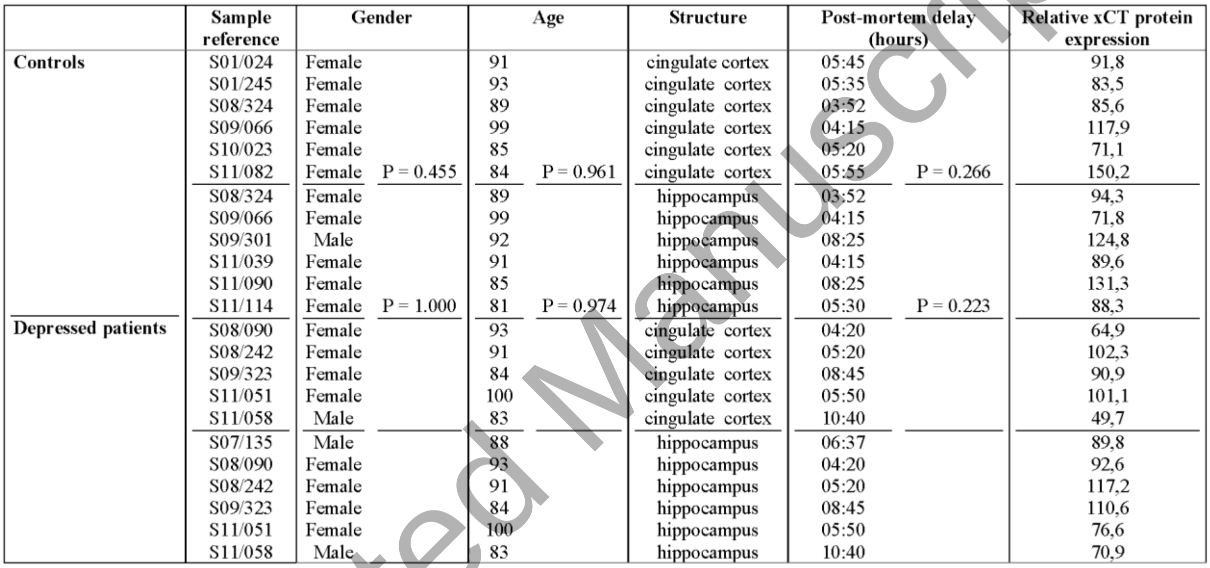
TABLES AND FIGURES

Table 1 Overview of *post-mortem* human samples provided by The Netherlands Brain Bank. Both controls and depressed patients are depicted with corresponding sample reference, gender, age at death, dissected brain structure, *post-mortem* delay and relative xCT protein expression per sample. Age and *post-mortem* delay, and gender are not significantly different between test groups, as analysed by means of respectively the two-sided Mann–Whitney U-test and Fisher's exact test, P-values indicated.



	Sample reference	Gender		Age		Structure	Post-mortem delay (hours)		Relative xCT protein expression
Controls	S01/024	Female		91		cingulate cortex	05:45		91,8
	S01/245	Female		93		cingulate cortex	05:35		83,5
	S08/324	Female		89		cingulate cortex	03:52		85,6
	S09/066	Female		99		cingulate cortex	04:15		117,9
	S10/023	Female		85		cingulate cortex	05:20		71,1
	S11/082	Female	P = 0.455	84	P = 0.961	cingulate cortex	05:55	P = 0.266	150,2
	S08/324	Female		89		hippocampus	03:52		94,3
	S09/066	Female		99		hippocampus	04:15		71,8
	S09/301	Male		92		hippocampus	08:25		124,8
	S11/039	Female		91		hippocampus	04:15		89,6
	S11/090	Female		85		hippocampus	08:25		131,3
	S11/114	Female	P = 1.000	81	P = 0.974	hippocampus	05:30	P = 0.223	88,3
Depressed patients	S08/090	Female		93		cingulate cortex	04:20		64,9
	S08/242	Female		91		cingulate cortex	05:20		102,3
	S09/323	Female		84		cingulate cortex	08:45		90,9
	S11/051	Female		100		cingulate cortex	05:50		101,1
	S11/058	Male		83		cingulate cortex	10:40		49,7
	S07/135	Male		88		hippocampus	06:37		89,8
	S08/090	Female		93		hippocampus	04:20		92,6
	S08/242	Female		91		hippocampus	05:20		117,2
	S09/323	Female		84		hippocampus	08:45		110,6
	S11/051	Female		100		hippocampus	05:50		76,6
	S11/058	Male		83		hippocampus	10:40		70,9

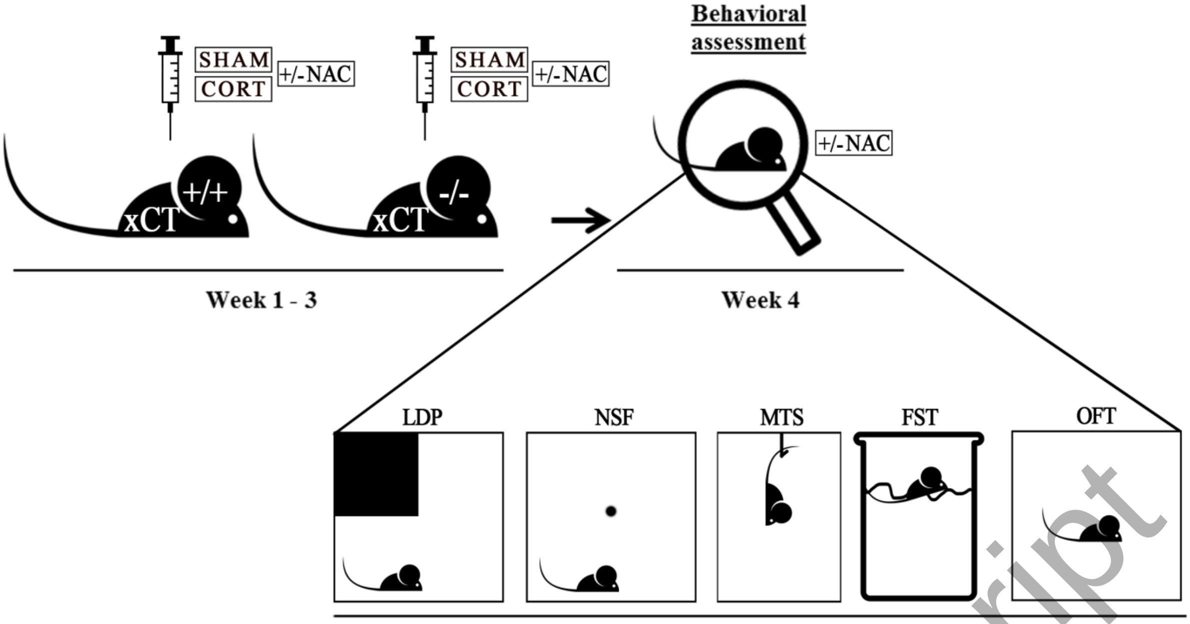

SHAM
CORT
+/-NAC
xCT
+/+
SHAM
CORT
+/-NAC
xCT
-/-
Week 1 - 3
Behavioral assessment
+/-NAC
Week 4
LDP
NSF
MTS
FST
OFT

Figure 1

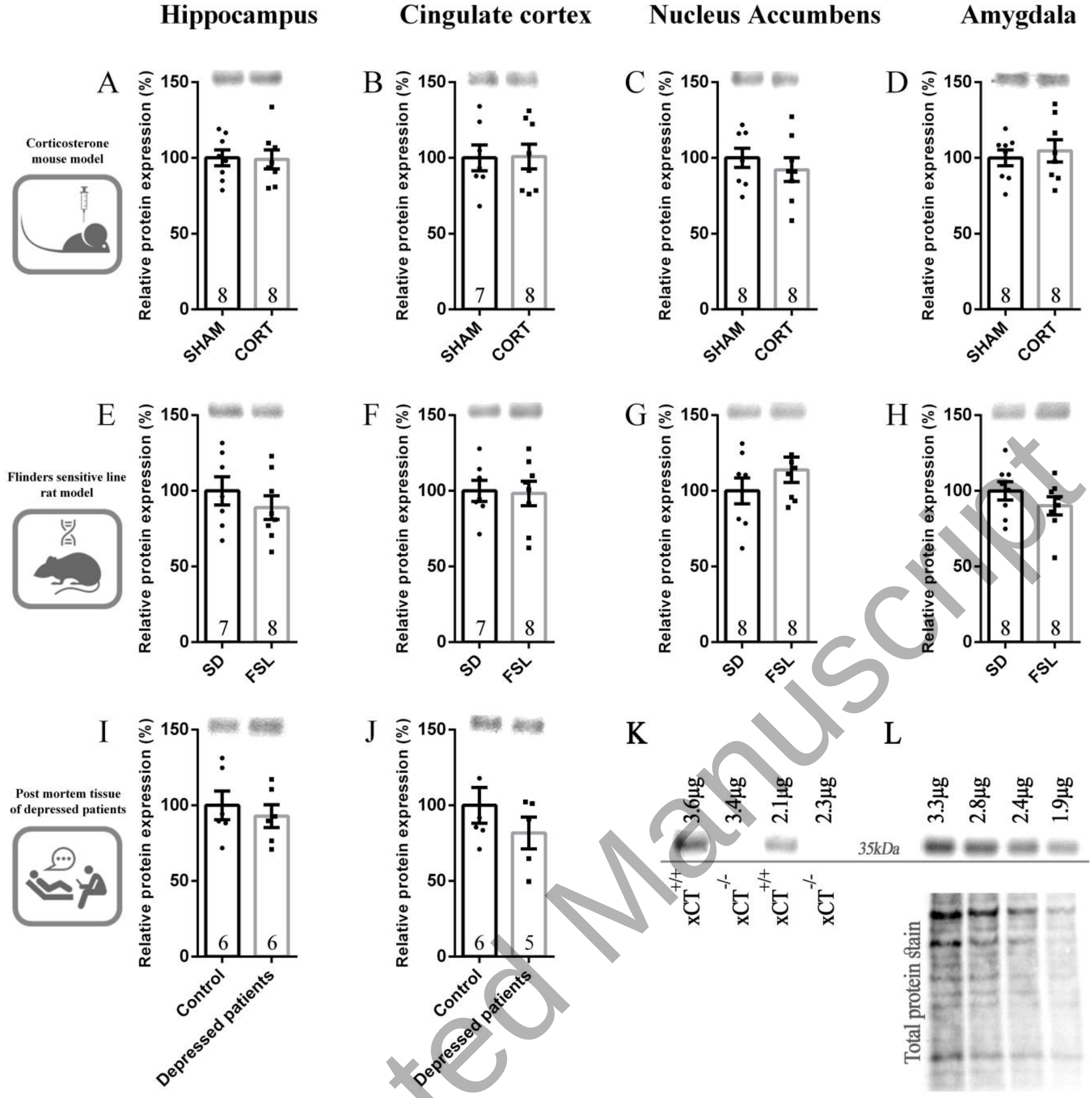

Hippocampus
Cingulate cortex
Nucleus Accumbens
Amygdala
A
B
C
D
E
F
G
H
I
J
K
L
Corticosterone mouse model
Flinders sensitive line rat model
Post mortem tissue of depressed patients
Relative protein expression (%)
0
50
100
150
SHAM
CORT
SD
FSL
Control
Depressed patients
8
7
6
5
3.6µg
3.4µg
2.1µg
2.3µg
xCT+/+
xCT-/-
xCT+/+
xCT-/-
35kDa
3.3µg
2.8µg
2.4µg
1.9µg
Total protein stain

Figure 2

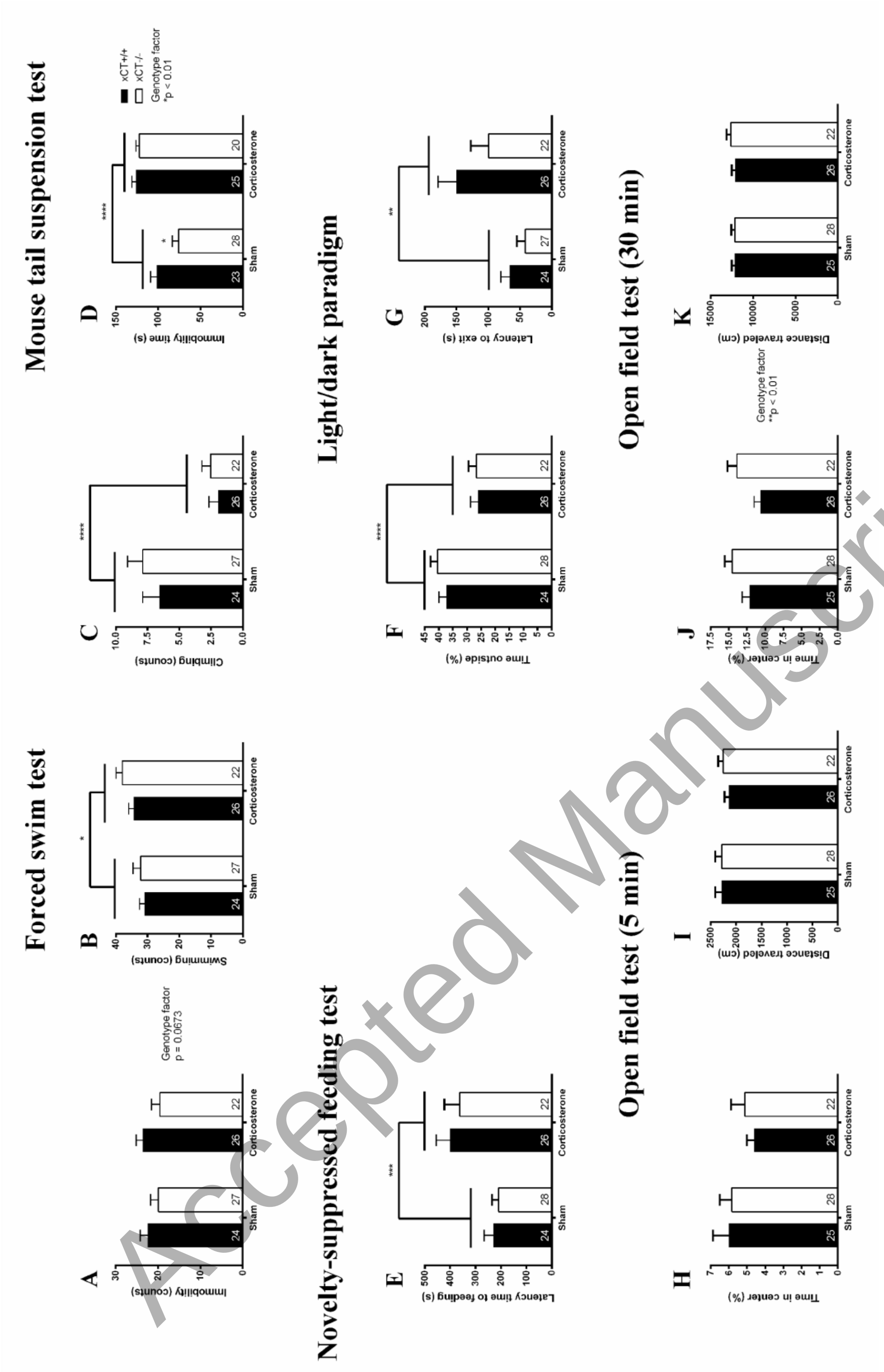

Forced swim test
A
Immobility (counts)
30
20
10
0
24
27
26
22
Sham
Corticosterone
Genotype factor
p = 0.0673
B
Swimming (counts)
40
30
20
10
0
*
24
27
26
22
C
Climbing (counts)
10.0
7.5
5.0
2.5
0.0
****
24
27
26
22
Mouse tail suspension test
D
Immobility time (s)
150
100
50
0
****
*
23
28
25
20
xCT+/+
xCT-/-
Genotype factor
*p < 0.01
Novelty-suppressed feeding test
E
Latency time to feeding (s)
500
400
300
200
100
0
***
24
28
26
22
Light/dark paradigm
F
Time outside (%)
45
40
35
30
25
20
15
10
5
0
****
24
28
26
22
G
Latency to exit (s)
200
150
100
50
0
**
24
27
26
22
Open field test (5 min)
H
Time in center (%)
7
6
5
4
3
2
1
0
25
28
26
22
I
Distance traveled (cm)
2500
2000
1500
1000
500
0
25
28
26
22
Open field test (30 min)
J
Time in center (%)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
25
28
26
22
Genotype factor
**p < 0.01
K
Distance traveled (cm)
15000
10000
5000
0
25
28
26
22

Figure 3

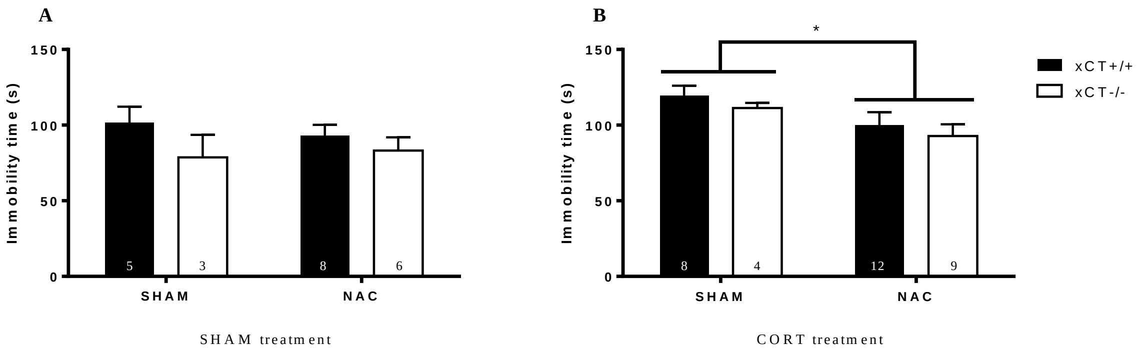

A
B
150
100
50
0
Immobility time (s)
5
3
8
6
SHAM
NAC
SHAM treatment
*
8
4
12
9
CORT treatment
xCT+/+
xCT-/-

Figure 4

Fig. 1 Graphical schedule of treatments and behavioural experiments

On day one of the protocol, $xCT^{+/+}$ and $xCT^{-/-}$ littermates were randomly assigned to one of the test groups (i.e. SHAM or CORT treated). After three weeks of CORT treatment and single housing or SHAM treatment and group housing, all mice were subjected to a series of tests for depression- and anxiety-like behaviour. In mice that were included in the NAC study, the last 10 days of CORT treatment were combined with an additional NAC or SHAM injection. These injections continued during behavioural assessment, with a final SHAM/NAC injection administered 30 min before the test.

(LDP: light/dark paradigm, NSF: novelty-suppressed feeding test, MTS: mouse-tail suspension test, FST: forced swim test, OFT: open field test, NAC: N-acetylcysteine)

Fig. 2 xCT protein expression levels in depression versus appropriate control samples

(A – D) in the corticosterone mouse model, (E – H) in the Flinders Sensitive Line rat model and (I, J) in *post-mortem* tissue of depressed patients. Several depression-related brain areas are depicted: hippocampus, cingulate cortex, nucleus accumbens and amygdala. Each graph is accompanied by a representative example of a Western blot (xCT ~35 kDa). xCT expression levels of the respective control groups were set to 100% and the expression levels of the “depressed” groups were expressed as a percentage of the corresponding controls (similar protein concentrates were loaded). Data are presented as mean ± SEM and were analysed by means of the two-sided Mann–Whitney U-test, sample size indicated in the graph. Technical controls: hippocampal tissue from $xCT^{+/+}$ and $xCT^{-/-}$ mice depicting the specificity of the xCT antibody (K) and a dilution series of hippocampal mouse tissue (L), total protein loaded is indicated in the graph.

Fig. 3 Behavioural analysis of xCT-/- and xCT+/+ mice, subjected to the corticosterone mouse model for depression and anxiety.

In the forced swim test, a trend towards a genotype effect was seen in the immobility mean counts (A). Furthermore a significant treatment effect was observed in the swimming (B) and climbing counts (C). In the mouse tail suspension test, a significant genotype and treatment effect are observed, but only in the SHAM treated mice the Tukey's post hoc test revealed a significant effect of xCT deletion (D). In the novelty-suppressed feeding test (E) and light/dark paradigm (F, G) only a significant treatment (but no genotype) effect is observed. Finally, in the open field test a significant genotype effect was observed only in the 30 min trial (J) but not in the first 5 min trial (H). No genotype nor treatment effect was furthermore observed in the total distance travelled (I, K). Data are presented as mean + SEM ****P < 0.0001, ***P < 0.001, ***P* < 0.01, **P* < 0.05 (2-way ANOVA and Tukey's post-hoc test for comparison of xCT-/- versus xCT+/+ mice), sample size indicated in the figure.

Fig. 4 Behavioural analysis in the mouse-tail suspension test of xCT-/- and xCT+/+ mice, subjected to the corticosterone (CORT) mouse model and treated with N-Acetylcysteine (NAC).

NAC significantly reduces immobility time in the CORT (B) (but not in SHAM (A)) treated animals, independent of genotype. Data are presented as mean + SEM **P* < 0.05 (2-way ANOVA), sample size indicated in the figure.