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Impaired long-term potentiation and long-term memory deficits in xCT-deficient *sut* mice

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Synopsis

xCT is the functional subunit of the cystine/glutamate antiporter system x_c^-, which exchanges intracellular glutamate with extracellular cystine. xCT has been reported to play roles in the maintenance of intracellular redox and ambient extracellular glutamate, which may affect neuronal function. To assess a potential role of xCT in the mouse hippocampus, we performed fear conditioning and passive avoidance for long-term memories and examined hippocampal synaptic plasticity in wild-type mice and xCT-null mutants, *sut* mice. Long-term memory was impaired in *sut* mice. Normal basal synaptic transmission and short-term presynaptic plasticity at hippocampal Schaffer collateral–CA1 synapses were observed in *sut* mice. However, LTP (long-term potentiation) was significantly reduced in *sut* mice compared with their wild-type counterparts. Supplementation of extracellular glutamate did not reverse the reduction in LTP. Taken together, our results suggest that xCT plays a role in the modulation of hippocampal long-term plasticity.

Key words: glutamate, long-term memory, long-term potentiation, xCT

INTRODUCTION

System x_c^- is a Na^+-independent amino acid transporting system, which exchanges intracellular glutamate for extracellular cystine. This antiporter consists of two subunits, xCT, which is encoded by *Slc7a11* (solute carrier family 7 member 11) and is responsible for transport activity and substrate specificity, and 4F2hc, a regulatory heavy chain [1]. In embryonic and adult rat brain, xCT protein is enriched at the CSF (cerebrospinal fluid)–brain barrier (i.e. meninges) and is also expressed in the cortex, hippocampus, striatum and cerebellum [2]. xCT and 4F2hc are localized to both neurons and glial cells [3].

Its bidirectional transfer of amino acids renders the dual roles of system x_c^- related to cystine uptake and glutamate output. Cystine taken up by x_c^- is rapidly reduced to cysteine, which is a rate-limiting precursor for glutathione synthesis. Thus the intracellular level of glutathione is regulated by x_c^- [4,5]. Since glutathione has been found to play a prominent role in cellular defence against ROS (reactive oxygen species) [6], x_c^- has been implicated in the maintenance of redox balance [2].

Another function of x_c^- is its involvement in non-vesicular release of glutamate. x_c^- has been demonstrated to be the major source of extracellular glutamate in several regions of the nervous system. Previous studies on xCT-knockout mice and pharmacological studies on rats have demonstrated that, in the mouse striatum and hippocampus or in the rat striatum and nucleus accumbens, x_c^- is the primary source of extracellular glutamate and participates in the regulation of glutamatergic neurotransmission [7–11]. Moreover, xCT is implicated in modulating synaptic plasticity. *Drosophila gb* (genderblind; an *Slc7a11* homologue) mutants show an increase in postsynaptic ionotropic glutamate receptors in the neuromuscular junction due to a reduction in extracellular glutamate [12]. Activation of xCT by *N*-acetylcysteine and subsequent elevation of extracellular glutamate restores the ability in the nucleus accumbens core subregion to develop LTP (long-term potentiation) and LTD (long-term depression) that is impaired by administration of cocaine [13].

However, whether xCT is involved in hippocampal synaptic plasticity under physiological conditions has not been reported yet. In the present study, we have used *sut* mice, which harbour a naturally occurring null mutation in *Slc7a11* [14], to investigate the potential role of xCT in synaptic plasticity.

Abbreviations used: CPG, (S)-4-carboxyphenylglycine; CS, conditioning stimulus; CSF, cerebrospinal fluid; aCSF, artificial CSF; EAAC1, excitatory amino acid carrier 1; EM, electron microscopy; fEPSP, field excitatory postsynaptic potential; H&E, haematoxylin and eosin; LTP, long-term potentiation; PFA, paraformaldehyde; PPF, paired pulse facilitation; pre-CS, pre-conditioning stimulus; PSD, postsynaptic densities; *Slc7a11*, solute carrier family 7 member 11; WT, wild-type.

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MATERIALS AND METHODS

Animals

The *sut* mutant mice (homozygous *sut/sut*) and WT (wild-type) control C3H/HeSnJ mice were originally obtained from The Jackson Laboratory, transferred from Dr Richard T. Swank's laboratory at Roswell Park Cancer Institute and bred in the animal facility at the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. All experimental procedures were approved by the Committee on Animal Health and Care of the Institute of Genetics and Developmental Biology. Mice in all experiments were 3 months of age. The genotypes were confirmed using a PCR-based method [14]. No more than five mice were housed in a cage for at least 20 days before starting the experiments and were kept under an inverse 12-h light/dark cycle (lights on at 08:00 h). Each individual animal was used in only one behavioural test to avoid the potential effect of the other tests. Only male mice were used in these experiments.

Learning and memory tests

Fear conditioning

The fear conditioning test consists of two phases: a training phase (day 1) and a testing phase (day 2). On day 1, mice were placed in operant chambers with a metal grid floor and were conditioned to the context (the chambers) and the auditory cue. Mice were kept in the chamber for 2 min before presentation of a clicker sound for 30 s, followed by a foot shock of 0.35 mA for 2 s. On day 2, for the context test, mice were placed into the same conditioning chamber that was used for the training and were kept for 5 min. Then the context was altered: the grid floor was covered using a white plastic plate with bedding material on it, the chamber wall was coloured white and chambers were made odorous with orange peel. Mice were then moved to the altered chamber and kept for 3 min as the pre-conditioning stimulus (pre-CS) stage, after which a clicker sound was present for 30 s without a foot shock. The mice were then kept for another 3 min as the conditioning stimulus (CS) stage. The programme was controlled by software and all of the procedures were recorded on a camera (Anilab). For the three tests (context, pre-CS and CS), mice were scored every 10 s as being rendered immobile by fear ('freezing') or not, and the number of bouts of freezing/30 (for the context test) or the number of bouts of freezing/18 (for pre-CS and CS tests) were calculated as indices.

Passive avoidance

The operant chamber for passive avoidance is composed of two compartments (a lit one and a dark one) separated by a trap door (Anilab). On the training day, mice were placed in the lit compartment facing away from the door. After 10 s, the door was opened and a mild foot shock (0.7 mA for 2 s) was delivered once the mice entered the dark compartment. At 10 s after the foot shock, the mice were transferred to the home cage. On the testing day (24 h after training), mice were placed in the lit compartment again with the door opened, but no foot shock was delivered. The latency to enter the dark compartment was recorded.

Extracellular field potential recording

Mice were anaesthetized with sodium pentobarbital and, after heart perfusion with ice-cold sectioning aCSF (artificial CSF; 110 mM choline chloride, 2.5 mM KCl, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 1.3 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 20 mM glucose, pH 7.4) gassed with 95 % O_2 and 5 % CO_2, hippocampal formation was rapidly dissected and immersed into sectioning aCSF. Transverse slices (350-μm thick) were prepared with a Vibratome (Microm HM 650V) and then equilibrated in recording aCSF (125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.3 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 10 mM glucose, pH 7.4). During recording, slices were fully immersed in a recording chamber and perfused continuously with the recording aCSF bubbled with 95 % O_2 and 5 % CO_2 in the presence of the GABA (γ-aminobutyric acid)$_A$ receptor antagonist picrotoxin (100 μM).

After severing the connections between CA1 and CA3 to avoid epileptiform activity, a stimulating bipolar electrode and a recording electrode were placed on the stratum radiatum of the hippocampal slices 100–200 μm apart from each other. After a stable recording of the population fEPSP (field excitatory postsynaptic potential) for at least 20 min, input–output characteristics were determined by varying the stimulus intensity and determining the amplitude of the fEPSP. PPF (paired pulse facilitation) was obtained by delivering two stimuli at various intervals (20, 50, 100, 150, 200, 250, 300 and 400 ms). LTP was induced by tetanic stimulus (100 Hz, 100 pulses, two trains with 30 s interval). CPG [(*S*)-4-carboxyphenylglycine; Tocris Bioscience] was added to a final concentration of 50 μM to inhibit the activity of xCT.

Morphological studies

Histological staining

Mouse hippocampal sections were prepared and stained with H&E (haematoxylin and eosin) as described previously [15]. In brief, mice were transcardially perfused with 4 % PFA (paraformaldehyde) in 0.1 M phosphate buffer (pH 7.4). Brains underwent 12 h fixation in 4 % PFA, followed by cryoprotection in 30 % sucrose. Then brains were cut into 10-μm-thick sections followed by H&E staining.

EM (electron microscopy)

Mouse hippocampal EM to examine the CA1 pyramidal cell bodies and their apical dendrites in the stratum radiatum was performed using our method described previously [16]. The asymmetric synapses, which are presumably excitatory, were examined. These synapses have clearly identifiable presynaptic terminals, synaptic clefts, postsynaptic membranes and PSD (postsynaptic densities). The synapse number, the width of the synaptic cleft and the thickness of PSD were measured using ImageJ (NIH) software, as described previously [16].

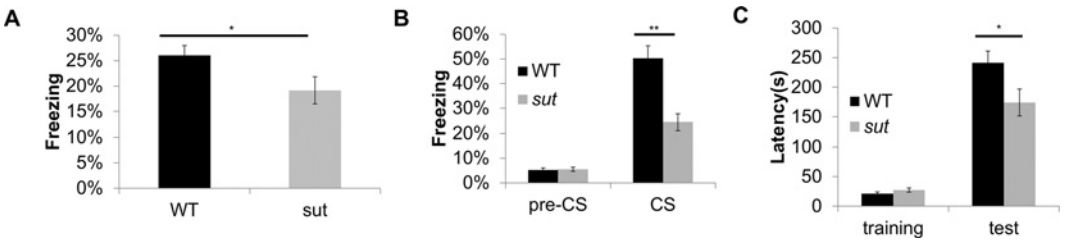

Figure 1 ***Sut* mice exhibit impaired long-term memories**

(**A**) In context fear conditioning, *sut* mice ($19.2 \pm 2.7\%$, $n = 16$) had a lower freezing response than WT mice ($26.1 \pm 1.9\%$, $n = 22$). (**B**) In cued fear conditioning, *sut* mice ($5.6 \pm 0.8\%$) had a comparable freezing response with WT mice ($5.3 \pm 0.8\%$) at the pre-CS stage, but had a lower freezing response than WT mice at the CS stage (24.5 ± 3.3 compared with $50.3 \pm 5.0\%$ respectively; WT, $n = 20$; *sut*, $n = 17$). (**C**) In the passive avoidance test, *sut* and WT mice had similar latencies to enter the dark compartment on the training day ($P > 0.05$). On the testing day, *sut* mice had a shorter latency than WT (173.9 ± 22.7 compared with 241.3 ± 19.6 s respectively; WT, $n = 19$; *sut*, $n = 20$).

Statistics

For behavioural tests and EM results, statistical significance was determined using a Student's *t* test. For electrophysiological assays, statistical analyses of the data were carried out using ANOVA, followed by a post-hoc test. In the Figures, all results are means $\pm$ S.E.M.; $^*P < 0.05$ and $^{**}P < 0.005$.

RESULTS

Impaired fear memory in *sut* mice

In order to characterize the role of xCT in hippocampus- and amygdala-dependent fear learning, we performed fear conditioning experiments with *sut* mice and their WT counterparts. Contextual fear memory involves both the hippocampus and amygdala [17], whereas the formation of auditory-cued fear memory is primarily amygdala-dependent [18]. After the mice were conditioned to both the context and auditory cue on day 1, mice were tested for their freezing response to the context or auditory cue on day 2. We found that *sut* mice had a lower freezing response in both contextual and cued fear conditioning than that of WT mice (Figures 1A and 1B), suggesting an impaired fear memory in *sut* mice.

Impaired passive avoidance memory in *sut* mice

We measured long-term memory of WT and *sut* mice in another hippocampus-dependent paradigm, namely a passive avoidance task [19]. On the training day, mice must learn to avoid entry into the dark compartment of the operant chamber where a foot shock was delivered. Both genotypes displayed a similar latency to enter the dark compartment. At 24 h later, mice were placed into the light compartment and the latency to enter the dark compartment served as an index of their ability to associate context with foot shock. *Sut* mice exhibited a shorter latency than WT mice (Figure 1C), demonstrating that fear learning and memory was affected by a loss of xCT.

Basal synaptic transmission is normal in *sut* mice

To investigate the cause of memory deficits in *sut* mice, we examined basal synaptic function in hippocampal CA1 stratum radiatum as an initial step towards characterizing synaptic plasticity. We found no differences in the input–output curves between hippocampal slices from WT and *sut* mice (Figure 2A), suggesting that the loss of xCT does not alter basal synaptic transmission. Similarly, no significant difference was observed in PPF between the two genotypes (Figure 2B). PPF is mediated by a presynaptic mechanism [20], suggesting that the presynaptic plasticity is unchanged in *sut* mice.

LTP is reduced in *sut* mice

Next we examined LTP induced by tetanic stimulus (100 Hz, 100 pulses and two trains) at the Schaffer collaterals–CA1 synapses. The average fEPSP amplitude at 20–40 min after tetanic stimulus increased to $175.6 \pm 3.3\%$ of baseline in WT slices, whereas in *sut* mice the fEPSP was $140.4 \pm 2.6\%$ of baseline (Figures 3A and 3B), demonstrating that long-term synaptic plasticity is impaired by a loss of xCT.

Since CPG has been shown to inhibit xCT in hippocampal slices [21], we investigated further the effect of xCT on LTP by inhibiting xCT with CPG in hippocampal slices from WT mice. However, we did not observe any significant changes in LTP after xCT was blocked (Figures 3A and 3B).

As xCT is involved in non-vesicular glutamate release and extracellular glutamate levels are decreased in the hippocampus of xCT-knockout mice [11], we investigated whether the impaired LTP in *sut* mice results from reduced glutamate levels in the hippocampus. Given that the extracellular glutamate concentration in mammalian brains is in the range 0.5–5 μM [22], hippocampal slices from *sut* mice were infused with aCSF supplemented with glutamate (2.5 and 5 μM). No significant change was observed after glutamate infusion in hippocampal slices from *sut* mice (Figures 4A and 4B), suggesting that a transient change in extracellular glutamate has no effect on the induction or expression of LTP in hippocampal slices from *sut* mice.

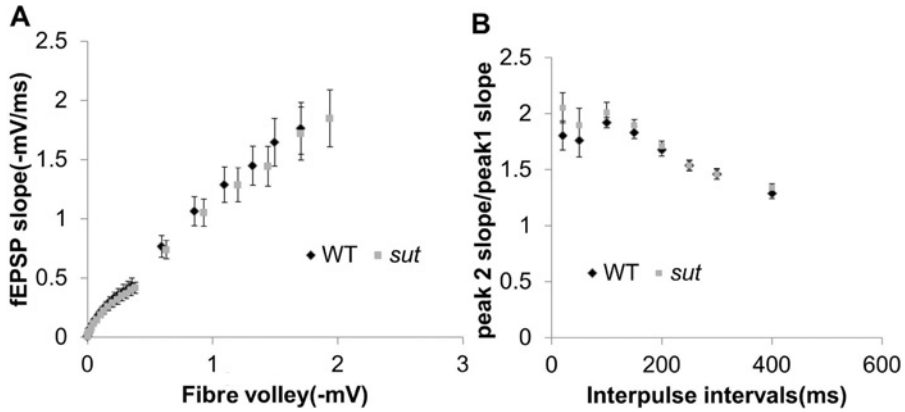

Figure 2 ***sut* mice have normal basal synaptic transmission and presynaptic short-term plasticity**
(**A**) No significant difference in the input–output curve was found between *sut* and WT mice ($n = 14$ slices from WT mice; $n = 17$ slices from *sut* mice). (**B**) The PPF ratio (fEPSP2/fEPSP1 slope) at intervals of 20, 50, 100, 150, 200, 250, 300 and 400 ms was similar between *sut* and WT mice ($n = 17$ slices from WT mice; $n = 19$ slices from *sut* mice).

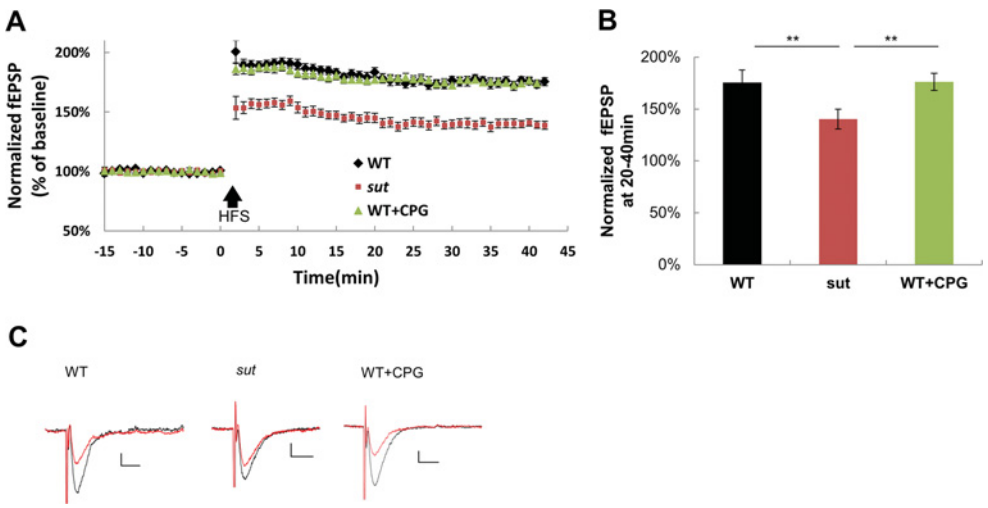

Figure 3 ***sut* mice have impaired LTP**
(**A**) fEPSP was recorded in hippocampal slices from WT and *sut* mice ($n = 13$ slices from WT mice in the absence of CPG; $n = 14$ slices from *sut* mice; and $n = 12$ slices from WT mice in the presence of CPG). (**B**) Comparison of the magnitude of LTP at 20–40 min. LTP in hippocampal slices from *sut* mice ($140.4 \pm 2.6\%$) was decreased compared with that of WT mice ($175.6 \pm 3.3\%$). Blocking xCT by CPG did not decrease LTP in hippocampal slices from WT mice ($176.2 \pm 2.4\%$). (**C**) Average of ten individual fEPSPs from representative recordings before the tetanic stimulus (red traces) and at 30 min after LTP induction (black traces). Axes represent 0.1 mV (x) and 10 ms (y).

No apparent morphological changes observed in *sut* mice

To investigate the possible mechanism for the reduction in LTP in *sut* mice, we examined whether there was any morphological change in the hippocampus. No significant defects were detected in the histological sections of the hippocampus from *sut* mice (Figure 5A). This agrees with the Nissl staining results in a previous study [2]. Furthermore, we examined ultrastructural morphology relevant to synaptic plasticity. In the CA1 asymmetric synapses of *sut* mice, there were no apparent changes in the synapse number, the width of synaptic cleft and the thickness of PSD compared with WT mice (Figures 5B and 5C), excluding the possibility that the reduced LTP in *sut* mice is caused by abnormal synaptic structures.

DISCUSSION

The function of xCT in cystine uptake has been intensively studied. The importance of cysteine in glutathione synthesis qualifies xCT as a key player in maintaining the intracellular redox balance. Many cultured cell lines undergo glutathione insufficiency

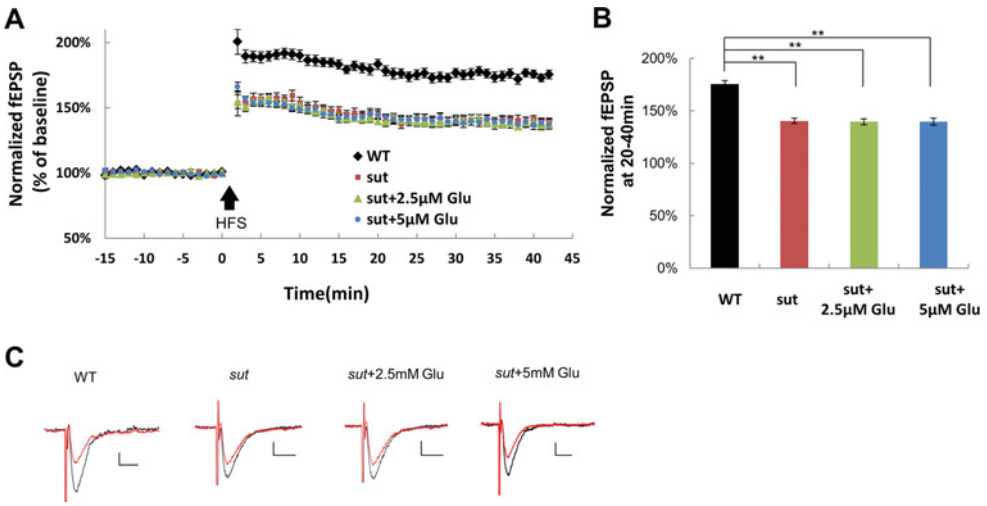

Figure 4 **Supplementation of extracellular glutamate does not restore the LTP reduction in *sut* mice**

(**A**) fEPSP was recorded in hippocampal slices from *sut* mice infused with added glutamate (2.5 and 5 μM) ($n = 13$ slices from WT mice; $n = 14$ slices from *sut* mice; $n = 12$ slices from *sut* mice in the presence of 2.5 μM glutamate; $n = 13$ slices from *sut* mice in the presence of 5 μM glutamate). (**B**) Comparison of the magnitude of LTP at 20–40 min. Elevation of extracellular glutamate levels did not increase LTP in hippocampal slices of *sut* mice (2.5 μM, $139.5 \pm 3.3\%$; 5 μM: $139.7 \pm 3.2\%$). (**C**) Average of ten individual fEPSPs from representative recordings before the tetanic stimulus (red traces) and at 30 min after LTP induction (black traces). Axes represent 0.1 mV (x) and 10 ms (y).

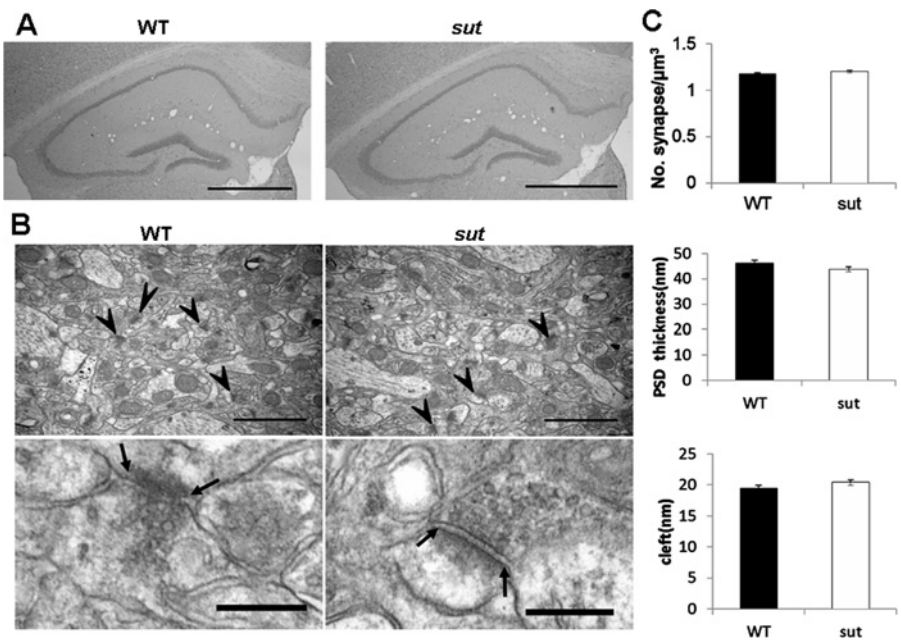

Figure 5 **Morphological studies in the hippocampus of *sut* mice**

(**A**) Low-power images of the hippocampus from WT and *sut* mice. H&E staining revealed no abnormality of gross morphology of the hippocampus. Scale bar, 0.8 mm. (**B**) Representative electron micrographs showing the asymmetrical synapses in the CA1 stratum radiatum of *sut* mice and WT controls. Arrowheads indicate individual asymmetrical synapses. Arrows mark the edges of the PSD. Scale bars, 1.5 μm (upper panels) and 0.3 μm (bottom panels). (**C**) Statistical analyses show no difference in synapse number (WT, $1.18 \pm 0.05/\mu m^3$; and *sut*, $1.20 \pm 0.17/\mu m^3$), the thickness of PSD (WT: 46.1 ± 1.2 nm, $n = 102$ synapses for three mice; and *sut*: 43.7 ± 9.7nm, $n = 103$ synapses for three mice) and the synaptic cleft (WT: 19.4 ± 0.5 nm, $n = 102$ synapses for three mice; and *sut*: 4.5 ± 0.5 nm, $n = 90$ synapses for three mice) between WT and *sut* mice.

upon pharmacological inhibition or genetic disruption of xCT, thus exhibiting impaired proliferation and the induction of apoptosis [2,23–25].

However, several independent lines of evidence suggest the role of xCT in redox maintenance is negligible *in vivo*, as both *sut* mice and xCT-knockout mice do not show apparent oxidative stress, and xCT deficiency does not increase vulnerability to oxidative stress in mouse brains and red blood cells [10,11,26]. This could be explained by the compensatory role of other cysteine transporters. For instance, the neuronal glutamate transporter EAAC1 (excitatory amino acid carrier 1), one of the members in the high-affinity Na^+-dependent glutamate transport system X_{AG}^- family, co-transports cysteine intracellularly. This transporter may play an important role in neuronal redox balance, as *EAAC1*$^{-/-}$ mice exhibit increased oxidant levels and increased susceptibility to oxidant injury [27]. This indicates that xCT is not the major regulator of redox balance in the neurons.

On the other hand, it is known that xCT plays a pivotal role in the maintenance of extracellular glutamate [7,8]. xCT deficiency results in a dramatic reduction in extracellular glutamate in xCT-knockout mice [10,11], which may lead to defects in glutamatergic neurotransmission [12,28]. Furthermore, xCT has been implicated in the dysfunction of metaplasticity caused by cocaine addiction [13]. In contrast with our expectation that impaired LTP in *sut* mice results from a reduction in extracellular glutamate levels, supplementation of extracellular glutamate did not restore LTP in *sut* mice. Moreover, blocking xCT by CPG did not mimic impaired LTP in *sut* mice. Although CPG might have an effect on LTP as an antagonist for the group I metabotropic glutamate receptor, we did not observe any obvious changes in the LTP of hippocampal slices from WT mice after treatment with CPG, excluding the possibility of a potential interference of CPG with LTP. These results indicate that the acute disruption of xCT in WT mice or supplementation of extracellular glutamate in *sut* mice did not alter synaptic plasticity.

We thus hypothesize that xCT may affect synaptic plasticity developmentally through modulating extracellular glutamate. Ambient extracellular glutamate has been shown to have multiple functions in early brain development. Migration of hippocampal pyramidal neurons and interneurons are partially modulated through glutamate receptors by paracrine glutamate at an early developmental stage in mice [29,30]. In addition, excess extracellular glutamate causes multiple brain defects in *Glast/Glt1* (glutamate aspartate transporter/ glutamate transporter 1)-double-knockout mice, including cortical and hippocampal disorganization with perinatal mortality, which may be attributable to an impaired radial glial fibre system as a result of the overactivation of NMDA (*N*-methyl-D-aspartate) receptors [31]. As xCT is also expressed prenatally during the development of the brain [32], it is possible that the level of extracellular glutamate remains consistently low in the brains of fetal *sut* mice as observed in the adult brain [10,11]. This might affect the development of the nervous system owing to the chronic shortage of extracellular glutamate. However, we did not observe any morphological defects in the hippocampal organization or synaptic ultrastructures in *sut* mice. We speculate that the insufficiency of extracellular glutamate may developmentally affect the synaptic circuitry required for LTP, thus leading to impaired long-term memories, or the spatial memory as observed in the xCT-knockout mice [11]. The results of the present study provide evidence that xCT is a regulator of synaptic plasticity. The underlying molecular mechanism awaits further investigation.

AUTHOR CONTRIBUTION

Wei Li, Shumin Duan and Zhongsheng Sun designed the experiments. Yan Li, Zhigang Li and Zhibing Tan performed the assays. Yan Li, Shumin Duan and Wei Li analysed the data. Wei Li and Yan Li wrote the paper, and all of the authors approved the submission.

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