Journal of Drug Metabolism and Toxicology

Yu et al., J Drug Metab Toxicol 2015, 6:4
DOI: 10.4172/2157-7609.1000190

Research Article Open Access

Effect of Glutamate on Brain Iron Metabolism and the Regulation Mechanism

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Received date: August 24, 2015; **Accepted date**: September 19, 2015; **Published date**: September 28, 2015

Abstract

Glutamate is an excitatory transmitter and can induce neurotoxicity, it can also increase the iron concentrations in the brain, but little is known about the detailed molecular regulation mechanism of iron metabolism by Glu. Based on our previous data, iron metabolism related proteins might be associated with the increase of brain iron contents induced by neurotransmitter. To investigate the issues, the iron contents, non-transferrin-bound iron (NTBI) uptake and the expression of iron uptake and iron release proteins were firstly examined in vivo and in vitro with iron histochemistry, inductively coupled plasma mass spectroscopy (ICP-MS), ^{55}Fe radioactive liquid scintillation counting and western blot methods. Data showed that glutamate induced the increase of total iron contents, storage iron contents and NTBI uptake activity. Moreover, only divalent metal transporter 1, one of iron uptake proteins, was increased in rat brain and PC12 cells treated with glutamate. Further investigations revealed that nuclear factor κB (NF-κB) and protein kinase C (PKC) were involved in the regulation of DMT1 in PC12 cells treated with glutamate. These findings demonstrate that glutamate increases iron contents in the brain through increased NTBI, and that DMT1 is the key molecule underlying regulation of iron metabolism by glutamate, Furthermore, NF-κB and PKC play important roles in the regulatory pathway of DMT1 expression by glutamate. Thus, it implicates that inhibiting the expression of DMT1 and disruption of its regulation pathway might be effective strategies in attenuating glutamate neurotoxicity through decreased iron contents.

Keywords: Divalent metal transporter 1; Nuclear factor κB; Protein kinase C; Glutamate; Iron; Rat

Abbreviations:

AD: Alzheimer's Disease; ALS: Amyotrophic Lateral Sclerosis; CLSM: Confocal Laser Scanning Microscope; DMT1: Divalent Metal Transporter 1; FPN1: Ferroportin 1; Glu: Glutamate; HD: Huntington's Disease; ICP-MS: Inductively Coupled Plasma Mass Spectroscopy; IRE: Iron Responsive Element; FTL: L-Ferritin; NDs: Neurodegenerative Diseases; NF-κB: Nuclear Factor κB; NTBI: Non-Transferrin-Bound Iron; PD: Parkinson's Disease; PDL: Poly-D-Lysine; PKC: Protein Kinase C; SFM: Serum-Free Medium; TfR1: Transferrin Receptor 1

Introduction

It has been well known that glutamate (Glu) is an abundant excitatory transmitter in the brain [1,2]. However, high concentrations of glutamate and excessive stimulation of Glu-gated cation channels were also correlated closely with neurodegenerative diseases (NDs) including Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) due to the neurotoxicity of glutamate [2-5]. It has been accepted that Glu induces excessive influx of Ca^{2+}, which leads to subsequent intracellular cascade of cytotoxic events (such as nitric oxide production) and further exacerbates oxidative damage [6,7]. However, it is unknown if there are other mechanisms accounting for the excitotoxicity of glutamate.

Over the last decades, it has been confirmed that iron is essential for normal biochemical processes in the brain, such as oxygen transport, electron delivery, neurotransmitter synthesis, myelin production and maintenance [8]. However, in cases of PD, there is an increase in the total iron content of the substantia nigra, accompanied by the loss of nerve cells in the same regions [9,10]. The levels of iron are also significantly increased in the globus pallidus in Alzheimer's disease (AD) and PD [11,12]. Iron overload and iron-induced oxidative stress constitute one of the mechanisms that are involved in the development of NDs such as PD and AD [13].

Earlier studies have recorded the relationship between iron and glutamate. It is reported that microinjection of excitatory amino acids inhibitor into the striatum of brain leads to alterations of iron concentrations in the ipsilateral globus pallidus and substantia nigra pars reticulate [14-16]. Our previous data also showed that the iron content was increased in the caudate putamen after microinjection of glutamate into substantia nigra [17]. While increased iron could induce oxidative damage to cells through Fenton reaction. These reports predicted new evidences for glutamate neurotoxicity from the role of iron metabolism. However, it is not well known how glutamate regulates the iron concentrations and the molecular mechanism in central nervous system.

Divalent Metal Transporter 1 (DMT1) is an important iron uptake protein in the brain [18,19], and its identification makes the investigation go into the molecular level. Ke et al. [20] found that DMT1 is distributed in different regions of rat brain and that development can significantly affect DMT1 expression in these areas. Our previous data [21] found that L-DOPA can significantly increase ferrous iron uptake and iron influx protein DMT1 expression in C6 cells, and that intranigral injection of glutamate induced the elevation of DMT1 protein level in the caudate putamen. All these seem to suggest that DMT1 might be a key molecule connecting iron and neurotransmitters. However, how glutamate regulates the expression of DMT1 in neuronal cells needs to be clarified.

PC12 cells, derived from rat pheochromocytoma, have been widely used as a neuron model in neurobiology and neuropharmacology because they have many properties in common with primary sympathetic neurons and chromaffin cell cultures [22]. Therefore, the present experiments were performed to detect the effect of glutamate on the iron contents, DMT1 expression and its regulatory mechanism in rat and PC12 cells. Results in this paper showed that glutamate can significantly increase iron contents through increased DMT1 expression accompanied by NTBI, and that NF-κB and PKC are involved in the regulation of DMT1 by glutamate.

Materials and Methods

Materials and reagents

The primary DMT1(+IRE)DMT1(-IRE) and FPN1 antibodies were purchased from ADI (ADI, San Antonio, TX, USA), the primary TfR1 antibody was from ZYMED (Invitrogen, Carlsbad, CA, USA), the primary FTL antibody was from Epitomics (Epitomics, Burlingame, CA, USA), the primary β-actin antibody was from Sigma (Sigma Chemical Co. St. Louis. Mo, USA), the primary NF-κB-p65 antibody was from Thermo-Fisher (Thermo-Fisher Scientific Inc., Rockford, USA), the primary H3 antibody was from Bioworld (1:500; bioworld, Louis park, USA). The secondary antibodies conjugated to horseradish peroxidase were purchased from Vector Labs (Burlingame, CA, USA); Dylight 488-conjugated secondary antibody was from Millipore (Temecula, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and horse serum were from Gibco (Gibco, Grand Island, NY, USA). BAY 11–7082 was from Enzo (ENZO Life Sciences, Inc., Farmingdale, NY, USA), Phorbol 12-myristate 13-acetate (PMA) and Chelerythrine chloride (CH) were from Amresco (Solon, OH, USA). All other chemicals and regents were purchased from Sigma and local commercial sources.

Animals and treatments

Male SD rats, purchased from HEB LAC (Hebei Medical University, China), were housed in cages at 21 ± 2°C and provided free access to food and water. Rooms were humidity controlled under a cycle of 12 h light and 12 h dark. All rats were allowed to adapt to their living conditions for 3 days before experiments.

For intracerebroventricular injection (ICV) of Glu, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.), placed in a stereotaxic instrument (SR-6N, Tokyo, Japan) and Glu was injected as previously described, rostral~caudal: 0.8 mm posterior to Bregma, medial ~ lateral: 1.6 mm from the midline, and dorsal~ventral: 3.2 mm from the surface of the dura [23]. Sodium glutamate (Sigma) was administrated into rat lateral ventricle (60 μg and 120 μg, respectively), and the control group received the same volume of artificial cerebrospinal fluid (ACSF). 24 h later, cerebral cortex (CC), hippocampus (Hippo), substantia nigra (SN), cerebellum and striatum were dissected and collected for the following western blots and iron contents detection after rats were anesthetized and perfused with ice-cold normal saline.

All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of Hebei Science and Technical Bureau in PRC.

Determination of non-heme iron contents in brain

CC, Hippo, cerebellum and striatum were dried for determining the non-heme iron contents as described previously [24,25]. Briefly, 0.6 M trichloroacetic acid in hydrochloride was added to brain samples at a ratio of 12.5 mL/g tissue (dry weight), and the tube was maintained at 65°C for 20 h. A clear acid extract was obtained after centrifugation at 12,000 g for 10 min. Then 4 μL of the acid extract and an equal volume of standard iron solution (1 mg/mL) were mixed with 200 μL of a Chromogen solution containing 0.2% thioglycolic acid and incubated for 10 min in a 96-well plate. Finally, the absorbance was read at 540 nm and iron levels were as previously described [25].

SN is small in sample size, so its iron contents was detected in cryostat sections with a modified Perl's staining as previously described [23]. SN was cut at thickness of 20 μm and incubated at room temperature for 20 min in 1% H_2O_2 in 0.01 M phosphate-buffered saline (PBS) to quench endogenous peroxidase activity, then incubated at room temperature for 15 hr in a freshly made Perl's solution [1% potassium ferrocyanide in 1% aqueous hydrochloric acid for ferric iron], and the negative control sections were incubated in Perl's solution with PBS in place of potassium ferrocyanide. Finally, the sections were developed in a diaminobenzidine (DAB substrate kit SK-4100, Vector Laboratories) solution to enhance the staining of iron. To examine the morphology and detect the integrity of tissues, SN sections were stained in Nissle solution (1% cresyl violet, 0.5% toluidine blue and 0.25% thionine) at 50°C or 100 s.

Cell culture

All experiments in vitro were performed using PC12 cells (ATCC No. 1721). The cells were maintained in DMEM culture medium including 10% horse serum, 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and subcultured every 5 ~ 6 d at a ratio of 1:5.

Measurement of the total iron contents in PC12 cells with ICP-MS

The total iron content was assayed in PC12 cells treated with different concentrations of Glu by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, Thermo Fisher, X Series, FL, USA) [26]. After PC12 cells were incubated with different concentrations of glutamate, the medium was removed and the cells were washed with cold PBS. Then the cells were collected and added to 1 mL ultra-pure nitric acid (69.9–70.0%, J.T. Baker, USA) in Teflon digestion tubes, digested in the microwave digestion system for 2 h at 100°C, and then 4 h at 200°C. Standard curves ranging from 0 to 200 ppb were prepared by diluting iron standard with blanks prepared from homogenization reagents in

0.2% nitric acid. Standards and digested samples were read in triplicate by ICP-MS.

Measurement of non-transferrin-bound iron uptake with radioactive liquid scintillation counting

Iron-55 ($^{55}FeCl_3$, Perkin-Elmer Life Sciences Company, MA, USA) solution was prepared by mixing $^{55}FeCl_3$ (10 mM in 0.5 M HCl) with $FeSO_4$ (2 mM in 0.1 M HCl) in a molar ratio of 1:10, followed by a 50-fold molar excess of 2-mercaptoethanol and 0.27 M sucrose to give an iron concentration of 62.5 μM, as described previously [27,28]. After PC12 cells were incubated with Glu for 16 h in a CO_2 incubator at 37°C, the cells (about 0.5×10^6 cells) were washed three times with ice-cold PBS (pH 7.4). The culture medium was then replaced with 0.5 mL solution which consisted of 0.27 M sucrose buffered to pH 6.4 ~ 6.5 with 4 mM Pipes. Then the radioactive iron solution was added into the sucrose solution to make a final iron concentration of 1.0 μM as determined previously [27]. Following our previous results, the incubations were performed at 37°C in a shaking water bath for 40 min. Then the cells were removed from the incubation solution, promptly washed three times with ice-cold PBS, and homogenized with buffer containing 1% SDS (500 μL) for 10 min at room temperature with shaking. 300 μL of the homogenate was taken for counting radioactivity with a Liquid Scintillation Analyzer (Packard, 2200 CA USA) and the remainder was used for protein assay with the Lowery method. The results were expressed as pmol Fe/min/mg of protein.

Preparation of the whole cell lysate and nuclear protein extraction

To extract the whole protein of cell lysate, rat brain tissues and PC12 cells were homogenized in RIPA buffer (containing 0.15 mM NaCl, 10 mM NaPO4, 1% Triton X-100 for cells or 1% NP-40 for tissues, 0.1% SDS, 0.1 mg/mL PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin) with a sonicator (Soniprep 150, MSE Scientific Instruments, England). After centrifugation of the homogenate at 10 000 g for 30 min at 4°C, the supernatant was collected and the total cell extract was obtained.

The nuclear isolation and extraction of PC12 cells was performed according to the method previously described [29]. Briefly, after PC12 cells were incubated with glutamate for 30 min, they were collected and re-suspended in Hypotonic Lysis Buffer (HLB, 10 mM Tris-Cl pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 1 mg/mL pepstatin, 1 mg/mL leupeptin and 1 mg/mL aprotinin). After a strong vortex for 10 s, the tubes were incubated for 10 ~ 15 min on ice to promote lysis, at this time the samples were stained with trypan blue to check with a microscope for >90% lysis. Then the tubes were centrifuged (15 min, 1 250 g, 4°C) and the nuclear fraction pellet was at the bottom of the centrifuge tube. Afterwards, the pellets were washed once with HLB, 1/2 packed nuclear volume of Low Salt Buffer (20 mM Tris-Cl pH 7.9, 25% Glycerol, 20 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1% NP40, 1 mg/mL pepstatin, 1 mg/mL leupeptin and 1 mg/mL aprotinin) was added and stirred gently, then 1/2 packed nuclear volume of High Salt Buffer (20 mM Tris-Cl pH 7.9, 25% glycerol, 1.2 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1% NP-40, 1 mg/mL pepstatin, 1 mg/mL leupeptin and 1 mg/mL aprotinin) was added and kept on ice for 40 min, during which time strong vortex was carried out every 5 min. Finally, the tube was centrifuged at 14,000 g for 20 min at 4°C. The supernatants containing the nuclear extracts were obtained.

Western blot

About 30 μg of total proteins were separated using 10% SDS–PAGE gels and then transferred onto PVDF membranes (Hybond-P, Amersham Pharmacia Biotech) as previously described [30]. The blots were incubated with rabbit anti-rat DMT1 (–IRE)/ DMT1 (+IRE) / FPN1 antibody (1:5, 000), rabbit anti-rat FTL monoclonal antibody, rabbit anti-rat NF-κB-p65 antibody (1:1, 000), mouse anti-human TfR1 antibody (1:2,000) for 12 ~ 16 h at 4°C. Then the blots were incubated with anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxide (1:5,000) for 1h at room temperature. Immunoreactive proteins were detected by enhanced chemiluminescence method (ECL kit, Pierce, USA). To ensure even loading of the samples, the same membrane was probed with rabbit anti-human β-actin antibody (1:5,000) for whole cell lysate or rabbit anti-rat H_3 antibody (1:500) for nuclear extract protein. All raw values obtained were standardized to the endogenous control.

Immunocytochemistry

After PC12 cells climbed onto the glass slides coated with PDL, they were incubated with 1000 μM Glu for 30 min, 1 h, 2 h and 4 h. Then the medium were discarded and the PC12 cells on the slides were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.2 ~ 7.4) overnight at 4°C. Next, the slides were washed in 0.01 M PBS (pH 7.4) and blocked with normal goat serum for 30 min at 37°C. After that, the slides were incubated in a primary mixture antibody containing rabbit anti-rat NF-κB-p65 antibody (1:150) for 24 h at 4°C. Then Dylight 488-conjugated secondary antibody (1:200) was added and kept in the dark for 60 min at 37°C. Subsequently DAPI was incubated for 5 min at room temperature. The slides were rinsed in PBS three times (5 min each) after every step. Finally, the slides were cover-slipped with glycerol and PBS at a ratio of 7:3. The slides were observed with a Confocal Laser Scanning Microscope (CLSM, Carl Zeiss LSMPascal 710, Zeiss, Oberkochen, Germany), and all images were digitally acquired using LSM software (ZEN2010).

Statistical Analysis

The data were expressed as means ± SD of 6 separate experiments. The differences between the means were determined using Statistical Product and Service Solutions (SPSS 21.0) followed by Post-Hoc Multiple Comparisons. The results were considered statistically significant with $P<0.05$.

Results

Glutamate increased iron contents in rat brain

To directly detect the effect of Glu on the iron levels in rat brain, striatum, Hippo, CC, cerebellum and SN were dissected for detection of non-haem iron contents. Data showed that Glu increased non-haeme iron contents in Hippo, CC, cerebellum and SN (Figure 1A and 1B). We also detected the expression of iron storage protein L-ferritin (FTL), and found that Glu increased FTL expression in the brain (Figure 1C and 1D). This clearly implicated that glutamate increased the iron contents in the brain.

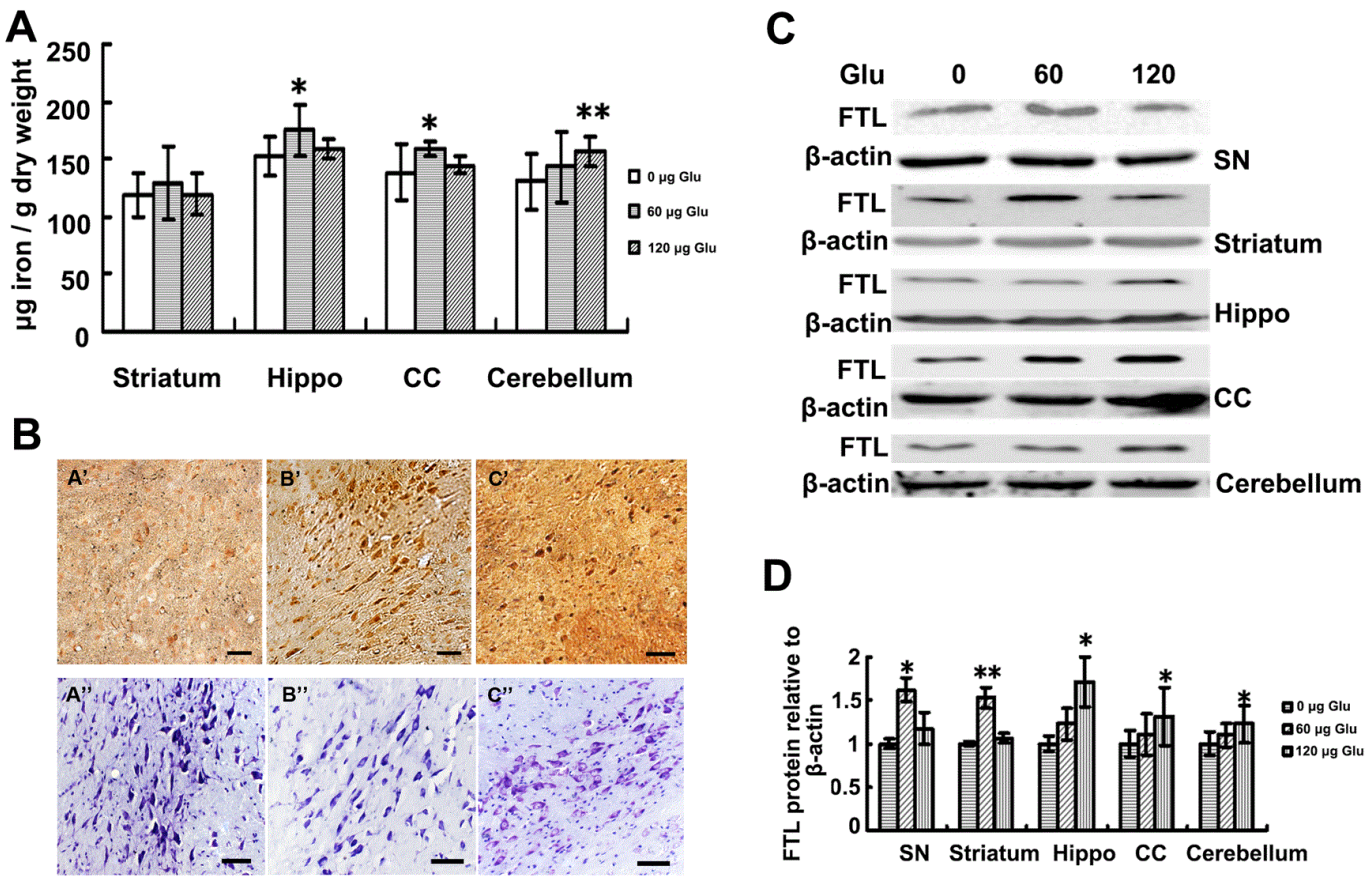

Figure 1: Iron contents were increased in rat brain after ICV of Glu. A shows the non-haem iron contents in striatum, hippocampus, CC and cerebellum. B shows the non-haem iron staining (A' B') and nissle staining (A" B") in SN after Glu treatment. A', A": ACSF, B', B": 60 μg Glu, C', C": 120 μg Glu. C shows the expression of FTL protein (iron storage) with western blot. D shows the analysis of immunostained bands in C and its statistics. The expression of FTL was normalized to β-actin and all represented data was the relative values to ACSF group (0 μg Glu). All Data were expressed as means ± SD. * $p<0.05$ vs. 0 μg Glu, ** $p<0.01$ vs. 0 μg Glu (n=6).

Glutamate increased the expression of DMT1 in rat brain

Iron homeostasis depends on iron uptake and iron export. Ferroportin 1 (FPN1) is commonly recognized as the iron release protein, transferrin Receptor 1 (TfR1) and DTM1 are responsible for iron uptake. Depending on the alternative splicing of iron responsive element (IRE) motif in the C-terminal, there are two isoforms of DMT1 protein named DMT1 (+IRE) and DMT1 (-IRE). To detect the mechanism underlying the increased iron contents in the brain, we detected the expression of TfR1, DMT1 and FPN1 in the brain after ICV of Glu. Data showed that DMT1 (+IRE) expression was increased significantly in SN, striatum, Hippo, CC and cerebellum (Figure 2). This indicated that the increased iron content in the brain might be mainly due to the increased expression of DMT1 protein after Glu treatment.

Glutamate increased the iron contents in PC12 cells

To detect the regulation mechanism of DMT1 expression by glutamate, PC12 cells were used as a neural model and incubated with different concentrations of glutamate. First, the iron storage protein FTL was detected with western blot analysis. Data showed that FTL was obviously increased after Glu treatment (Figure 3A). The total iron content of the PC12 cells was also determined by ICP-MS after they were treated with Glu. Data also showed that the total iron contents were increased significantly after treatment with 100 μM and 1000 μM concentrations of Glu (Figure 3B). The results were in accordance with data *in vivo*.

Glutamate enhanced the non-transferrin-bound iron (NTBI) uptake activity in PC12 cells

The results showed that the iron contents and the expression of DMT1 protein were both obviously increased in rat brain after Glu treatment. As is known, DMT1 plays the main role in NTBI uptake. To investigate or confirm if the increased iron contents in rat brain and PC12 cells was caused by the increase of iron uptake, ^{55}Fe liquid scintillation counting method was used to detect the NTBI uptake in PC12 cells incubated with Glu. Data showed that there were significant increases in NTBI uptake activity in cells treated with 1 μM, 10 μM, 100 μM, 1000 μM Glu, compared with that of control groups (0 μM

treatment) (Figure 4). This implied that the increased iron level in rat brain and PC12 cells induced by Glu was due to the increased NTBI uptake thus leading to the decreased cell viability of PC12 cells (Figure A1).

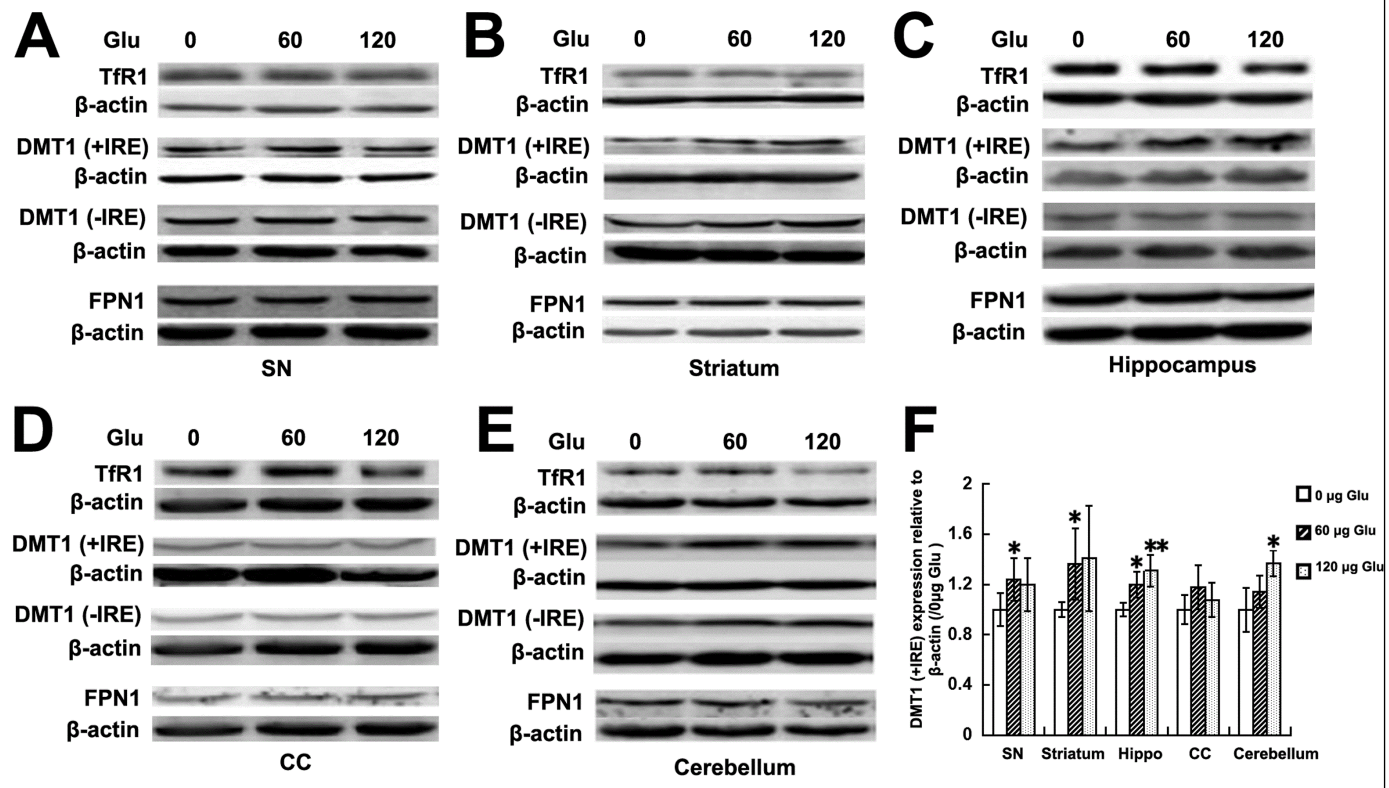

Figure 2: The expression of TfR1, DMT1 (+IRE), DMT1 (-IRE) and FPN1 protein was detected in rat brain after Glu treatment with western blot. A, B, C, D, E show the protein expression in SN, striatum, Hippo, CC and cerebellum respectively. F shows the analysis of immunostained bands and its statistics. The expression of DMT1 (+IRE) protein was normalized to β-actin and all represented data was the relative values to ACSF group (0 μg Glu). All Data were expressed as means ± SD. * $p<0.05$ vs. 0 μg Glu, ** $p<0.01$ vs. 0 μg Glu (n=6).

Glutamate increased the expression of DMT1 in PC12 cells

To further determine if the increased iron contents and iron uptake were induced by the altered expression of iron metabolism related proteins, DMT1 (+IRE), DMT1 (-IRE), TfR1 and FPN1 were detected with western blot in PC12 cells. Data showed that there were significant increases in the expression of both DMT1 (+IRE) and DMT1 (-IRE) proteins in cells treated with 1 μM, 10 μM, 100 μM Glu (Figure 5A and 5B). While there were no changes in the expression of TfR1 and FPN1 protein (Figure 5C and 5D). These indicated that the elevated iron contents resulted from the increased iron uptake through increased expression of DMT1 protein.

The translocation of NF-κB was involved in the increased DMT1 expression of PC12 cells after glutamate treatment

Both DMT1 (+IRE) and DMT1 (-IRE) expression were increased in PC12 cells after they were treated with high concentrations of Glu. To investigate the regulation mechanism of DMT1 by Glu, the distribution and expression of NF-κB was examined in the nucleus of PC12 cells treated with 100 μM Glu for 15 min, 30 min, 1 h, 2 h and 4 h, respectively. The distribution of NF-κB was observed with CLSM after immunofluorescence staining. It was found that the staining of NF-κB appeared mainly in the cytoplasm in the control group, and its staining was partly appeared in the nuclei of PC12 cells treated with Glu (except for 4 h), especially the staining of NF-κB was most obvious after Glu treatment for 30 min. This means the distribution of NF-κB was translocated from the cytoplasm to the nucleus of PC12 cells (Figure 6). This indicated that glutamate induced the translocation of NF-κB in PC12 cells, which might suggest the activation of NF-κB signal pathway.

To confirm the activation of NF-κB in the up-regulation of DMT1 by glutamate, BAY 11-7082 was used as an irreversible inhibitor of IκBα phosphorylation and can inhibit cytokine-induced NF-κB activation (Figure A2). PC12 cells were pre-incubated with 5 μM Bay 11-7082 for 2 h, and then incubated with 100 μM Glu for 12 h, or along with 100 μM Glu for another 12 h. Data showed that Bay could inhibit the increased expression of DMT1 (+IRE) and DMT1 (-IRE) induced by glutamate through inhibition of NF-κB (Figure 7A). This suggested that glutamate might increase the DMT1 expression through NF-κB pathway.

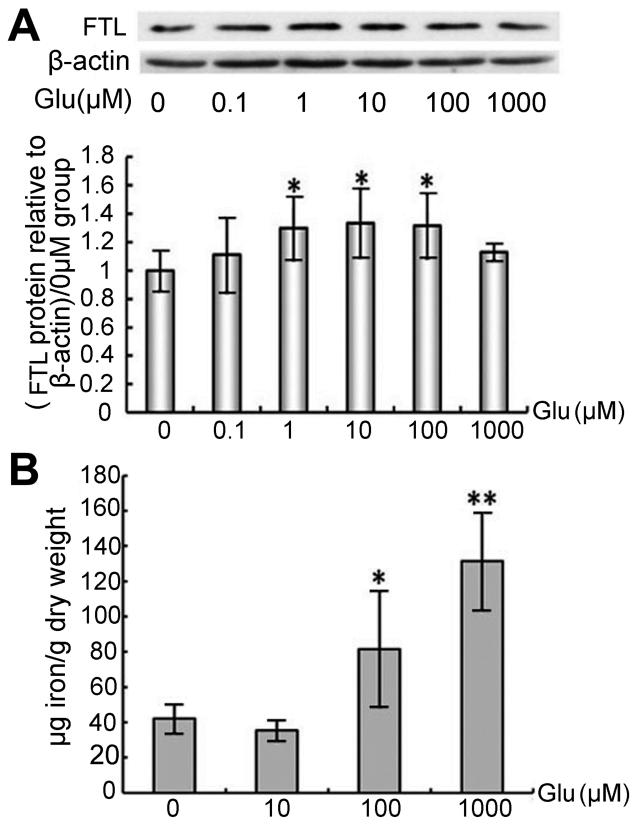

Figure 3: The iron levels were increased in PC12 cells treated with different concentrations of Glu. A shows the expression of FTL in PC12 cells determined by western blot, and the histogram was the statistical relative values of FTL protein relative to control groups (0 μg Glu) after normalization by β-actin. B shows the total iron contents detected by ICP-MS in PC12 cells incubated with 0, 10, 100 and 1000 μM of Glu. All Data were expressed as means ± SD. * p<0.05 vs. 0 μg Glu, **p<0.01 vs. 0 μg Glu (n=6).

PKC was also involved in the regulation of DMT1 by glutamate in PC12 cells

It is reported PKC regulate the expression of DMT1 through stabling DMT1 mRNA [31], so we detected the role of PKC in the regulation of DMT1 expression in PC12 cells treated with Glu. PMA was used as a PKC activator, and CH was used as a PKC inhibitor. Data found that PMA could increase DMT1 expression and CH could decrease DMT1 (+IRE) expression in PC12 cells, compared with that of Glu treatment (Figure 7B). This suggested that PKC might be involved in the regulation of DMT1 in PC12 cells treated with glutamate.

Discussion

It is reported that excessive glutamate can induce the burst influx of Ca^{2+} into cells and subsequent signal cascade events, which might cause oxidative damage and cell apoptosis even cell death. Several lines of evidences have showed that excitatory amino acid could affect iron accumulation in the brain [16,17]. It is known that iron can also cause oxidative damage through Fenton reaction, which might be another mechanism for glutamate neurotoxicity, but the detailed mechanism of the regulation has not been reported.

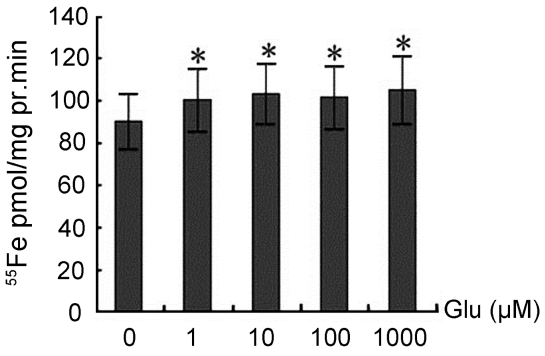

Figure 4: The non-transferrin-bound iron (NTBI) uptake activity was increased in PC12 cells treated with Glu. The PC12 cells treated with different concentrations of Glu were incubated with $^{55}FeCl_3$ iron solution (1.0 μM) for 40 min at 37°C and then the NTBI iron uptake was detected with ^{55}Fe liquid scintillation counting method. All data were expressed as means ± SD. * P<0.05 vs. 0 μM Glu group (n=6).

In order to investigate the possibility of the regulation of iron metabolism by glutamate, the iron concentrations, NTBI activity and iron transport protein expressions were examined in rat brain and PC12 cells. Data showed that Glu could increase iron levels in rat brain. Data also showed that glutamate decreased the cell viability obviously and that the total iron content was increased significantly in PC12 cells treated with Glu, which is in accordance with our results in vivo. Furthermore, the cellular storage of iron was also clearly increased. These data seem to imply that glutamate makes it much easier for more iron to enter cells. However, how glutamate induced the iron accumulation was still not clear.

Iron homeostasis mainly depends on the balance between iron uptake and iron release in a mammalian cells. DMT1 and TfR1 are key transporters for ferrous and ferric iron uptake, and FPN1 is the main iron exporter. The above data showed that iron was accumulated in rat brain and PC12 cells after glutamate treatment, so the expression of these iron transport proteins was examined with western blot analysis. Data showed that there were no changes in the expression of FPN1 and TfR1 protein, and that only the expression of DMT1 was significantly increased in brain and PC12 cells after glutamate treatment. At the same time, further experiment with radioactive ^{55}Fe revealed that NTBI uptake was increased significantly as well. DMT1 has been well determined in the brain [20,32], and plays important roles in translocation of iron from endosome to cytosol in the transferrin-bound iron uptake pathway and acquisition of NTBI by neurons [13,33]. Therefore, we deduced that the iron accumulation was the result of increased NTBI through increased DMT1 induced by glutamate. Studies by Cheah [34,35] revealed that DMT1 mediated the neurotoxicity of NMDA, and other reports also indicated that NMDA could increase DMT1 protein content after synaptic stimulation and improve their iron uptake capacity of primary hippocampus cultures

[36]. These reports are in accordance with our results. However, how glutamate could regulate DMT1 is not fully elucidated.

It has been known that DMT1 expression depends on the IRE/IRP (iron responsive element-iron regulatory protein) regulatory system [37,38]. It is also reported that 6-OHDA can regulate the expression of DMT1 through the IRE/IRP system [39]. While our data did not show any evidence that DMT1 expression was regulated by glutamate through IRE/IRP system due to the following two reasons. First, there is no IRE motif in the 3'-untranslated region of DMT1 (-IRE) mRNA, so its expression cannot be regulated by the IRE/IRP regulatory system. Second, our data about DMT1 expression was not in accordance with the IRE/IRP regulatory theory. Because the iron content was also increased in PC12 cells treated with Glu, if this theory does work, the expression of DMT1 (+IRE) and DMT1 (-IRE) should be decreased, not increased obviously as indicated in our results. Therefore, the regulation of DMT1 expression by glutamate might not be dependent on IRE/IRP system, there must exist other regulatory mechanisms underlying the effect of glutamate on DMT1.

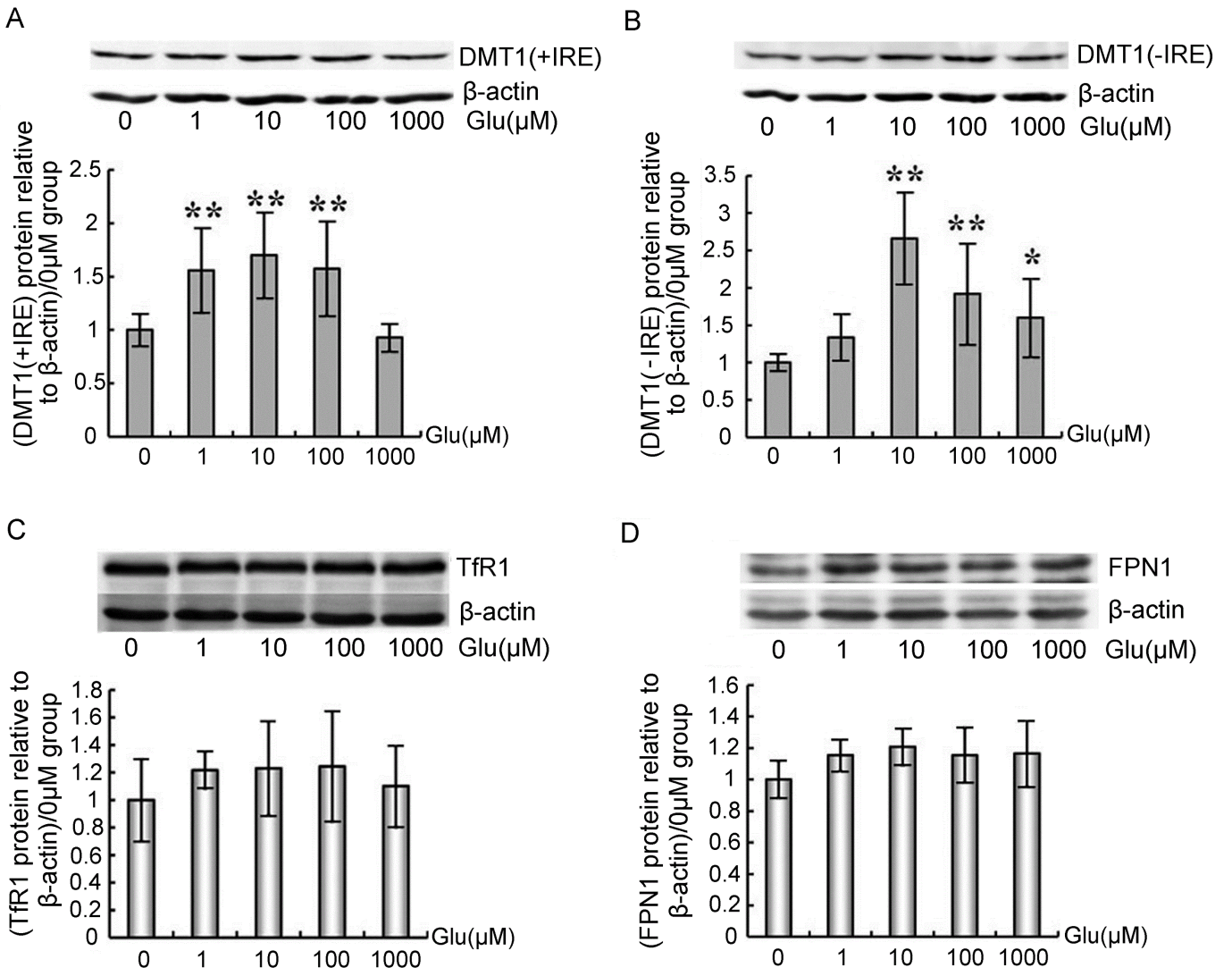

Figure 5: The expressions of iron transport proteins were detected in PC12 cells after Glu treatment with western blot analysis. After PC12 cells were incubated with Glu (0, 1, 10, 100, 1000 μM) for 16 h as described in Materials and Methods, the expression of DMT1 (+IRE) (A) and DMT1 (-IRE) (B) protein was increased at concentrations of 1 μM, 10 μM and 100 μM Glu, compared with that of control (0 μM Glu). While there were no changes of TfR1 (C) and FPN1 (D) expression in PC12 cells incubated with Glu. All histograms represent the relative values to the control group's (0μM Glu) after normalization by β-actin. Data were expressed as means ± SD (relative to that of the 0 μM Glu group). * P<0.05 vs. 0 μM Glu group; **P<0.01 vs. 0 μM Glu group (n=6).

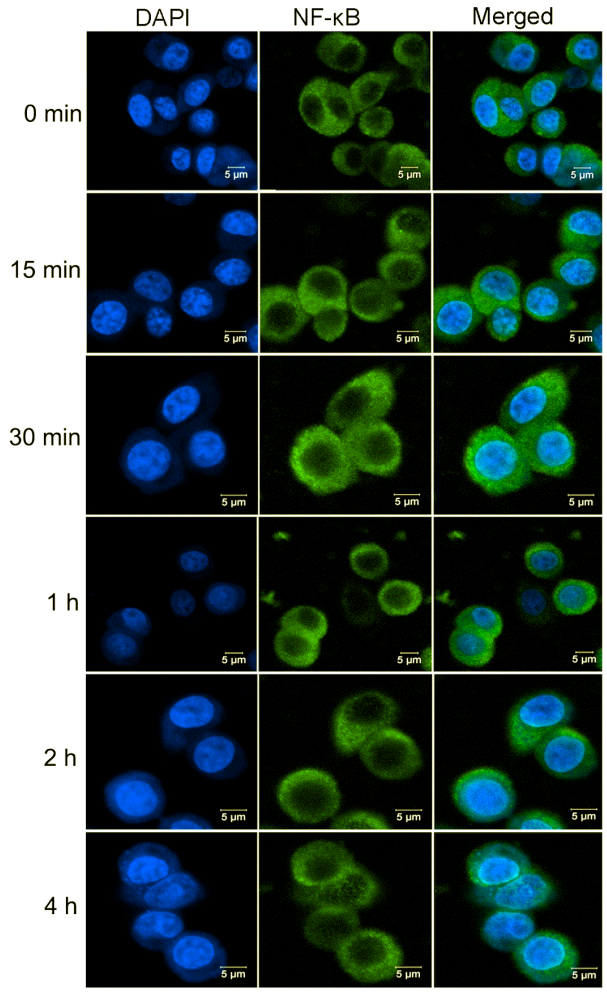

Figure 6: The distribution of NF-κB was translocated from cytoplasm into nucleus in PC12 cells after Glu treatment with immunocytochemistry. PC12 cells were treated with 100 μM Glu for 15 min, 30 min, 1 h, 2 h and 4 h. The left showed the cell nuclei staining with DAPI (blue), the middle showed the immunostaning of NF-κB (green), and the right showed the merged images. The upper three photomicrographs showed the staining of the control group, and the lowered parts showed the staining of Glu-treated group as indicated. The staining of NF-κB was observed obviously in the nucleus after Glu treatment for 30min. Scale bar: 5 μm (n=4).

As is known, there are four isoforms of DMT1 [18], and the translocation of NF-κB from cytoplasm to nucleus and subsequent binding to the putative NF-κB response element within the 1B promoter of DMT1 increased the expression of DMT1 in undifferentiated P19 embryonic carcinoma cells [40-42]. Our data showed that glutamate can induce the translocation of NF-κB into the nucleus, which implicated the activation of NF-κB. The increased expression of DMT1 might be regulated by NF-κB. Further investigations were done by the application of BAY 11-7082, which can inhibit the activation of NF-κB due to its inhibition of IκBα phosphorylation and degradation. As expected, pre-treatment with BAY 11-7082 decreased the Glu-induced up-regulation of DMT1. Therefore, NF-κB was involved in the up-regulation of DMT1 expression induced by Glu in PC12 cells. PKC can enhance the expression of DMT1 protein through inhibition of DMT1 mRNA degradation. Our data also showed that activation and inhibition of PKC resulted in the changes of DMT1 expression.

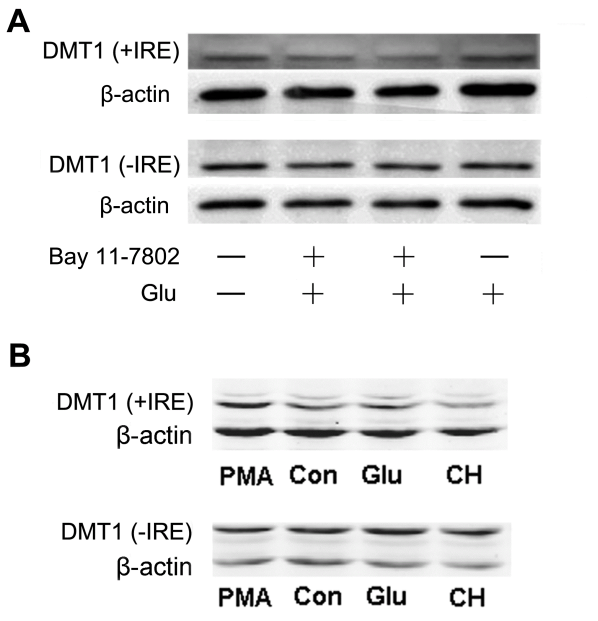

Figure 7: NF-κB and PKC were involved in the regulation of DMT1 in PC12 cells treated with Glu. A shows the expression of DMT1 (+IRE) and DMT1 (-IRE) after PC12 cells were pre-incubated with 5 μM Bay 11-7082 (an NF-κB inhibitor) for 2 h, and then incubated with 100 μM Glu for 12 h (the second column from the left), or then they were continued incubating with 100 μM Glu for another 12 h (the third lane from the left). B PKC activator (PMA) and inhibitor (CH) obviously altered DMT1 (+IRE) expression in PC12 cells (n=6).

In conclusion, glutamate can increase iron contents in the brain, and the increased iron contents was due to the increased NTBI through increased DMT1. Moreover, NF-κB and PKC were involved in the regulation of DMT1 by glutamate. The induced iron accumulation in the brain would aggravate the oxidative damage through Fenton reaction, so as to result in glutamate excitotoxicity. This not only contribute to the understanding the mechanism of glutamate neurotoxicity from the view of iron metabolism, but also provide new molecular regulation evidences for the effect of glutamate on iron metabolism, which might give a new strategy in attenuating Glu neurotoxicity by inhibiting the expression of DMT1 through inactivation of NF-κB and PKC.

Acknowledgements

We are grateful to Professor Norman Davison, University of Manitoba, Winnipeg, Manitoba, Canada, for helpful discussions regarding the language.

The studies are supported by National Natural Science Foundation of China (31100822, 30871260 and 30870265), Natural Science

Foundation of Hebei Province (C2012205068), Science Foundation of Hebei Education Department (2010145), and Doctoral Foundation of Hebei Normal University (L2006B22).

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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