Absence of system xc- in mice decreases anxiety and depressive-like behavior without affecting sensorimotor function or spatial vision

Eduard Bentea[1*], Thomas Demuyser[2*], Joeri Van Liefferinge[2], Giulia Albertini[2], Lauren Deneyer[1], Julie Nys[3], Ellen Merckx[1], Yvette Michotte[2], Hideyo Sato[4], Lutgarde Arckens[3], Ann Massie[1*], Ilse Smolders[2*#]

[1]Department of Pharmaceutical Biotechnology and Molecular Biology, Center for Neurosciences, Vrije Universiteit Brussel, Brussels, Belgium

[2]Department of Pharmaceutical Chemistry and Drug Analysis, Center for Neurosciences, Vrije Universiteit Brussel, Brussels, Belgium

[3]Laboratory of Neuroplasticity and Neuroproteomics, Department of Biology, KU Leuven, Leuven, Belgium

[4]Laboratory of Biochemistry and Molecular Biology, Department of Medical Technology, Faculty of Medicine, Niigata University, Niigata, Japan

* These authors contributed equally to the work

#Correspondence:
Prof. Dr. Ilse Smolders
Vrije Universiteit Brussel
Center for Neurosciences
Department of Pharmaceutical Chemistry and Drug Analysis
Laarbeeklaan 103
Brussels, Belgium
Ilse.Smolders@vub.ac.be
+32 2 477 47 47

Double names: Joeri Van Liefferinge (first name “Joeri”, last name “Van Liefferinge”)

Abstract:

There is considerable preclinical and clinical evidence indicating that abnormal changes in glutamatergic signaling underlie the development of mood disorders. Astrocytic glutamate dysfunction, in particular, has been recently linked with the pathogenesis and treatment of mood disorders, including anxiety and depression. System xc- is a glial cystine/glutamate antiporter that is responsible for nonvesicular glutamate release in various regions of the brain. Although system xc- is involved in glutamate signal transduction, its possible role mediating anxiety or depressive-like behaviors is currently unknown. In the present study, we phenotyped adult and aged system xc- deficient mice in a battery of tests for anxiety and depressive-like behavior (open field, light/dark test, elevated plus maze, novelty suppressed feeding, forced swim test, tail suspension test). Concomitantly, we evaluated the sensorimotor function of system xc- deficient mice, using motor and sensorimotor based tests (rotarod, adhesive removal test, nest building test). Finally, due to the presence and potential functional relevance of system xc- in the eye, we investigated the visual acuity of system xc- deficient mice (optomotor test). Our results indicate that loss of system xc- does not affect motor or sensorimotor function, in either adult or aged mice, in any of the paradigms investigated. Similarly, loss of system xc- does not affect basic visual acuity, in either adult or aged mice. On the other hand, in the open field and light/dark tests, and forced swim and tail suspension tests respectively, we could observe significant anxiolytic and antidepressive-like effects in system xc- deficient mice that in certain cases (light/dark, forced swim) were age-dependent. These findings indicate that, under physiological conditions, nonvesicular glutamate release via system xc- mediates aspects of higher brain function related to anxiety and depression, but does not influence sensorimotor function or spatial vision. As such, modulation of system xc- might constitute the basis of innovative interventions in mood disorders.

Keywords:

system xc-, xCT, anxiety, depression, sensorimotor, vision

Abbreviations:

NMDA, N-Methyl-D-Aspartate; xCT-/-, xCT knock-out mice; xCT+/+, xCT wild-type mice

1. Introduction

Depression and anxiety are among the most prevalent mood disorders in our current society. Therapeutic management of these psychiatric disorders relies on classical approaches targeting the monoaminergic neurotransmission systems, which although they have proven their usefulness, are limited in terms of efficacy and side-effects profile (Li et al., 2012). Lack of innovative therapies for anxiety and depression is related to an incomplete understanding of the pathophysiological basis of these complex disorders and the limited knowledge about the cellular downstream adaptations causing the slow onset of the current monoaminergic drugs (Li et al., 2012; Sanacora et al., 2008). Glutamate, the major excitatory neurotransmitter in the brain, has been implicated in the manifestation of stress and mood disorders (Popoli et al., 2012). The finding of altered glutamate levels in plasma and cerebrospinal fluid, as well as changes in glutamate content in brain tissue of patients with mood disorders, indicates a possible pathogenic contribution (Li et al., 2012; Sanacora et al., 2008). Clinical proof-of-concept studies show rapid antidepressant activity of N-Methyl-D-Aspartate (NMDA) receptor antagonists, such as ketamine, in patients with major depressive disorder (Li et al., 2012; Zarate et al., 2010). Alterations in astrocytic glutamate regulation, including perturbation in glutamate reuptake mechanisms and glutamate metabolism have been linked with depression (Gomez-Galan et al., 2013; Popoli et al., 2012). Finally, increasing evidence points to the involvement of glial cell pathology in disease processes associated with mood and anxiety disorders (Sanacora and Banasr, 2013; Sanacora et al., 2008).

System xc- is a glial plasma membrane antiporter that imports cystine and exports glutamate to the extracellular environment in a 1:1 ratio (Lewerenz et al., 2013). Structurally, system xc- is a heterodimer composed of 4F2hc and xCT, with xCT being the specific subunit and mediating the transport function of the antiporter (Lewerenz et al., 2013). We have previously demonstrated that in regions of the brain such as the striatum (Massie et al., 2011) or hippocampus (De Bundel et al., 2011), nonvesicular glutamate release by system xc- is the major source of extracellular glutamate, which in turn might mediate tonic activation of extrasynaptic glutamate receptors, including group I and group II metabotropic glutamate receptors as well as extrasynaptic NMDA receptors (Bridges et al., 2012). Via these pathways, system xc- is thought to play a modulatory role on the glutamatergic signaling in the brain. Nevertheless, the possible effect of system xc- on emotional features of behavior, such as those related to anxiety or depressive-like behavior, is currently unknown. These gaps in our understanding of the functional relevance of system xc- in the brain are also a result of

lack of selective pharmacological modulators (Lewerenz et al., 2013), consequently genetic approaches offer an alternative way to dissect the contribution of specific proteins to behavior (Cryan and Holmes, 2005). We have previously indicated that mice lacking xCT (xCT-/-) (Sato et al., 2005) do not demonstrate severe motor dysfunction, have an intact spatial reference memory, but demonstrate an impaired spatial working memory (in adult but not aged mice), potentially due to the decreased extracellular glutamate levels in the hippocampus (De Bundel et al., 2011). In the current study, we extend our behavioral characterization of system xc- deficient mice, with particular focus on anxiety and depressive-like behavior. Furthermore, as system xc- is potentially expressed in areas of the brain regulating sensorimotor function, such as the basal ganglia (Baker et al., 2002; Massie et al., 2011) and cerebellum (Sato et al., 2002; Shih et al., 2006), we evaluated differences in sensorimotor function in xCT-/- mice compared to xCT+/+ littermates. For both affective and sensorimotor function, we employed a battery of behavioral tests to reduce false negative results and evaluate specific aspects of behavior (Crawley, 2008). Finally, due to the presence and potential functional relevance of system xc- in the eye (Bridges et al., 2001; Langford et al., 2010; Lim et al., 2005), we investigated whether loss of xCT would lead to impairment in visual acuity. We included in our phenotypic screen both adult (16-20 weeks old) as well as aged (19-23 months old) mice, in order to evaluate whether changes in behavior would be consistently observed across aging, as well as to investigate whether loss of system xc- affects the aging process in terms of behavior.

2. Materials and Methods

2.1. Animals

This phenotyping study was performed on adult (16-20 weeks old) and aged (19-23 months old) male xCT-/- mice and xCT+/+ littermates. The mice used in this study are high-generation descendants of the strain described previously (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. Mice were group-housed (2-6 mice per cage) under standardized conditions (25^{o}C, 10/14 h dark/light cycle), with free access to food and water. Studies were performed according to national guidelines on animal experimentation and were approved by the Ethical Committee for Animal Experimentation of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel.

2.2. Genotyping

Genotypes were confirmed by PCR amplification of tail DNA using REDExtract-N-Amp Tissue PCR Kit (Sigma), and the following primers: 5′-GATGCCCTTC AGCTCGATGCGGTTCACCAG-3′ (GFPR3); 5′-CAGAGCAGCCCTAAGGCACTTTCC-3′ [mxCT5′flankF6]; 5′-CCGATGACGCTGCCGATGATGATGG-3′ [mxCT(Dr4)R8].

2.3. Phenotyping

In this retrospective study, behavioral phenotyping was performed at various time points during a two-year period, using seven different breeding batches of naive mice (no experimental history prior to behavioral assessment) matched by genotype and age. Tests were performed sequentially during a period of one month (starting from the least stressful test, e.g. open field test, to the more stressful, e.g. tail suspension test), with each test performed on a different day. The age of the mice at the beginning of the tests was 16-20 weeks old (for adult mice) or 19-23 months old (for aged mice). Not all mice included in this study were tested in all of the paradigms described. The initial batches of mice were tested for spontaneous activity (open field test), while subsequent batches also included specific tests evaluating sensorimotor function, vision, anxiety- or depressive-like behavior. Behavioral assessment was performed between 9:00 AM and 6:00 PM (during the light phase), with alternate testing of xCT-/- and xCT+/+ mice to ensure evaluation of both genotypes during the same time of the day. For tests requiring real-time behavioral scoring (nest building test,

adhesive removal test, optomotor test, novelty-suppressed feeding test), blinding for genotype was ensured by the presence of an additional researcher blinded to test order during the experiment. For tests integrated off-line (light/dark test, elevated plus maze test, forced swim test, tail suspension test), blinding for genotype was ensured by integrating the acquired video files in a blinded manner. For the remaining tests (rotarod, and open field tests), blinding for genotype was ensured by employing objective and automated integration systems (TSE RotarRod Systems, and Noldus Ethovision respectively). For each test, mice were acclimatized to the testing room at least 1 hour prior to assessment.

2.3.1. Open field test

The open field set-up consisted of a square box (60 cm x 60 cm) with surrounding walls (height 60 cm) that prevent escape, manufactured in clear poly(methyl-methacrylate) (Plexiglas), with black opaque walls that prevent observation of visual cues outside the arena. The center of the arena was defined as the central 40 cm x 40 cm zone. The light levels in the room created an illuminance of 150 lux at the center of the open field. Total distance traveled and frequency of rearing (as measures of exploratory behavior), as well as time spent in the center zone (as measure of anxiety-like behavior), were calculated. The experiment was recorded by a video tracking system (Ethovision software, Noldus, The Netherlands) for 60 minutes.

2.3.2. Rotarod test

Motor functions were investigated using an accelerating rotarod system (TSE RotaRod Advanced, TSE Systems). First, mice were trained for 5 minutes at a constant speed of 5 rpm. During this initial training phase, mice were placed immediately back on the rod after falling, allowing them to get familiarized to the test. In the second phase of training, mice underwent 3 repeated trials of 1 minute at a fixed speed of 5 rpm, with 3 minutes of rest between trials. For the test, mice underwent 5 repeated trials that started at constant speed of 5 rpm for 30 seconds, and continued with a 5 rpm – 25 rpm accelerating protocol during 200 seconds, leading to a maximum total rod time of 230 seconds. Mice were allowed 3 minutes of rest between trials. We applied statistical analysis on the mean of the 5 test trials.

2.3.3. Nest building test

Sensorimotor performance and skilled forelimb use were investigated using the nest building test, as described previously (Deacon, 2006). In this paradigm, mice were individually housed

overnight in a non-enriched cage, and challenged to build a nest starting from nesting material in order to provide shelter and heat insulation. The following morning, the quality of the nest was scored on a 0-5 scale, with 0 representing no nest, and 5 a perfect nest (Deacon, 2006). Furthermore, in order to provide a semi-independent objective assay of nesting ability, the amount of nesting material shredded was quantified, by weighing the complete nesting material (one pressed cotton Nestlet™ square) before the test, and weighing the untorn material at the end of the test.

2.3.4. Adhesive removal test

Sensorimotor performance was assessed using the adhesive removal test, as described previously (Bouet et al., 2009). After mice were habituated to a transparent test box for 60 seconds, small adhesive strips (0.3 cm x 0.4 cm) were taped on the plantar surface of both forelimbs, by applying equal pressure. Next, the mice were placed back in the test box. Two parameters were counted: time-to-contact, defined as the time required for the mouse to sense the presence of the adhesive (i.e. mouth to paw contact) and being indicative of correct paw and mouth sensitivity; and time-to-remove, defined as the time required to completely remove the adhesive from the paw, reflecting sensorimotor performance (Bouet et al., 2009). If the mouse did not feel or remove an adhesive during the trial, a maximum time of 120 seconds was given. The shortest time-to-contact and time-to-remove from the two forepaws underwent statistical analyses. Initially, mice were trained for 5 days by performing the test in identical conditions as the test condition. The adhesive placement order (left forepaw or right forepaw first) was alternated for each day of training during the first 4 days, and randomized for the last day of training, and for the test session.

2.3.5. Virtual-reality optomotor system

Visual acuity of mice was estimated using the detection of optokinetic head movements in the virtual-reality optomotor system developed by Prusky et al. (Prusky et al., 2004). Briefly, the testing apparatus (OptoMotry, CerebralMechanics) consisted of a box made of four computer screens (Four 20-inch LCD monitors) onto which a virtual cylinder comprised of a vertical sine wave grating was projected and a platform was situated at the epicenter of the arena. A video camera was secured to the top lid of the box, directly above the platform to observe the behavior of untrained and freely moving animals. Individual mice were exposed to moving sine wave gratings of different spatial frequencies at 100% contrast and at a fixed speed (12 degrees per second). The experimenter judged the presence of head movements in concert

with the cylinder rotation that represent their slow reflexive tracking of the gratings and a feeling of self-motion (Douglas et al., 2005). Marking the location between the eyes of the mouse with a crosshair provided positional information to the software to center the rotation of the cylinder at the mouse's viewing position, thereby maintaining the virtual walls of the cylinder at a constant distance from the mouse. Visual thresholds for spatial vision were obtained with a staircase procedure and drum rotation was random from trial to trial. By varying the spatial frequencies randomly and separate for each eye, the ability of the mouse visual system to detect the visual stimuli was determined and the highest spatial frequency capable of driving a response was adopted as the threshold.

2.3.6. Light/dark paradigm

The light/dark paradigm investigates the spontaneous exploratory behavior of rodents in response to mild stress, in this case being a novel and well-illuminated environment. The test apparatus consisted of an open field arena (60 cm x 60 cm; height 60 cm) manufactured in clear Plexiglas, with black opaque walls that prevent observation of visual cues outside the arena. A small dark compartment (one fourth of the total area), manufactured in black high-pressure laminate (Volkern-Trespa), was positioned in one of the corners of the open field arena. The light/dark test was performed in a dark room in which the open field arena was illuminated with three overhead lamps, creating a light contrast (illuminance outside the shelter 700 lux, inside the shelter 0.5 lux). In this conflict test, anxious behavior is investigated by comparing the innate exploratory activity of the mice with the preference for an enclosed, safe shelter. Mice demonstrate an aversion to brightly illuminated areas, and therefore prefer to stay in the small dark compartment during the trial. The test takes 5 minutes in total and mice were placed in the dark zone at the start. The trial was videotaped and timed manually by a blinded researcher. Anxiolytic behavior is characterized as the time the subject spends outside the shelter and the latency time before the first exit (Pogorelov et al., 2007).

2.3.7. Novelty-suppressed feeding test

The novelty suppressed feeding test is a conflict test that evokes competing motivations: the drive to eat versus the fear to enter the center of a brightly lit box. In this way hyponeophagia can be considered a parameter for both depressive- and anxiety-like behavior. The procedure was slightly adapted from Mineur et al. (Mineur et al., 2007). For this test, mice were deprived from food for 24 hours before the start of the experiment, water remained available

ad libitum. Each subject was placed in a corner of an open Plexiglas box (60 cm x 60 cm; height 60 cm) with a one cm layer of bedding and one food pellet in the center. The illuminance in the center of the open field arena was 150 lux. The time to the first feeding episode was recorded by a researcher blinded to the genotype.

2.3.8. Elevated plus maze

The elevated plus maze protocol is based on the aversive nature of mice for an open, elevated, unprotected area. We used a cross-shaped maze manufactured in black high-pressure laminate (Volkern-Trespa), and elevated to a height of 37 cm from the ground. The elevated plus maze consisted of two arms without walls and two enclosed by walls (32.5 cm length x 6 cm width x 17 cm height), with a center area of 6 cm x 6 cm (Rodgers et al., 1995). The illuminance at the level of the open arms was 150 lux. Mice were placed at the junction of the maze, facing a closed arm and allowed to explore the maze for 10 minutes. Each mouse was video-recorded during the test, and the researcher left the room after the start of the trial. Time spent in the open arms and the number of open arm entries are parameters for anxiolytic behavior. The duration and entries in the open and closed arms were timed manually by a blinded researcher.

2.3.9. Forced swim test

A modified version of the forced swim test, originally described by Porsolt et al., was used to assess depressive-like behavior (Porsolt et al., 1977). Mice were placed in a glass tank cylinder filled with 30 cm of water (25 ± 1 °C) and videotaped during 5 minutes. The inescapability of the set-up induces a state of helplessness. The light levels in the room created an illuminance of 400 lux at the level of the forced swim test. Cryan et al. described a time sampling technique, whereby the predominant behavior in each 5 second period of the 300 seconds test was recorded by a blinded researcher (Cryan et al., 2002). Three types of behavior were distinguished: climbing behavior consisted of upward-directed movements of the forepaws along the side of the swim tank (also called thrashing). Swimming behavior 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., 2002). 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.

306 *2.3.10. Tail suspension test*

307 Besides the forced swim test, the tail suspension test is one of the most widely used tests for
308 the examination of depressive-like behavior in mice. The set-up was similar to that described
309 by Steru et al. (Steru et al., 1985). Mice were suspended by the tip of their tail for 5 minutes to
310 induce an inescapable, short-term stress situation. The light levels in the room created an
311 illuminance of 400 lux at the level of the tail suspension test. The time of immobility was
312 measured by a blinded researcher and is considered a parameter for depressive-like behavior.
313 Mice that climbed their tail were excluded from the experiment.

314

315 *2.4. Statistical analysis*

316 Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were
317 performed using GraphPad Prism 4.0 software. For all analyses, we employed a two-way
318 ANOVA followed by Bonferroni post hoc tests. The α value was set at 0.05.

319

320

321

322

323

324

325

326

327

328

329

330

331

332

333

334

335

336

337

338

339

3. Results

3.1. Effect of xCT deletion on motor and sensorimotor function

The spontaneous horizontal and vertical motor activity of the xCT-/- and xCT+/+ mice was measured in a 60 minute open field test. Distance traveled, as measure of horizontal activity (see Fig. 1A for 5 minute time bins), was unaffected by the loss of xCT, as xCT-/- mice demonstrated similar activity levels compared to their xCT+/+ littermates irrespective of age, for either the first 5 minutes of the test [genotype factor: $F(1,128)=3.796$, $p=0.053$; Fig. 1B], or the entire 60 minute trial [genotype factor: $F(1,128)=0.460$, $p=0.499$; Fig. 1C]. Total distance traveled decreased with aging independent of genotype during the first 5 minutes of the test [age factor: $F(1,128)=62.96$, $p<0.0001$], but not during the entire 60 minute trial [age factor: $F(1,128)=1.473$, $p=0.227$]. Rearing, as measure of vertical activity (see Fig. 1D for 5 minute time bins), was similarly unaffected by the loss of xCT, as xCT-/- mice demonstrated similar activity levels compared to their xCT+/+ littermates irrespective of age, for either the first 5 minutes of the test [genotype factor: $F(1,128)=1.318$, $p=0.253$; Fig. 1E], or the entire 60 minute trial [genotype factor: $F(1,128)=1.376$, $p=0.243$; Fig. 1F]. The frequency of rearing decreased with aging, independent of genotype, for both the first 5 minutes of the test [age factor: $F(1,128)=84.98$, $p<0.0001$], as well as for the entire 60 minute trial [age factor: $F(1,128)=16.02$, $p=0.0001$]. Altogether, the open field data reveal age-related decreases in horizontal and vertical activity that occur in the absence of significant genotype effects, indicating that loss of xCT does not affect spontaneous motor activity.

In the rotarod test (Fig. 2A), xCT-/- mice performed equally as their xCT+/+ littermates, irrespective of age [genotype factor: $F(1,83)=0.615$, $p=0.435$], demonstrating intact motor coordination and balance. Concomitantly, we could notice an age-related decrease in motor function independent of genotype [age factor: $F(1,83)=17.08$, $p<0.0001$]. In the nest building test (Figs. 2B and C), xCT-/- mice performed equally well when compared to their xCT+/+ littermates, irrespective of age, both when comparing the nest score [genotype factor: $F(1,62)=0.152$, $p=0.697$], as well as the amount of nest building material shredded [genotype factor: $F(1,62)=0.169$, $p=0.682$]. As with the rotarod and open field tests, we could notice age-related decreases in motor function in the nest building test irrespective of genotype, both when evaluating the nest score [age factor: $F(1,62)=16.78$, $p=0.0001$], as well as the amount of nest building material shredded [age factor: $F(1,62)=19.00$, $p<0.0001$]. Finally, in the adhesive removal test (Figs. 3A and B), xCT-/- mice demonstrated equal sensorimotor

function compared to xCT+/+ littermates, both when evaluating time-to-contact [genotype factor: F(1,54)=0.636, p=0.428] as well as time-to-remove [genotype factor: F(1,54)=0.190, p=0.664].

3.2. Effect of xCT deletion on visual acuity

To assess the effect of xCT deficiency and age on basic mouse visual function, we examined the visual acuity of adult and aged xCT+/+ and xCT-/- littermates (Fig. 4) using the virtual-reality optomotor system established by Prusky and colleagues (Prusky et al., 2004). The maximal spatial frequency that elicits optokinetic tracking or head movements to follow the drifting gratings provides a proxy for mouse visual acuity in cycles per degree (c/d). Visual acuity values (maximal spatial frequency; c/d) of the left and right eye were not different within each group (adult xCT+/+ mice: p=0.706, t-test; adult xCT-/- mice: p=0.591, t-test; aged xCT+/+ mice: p=0.704, t-test; aged xCT-/- mice: p=0.227, t-test) and were therefore averaged for display. There was no significant genotype effect [genotype factor: F(1,27)=0.125, p=0.726], and no significant interaction between age and genotype [age x genotype factor: F(1,27)=0.026, p=0.872], hence no genotype-dependent effect on the visual acuity was observed in either adult or aged mice. In contrast, a significant decline in acuity was detected with aging in both genotypes [age factor: F(1,27)=34.84, p<0.0001]. This age-dependent decrease in spatial vision is consistent with previous findings in old C57BL/6-mice (Lehmann et al., 2012). Together, the behavior effects observed in xCT-/- mice in this study cannot be due to decreased or altered visual acuity since they show similar responses and age-dependency as xCT+/+ littermates in the optomotor test.

3.3. Effect of xCT deletion on anxiety-like behavior

In the 60 minute open field test (Fig. 5C; see Fig. 5A for 5 minute time bins), xCT-/- mice spent more time in the center of the arena compared to their xCT+/+ littermates [genotype factor: F(1,128)=14.45, p=0.0002], an anxiolytic-like effect confirmed for adult (p=0.003) as well as aged (p=0.017) mice. This effect was not observed, however, when evaluating the first 5 minutes of the test (Fig. 5B), during which xCT-/- mice spent a similar amount of time in the center compared to their xCT+/+ littermates [genotype factor: F(1,128)=1.196, p=0.276]. These findings indicate that, although the immediate reaction to the open field arena was similar between genotypes, xCT-/- mice habituated quicker to the novel environment (Fig. 5A). In the light/dark paradigm (Figs. 5D and E), xCT-/- mice spent more time outside the shelter compared to their xCT+/+ littermates [genotype factor: F(1,62)=4.835, p=0.031], an

effect that seemed to be mainly due to a significant difference observed in adult mice (p=0.013). On the other hand, no significant changes could be observed in the latency to exit the shelter between the two genotypes [genotype factor: F(1,62)=2.627, p=0.110]. Also in the light/dark paradigm, we could observe a global increase in anxiety-like behavior with aging independent of genotype, that seemed to be observed both when evaluating time spent outside the shelter [age factor: F(1,62)=14.60, p=0.0003] as well as the latency to exit the shelter [age factor: F(1,62)=7.242, p=0.0091]. This age-dependent increase in anxiety-like behavior confirms previous observations in rodents (Pietropaolo et al., 2014). In the novelty-suppressed feeding test (Fig. 4F), we could also notice a global anxiolytic-like effect in the xCT-/- mice independent of age [genotype factor: F(1,34)=4.395, p=0.043], as well as an increase in anxiety-like behavior with age independent of genotype [age factor: F(1,34)=8.434, p=0.006]. The decreased latency to feed in xCT-/- mice occurred in the absence of significant effects of the xCT deletion on feeding behavior, as evaluated over 24 hours in the home cage or in a metabolic cage (data not shown). Finally, in the elevated plus maze, no significant differences could be observed either in time spent in the open arms [genotype factor: F(1,32)=1.478, p=0.233; age factor: F(1,32)=0.919, p=0.345], or in the number of open arm entries [genotype factor: F(1,32)=2.855, p=0.101; age factor: F(1,32)=1.505, p=0.229]. The mice included in this study, however, demonstrated extreme low scores for elevated plus maze activity, independent of genotype or age, despite our best efforts to use a standardized protocol. The mean time spent in the open arms was 11.66 seconds out of a total trial time of 600 seconds (approximately 2% of the total time), while the average number of total arm entries was 14.69 (average of 1.44 open arm entries and 13.64 closed arm entries; n = 36). Importantly, it has been argued that low elevated plus maze activity could render the test unstable (Browne and Lucki, 2013; Crabbe et al., 1999; Wahlsten et al., 2003). Furthermore, adult xCT-/- mice demonstrate impairment in spatial working memory in the Y maze (De Bundel et al., 2011) that might have influenced the alternation between closed and open arms of the maze. Especially as the mice underperformed, we believe that interpretation of the elevated plus maze data is not suited and will not be discussed further.

3.4. Effect of xCT deletion on depressive-like behavior

Depressive-like behavior was evaluated in two behavioral despair paradigms, the forced swim test and the tail suspension test. In the tail suspension test (Fig. 6A), xCT-/- mice spent less time immobile compared to their xCT+/+ littermates [genotype factor: F(1,62)=25.14, p<0.0001], an antidepressive-like effect visible for both adult (p=0.001) as well as aged

(p=0.001) mice. In the forced swim test (Figs. 6B, C and D), loss of system xc- also lead to a global decrease in immobility time [genotype factor: $F(1,72)=9.420$, $p=0.003$] that seemed to be particularly due to an antidepressive-like effect observed in aged mice ($p<0.0001$). Because of this particular difference, we could observe a global decrease in immobility time with aging irrespective of genotype [age factor: $F(1,72)=27.63$, $p<0.0001$], as well as a significant interaction effect [age x genotype factor: $F(1,72)=11.25$, $p=0.0013$]. The global decrease in immobility in the xCT-/- mice seemed to be associated with an increase in climbing but not swimming behavior [genotype factor: $F(1,72)=6.286$, $p=0.0144$], again mainly due to a significant increase in climbing behavior in aged xCT-/- mice ($p=0.005$).

4. Discussion

Current theories for mood disorders focus mainly on biogenic amines and still all marketed antidepressant drugs target monoamine reuptake transporters, monoamine oxidase and/or monoamine receptors. Even though these drugs are the standard of treatment today, they have significant limitations with regard to their slow onset of action and the substantial proportion of refractory patients (Niciu et al., 2014; Zarate et al., 2013). Glutamate, the major excitatory neurotransmitter in the brain, has been linked with the pathogenic process occurring in depression and anxiety disorders (for review see (Popoli et al., 2012; Sanacora et al., 2008)). Pre-clinical evidence indicates that modulation of glutamatergic signaling using metabotropic glutamate receptor ligands (Swanson et al., 2005) or NMDA receptor antagonists (Pilc et al., 2013; Sanacora et al., 2008) leads to anxiolytic and anti-depressive-like effects. Administration of ketamine to rodents, for instance, was found to reduce immobility in the forced swim and tail suspension tests, while also acting as anxiolytic in the novelty-suppressed feeding test and elevated plus maze (for review see (Browne and Lucki, 2013)). In addition to effects on glutamate receptors, there has been recent interest on glutamate metabolism and clearance as mechanisms in mood regulation. Glial astrocytes play an important role in modulation of both actions. They control glutamatergic neurotransmission by rapidly clearing synaptic glutamate thereby preventing spillover to the extrasynaptic space, and also serve a central role in amino acid neurotransmitter metabolism by converting glutamate into glutamine for the glutamate/glutamine cycle (Danbolt, 2001). Reduced brain expression of excitatory amino acid transporters in depressed humans (Valentine et al., 2011) as well as in a rat model for depression (Gomez-Galan et al., 2013) was in line with a rodent study showing that enhanced glutamate uptake by ceftriaxone had antidepressant-like effects (Mineur et al., 2007).

System xc- is a plasma membrane transporter, believed to be located on glial cells, mediating uptake of cystine and release of glutamate in a 1:1 ratio (Lewerenz et al., 2013). Cystine imported by system xc- is converted to cysteine that can either participate in the synthesis of glutathione, or be exported back to the extracelluar space via system ASC, and as such regulate the extracellular cystine/cysteine redox couple (Lewerenz et al., 2013). In turn, nonvesicular glutamate release via system xc- has been indicated in various regions of the brain (Baker et al., 2003; Baker et al., 2002; De Bundel et al., 2011; Massie et al., 2011). Although system xc- participates in the modulation of glutamatergic signaling (Bridges et al., 2012), its role in behavioral regulation is poorly understood.

The precise distribution of system xc- in the brain has been difficult to evaluate, due to the paucity of specific antibodies recognizing xCT (the specific subunit of system xc-) appropriate for immunohistochemistry. Nevertheless, previous *in situ* hydbridization studies indicated robust xCT expression in regions facing the cerebral ventricles as well as in meninges, linking system xc- with maintenance of the redox state of the cerebrospinal fluid (Sato et al., 2002). Furthermore, strong xCT expression was observed in the area postrema, the medial habenular nucleus as well as the paraventricular thalamic nuclei (Sato et al., 2002). Expression of system xc- in such discrete areas of the brain is intriguing, as it could be linked with particular roles in the brain. Interestingly, lesions of the area postrema have been previously found to decrease anxiety like behavior in rats (Miller et al., 2002). Furthermore, the medial habenula has been linked with mediating anxiety and fear responses, while increased activity has been linked with depression (Viswanath et al., 2013). Finally, the paraventricular thalamic nuclei were recently shown to be activated in depressive-like states (Zhu et al., 2011). Altogether, the strong expression of xCT in discrete regions mediating stress, anxiety and depressive-like responses could indicate that this antiporter might be relevant for the manifestation of such behaviors *in vivo* (Crawley and Paylor, 1997). Furthermore, expression of system xc- is also present in other areas of the brain including the hippocampus (De Bundel et al., 2011), striatum (Baker et al., 2002; Massie et al., 2011), nucleus accumbens (Baker et al., 2003), as well as in the cortex and cerebellum (Burdo et al., 2006; Sato et al., 2002; Shih et al., 2006), while recently Lutgen et al. described the presence of xCT mRNA in the basolateral amygdala and bed nucleus of the stria terminalis (Lutgen et al., 2014). In the current phenotypic study we embarked on the characterization of system xc- deficient mice using a battery of tests assaying anxiety and depressive-like behavior, with the hope of gaining further insight on the role of nonvesicular glutamate released by system xc- on behavior.

Due to the presence of system xc- in areas mediating sensorimotor control such as the basal ganglia (Patel et al., 2014) or cerebellum (Proville et al., 2014), we first carefully evaluated the potential impact of xCT deletion on sensorimotor function. In order to do so, we evaluated different aspects of motor performance, ranging from motor coordination and balance (rotarod), spontaneous behavior and exploration (open field) to fine movement skills and correct sensorimotor integration (nest building and adhesive removal). Our results consistently indicate that loss of xCT has no significant effect on motor and sensorimotor

function, while observing age-related genotype-independent decreases in motor function in the open field, rotarod and nest building tests. Our findings are in agreement with a recent behavioral characterization of xCT-/- mice that similarly did not observe changes in motor coordination or motor learning in the rotarod task, or in spontaneous motor activity in the open field test (McCullagh and Featherstone, 2014).

System xc- has also been found to be present and potentially functionally relevant in the eye. Expression of system xc- has been described in the retina (Bridges et al., 2001; Hu et al., 2008; Kato et al., 1993), lens (Lim et al., 2005) and cornea (Langford et al., 2010), where it might act to protect against oxidative stress. Indeed, various oxidative stress stimuli such as xanthine/xanthine oxidase or hydrogen peroxide leads to increased xCT expression (Dun et al., 2006; Mysona et al., 2009), while intravitreal injection of system xc- inhibitor DL-α-aminoadipate leads to a decline in retinal GSH levels and retinal dysfunction (Kato et al., 1993). At the same time, increased glutamate release via an overactive system xc- might be detrimental to retinal neurons, due to excitotoxic damage (Lewerenz et al., 2013; Lim and Donaldson, 2011). Conclusive studies on the functional involvement of system xc- in the eye are lacking, and it is currently unclear whether system xc- contributes to visual function *in vivo*. In the present study, we evaluated the basic visual acuity of system xc- deficient mice using the optomotor test. Although we observed an age-related decline of visual acuity, in line with previous observations in aged C57BL/6 mice (Lehmann et al., 2012), we did not detect any significant differences between genotypes, in either adult or aged mice. Our data, thus, suggest that system xc- might not play a major role in the subcortical-mediated visual acuity in physiological conditions. At the same time, the age-related decline in visual acuity might have contributed, at least partly, to the age-related effects observed in other behavioral tests evaluating motor function or anxiety-like behavior. Further studies evaluating ocular GSH and extracellular glutamate levels in xCT-/- mice, as well as the response of system xc- deficient mice in animal models of visual deprivation, would be extremely important in further defining the role of system xc- in visual function.

The absence of significant effects on motor function or visual acuity in system xc- deficient mice in each age group provided important controls to evaluate these mice in assays for anxiety- and depressive-like behavior. In order to evaluate anxiety-like behavior, we tested xCT-/- mice and their xCT+/+ littermates in various approach-avoidance paradigms. These tests are based on the conflict between the innate exploratory behavior of rodents and their

aversion towards open, bright, or elevated spaces, at risk for predation (Griebel and Holmes, 2013). In the 60 minute open field test, we could notice that system xc- deficient mice showed decreased avoidance of the center area in both age groups, indicating an age-independent anxiolytic effect of xCT deletion. This effect, however, could not be observed during the initial 5 minutes of the trial, in line with previous findings evaluating xCT-/- mice during short open field trials (De Bundel et al., 2011; McCullagh and Featherstone, 2014). It is believed that the initial stages of the open field test reflect the immediate reaction to the mildly stressful novel environment, while long term observation periods reflect habituation effects (Fonio et al., 2012). Our data, therefore, suggest that although xCT-/- mice show a similar reaction upon the introduction to the open field arena, they habituate faster to this novel environment compared to xCT+/+ littermates. In the light-dark paradigm, adult xCT-/- mice spent significantly more time outside the shelter compared to their xCT+/+ littermates, further reinforcing an anxiolytic behavior. Interestingly, this anxiolytic effect could not be observed in aged xCT-/- mice, indicating an age-dependent effect. In the novelty-suppressed feeding test, loss of system xc- globally decreased the latency to feed, also indicating a trend for decreased anxiety-like behavior. In conclusion, our findings indicate an anxiolytic effect of loss of system xc- in mice, observed in diverse paradigms tapping into distinct or overlapping domains of anxious behavior (Ramos, 2008; Turri et al., 2001).

Next, we evaluated the effect of loss of system xc- on depressive-like behavior using two behavioral despair tests. In these paradigms, mice are faced with an inescapable situation, with their total test immobility time indicating reluctance to maintain an active escape-oriented behavior. An increase in immobility time is considered to indicate depressive-like behavior (Cryan and Holmes, 2005). In the mouse tail suspension test, we could observe that xCT-/- mice consistently demonstrated decreased immobility time compared to xCT+/+ littermates, highlighting an age-independent antidepressive-like effect. In the forced swim test, we could notice that aged, but not adult, xCT-/- mice had decreased immobility time, with a concomitant increase in climbing but not swimming behavior. Importantly, aged xCT-/- mice did not demonstrate differences in performing complex and challenging motor tasks, such as the rotarod task, and as such changes observed in the forced swim test are most likely attributed to changes in mood, and not to a shift in motor phenotype. The finding that adult xCT-/- mice demonstrate an antidepressant effect in the tail suspension test, but not in the forced swim test, is intriguing, and might indicate different neurobiological pathways mediating the response in the two tests. Indeed, in spite of the similarity at face value, the

612 neurochemical pathways mediating behavior in the two paradigms are not believed to be the
613 same (Bai et al., 2001). This finding reinforces the antidepressive-like phenotype observed
614 after loss of system xc-, especially in the aged mice.
615
616 Although assigning specific mouse tests to various human mood symptoms is understandably
617 difficult (Ramos, 2008), attempts have been made to model various manifestations of
618 depression in mice (Cryan and Holmes, 2005). In particular, immobility in behavioral despair
619 paradigms could be related to depressed mood, inappropriate reaction to a stressful situation,
620 helplessness, as well as psychomotor retardation (Cryan and Holmes, 2005). The latency to
621 feed in the novelty suppressed feeding, besides indicating anxiety, might also be related to
622 hedonic processes (Dulawa et al., 2004). Similarly, the quality of the nest built during the nest
623 building test, besides reflecting motor activity, could also be related to fatigue or loss of
624 energy as seen in major depression (Cryan and Holmes, 2005). Overall, our findings indicate
625 an antidepressive-like effect of xCT deletion, particularly regarding measures of behavioral
626 despair. The slightly decreased latency to feed in the novelty suppressed feeding test in
627 system xc- deficient mice indicates a potential implication in anhedonia, and merits further
628 investigation in more specific tests, such as the intracranial self-stimulation paradigm (Cryan
629 and Holmes, 2005).
630
631 Geriatric depression is considered to entail a unique set of neurochemical and pathological
632 changes that distinguishes it from middle aged depression (Smith et al., 2007). As a
633 consequence, geriatric depression faces a greater variability in antidepressant treatment
634 response, an increased relapse rate, and a significant number of treatment resistant patients, as
635 well as an important impact of co-morbid anxiety on treatment response (Flint, 2005; Smith
636 et al., 2007). Furthermore, minor depression is more frequent in older adults, and is more
637 likely to present with co-morbid anxiety (Byrne and Pachana, 2010). Interestingly, Slotkin et
638 al. indicate distinct effects of olfactory bulbectomy in aged versus young rats, both on
639 behavior, as well as on the serotonergic and catecholaminergic systems, and such
640 particularities in geriatric depression should be taken into account for antidepressant treatment
641 (Slotkin et al., 1999). In our phenotyping study, we observed age-related interactions between
642 loss of system xc- and both anxiety and depressive-like behavior, further reinforcing the idea
643 of a differential involvement of neurotransmitter systems, such as the glutamatergic system,
644 during late life. This finding is consistent with a recent behavioral evaluation of mGlu5-/-
645 mice, which showed differential effects of the mutation on anxiety and depressive-like

behavior with age (Inta et al., 2013). In particular, due to a strong antidepressive-like effect observed in the aged xCT-/- mice, we believe that system xc- could be an interesting target for treatment of geriatric depression, especially when co-morbid with anxiety. Furthermore, as loss of system xc- protects against nigrostriatal dopaminergic neurodegeneration (Massie et al., 2011), this strategy could be of interest in treating age-related neurodegenerative disorders such as Parkinson's disease that have important co-occurrence of anxiety and depression (Chaudhuri and Schapira, 2009). Finally, changes in affective behavior in aged system xc- deficient mice occur in absence of impairment in either spatial reference memory or working memory (De Bundel et al., 2011), which might favor such interventions in late-life psychiatric disorders that are strongly associated with dementia and cognitive impairment (Laks and Engelhardt, 2010; Smith et al., 2007).

Astrocytic glutamate release via system xc- can activate extrasynaptic NMDA receptors, as well as group I and group II metabotropic glutamate receptors (Bridges et al., 2012). As discussed above, antagonists for NMDA, as well as for some metabotropic glutamate receptors can induce antidepressive- and/or anxiolytic-like effects (Pilc et al., 2013; Sanacora et al., 2008; Swanson et al., 2005). Furthermore, overactivation of extrasynaptic NMDA receptors can induce excitotoxicity (McCullagh and Featherstone, 2014) and might be involved in the cellular and morphological changes observed in the prefrontal cortex or hippocampus of patients with mood disorders (Popoli et al., 2012). Loss of system xc- has been shown to be neuroprotective, possibly by reducing the extracellular glutamate levels and the potential for excitotoxicity (Massie et al., 2011). Although highly speculative, the observed behavioral changes in xCT-/- mice might be linked with a decreased activation of extrasynaptic NMDA receptors, and/or reduced tonic stimulation of metabotropic glutamate receptors, such as group II metabotropic glutamate receptors (Baker et al., 2002; Moran et al., 2005). Further studies are warranted investigating the molecular mechanisms underlying the anxiolytic and antidepressive-like behavior observed in xCT-/- mice. Still, by modulating rather than strongly interfering with glutamatergic neurotransmission, inhibition of system xc- might represent a safe approach in targeting glutamatergic dysfunction, especially when associated with chronic disorders such as mood disorders (Bergink et al., 2004; Pilc et al., 2013).

Although our current findings indicate that system xc- mediates aspects of anxiety and depressive-like behavior, certain limitations should be acknowledged. First, the retrospective

nature of our study reflects a more heterogeneous experimental design, with different tests performed for different batches of mice. Test history has been found to influence the results of behavioral testing (Voikar et al., 2004). However, by ensuring that all individual batches were matched by genotype and age, we believe that the potential bias of reporting genotype effects due to test history is small. On the other hand, the retrospective nature of our study allowed for a large sample size (which is sometimes limiting in behavioral research, see (Button et al., 2013)) and the evaluation of the xCT -/- mice in a large variety of paradigms that is sometimes difficult to perform in a prospective study. Furthermore, employing different batches of mice might also decrease the chance of reporting batch-related effects on behavior. A second limitation of our study relates to the exclusive characterization of male system xc- deficient mice. While our findings provide a starting point regarding the effects of system xc- on behavior, it would be of interest to perform a similar phenotyping study on female mice, in order to see if the effects of system xc- would be consistent across genders. Such studies would be especially insightful, in light of the known differences that exist between genders regarding regulation of mood and stress responses and the increased incidence of depression and anxiety disorders in women (Young and Korszun, 2010).

In a very recent report, Lutgen et al. indicate that system xc- inhibitor sulfasalazine leads to increased anxiety-like behavior in rats in the open field and elevated plus maze tests, without affecting depressive-like behavior in the forced swim test (Lutgen et al., 2014). These findings are in contrast with our current observations, and widen the perspective and complexity of behavioral modulation by system xc-. It is conceivable that these contrasting results are due to different levels of system xc- inhibition (acute and partial with sulfasalazine, versus chronic and complete in xCT-/- mice), or the species of animals used. At the same time, however, sulfasalazine is known to have poor blood-brain barrier permeability in intact animals (Liu et al., 2012), and peripheral and central off-target effects, such as anti-inflammatory properties (inhibition of nuclear factor kappa B, see (Wahl et al., 1998)) and blockade of NMDA receptors (Ryu et al., 2003), that could influence acute effects on behavior. Future studies will be of particular importance evaluating acute versus chronic modulation of system xc- and its relation to emotional behavior.

5. Conclusion

Our present findings indicate that system xc- mediates aspects of anxiety and depressive-like behavior in mice. Inhibition of system xc-, in particular, might be an interesting and novel

approach in the management of mood disorders, including those with late life onset. Further studies are eagerly awaited evaluating this hypothesis in animal models of anxiety and depression, and using selective pharmacological inhibitors of system xc- or conditional xCT mutants.

Acknowledgements

The authors acknowledge the Fund for Scientific Research Flanders (FWO, grant G.038412N), the Queen Elisabeth Medical Foundation (G.S.K.E.), and the Vrije Universiteit Brussel (Strategic Research Program, grant SRP40) for financial support. E. B. is funded by the FWO. J. V. L. and T. D. are funded by the agency for Innovation by Science and Technology (IWT).

References

Bai F, Li X, Clay M, Lindstrom T, Skolnick P. Intra- and interstrain differences in models of "behavioral despair". Pharmacol Biochem Behav 2001;70:187-92.

Baker DA, McFarland K, Lake RW, Shen H, Tang XC, Toda S, et al. Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse. Nat Neurosci 2003;6:743-9.

Baker DA, Xi ZX, Shen H, Swanson CJ, Kalivas PW. The origin and neuronal function of in vivo nonsynaptic glutamate. J Neurosci 2002;22:9134-41.

Bergink V, van Megen HJ, Westenberg HG. Glutamate and anxiety. Eur Neuropsychopharmacol 2004;14:175-83.

Bouet V, Boulouard M, Toutain J, Divoux D, Bernaudin M, Schumann-Bard P, et al. The adhesive removal test: a sensitive method to assess sensorimotor deficits in mice. Nat Protoc 2009;4:1560-4.

Bridges CC, Kekuda R, Wang H, Prasad PD, Mehta P, Huang W, et al. Structure, function, and regulation of human cystine/glutamate transporter in retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2001;42:47-54.

Bridges R, Lutgen V, Lobner D, Baker DA. Thinking outside the cleft to understand synaptic activity: contribution of the cystine-glutamate antiporter (System xc-) to normal and pathological glutamatergic signaling. Pharmacol Rev 2012;64:780-802.

Browne CA, Lucki I. Antidepressant effects of ketamine: mechanisms underlying fast-acting novel antidepressants. Front Pharmacol 2013;4:161.

Burdo J, Dargusch R, Schubert D. Distribution of the cystine/glutamate antiporter system xc- in the brain, kidney, and duodenum. J Histochem Cytochem 2006;54:549-57.

Button KS, Ioannidis JP, Mokrysz C, Nosek BA, Flint J, Robinson ES, et al. Power failure: why small sample size undermines the reliability of neuroscience. Nat Rev Neurosci 2013;14:365-76.

Byrne GJ, Pachana NA. Anxiety and depression in the elderly: do we know any more? Curr Opin Psychiatry 2010;23:504-9.

Chaudhuri KR, Schapira AH. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. Lancet Neurol 2009;8:464-74.

Crabbe JC, Wahlsten D, Dudek BC. Genetics of mouse behavior: interactions with laboratory environment. Science 1999;284:1670-2.

Crawley JN. Behavioral phenotyping strategies for mutant mice. Neuron 2008;57:809-18.

Crawley JN, Paylor R. A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Horm Behav 1997;31:197-211.

Cryan JF, Holmes A. The ascent of mouse: advances in modelling human depression and anxiety. Nat Rev Drug Discov 2005;4:775-90.

Cryan JF, Markou A, Lucki I. Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharmacol Sci 2002;23:238-45.

Danbolt NC. Glutamate uptake. Prog Neurobiol 2001;65:1-105.

De Bundel D, Schallier A, Loyens E, Fernando R, Miyashita H, Van Liefferinge J, et al. Loss of system x(c)- does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility. J Neurosci 2011;31:5792-803.

Deacon RM. Assessing nest building in mice. Nature Protocols 2006;1:1117-9.

Douglas RM, Alam NM, Silver BD, McGill TJ, Tschetter WW, Prusky GT. Independent visual threshold measurements in the two eyes of freely moving rats and mice using a virtual-reality optokinetic system. Vis Neurosci 2005;22:677-84.

Dulawa SC, Holick KA, Gundersen B, Hen R. Effects of chronic fluoxetine in animal models of anxiety and depression. Neuropsychopharmacology 2004;29:1321-30.

Dun Y, Mysona B, Van Ells T, Amarnath L, Ola MS, Ganapathy V, et al. Expression of the cystine-glutamate exchanger (xc-) in retinal ganglion cells and regulation by nitric oxide and oxidative stress. Cell Tissue Res 2006;324:189-202.

798 Flint AJ. Anxiety and its disorders in late life: moving the field forward. Am J Geriatr Psychiatry
799 2005;13:3-6.
800 Fonio E, Benjamini Y, Golani I. Short and long term measures of anxiety exhibit opposite results. PLoS
801 One 2012;7:e48414.
802 Gomez-Galan M, De Bundel D, Van Eeckhaut A, Smolders I, Lindskog M. Dysfunctional astrocytic
803 regulation of glutamate transmission in a rat model of depression. Mol Psychiatry 2013;18:582-94.
804 Griebel G, Holmes A. 50 years of hurdles and hope in anxiolytic drug discovery. Nat Rev Drug Discov
805 2013;12:667-87.
806 Hu RG, Lim J, Donaldson PJ, Kalloniatis M. Characterization of the cystine/glutamate transporter in
807 the outer plexiform layer of the vertebrate retina. Eur J Neurosci 2008;28:1491-502.
808 Inta D, Vogt MA, Luoni A, Filipovic D, Lima-Ojeda JM, Pfeiffer N, et al. Significant increase in anxiety
809 during aging in mGlu5 receptor knockout mice. Behav Brain Res 2013;241:27-31.
810 Kato S, Ishita S, Sugawara K, Mawatari K. Cystine/glutamate antiporter expression in retinal Muller
811 glial cells: implications for DL-alpha-aminoadipate toxicity. Neuroscience 1993;57:473-82.
812 Laks J, Engelhardt E. Peculiarities of geriatric psychiatry: a focus on aging and depression. CNS
813 Neurosci Ther 2010;16:374-9.
814 Langford MP, Redmond P, Chanis R, Misra RP, Redens TB. Glutamate, excitatory amino acid
815 transporters, Xc- antiporter, glutamine synthetase, and gamma-glutamyltranspeptidase in human
816 corneal epithelium. Curr Eye Res 2010;35:202-11.
817 Lehmann K, Schmidt KF, Lowel S. Vision and visual plasticity in ageing mice. Restor Neurol Neurosci
818 2012;30:161-78.
819 Lewerenz J, Hewett SJ, Huang Y, Lambros M, Gout PW, Kalivas PW, et al. The cystine/glutamate
820 antiporter system x(c)(-) in health and disease: from molecular mechanisms to novel therapeutic
821 opportunities. Antioxid Redox Signal 2013;18:522-55.
822 Li X, Frye MA, Shelton RC. Review of pharmacological treatment in mood disorders and future
823 directions for drug development. Neuropsychopharmacology 2012;37:77-101.
824 Lim J, Lam YC, Kistler J, Donaldson PJ. Molecular characterization of the cystine/glutamate exchanger
825 and the excitatory amino acid transporters in the rat lens. Invest Ophthalmol Vis Sci 2005;46:2869-
826 77.
827 Lim JC, Donaldson PJ. Focus on molecules: the cystine/glutamate exchanger (System x(c)(-)). Exp Eye
828 Res 2011;92:162-3.
829 Liu X, Ding X, Deshmukh G, Liederer BM, Hop CE. Use of the cassette-dosing approach to assess brain
830 penetration in drug discovery. Drug Metab Dispos 2012;40:963-9.
831 Lutgen V, Resch J, Qualmann K, Raddatz NJ, Panhans C, Olander EM, et al. Behavioral assessment of
832 acute inhibition of system xc (-) in rats. Psychopharmacology (Berl) 2014;231:4637-47.
833 Massie A, Schallier A, Kim SW, Fernando R, Kobayashi S, Beck H, et al. Dopaminergic neurons of
834 system x(c)(-)-deficient mice are highly protected against 6-hydroxydopamine-induced toxicity.
835 FASEB J 2011;25:1359-69.
836 McCullagh EA, Featherstone DE. Behavioral characterization of system xc- mutant mice. Behav Brain
837 Res 2014;265:1-11.
838 Miller CC, Holmes PV, Edwards GL. Area postrema lesions elevate NPY levels and decrease anxiety-
839 related behavior in rats. Physiol Behav 2002;77:135-40.
840 Mineur YS, Picciotto MR, Sanacora G. Antidepressant-like effects of ceftriaxone in male C57BL/6J
841 mice. Biol Psychiatry 2007;61:250-2.
842 Moran MM, McFarland K, Melendez RI, Kalivas PW, Seamans JK. Cystine/glutamate exchange
843 regulates metabotropic glutamate receptor presynaptic inhibition of excitatory transmission and
844 vulnerability to cocaine seeking. J Neurosci 2005;25:6389-93.
845 Mysona B, Dun Y, Duplantier J, Ganapathy V, Smith SB. Effects of hyperglycemia and oxidative stress
846 on the glutamate transporters GLAST and system xc- in mouse retinal Muller glial cells. Cell Tissue
847 Res 2009;335:477-88.

Niciu MJ, Ionescu DF, Richards EM, Zarate CA, Jr. Glutamate and its receptors in the pathophysiology and treatment of major depressive disorder. J Neural Transm 2014;121:907-24.
Patel N, Jankovic J, Hallett M. Sensory aspects of movement disorders. Lancet Neurol 2014;13:100-12.
Pietropaolo S, Feldon J, Yee BK. Environmental enrichment eliminates the anxiety phenotypes in a triple transgenic mouse model of Alzheimer's disease. Cogn Affect Behav Neurosci 2014;14:996-1008.
Pilc A, Wieronska JM, Skolnick P. Glutamate-based antidepressants: preclinical psychopharmacology. Biol Psychiatry 2013;73:1125-32.
Pogorelov VM, Lanthorn TH, Savelieva KV. Use of a platform in an automated open-field to enhance assessment of anxiety-like behaviors in mice. J Neurosci Methods 2007;162:222-8.
Popoli M, Yan Z, McEwen BS, Sanacora G. The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. Nat Rev Neurosci 2012;13:22-37.
Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. Nature 1977;266:730-2.
Proville RD, Spolidoro M, Guyon N, Dugue GP, Selimi F, Isope P, et al. Cerebellum involvement in cortical sensorimotor circuits for the control of voluntary movements. Nat Neurosci 2014.
Prusky GT, Alam NM, Beekman S, Douglas RM. Rapid quantification of adult and developing mouse spatial vision using a virtual optomotor system. Invest Ophthalmol Vis Sci 2004;45:4611-6.
Ramos A. Animal models of anxiety: do I need multiple tests? Trends Pharmacol Sci 2008;29:493-8.
Rodgers RJ, Cole JC, Aboualfa K, Stephenson LH. Ethopharmacological analysis of the effects of putative 'anxiogenic' agents in the mouse elevated plus-maze. Pharmacol Biochem Behav 1995;52:805-13.
Ryu BR, Lee YA, Won SJ, Noh JH, Chang SY, Chung JM, et al. The novel neuroprotective action of sulfasalazine through blockade of NMDA receptors. J Pharmacol Exp Ther 2003;305:48-56.
Sanacora G, Banasr M. From pathophysiology to novel antidepressant drugs: glial contributions to the pathology and treatment of mood disorders. Biol Psychiatry 2013;73:1172-9.
Sanacora G, Zarate CA, Krystal JH, Manji HK. Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. Nat Rev Drug Discov 2008;7:426-37.
Sato H, Shiiya A, Kimata M, Maebara K, Tamba M, Sakakura Y, et al. Redox imbalance in cystine/glutamate transporter-deficient mice. J Biol Chem 2005;280:37423-9.
Sato H, Tamba M, Okuno S, Sato K, Keino-Masu K, Masu M, et al. Distribution of cystine/glutamate exchange transporter, system x(c)-, in the mouse brain. J Neurosci 2002;22:8028-33.
Shih AY, Erb H, Sun X, Toda S, Kalivas PW, Murphy TH. Cystine/glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation. J Neurosci 2006;26:10514-23.
Slotkin TA, Miller DB, Fumagalli F, McCook EC, Zhang J, Bissette G, et al. Modeling geriatric depression in animals: biochemical and behavioral effects of olfactory bulbectomy in young versus aged rats. J Pharmacol Exp Ther 1999;289:334-45.
Smith GS, Gunning-Dixon FM, Lotrich FE, Taylor WD, Evans JD. Translational research in late-life mood disorders: implications for future intervention and prevention research. Neuropsychopharmacology 2007;32:1857-75.
Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology (Berl) 1985;85:367-70.
Swanson CJ, Bures M, Johnson MP, Linden AM, Monn JA, Schoepp DD. Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. Nat Rev Drug Discov 2005;4:131-44.
Turri MG, Datta SR, DeFries J, Henderson ND, Flint J. QTL analysis identifies multiple behavioral dimensions in ethological tests of anxiety in laboratory mice. Curr Biol 2001;11:725-34.
Valentine GW, Mason GF, Gomez R, Fasula M, Watzl J, Pittman B, et al. The antidepressant effect of ketamine is not associated with changes in occipital amino acid neurotransmitter content as measured by [(1)H]-MRS. Psychiatry Res 2011;191:122-7.

Viswanath H, Carter AQ, Baldwin PR, Molfese DL, Salas R. The medial habenula: still neglected. Front Hum Neurosci 2013;7:931.
Voikar V, Vasar E, Rauvala H. Behavioral alterations induced by repeated testing in C57BL/6J and 129S2/Sv mice: implications for phenotyping screens. Genes Brain Behav 2004;3:27-38.
Wahl C, Liptay S, Adler G, Schmid RM. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 1998;101:1163-74.
Wahlsten D, Rustay NR, Metten P, Crabbe JC. In search of a better mouse test. Trends Neurosci 2003;26:132-6.
Young E, Korszun A. Sex, trauma, stress hormones and depression. Mol Psychiatry 2010;15:23-8.
Zarate C, Duman RS, Liu G, Sartori S, Quiroz J, Murck H. New paradigms for treatment-resistant depression. Ann N Y Acad Sci 2013;1292:21-31.
Zarate C, Jr., Machado-Vieira R, Henter I, Ibrahim L, Diazgranados N, Salvadore G. Glutamatergic modulators: the future of treating mood disorders? Harv Rev Psychiatry 2010;18:293-303.
Zhu L, Wu L, Yu B, Liu X. The participation of a neurocircuit from the paraventricular thalamus to amygdala in the depressive like behavior. Neurosci Lett 2011;488:81-6.

Figure Legends

Fig. 1. Spontaneous motor behavior. Loss of system xc- does not affect horizontal (C) or vertical (F) spontaneous motor activity as assessed in a 60 minute open field test (see A and D for corresponding 5 minute time bins). Activity levels were similarly unaffected in xCT-/- mice during the first 5 minutes of the trial (B, E). Data are presented as mean ± SEM *** $p<0.001$ (2-way ANOVA), sample size indicated in figure.

Fig. 2. Motor coordination and fine motor skills. Loss of system xc- does not affect motor coordination and balance as assessed using an accelerating rotarod protocol (A), nor does it influence fine motor skills and the capacity to build a nest (B, C). Data are presented as mean ± SEM *** $p<0.001$ (2-way ANOVA), sample size indicated in figure.

Fig. 3. Sensorimotor behavior. Loss of system xc- does not affect sensorimotor function as evaluated in the adhesive removal test; xCT-/- mice demonstrate intact sensory function (time-to-contact, A), as well as intact fine motor skills (time-to-remove, B). Data are presented as mean ± SEM, sample size indicated in figure.

Fig. 4. Visual acuity thresholds. Loss of system xc- does not affect spatial frequency thresholds or visual acuity in either adult or aged mice tested in the optomotor setup. Data are presented as mean ± SEM *** $p<0.001$ (2-way ANOVA), sample size indicated in figure.

Fig. 5. Anxiety-like behavior. Loss of system xc- leads to age-independent anxiolytic effects in the 60 minute open field test (C), that are not observed however in the first 5 minutes of the trial (B), and potentially associated with facilitated habituation to the open field arena (A). Loss of system xc- leads to age-dependent anxiolytic effects in the light/dark paradigm (D, E), and global anxiolytic effects in the novelty suppressed feeding test (F). Data are presented as mean ± SEM *** $p<0.001$, ** $p<0.01$, * $p<0.05$ (2-way ANOVA), $^{\#\#}$ $p<0.01$, $^{\#}$ $p<0.05$ (Bonferroni *post-hoc* versus age-matched xCT+/+), sample size indicated in figure.

Fig. 6. Depressive-like behavior. Loss of system xc- leads to age-independent antidepressive effects in the tail suspension test (A), and age-dependent antidepressive effects in the forced swim test (B), with increased climbing (D) but not swimming (C) behavior. Data are presented as mean ± SEM *** $p<0.001$, ** $p<0.01$, * $p<0.05$ (2-way ANOVA), $^{\#\#\#}$ $p<0.001$,

$p<0.01$, # $p<0.05$ (Bonferroni *post-hoc* versus age-matched xCT+/+), sample size indicated in figure.

Figures

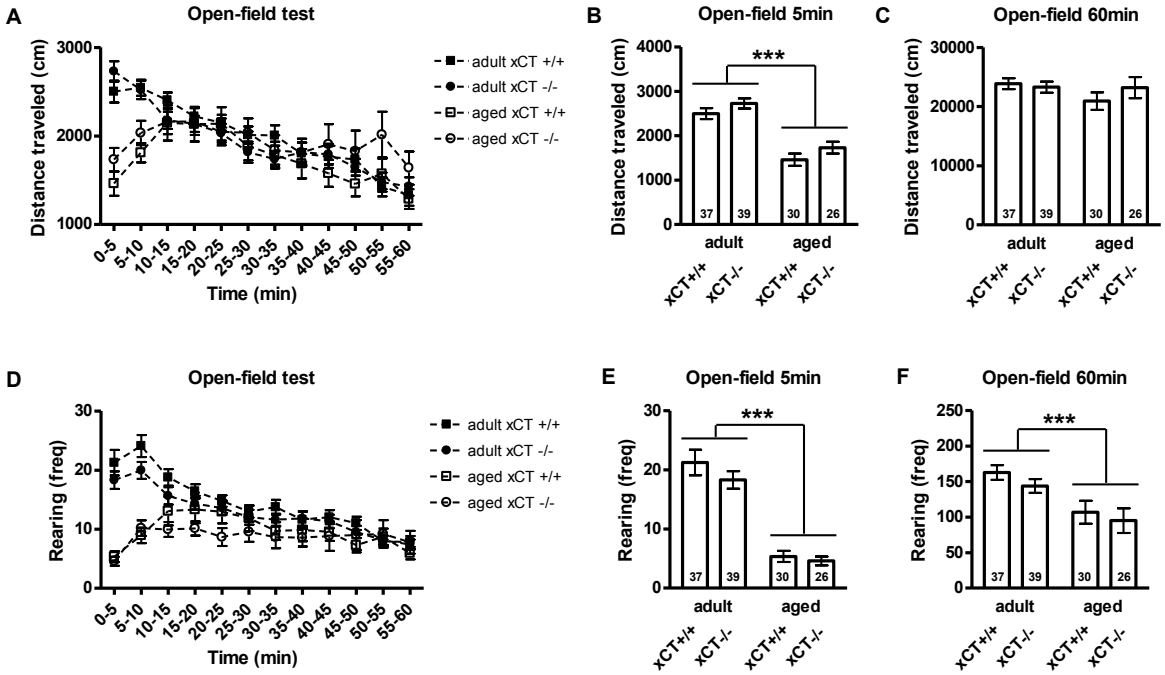


Fig.1

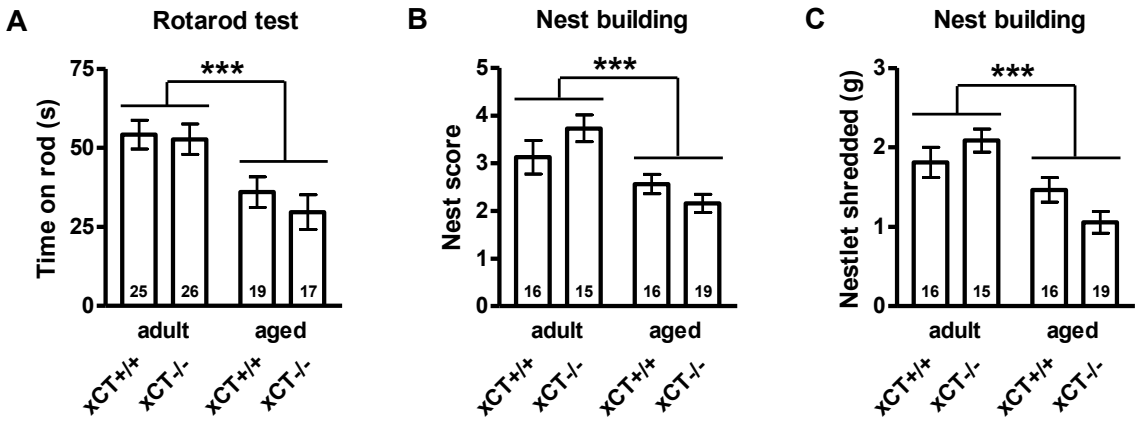

A
Rotarod test
Time on rod (s)
75
50
25
0
***
25
26
19
17
adult
aged
xCT+/+
xCT-/-
xCT+/+
xCT-/-
B
Nest building
Nest score
5
4
3
2
1
0
***
16
15
16
19
adult
aged
xCT+/+
xCT-/-
xCT+/+
xCT-/-
C
Nest building
Nestlet shredded (g)
3
2
1
0
***
16
15
16
19
adult
aged
xCT+/+
xCT-/-
xCT+/+
xCT-/-

Fig. 2

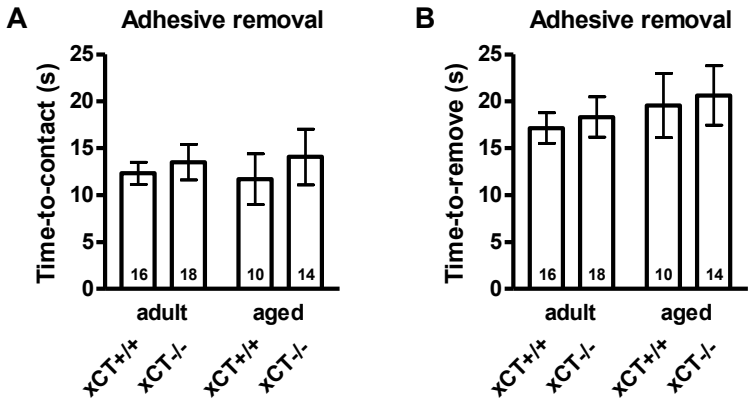

A
Adhesive removal
Time-to-contact (s)
25
20
15
10
5
0
16
18
10
14
adult
aged
xCT+/+
xCT-/-
xCT+/+
xCT-/-
B
Adhesive removal
Time-to-remove (s)
25
20
15
10
5
0
16
18
10
14
adult
aged
xCT+/+
xCT-/-
xCT+/+
xCT-/-

Fig.3

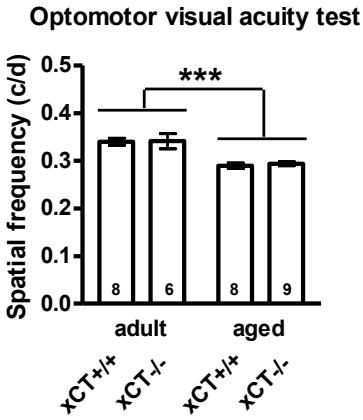

Optomotor visual acuity test
Spatial frequency (c/d)
0.5
0.4
0.3
0.2
0.1
0.0
***
8
6
8
9
adult
aged
xCT+/+
xCT-/-
xCT+/+
xCT-/-

Fig. 4

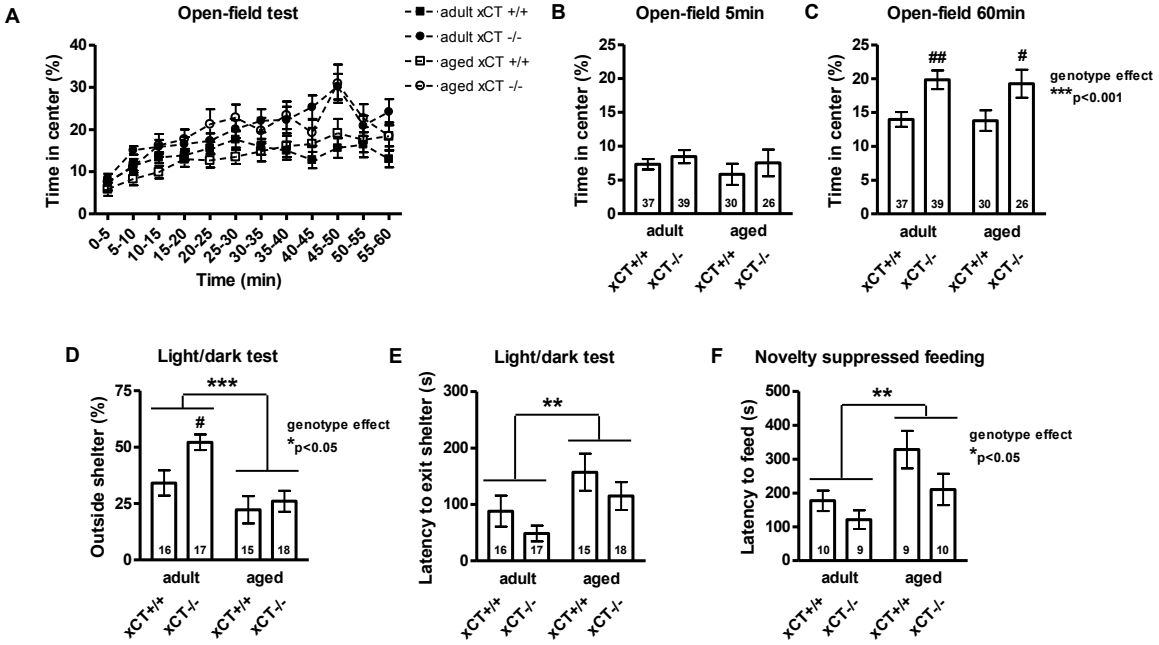
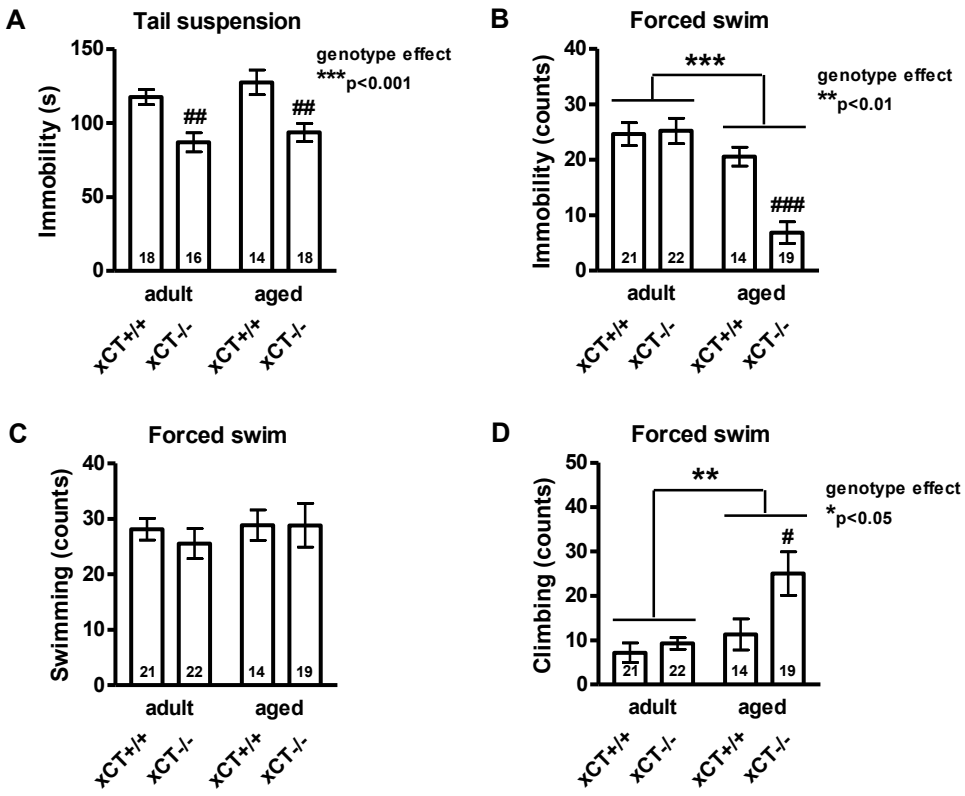

A
Open-field test
adult xCT +/+
adult xCT -/-
aged xCT +/+
aged xCT -/-
Time in center (%)
Time (min)
0-5
5-10
10-15
15-20
20-25
25-30
30-35
35-40
40-45
45-50
50-55
55-60
B
Open-field 5min
Time in center (%)
adult
aged
xCT+/+
xCT-/-
C
Open-field 60min
##
#
genotype effect
***p<0.001
D
Light/dark test
Outside shelter (%)
***
#
genotype effect
*p<0.05
E
Light/dark test
Latency to exit shelter (s)
**
F
Novelty suppressed feeding
Latency to feed (s)
**
genotype effect
*p<0.05

Fig. 5

1098



1099

1100

1101 **Fig. 6**