Implication of the glutamate–cystine antiporter xCT in schizophrenia cases linked to impaired GSH synthesis

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xCT is the specific chain of the cystine/glutamate antiporter, which is widely reported to support anti-oxidant defenses in vivo. xCT is therefore at the crossroads between two processes that are involved in schizophrenia: oxidative stress and glutamatergic neurotransmission. But data from human studies implicating xCT in the illness and clarifying the upstream mechanisms of xCT imbalance are still scarce. Low glutathione (GSH) levels and genetic risk in GCLC (Glutamate–Cysteine Ligase Catalytic subunit), the gene of limiting synthesizing enzyme for GSH, are both associated with schizophrenia. In the present study, we aimed at determining if xCT regulation by the redox system is involved in schizophrenia pathophysiology. We assessed whether modulating GCLC expression impact on xCT expression and activity (i) in fibroblasts from patients and controls with different GCLC genotypes which are known to affect GCLC regulation and GSH levels; (ii) in rat brain glial cells, i.e., astrocytes and oligodendrocytes, with a knock-down of GCLC. Our results highlight that decreased GCLC expression leads to an upregulation of xCT levels in patients’ fibroblasts as well as in astrocytes. These results support the implication of xCT dysregulation in illness pathophysiology and further indicate that it can result from redox changes. Additionally, we showed that these anomalies may already take place at early stages of psychosis and be more prominent in a subgroup of patients with GCLC high-risk genotypes. These data add to the existing evidence identifying the inflammatory/redox systems as important targets to treat schizophrenia already at early stages.

INTRODUCTION

The system xc⁻ is a sodium-independent antiporter, which imports cystine and exports glutamate in a 1:1 ratio.¹ Intracellular cystine is readily reduced to cysteine, the limiting precursor for glutathione (GSH) synthesis. Accordingly, xc⁻ is widely reported to support anti-oxidant defenses in vivo.²,³ xc⁻ is a heterodimer formed by the association of xCT (coded by SLC7A11) and 4F2hc (SLC3A2). xCT is the specific chain and an increase of gene expression often reflects an enhancement of cystine transport.³–⁵ xCT is stabilized at the membrane by CD44, a receptor for hyaluronic acid, whose expression increases intracellular levels of cysteine and GSH.⁶

Oxidative stress induces the expression of xCT.⁷ The transcription factor Nrf2 is well described as a master regulator for the up-regulation of genes in response to oxidative stress.⁷ In basal conditions, Keap1 binds to Nrf2 and promotes its degradation by the ubiquitin-proteasome system.⁸ In case of oxidative stress, Keap1 dissociates, allowing Nrf2 accumulation, translocation to the nucleus, and binding to antioxidant response elements (ARE) in the promoter regions of target genes.⁹–¹¹ The promoter of SLC7A11 contains four ARE¹²,¹³ and activation of SLC7A11 expression by oxidation depends on Nrf2 as shown in Nrf2⁻/⁻ mice.¹² The Nrf2 inducer tert-butyl-hydroquinone (tBHQ), as well as the inhibitor of GSH synthesis buthionine sulfoximine (BSO), robustly increase xCT protein levels in cell culture.¹⁴,¹⁵

xCT expression is high in the brain¹⁶,¹⁷ where it is expressed by astrocytes¹⁸ while mature neurons show no or little expression.¹⁸ xCT in rodent brain modulates extracellular glutamate levels through non vesicular release: over half of the non synaptic release of glutamate is attributed to the antiporter.¹⁹–²¹ Changes in xCT levels are linked to many neurological and psychiatric disorders, including schizophrenia based on two human studies.²²,²³ In ‘postmortem’ tissue of dorso lateral-prefrontal cortex, xCT protein levels are increased in schizophrenia patients compared to control individuals.²⁴ A recent study reported that SLC7A11 gene expression is decreased compared to controls in peripheral white blood cells from Chinese Han patients.²⁵ The authors of both studies excluded a confounding effect of antipsychotic treatment but did not identify potential upstream pathways which may lead to xCT impairment in patients.

Mounting evidence suggests oxidative stress and impairment of fast-spiking GABAergic interneurons as interdependent mechanisms forming a hub in schizophrenia physiopathology on which genetic and environmental factors converge.²⁶–²⁸ Many studies revealed markers of oxidative stress in patients, both in the brain and in peripheral samples such as blood or fibroblasts.²⁹ In line with these observations, levels of the anti-oxidant defenses differ between patients and control individuals.³⁰ These data indicate that induction of the response to oxidative stress, despite being present to some extent, is not efficiently regulated in patients. The gene coding for the limiting enzyme for GSH synthesis (GCLC: Glutamate-Cysteine Ligase Catalytic subunit) was associated with schizophrenia and variants of the tri-nucleotide repeat polymorphism in GCLC were more frequent in schizophrenia patients than in controls (GCLC high-risk genotypes).³¹ The GCLC high-risk genotypes are associated with a decrease of GSH levels in medial prefrontal cortex³² and in fibroblasts.³³ Moreover a metabolomic

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study with patients’ fibroblasts showed altered reactivity to oxidative stress in GCLC high-risk genotypes. Therefore xCT is at the crossroads between oxidative stress and glutamatergic neurotransmission, two processes that are involved in schizophrenia. But data from human studies implicating xCT in oxidative stress in schizophrenia. Two processes that are involved in schizophrenia. The oxidative stress induces xCT function, which subsequently participates in the dysregulation of glutamatergic signaling. In the present study, we aimed at determining if xCT regulation by the redox system is involved in schizophrenia physiopathology. We focused on patients and controls to assess the impact of GCLC high-risk genotypes on xCT expression and activity, either in control conditions or by tBHQ-induced anti-oxidant response. We investigated whether impairment of GCLC expression may alter xCT function in rat brain glial cells, astrocytes and oligodendrocytes.

RESULTS

GCLC high-risk genotypes are associated with increased levels of xCT mRNA. Fibroblasts from schizophrenia patients or control individuals, with GCLC high-risk or GCLC low-risk genotypes (Table 1), were treated or not by tBHQ. Gene expression was assessed by microarray in vehicle and tBHQ-treated conditions.

In the top five genes that were up-regulated by tBHQ both in patients and controls, SLC7A11 (coding for xCT) expression showed a 7.9-fold increase (false discovery rate for paired comparisons, FDR < 1.10⁻⁵; Fig. 1a). Expression of the gene SLC3A2 (coding for 4F2hc) was also increased by tBHQ, suggesting an overall enhancement of amino-acid uptake (fold change, FC = 3.10; FDR = 1.10⁻⁵).

Levels of SLC7A11 and SLC3A2 were similar between patients and controls both in vehicle (FC = −1.03; p-value = 0.85; FC = 1.04; p-value = 0.73) and tBHQ-treated condition (FC = 1.06; p-value = 0.72; FC = 1.04; p-value = 0.70).

When comparing individuals (patients and controls) with GCLC high-risk and GCLC low-risk genotypes, SLC7A11 was the most up-regulated gene associated with GCLC high-risk variants, with a 2-fold increase of expression already at basal level (p-value = 1.2,10⁻⁵; Fig. 1b). Expression of the subunit SLC3A2 was not modified (FC = 1.22; p-value = 0.08), however the gene CD44 was slightly increased (FC = 1.16; p-value = 0.002). For SLC7A11, the interaction between tBHQ treatment and genotype was significant (t = −3.81; p-value = 0.0004); examining the means of expression indicates that the up-regulation in response to tBHQ was less in the GCLC high-risk genotypes than in the GCLC low-risk genotypes (Fig. 1c).

Table 1. Demographics for the microarray study

| Schizotype          | Patients | Controls |
|---------------------|----------|----------|
| Low-risk            | 11       | 8        |
| High-risk           | 11       | 9        |

Age in years (s.d) 22.6 (3.5) 22.3 (3.2) 23.5 (3.6)

| Sex (males/females) | Patients | Controls |
|---------------------|----------|----------|
| Low-risk            | 11 / 0   | 8 / 0    |
| High-risk           | 11 / 0   | 9 / 0    |

| Diagnostic          | Patients | Controls |
|---------------------|----------|----------|
| Schizophrenia       | 9        | 6        |
| Schizo-affective    | 1        | 1        |
| Schizophreniform    | 1        | 1        |

| Illness duration in years (s.d) | Patients | Controls |
|---------------------------------|----------|----------|
| Low-risk                         | 3.8 (3.2)| 2.0 (1.2)|
| High-risk                        | 3.8 (3.2)| 2.0 (1.2)|

Medication in CPZ (mg/day; chlorpromazine equivalents)

| Patients | Controls |
|----------|----------|
| Low-risk | 303 (247) |
| High-risk| 301 (265) |

Table 2. Demographics for the uptake experiments

| Gene ID | FC  | FDR  | Protein name | short name |
|---------|-----|------|--------------|------------|
| SLC7A11 | 1.78| 0.002| xCT          | xCT        |
| SLC14A1 | 1.77| 0.005| xCT          | xCT        |
| B2L/D   | 1.72| 0.007| xCT          | xCT        |

Fig. 1 SLC7A11 expression in skin fibroblasts with GCLC high-risk or GCLC low-risk genotypes. Top-5 genes up-regulated in fibroblasts treated by tBHQ versus vehicle (a), and up-regulated in fibroblasts with GCLC high-risk versus GCLC low-risk genotypes (b) both in patients and controls. FC fold of change, FDR false discovery rate (paired comparisons). c Plot illustrating microarray data for SLC7A11. Data are represented as mean ± standard error of the mean.

Table 3. Top-5 genes up-regulated in tBHQ vs vehicle conditions

| Gene ID | FC  | FDR  | Protein name | short name |
|---------|-----|------|--------------|------------|
| MHC0T   | 19.1| 5.25E-32| Heme oxygenase 1, HO-1 |
| EDNRB   | 9.6 | 7.26E-25| Endothelin B receptor, ET-B |
| SLC6A15 | 9.1 | 1.98E-26| Sodium-dependent neutral amino acid transporter, B0T1 |
| SLC7A11 | 7.9 | 6.26E-22| Cysteine/glutamate transporter, xCT |
| MNK1    | 7.8 | 1.50E-22| Interferon gamma |

Altogether these data indicated that regulation of xCT expression was altered in individuals with GCLC high-risk genotypes. Therefore GCLC high-risk schizophrenia patients may represent a distinct subgroup with more pronounced anomalies of xCT regulation than GCLC low-risk patients. In a next step, we aimed at validating this finding at the functional level in early psychosis patients. In order to maximize the power and to avoid bias due to sex, we analyzed only male early psychosis patients.

GCLC high-risk genotypes are associated with increased cystine uptake

We quantified cystine uptake by xCT system in fibroblasts from GCLC low-risk, high-risk early psychosis patients and age-matched GCLC low-risk controls (Table 2), in vehicle and tBHQ treated conditions.

In vehicle conditions, cystine uptake was higher in GCLC high-risk patients than in GCLC low-risk patients and GCLC low-risk
controls (respectively 1.4-fold; p = 0.010 and 1.2-fold; p = 0.041, see Fig. 2a). The uptake was inhibited by the addition of glutamate or by the xCT inhibitor sulfasalazine, therefore indicating the specificity of the measurements (Supplementary Fig. 1). As expected, treatment with tBHQ increased cystine uptake by 4-fold (p < 0.01). The tBHQ-induced cystine uptake was comparable for the three groups. After tBHQ treatment, cystine uptake remained higher in GCLC high-risk patients compared with GCLC low-risk controls (1.6-fold; p = 0.040, Fig. 2b), but not compared with GCLC low-risk patients. Cystine uptake was not correlated with the levels of anti-psychotic treatment (Supplementary Fig. 2).

Because regulation of xCT system may differ according to cell types, we wanted to clarify whether these impairments are relevant for specific brain cells.

**GCLC down-regulation increases cystine uptake by astrocytes**

Because xCT system is mostly present in astrocytes and not in neurons,18 we assessed cystine uptake in glial cells from rat cortex (oligodendrocyte progenitor cells (OPCs) and astrocytes). To impair the regulation of GCLC, cells were transduced by lentivirus to overexpress shRNA as previously described.34

Knock-down with shRNA decreased GCLC protein levels by 49% in OPCs.34 OPCs had a slow uptake of cystine, which reached a maximum after 30 min and was inhibited by the addition of glutamate (Fig. 3). The knock-down of GCLC did not affect the level of cystine uptake after 15 min (Fig. 3a) nor after 30 min compared to either scrambled shRNA or non-infected cells (Fig. 3b).

In dividing astrocytes, knock-down with shRNA decreased GCLC mRNA by 27% (Fig. 4a). Cystine uptake was faster in astrocytes than in OPCs, and was also inhibited by sulfasalazine (Fig. 4b). After GCLC knock-down, the uptake was 1.6-fold higher than in control conditions with scrambled shRNA, both after 1 and 5 min of uptake (Fig. 4b).

Primary astrocytes were also cultured with di-butylryl cyclic AMP (dbcAMP) as dbcAMP-treated astrocytes may resemble more closely the differentiated astrocytes present in brain tissue.15,35 Cyclic AMP induces morphological changes, stops cell division and increases antioxidant defenses.35–37 dbcAMP treatment increased cystine uptake by 6-fold higher uptake of cystine than dividing astrocytes (see uptake at 5 min in Fig. 4b), and consistent with previous publications.35,38 In dbcAMP-treated astrocytes, knock-down with shRNA decreased GCLC mRNA by 46% (Fig. 4a). GCLC knock-down in dbcAMP-treated astrocytes led to a 1.2-fold and 1.5-fold increase of cystine uptake after 1 and 5 min respectively compared with scrambled shRNA (p = 0.028 and 0.045 respectively, Fig. 4c).

**Discussion**

We studied the regulation of xCT by genetic impairment of GSH synthesis. We found that fibroblasts with GCLC high-risk genotypes, which are associated with lower brain GSH brain levels and higher risk for schizophrenia31,32 displayed higher expression and higher activity of xCT than fibroblasts with GCLC low-risk genotypes. The tBHQ-induced increase of cystine uptake appeared to be similar across genotypes. In a translational approach, we confirmed that GCLC knock-down increased the activity of xCT in primary culture of rodent astrocytes. We did not observe the same response to GCLC knock-down in oligodendrocyte precursors, therefore underlining that xCT regulation by GCLC levels was cell type dependent. Altogether, the results indicate that impaired GSH synthesis leads to the upregulation of xCT activity in specific glial cells, a mechanism already relevant to early stages of schizophrenia.

These data add to the characterization of GCLC high-risk genotypes. Previous works indicate that these high-risk genotypes, without an additional oxidative stress, affect at least two metabolic pathways in cultured fibroblasts: the redox system31,33 and lysolipids levels.33 Importantly, the redox pathway is also affected in blood39 and in the brain as shown by the 14% decrease of GSH concentration in prefrontal cortex of GCLC high-risk individuals.32 Here we show that the GCLC high-risk genotypes are also associated with an increased activity of xCT in fibroblasts. Frequencies of GCLC high-risk genotypes are higher in schizophrenia patients than in controls31 and vary with the ethnicity.40

Controlling for this confounding factor in case-control studies is thus important as it may explain discrepancies between studies. The decrease of GCLC expression (by genetic variants or by knock-down) may increase xCT activity through the Nrf2 signaling pathway. Indeed GCLC expression tightly controls GSH levels, and the high-risk variants are associated with lower GSH.31,32 Depletion of cysteine or GSH may lead to oxidative stress, to the activation of Nrf2, and to the enhancement of xCT activity.12 Consistently, previous work showed that depletion of intracellular cysteine or GSH enhanced the activity of the cysteine-glutamate antiporter.15,41 Although Nrf2 is the most studied antioxidant regulator, other pathways may also be involved.15 For instance, SLC7A11 promoter also contains binding sites for ATF4, a

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**Fig. 2** Cystine uptake by skin fibroblasts: Cells were treated (a) with vehicle (0.05% DMSO) or (b) with tBHQ (50μM for 18 h) which induces the anti-oxidant response. Internalized Cystine was assessed after 5 min of uptake. Data are represented as mean ± standard error of the mean; *p-value < 0.05
transcription factor typically activated by amino acid starvation. Activation of ATF4 pathway up-regulates xCT, increases intracellular GSH levels, and confers resistance to oxidative stress.

An interesting downstream effect of enhanced xCT activity is the increased efflux of glutamate, which may participate to schizophrenia physiopathology by affecting the inhibitory/excitatory balance. Glutamate levels in various brain regions are higher in early psychosis patients than in matched healthy controls. Accordingly, impairment of glutamate transport has been suggested by ‘postmortem’ brain studies of schizophrenia patients. The upregulation of xCT may thus participate in the impairment of glutamate transport and in the increase of brain glutamate in schizophrenia. xCT activity has been shown to significantly affect glutamate levels as knock-out mice for Slc7a11 have decreased levels of extracellular glutamate. Gene deletion leads to minor spatial memory deficits, and impaired hippocampal LTP and acute inhibition of xCT is associated with anxiety-related behaviors. Inactivation of the glial glutamate transporter GLAST, which likely leads to an increase of extracellular glutamate, also leads to endophenotypes that are associated with schizophrenia such as memory deficits and impaired acoustic startle.

Interpretation of the results is limited by the use of rodent cells, as it is not clear to which extent rat primary glial cells reflect human brain physiology. Because xCT is induced by the higher O2 levels in culture compared to in vivo conditions, interpretation of this work is limited by the in vitro setting. Nevertheless, regulatory mechanisms of xCT expression have been largely deciphered by studies using cell culture, thus highlighting that our conclusions are transposable to in vivo conditions. Moreover, our observation that OPCs did not display an increase of xCT function following GCLC knock-down is also in favor of a specific regulation.

In conclusion, our data show that a decrease of GCLC expression, the limiting synthesizing enzyme for GSH, leads to an upregulation of xCT levels in patients’ fibroblasts as well as in astrocytes. These results from schizophrenia patients support the emerging data involving xCT dysregulation in illness physiopathology and further indicate that it can result from redox changes such as lower GSH levels, which have been previously associated with schizophrenia. According to our results, xCT dysregulation already takes place at early stages of psychosis and is more prominent in a subgroup of patients with GCLC high-risk genotypes. Investigating consequences of xCT dysregulation at the clinical level would shed light on the symptoms that may respond to molecules targeting the immune and/or redox system.

**METHODS**

**Recruitment**

All individuals were recruited in Lausanne area, Switzerland. Early psychosis patients were diagnosed according to DSM-IV criteria after a 3-years follow-up in the TIPP program (University Hospital Lausanne). Patients included in the TIPP program had less than 6 months of antipsychotic medication. Less female than male patients have been recruited in this cohort; therefore we focused on men the study of early psychosis patients. Control subjects were assessed and selected with the Diagnostic Interview for Genetic Studies. Individuals with a neurological, major mood, psychotic, or substance-use disorder and a first-degree relative with a psychotic disorder were excluded. All enrolled subjects provided a fully informed written consent; all procedures, including biopsy, were in accordance with the ethical standards of the Helsinki Declaration as revised in 1983, and was approved by the ethical committee of Lausanne University Hospital on human experimentation.

**Culture of fibroblast**

Secondary cultures of fibroblasts were established from skin biopsies. Skin-derived fibroblasts from patients with early psychosis and age-matched, sex-matched controls were processed in parallel as described previously. We could not match for GCLC genotypes as control individuals with GCLC high-risk genotypes were not frequent enough in our cohort. After thawing, cells were amplified in Dulbecco’s Modified Eagle Medium (DMEM, Gibco), 2% Ultroser (Pall Corp), 1% penicilline-streptomycine (Gibco). After five passages, we plated the fibroblasts at 5,10^5 cells/well (12-wells plate); we treated the cells the day after (18 h of treatment, 50uM BHQ (Sigma) or 0.05% dimethylsulfoxide (Sigma) for vehicle).

**Microarray**

RNA was purified with RNAeasy column (QIagen); quality was checked by Agilent 2100 bioanalyzer chips. Affymetrix, 1.0ST GeneChips were processed at Lausanne Genomic Technologies Facility according to manufacturer recommendation. Hybridization quality was assessed using the Bioconductor package affy in R (http://www.R-project.org; http://www.Bioconductor.org). Log2 normalized expression signals were calculated using RMA algorithm comprising background correction, quantile normalization and probe set summarization by robust regression. Subsequent analyses were based on Gene Ontology Annotation (UniProt-GOA).
plated at 1.2,10^5 cells/well in 12-wells plate (DMEM, 2.5 μM forskoline (Calbiochem), 50 μg/ml apotransferrin, 5 μg/ml insulin (Sigma), 30 mM sodium selenate (Sigma), 10 mM hydrocortisone (Sigma), 10 mM D-biotine (Sigma), 1 mg/ml bovine serum albumin (Sigma), 10 ng/ml PDGF-AA (Sigma), 10ng/ml human fibroblast growth factor-basic (Sigma)); experiments were performed 14 DIV. In parallel, astrocytes which remained attached after shaking were plated at 7,5,10^5 cells/well in 12-wells plates in normal culture media (DMEM, 10% FCS, 1% penicillin-streptomycin) or in differentiation media (DMEM, 2% FCS, 15μM di-butyl cyclic AMP (Enzo) for 8 days).

Cystine uptake
Xc- activity was assessed based on previously published protocol. \textsuperscript{53} Briefly, cells were washed with Hank’s balanced salt solution pH 7.4 (HBSS; 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 0.1% Glucose, 20 mM Hepes (Sigma)) and equilibrated in HBSS for 10 min at 37 °C, eventually with transporter inhibitor (sulfasalazine 1 mM (Sigma); glutamate 2.5 mM). Medium was then changed for uptake buffer (0.5 mM acivicin, 1 mM D-aspartate, 35μM cystine including 1uCi/mL of 14C-cystine (PerkinElmer) in HBSS). Uptake was done at 37 °C and terminated on ice by removing uptake buffer and adding cold phosphate buffer saline (PBS). Cells were lysed with 500 μL of warm PBS with 0.5% sodium-dodecyl-sulfate. Incorporated radioactivity was quantified by liquid scintillation counting (Tricarb 2900TR Packard). Levels of radioactivity are normalized for protein content assessed with Bicinchoninic acid assay, the mean of technical duplicates was calculated. Data presented are representative of two experimental replications.

Statistical analyses
For microarray data, we calculated for each probe set M-values (log base 2 of the fold change between two conditions), moderated t-statistic (ratio of the M-value to its standard error), p-values derived from moderated t, and adjusted p-value (FDR, Benjamini–Hochberg step-up procedure). For uptake experiments, we used student t-test in R on log-transformed data.

Data availability statement
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS
M.F., K.D. conceived the study. P.C., conceived the recruitment. C.F., P.S.B., P.C. evaluated and recruited the patients. M.F., A.M. performed the experiments, M.F., K.D. conceived the study. P.C., conceived the recruitment. C.F., P.S.B., P.C.

ADDITIONAL INFORMATION
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Fig. 4 Uptake of cystine by astrocytes: a Decrease of GCLC mRNA assessed by quantitative PCR in astrocytes transduced with GCLC shRNA is expressed as percentage of the condition with scrambled shRNA. b Uptake of cystine in dividing astrocytes transduced with scrambled (black) or GCLC shRNA (white). c Uptake of cystine in dBCAMP-treated astrocytes transduced with scrambled (black) or GCLC shRNA (white). Data are represented as mean ± standard error of the mean (n = 3 per group); *p-value < 0.05.

Primary cultures of glial cells
All animal procedures were approved by the Swiss cantonal veterinary office. Primary glial cells were dissociated from cortex of males and females Wistar Han rat pups at 3-days postnatal as previously described. Cells were cultured in DMEM, 10% fetal calf serum (FCS), 1% penicillin-streptomycin at 37 °C, 5% CO₂. After 7 days in vitro (DIV), cells were infected with lentiviruses (multiplicity of infection: 5) to overexpress GCLC shRNA: GGAGGCTACTTCTATATTA or scrambled shRNA: CCTACAATCA-GACTGGCGA. Puromycin was added 48 h post-infection (Calbiochem, 1ug/mL). After 10 DIV, oligodendrocytes progenitor cells (OPCs) were separated from astrocytes and microglia by overnight shaking. OPCs were

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Upregulation of xc- activity in schizophrenia

M Fournier et al.

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