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Expression and functions of glutamate and γ-aminobutyric acid transporters in ischemic models

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Abstract. Glutamate and γ-aminobutyric acid (GABA) transporters serve central roles in normal neuronal activity and are associated with numerous pathological brain conditions, including ischemia and epilepsy. However, the interplay between these transporters in ischemia remains unclear. In the present study, the expression levels of the excitatory amino acid carrier 1 (EAAC1) and GABA transporter 1 (GAT1) were analyzed *in vivo* and *in vitro* within ischemic models by immunofluorescence, western blot and RT-qPCR. Cell survival rates were analyzed following altered expression of these transporters within neuronal cells by flow cytometry. Expression levels of EAAC1 were reduced within the cerebrum of focal cerebral ischemic middle cerebral artery occlusion rat models as well as in primary neurons cultured under hypoxia. However, GAT1 expression levels were slightly elevated under ischemic conditions. The altered expression levels of EAAC1 and GAT1 were combined within neuron cells and the effects were investigated. Apoptotic analysis revealed that EAAC1 suppression and overexpression of GAT1 increased neuronal cell apoptosis under hypoxic conditions; however, EAAC1 overexpression combined with GAT1 knockdown reduced neuronal cell apoptosis under hypoxic conditions. The present study detected the expression levels of the glutamate and GABA transporters under hypoxia, in association with ischemia. The results indicated that, increased expression of EAAC1 combined with GAT1 suppression may provide protective effects in the treatment of epilepsy and ischemia.

Introduction

Cerebral hypoxic ischemia is a leading cause of numerous neurological diseases, including cerebral epilepsy, palsy and cognitive disabilities associated with high mortality and morbidity (1-3). Matsumoto *et al* (4) reported that ischemia may cause neuronal cell apoptosis in the central nervous system due to an excess of L-glutamate. However, ischemic-associated release of L-glutamate may be inhibited by the activation of γ-aminobutyric acid (GABA) receptors (4,5). GABA- and glutamate-associated transporters serve roles in the maintenance of extracellular GABA and glutamate levels (4). However, in abundance, these transporters have been associated with numerous pathological brain conditions, in particular ischemia and epilepsy.

Glutamate transporters serve crucial functions in the prevention of neuronal cell death by reducing glutamate-associated toxicity. Five associated glutamate transporters have been reported: Glutamate-aspartate transporter/excitatory amino acid transporter (EAAT)1, glial glutamate transporter (GLT)-1/EAAT2, EAA carrier (EAAC)1/EAAT3, EAAT4 and EAAT5 (6,7). The functional relevance of GLT-1 was clearly demonstrated in a study using GLT1 knockout mice, which developed severe epilepsy (8). However, the roles of EAAC1 in neuronal death have not been resolved compared with glial glutamate transporters, including GLT-1. In addition to maintaining extracellular glutamate, a previous study investigating EAAC1-deficient mice revealed that EAAC1 can function as a cysteine transporter and maintain neuronal glutathione homeostasis (9). Additionally, EAAC1 has been reported to protect injured motor neurons via interactions with holocytochrome c synthetase (10). EAAC1 may therefore perform additional functions in addition to glutamate homeostasis.

Sodium- and chloride-dependent GABA transporter 1 (GAT1) is primarily responsible for the removal of GABA from the synaptic cleft and termination of GABA-mediated neurotransmission (11). Chronic neurological abnormalities that develop following hypoxia at an early age may be associated with alterations of GAT functions (12-15). A previous study reported that the protein expression levels of GAT1 were reduced within the brains of thrombotic infarct rat models (16). The interplay between these transporters and ischemia require further investigation. The present study aimed to investigate the expression and associated functions of GAT1 and EAAC1 under hypoxic conditions *in vivo* and *in vitro*. However, the interplay between these transporters in ischemia remains unclear. The present study aimed to investigate the expression

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and associated functions of GAT1 and EAAC1 under hypoxia *in vivo* and *in vitro*.

Materials and methods

Middle cerebral artery occlusion (MCAO) animal models. 14 male Sprague-Dawley rats (250-300 g; Experimental Animal Center, Shanghai Jiao Tong University School of Medicine, Shanghai, China) were housed in a climate-controlled room (25˚C, 50% humidity), 5 rats per cage; food and water was provided *ad libitum* under a 12 h-light/dark cycle. The animal study protocol was approved by the ethics committee of Shanghai Pudong Hospital (Shanghai, China) and was conducted according to guidelines of the Animal Experimentation group of Shanghai Jiao Tong University School of Medicine.

Rats were randomly allocated into the following groups (n=6 in sham group, n=8 in MACO group): A sham-operated control group, which underwent the operation with no occlusion; and a 24 h post-MCAO/reperfusion group. Briefly, rats were anesthetized with an intraperitoneal (i.p.) injection of 50 mg/kg pentobarbital and placed in a stereotaxic instrument (Advanced Scientifics Inc.; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A 28-gauge stainless steel injection cannula was inserted into the right lateral ventricle as previously described (17). MCAO rat models were generated as previously described (18). Briefly, a 3-0 monofilament nylon suture (Beijing Shandong Technology Co., Ltd., Beijing, China) with a heat-treated rounded tip was introduced into the right internal carotid artery via the external carotid artery until slight resistance was achieved. The suture was maintained in place for 90 min and was then withdrawn to facilitate reperfusion.

24 h following the onset of reperfusion, the rats were deeply anesthetized using sodium pentobarbital (100 mg/kg, i.p.). For mRNA and protein expression analysis, rats were transcardially perfused with 4˚C normal saline; regions corresponding to the ischemic core and penumbra were dissected on ice using previously described methods (18). For immunostaining, the rats were transcardially perfused with 4˚C normal saline and fixed with 4% paraformaldehyde (PFA; pH=7.4). Brains were extracted and post-fixed in 4% PFA, the tissues were subsequently cryoprotected in 20% sucrose, followed by 30% sucrose at 4˚C for 48 h. Serial coronal sections (25 μm) were collected between -1.80 and -4.80 mm bregma levels. Every fifth section from a total of 24 sections was selected for staining.

Cells and cell culture. Primary neurons derived from rat were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (PAA Laboratories; GE Healthcare, Chicago, IL, USA). Briefly, brain tissue was removed from freshly euthanized rats into a cold, buffered salt solution. Using a dissecting microscope, cerebral cortex can be carefully isolated for further processing. The dissected tissue was first minced using a scalpel or scissors. The resulting tissue pieces were then transferred to a new container. A proteolytic enzyme solution including trypsin and papain were then added to digest the extracellular matrix proteins that bind cells together. Following a short incubation of 15 min in a warm incubator of 37˚C, tissue pieces were gently washed with buffer to remove the enzymes. The softened tissue pieces were dissociated by trituration, which involved passing the tissue through a pipet, multiple times so that cells become a single cell suspension. At this point, cells were counted and checked for viability by Trypan blue. Cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. To simulate a hypoxic environment, dissociated cells were seeded in a 10-cm dish with or without 250 μM $CoCl_2$ treatment for 24 or 48 h at 37˚C prior to analysis.

Immunofluorescence. Slides were fixed with 4% PFA at 4˚C for 15 min and incubated with PBS containing 0.1% saponin (Beyotime Institute of Biotechnology, Himen, China) and 1% normal goat serum or 2% normal donkey serum (Beyotime Institute of Biotechnology) at room temperature for 30 min. Slides were then incubated with primary mouse polyclonal anti-EAAC1 (1:500; cat. no. orb149931; Biorbyt, Ltd., Cambridge, UK), or GAT1 (1:500; cat. no. ab426; Abcam, Cambridge, UK) at 4˚C overnight. Slides were subsequently washed and incubated with secondary fluorescent Alexa-Fluor-488-conjugated goat anti-mouse or rabbit immunoglobulin G (cat. no. a24920/a24922; 1:100; Thermo Fisher Scientific Inc.) in a dark room for 2 h at room temperature. DAPI staining (Beyotime Institute of Biotechnology) was used for nuclear staining. Slides were observed with a laser-scanning confocal microscope (TCS SP5; Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Lentiviral-mediated short hairpin RNA (shRNA) gene knockdown or overexpression. Primary neurons exhibiting stable knockdown of EAAC1 or GAT1 were generated via transduction with a lentiviral-mediated expression-specific target shRNA (10^9 TU/ml; HanYin Biotech, Shanghai, China). Lentivirus containing an empty vector was used as a negative control (NC, HanYin Biotech). The targeted knockdown sequence for EAAC1 was 5'-caacaatgtctgagaaca a-3' and for GAT1 was 5'-ccaaatgacagatgggcta-3'. Cells were seeded in six-cm dishes at a density of $5x10^5$. Cells were then infected with the same titer virus with 8 μg/ml Polybrene® (HanYin Biotech) on the following day. Overexpression of EAAC1 or GAT1 was generated following infection of cells with EAAC1- or GAT1-expressing lentiviruses (10^8 TU/ml, HanYin Biotech) with 8 μg/ml Polybrene® (HanYin Biotech) on the following day. After 48 h, cells were harvested, and the knockdown or overexpression efficiency was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting.

RT-qPCR. Cellular RNA was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, DNA was removed from the samples via DNase treatment (DNA-free kit; Ambion; Thermo Fisher Scientific, Inc.) and cDNA was synthesized from the purified RNA using a Moloney murine leukemia virus RT kit (Promega Corporation, Madison, WI, USA). GAPDH primer sets were used as a normalization control. Primer sequences were as follows: GAT1 forward, 5'-GCAATCGCCGTGAAC TCTTC-3' and reverse, 5'-AGGAAATGGAGACACACT CAAAGA-3'; EAAC1 forward, 5'-CTCCACCACCGTCAT TGCT-3' and reverse, 5'-TGGCAGGCTTCACTTCTTCAC-3';

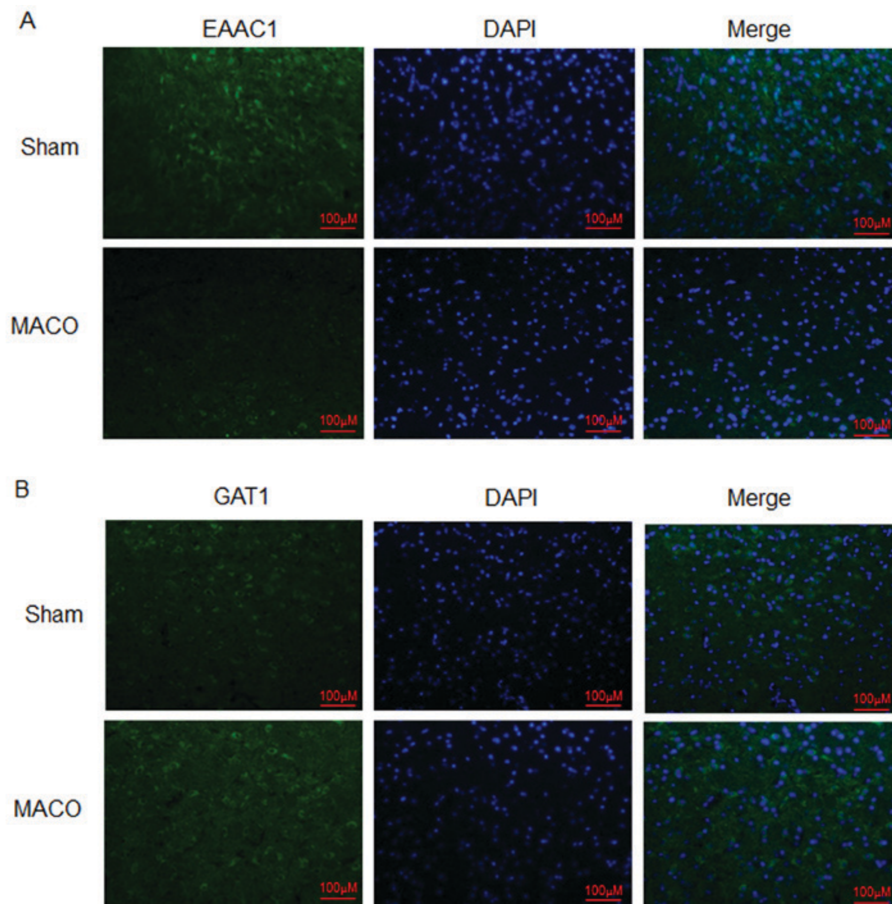

Figure 1. Detection of protein expression levels of EAAC1 and GAT1 within the penumbra in a MCAO/reperfusion-rat model. Immunofluorescence staining of (A) EAAC1 and (B) GAT1 in the penumbra area 24 h post-MCAO compared with the sham group. EAAC1, excitatory amino acid carrier 1; GAT1, γ-aminobutyric acid transporter 1; MCAO, middle cerebral artery occlusion.

GAPDH forward, 5'-GTATGTCGTGGAGTCTACTG-3' and reverse, 5'-CTTGAGGGAGTTGTCATATTTC-3'. RT-qPCR cycling conditions were: Initial denaturation for 3 min at 95˚C followed by 45 cycles of 95˚C (10 sec) and 58˚C (45 sec), and data were acquired at the end of the annealing/extension phase. RT-qPCR was performed in triplicate using the SYBR-Green PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR machine according to the manufacturer's protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative quantification method ($2^{-\Delta\Delta Cq}$) (19) was used to analyze quantitative RT-PCR data using GAPDH as normalizer. NC were served as a reference.

Western blot analysis. Radioimmunoprecipitation assay buffer, a protease inhibitor cocktail and a phosphorylation inhibitor cocktail (Beyotime Institute of Biotechnology) were used to extract total protein from cells. Protein concentration was determined by BCA method. A total of 30 µg protein was separated by 10-15% SDS-PAGE and was transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk 30 min at room temperature. The membrane was further incubated with primary antibodies [EAAC1, (1:1,000; cat. no. orb149931; Biobyt, Ltd.) GAT1 (1:1,000) and Actin (1:3,000; both Abcam)] overnight at 4˚C and subsequently incubated with HRP conjugated anti-mouse or rabbit immunoglobulin G (1:10,000) for 1 h at room temperature. The signal was observed and developed with a Kodak film (Kodak, Rochester, NY, USA) via enhanced chemiluminescence plus western blotting detection reagent (Amersham; GE Healthcare). β-actin was used as a control.

Apoptosis assay. An Annexin V-phycoerythrin (PE) Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) was employed to assess apoptosis according to the manufacturer's protocol. Briefly, cells treated with or without CoCl2 (control, CK) were resuspended in 1X Binding Buffer at a concentration of $1x10^6$ cells/ml, and 100 µl of this suspension was added to each of the following tubes: i) An empty tube, ii) a tube containing Annexin V-PE reagent (5 µl); iii) a tube containing 7-aminoactinomycin D (AAD) reagent (5 µl); and iv) a tube containing Annexin V-PE reagent (5 µl) and 7-AAD reagent (5 µl). The tubes were gently vortexed and were incubated for 15 min at room temperature in the dark. 1X Binding Buffer (400 µl) was then added to each tube and the cells were analyzed by flow cytometry (Beckman gallios, flowjo10.07).

Statistical analysis. Statistical analysis was performed using the Student's t-test for comparison between two groups and one-way analysis of variance followed by and a Tukey test for multiple comparisons. $P \leq 0.05$ was considered to indicate a statistically significant difference.

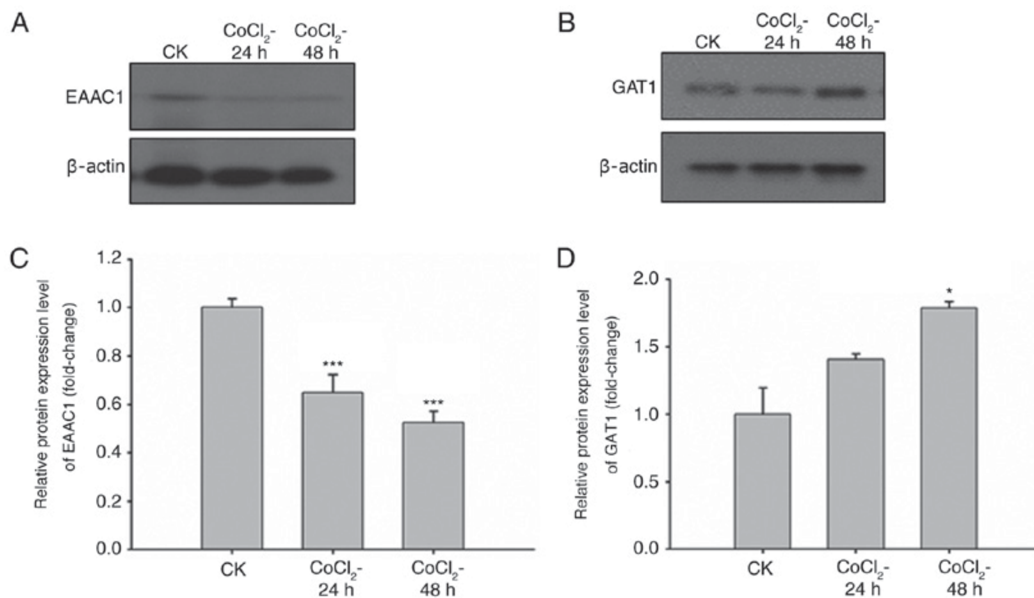

Figure 2. Expression levels of EAAC1 and GAT1 in response to hypoxic stimuli in primary neuronal cells. Protein was extracted 24 or 48 h post-$CoCl_2$ treatment within primary neuronal cells. (A) EAAC1 and (B) GAT1 protein expression levels were detected via western blot analysis; β-actin was used as the loading control. mRNA expression levels of (C) EAAC1 and (D) GAT1 within $CoCl_2$-treated primary neuronal cells were detected via reverse transcription-quantitative polymerase chain reaction; GAPDH served as the control. EAAC1, excitatory amino acid carrier 1; GAT1, γ-aminobutyric acid transporter 1. CK, Control; No $CoCl_2$ treatment. *P<0.05; ***P<0.001.

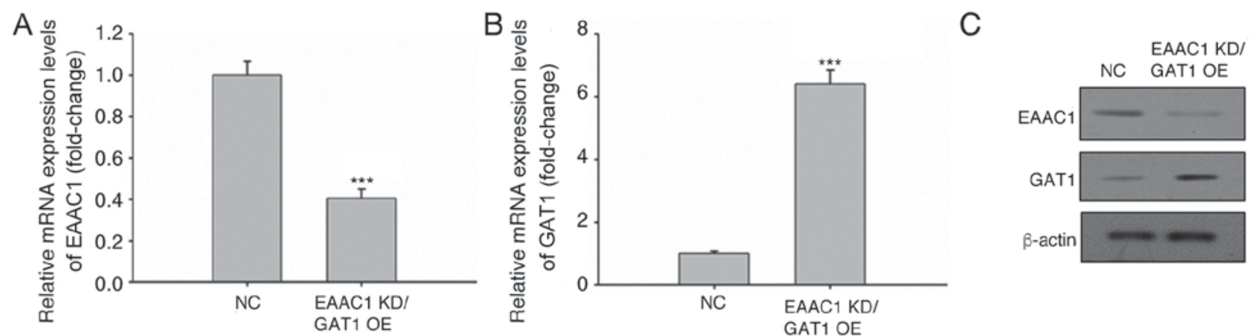

Figure 3. EAAC1 knockdown with GAT1 overexpression in primary neuronal cells. (A) mRNA expression levels of EAAC1 within primary neuronal cells transduced with EAAC1-control RNA or EAAC1 shRNA lentivirus were detected via RT-qPCR. (B) mRNA expression levels of GAT1 within transduced primary neuronal expressing GAT1 RNA/EAAC1-shRNA or GAT1-control RNA were detected via RT-qPCR. (C) Western blot analysis of EAAC1 and GAT1 protein levels were detected within transduced primary neuronal cells expressing control RNA or GAT1 RNA/EAAC1-shRNA. EAAC1, excitatory amino acid carrier 1; GAT1, γ-aminobutyric acid transporter 1; KD, knockdown; NC, control; OE, overexpression; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. ***P<0.001.

Results

Expression levels of EAAC1 and GAT1 within the penumbra in a rat model of MCAO/reperfusion. Expression levels of EAAC1 and GAT1 within the penumbra were analyzed via immunofluorescence. The results of the present study revealed that the protein expression levels of EAAC1 were markedly reduced at 24 h post-MCAO/reperfusion compared with the sham group (Fig. 1A). Protein expression levels of GAT1 were increased compared with the sham group (Fig. 1B). These results indicated that EAAC1 and GAT1 may serve different functions in hypoxia-induced ischemia.

Expression levels of EAAC1 and GAT1 are influenced by hypoxic stimuli within neuronal cells. The pathogenesis of ischemia-induced brain injury is mainly caused by neuronal death in oxygen- and energy-deficient environments. Therefore, the expression levels of EAAC1 and GAT1 under hypoxic conditions were analyzed. $CoCl_2$ is a commonly used agent to induce oxygen failure. Neuronal cells were treated with $CoCl_2$ for 24 or 48 h, and the expression levels of EAAC1 and GAT1 were detected through western blot analysis and RT-qPCR. The results revealed that the protein expression levels of EAAC1 were reduced in a time-dependent manner in response to $CoCl_2$ (Fig. 2A). GAT1 protein expression levels were slightly increased following $CoCl_2$ treatment (Fig. 2B). The mRNA expression levels of EAAC1 and GAT1 were detected via RT-qPCR following $CoCl_2$ treatment for 24 and 48 h. Compared with the untreated cells, mRNA levels of EAAC1 were significantly reduced and those of GAT1 were increased in response to $CoCl_2$ treatment (Fig. 2C and D). The expression levels of EAAC1 and GAT1 within primary

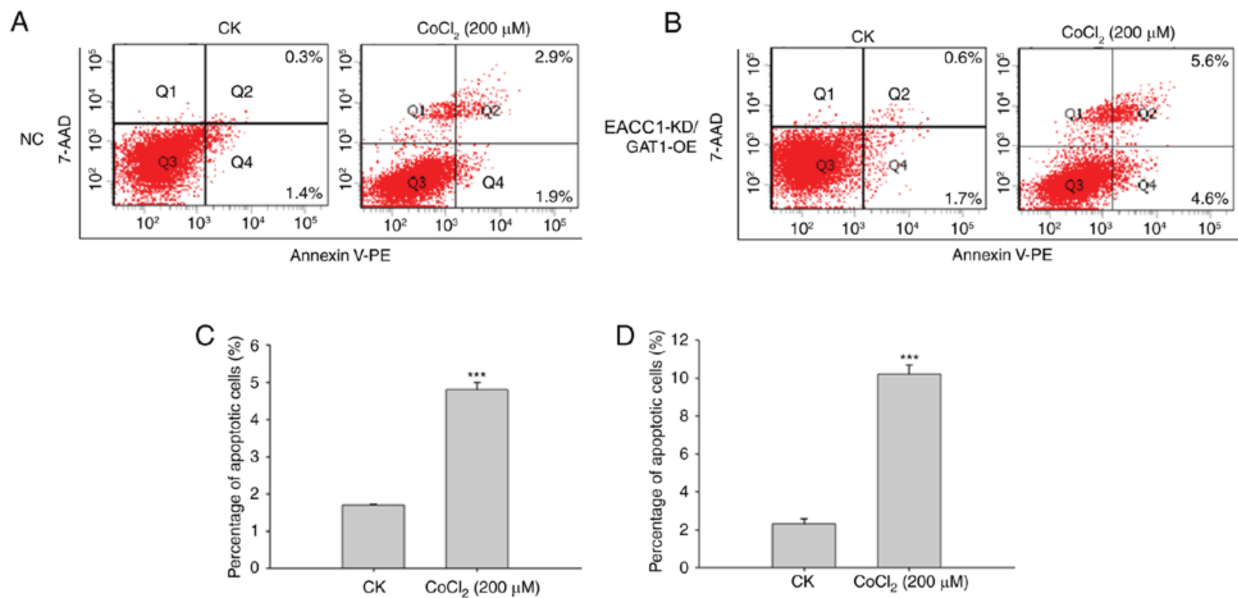

Figure 4. EAAC1 suppression with overexpression of GAT1 elevates neuronal death under hypoxia. Primary neuronal cells transduced with the (A) NC lentiviral vector or (B) EAAC1 shRNA/GAT1 RNA were treated with or without $CoCl_2$ for 48 h, apoptosis was detected by a fluorescence-activated cell sorting assay. Number of apoptotic cells following transduction with (C) negative control lentiviral vector or (D) EAAC1 shRNA/GAT1. Apoptotic rate is expressed as the mean percentage of apoptotic cells ± standard deviation. 7-AAD, 7-aminoactinomycin D; EAAC1, excitatory amino acid carrier 1; GAT1, γ-aminobutyric acid transporter 1; KD, knockdown; NC, negative control; OE, overexpression; CK, control: No $CoCl_2$ treatment; PE, phycoerythrin; shRNA, short hairpin RNA. ***P<0.001.

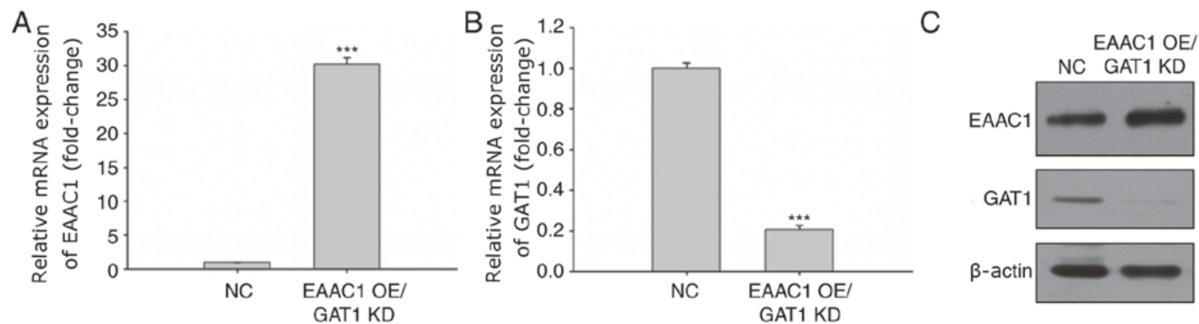

Figure 5. EAAC1 overexpression with GAT1 knockdown within primary neuronal cells. Reverse transcription-quantitative polymerase chain reaction analysis of (A) EAAC1 and (B) GAT1 mRNA expression levels within primary neuronal cells transduced with a negative control or EAAC1 RNA/GAT1-shRNA lentiviral vector. (C) Western blot analysis of EAAC1 and GAT1 protein levels within primary neuronal cells transduced with a negative control or EAAC1 RNA/GAT1-shRNA lentiviral vector. EAAC1, excitatory amino acid carrier 1; GAT1, γ-aminobutyric acid transporter 1; KD, knockdown; NC, negative control; OE, overexpression; shRNA, short hairpin RNA. ***P<0.001.

neuronal cells were altered following exposure to hypoxia; therefore, EAAC1 and GAT1 may serve important roles in the hypoxia-induced ischemia.

EAAC1 suppression and GAT1 overexpression increases apoptosis under hypoxic conditions. Following $CoCl_2$ treatment, the mRNA and protein expression levels of EAAC1 and GAT1 were reduced and elevated, respectively. The effects of EAAC1 knockdown and increased GAT1 expression on neuronal cell apoptosis were subsequently investigated. EAAC1 expression was targeted within neuronal cells via a lentiviral shRNA gene knockdown system, which demonstrated a >60% decrease in EAAC1 expression (Fig. 3A). Lentivirus-mediated gene overexpression significantly increased GAT1 expression (Fig. 3B). The protein expression levels of EAAC1 and GAT1 were also confirmed via western blotting (Fig. 3C).

Subsequently, cells were treated with $CoCl_2$ and analyzed with a fluorescence-activated cell sorting (FACS) apoptosis assay. Analysis indicated that EAAC1 suppression with GAT1 overexpression elevated neuronal death under hypoxic conditions compared with the negative control group. As presented in Fig. 4, the percentage of apoptotic cells was significantly increased compared with control cells following $CoCl_2$ treatment.

Overexpression of EAAC1 with GAT1 knockdown reduces neuronal cell apoptosis. To further investigate the effects of EAAC1 and GAT1 expression on neuronal cell apoptosis, EAAC1 overexpression and GAT1 knockdown was applied. The mRNA expression levels of EAAC1 were markedly upregulated within neuronal cells (Fig. 5A), whereas >80% of GAT1 mRNA expression was reduced (Fig. 5B). The protein

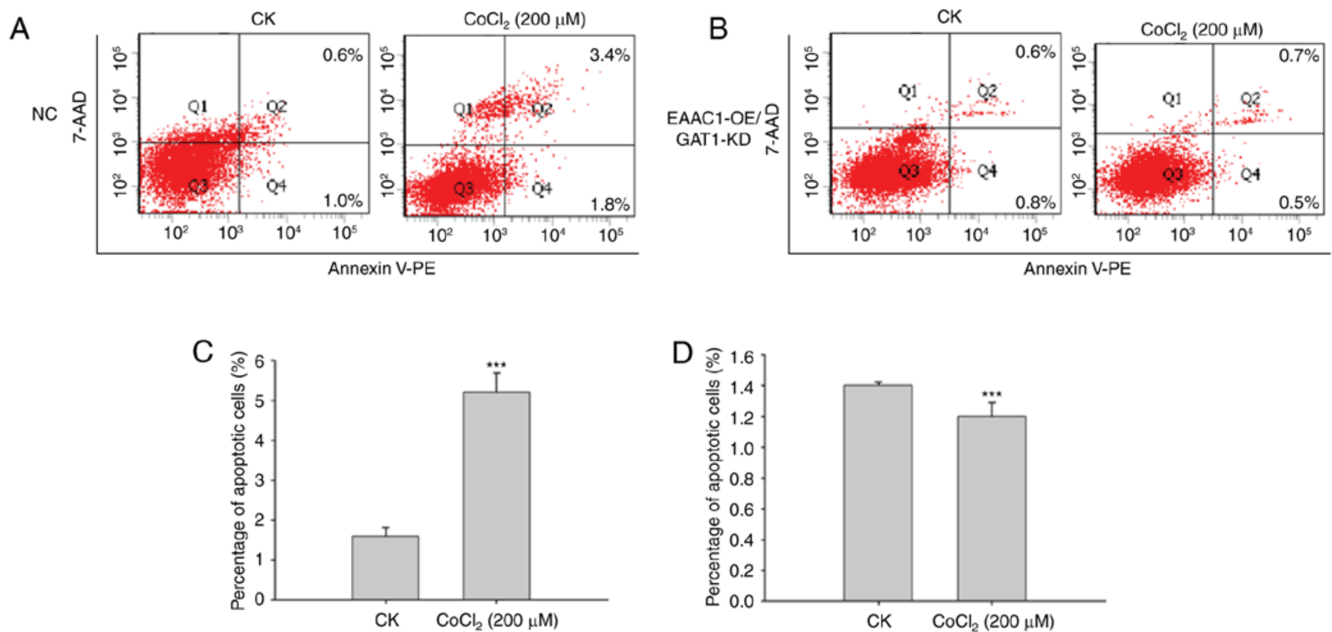

Figure 6. Overexpression of EAAC1 with GAT1 knockdown reduces neuronal death. (A) Primary neuronal cells transduced with (A) NC lentiviral vector or (B) EAAC1 RNA/GAT1-short hairpin RNA were treated with $CoCl_2$ for 48 h, apoptosis was detected by a FACS assay. (C and D) Apoptotic rate is expressed as the mean percentage of apoptotic cells ± standard deviation. 7-AAD, 7-aminoactinomycin D; EAAC1, excitatory amino acid carrier 1; FACS, fluorescence-activated cell sorting; GAT1, γ-aminobutyric acid transporter 1; GLT1, glutamate transporter 1; KD, knockdown; NC, negative control; OE, overexpression; CK, control: No $CoCl_2$ treatment; PE, phycoerythrin. $^{***}P<0.001$.

expression levels of EAAC1 and GAT1 were also confirmed via western blotting (Fig. 5C).

The FACS apoptosis assay suggested that EAAC1 overexpression with GAT1 knockdown reduced neuronal cell apoptosis under hypoxic conditions. As presented in Fig. 6, the percentage of apoptotic cells was significantly reduced compared with control cells following $CoCl_2$ treatment compared with the negative control group. These results demonstrated the importance of EAAC1 with GAT1 within hypoxic ischemia-induced brain injury and suggested that upregulation of EAAC1 with GAT1 suppression may provide benefits for the therapy of ischemia-associated diseases.

Discussion

Hypoxic ischemia-induced brain injury is a major cause of morbidity and mortality in infants and children (3,20-22); at present, effective treatments for these diseases are unavailable. Glutamate and GABA are important neurotransmitters in the human nervous system, however, whether glutamate/GABA transporters serve important functions in hypoxic ischemia remains unclear (23). In the present study, it was reported that EAAC1 expression was reduced within the cerebrum of focal cerebral ischemic MCAO rat models, as well as in primary neurons cultured under hypoxia. Conversely, the expression levels of GAT1 were slightly elevated under ischemic conditions. Additionally, analysis of apoptosis revealed that EAAC1 suppression with co-overexpression of GAT1 elevated neuronal cell apoptosis under hypoxia; however, EAAC1 overexpression with GAT1 knockdown reduced neuronal cell death. The present study indicated the expression of glutamate and GABA transporters may be associated with ischemia. Increasing the expression levels of EAAC1 and suppressing GAT1 expression may provide beneficial effects in the treatment of epilepsy or ischemia treatment.

Previous studies have demonstrated that the pathogenesis of ischemic injury may be due to cellular apoptosis in oxygen- and energy-deficient environments (24,25). Neuronal cell apoptosis increased following $CoCl_2$ treatment; however, apoptosis induced by $CoCl_2$ may be inhibited by increased expression levels of EAAC1 and suppressed GAT1.

In conclusion, the results of the present study demonstrated the importance of glutamate and GABA transporters in hypoxic-ischemic brain injury; therefore, targeting the functions of EAAC1 and GAT1 may provide advantages for the development of hypoxic ischemia therapies.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

ZQ contributed to acquisition, analysis and interpretation of data and wrote the main manuscript. YL, JX and YQ contributed to data analysis, and LR designed the study and contributed to revision of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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