Blood glutamate EAAT₂-cell grabbing therapy in cerebral ischemia

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Abstract

Background: Excitatory amino acid transporter 2 (EAAT₂) plays a pivotal role in glutamate clearance in the adult brain, thereby preventing excitotoxic effects. Considering the high efficacy of EAAT₂ for glutamate uptake, we hypothesized that the expression of this transporter in mesenchymal stem cells (MSCs) for systemic administration could yield a cell-based glutamate-grabbing therapy, combining the intrinsic properties of these cells with excitotoxic protection.

Methods: To address this hypothesis, EAAT₂-encoding cDNA was introduced into MSCs and human embryonic kidney 293 cells (HEK cells) as the control cell line. EAAT₂ expression and functionality were evaluated by in vitro assays. Blood glutamate-grabbing activity was tested in healthy and ischemic rat models treated with 3 × 10⁶ and 9 × 10⁶ cells/animal.

Findings: The expression of EAAT₂ in both cell types conferred the expected glutamate-grabbing activity in vitro and in vivo studies. The functional improvement observed in ischemic rats treated with EAAT₂–HEK at low dose, confirmed that this effect was indeed mediated by the glutamate-grabbing activity associated with EAAT₂ functionality. Unexpectedly, both cell doses of non-transfected MSCs induced higher protection than transfected EAAT₂–MSCs by another mechanism independent of the glutamate-grabbing capacity.

Interpretation: Although the transfection procedure most likely interferes with some of the intrinsic protective mechanisms of mesenchymal cells, the results show that the induced expression of EAAT₂ in cells represents a novel alternative to mitigate the excitotoxic effects of glutamate and paves the way to combine this strategy with current cell therapies for cerebral ischemia.

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1. Introduction

Ischemic stroke, resulting from the interruption of blood supply to the brain, is one of the most frequent causes of morbidity and mortality worldwide [1]. During the acute phase of stroke, glutamate homeostasis is disrupted, and glutamate levels rise above physiological concentrations in the extracellular fluid. This pathological situation leads to an overstimulation of glutamate receptors, followed by the excitotoxic death of neurons [2,3]. Under physiological conditions, once glutamate is released from excitatory neurons upon an action potential, the concentration of extracellular glutamate is tightly regulated by integral membrane electrogenic transporters, known as excitatory amino acid transporters (EAATs) [3]. Five subtypes of EAATs have been identified and characterized thus far [4]. Among these, the EAAT₂ protein, which is mainly expressed in astroglial cells, is responsible for 90% of total glutamate uptake, thereby preventing neuronal excitotoxicity. Inside the astrocytes, glutamate is metabolized to glutamine or used for energy production. Glutamine is subsequently transferred to neurons for the replenishment of the glutamate pool via the glutamate–glutamine cycle [2,5,6].

The expression of EAAT₂ has also been described on the antiluminal surface, i.e., the parenchymal side, of brain capillary endothelial cells. Here, the transporter assists in maintaining low glutamate levels.
Research in context

Evidence before this study

Ischemic stroke is a major cause of morbidity and mortality worldwide. During the acute phase of stroke, glutamate homeostasis is disrupted, and glutamate levels rise above physiological concentrations in the extracellular fluid. This pathological situation leads to an overstimulation of glutamate receptors, followed by the excitotoxic death of neurons. In brain, excitatory amino acid transporters (EAATs) maintain the balance between pathological and physiological conditions by limiting the extracellular concentration of glutamate within the brain and thus preventing excitotoxic effects. Five genes have been identified in mammals encoding EAATs. Of these, EAAT2 plays a pivotal role for glutamate clearance in the adult brain. The critical role of EAAT2 in controlling brain glutamate homeostasis has led to the development of different therapeutic strategies to reduce the excitotoxic damage by glutamate after stroke.

Added value of the study

Mesenchymal stem cells are currently the most promising candidates for stem cell therapy against ischemic stroke owing to their intrinsic capability to secrete growth factors and immunomodulatory cytokines. Based on the high efficacy of EAAT2 for glutamate uptake, we hypothesized that the induced expression of this membrane transporter in mesenchymal stem cells for systemic administration could lead to a cell-based glutamate-grabbing therapy, combining the intrinsic properties of these cells with the excitotoxic protection.

Implications of all the available evidence

Here, and for first time, we found that this cell-based therapy, using the administration of EAAT2-expressing cells for ischemic lesions, could serve as a novel alternative to reduce glutamate neuronal excitotoxicity. We believe that our study makes a significant contribution to the literature because this new strategy paves the way to devise promising cell therapies for cerebral ischemia.

2. Experimental procedures

2.1. Culture of MSCs

Commercially available rat MSCs (Trevigen; Cultiex, Gaithersburg, MD, USA) were cultured in a medium consisting of Iscove's modified Dulbecco's medium (Gibco-Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen), 10% horse serum (Gibco-Invitrogen), 1% penicillin-streptomycin (PS; Gibco-Invitrogen), and 1% antimitotic solution (amphotericin; Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained at 37 °C in a humidified atmosphere and 5% CO2.

2.2. Culture of HEK 293 cells

HEK 293 cells obtained from ECACC (Sigma-Aldrich, Taufkirchen, Germany) were cultured in minimum essential medium (MEM) GlutaMAX I (Gibco-Invitrogen) supplemented with 1% FBS, 1% MEM non-essential amino acids (Gibco-Invitrogen), 1% PS, and 1% amphotericin. The cells were maintained at 37 °C in a humidified atmosphere and 5% CO2.
2.3. Transfection and expression of EAAT2 in cell lines

To functionally express EAAT2 in MSCs and HEK cells, we generated recombinant adeno-associated virus serotype 2 (rAAV2) harboring the coding sequence for yellow-fluorescent protein (YFP). N-terminally fused in-frame to the coding sequence of EAAT2. We observed good transduction rates in HEK cells (≥50%) but rather low efficiencies in MSCs (approximately 5–10%). Changing the AAV serotype from AAV2 to AAV6 did not overcome the low transduction rate in MSCs. Therefore, we changed the expression strategy and used a plasmid containing the N-terminally YFP-tagged EAAT2 cDNA (pRcCMVmYPFEEAAT2) for cell transfection (see Fig. S1).

YFP-EAAT2 expression in MSCs was achieved using an electroporation method as previously described [26]. For electroporation, cells were resuspended in Human MSC Nucleofector Solution (Amazu Biosystems, Cologne, Germany) at a concentration of 1 × 10⁶ cells/100 μL. The cell suspension was mixed with 4 μg endotoxin-free plasmid DNA in a sterile electroporation cuvette (Amazu Biosystems) and electroporated with the U-23 program. After electroporation, the cells were incubated for 5 min at RT and then transferred to culture dishes precoated with poly-D-lysine (Sigma-Aldrich) containing prewarmed growth medium. After 24 h, the medium was changed to remove debris and dead cells.

For the expression of YFP-EAAT2 in HEK cells, the plasmid was transfected using calcium phosphate co-precipitation according to the protocol described by Chen and Okayama [27]. Since the plasmid contains a neomycin resistance gene, stably transfected cells were selected in medium containing 1.2 mg/mL geneticin (G-418; Gibco-Invitrogen). Antibiotic-resistant colonies were isolated and maintained in culture medium supplemented with 1 mg/mL G-418 and examined for homogeneity based on YFP fluorescence.

Viral transduction and plasmid transfection methods are described in detail in the Supporting Information.

2.4. Assessment of YFP-EAAT2 expression in MSCs and HEK cells

The expression of YFP-tagged EAAT2 was evaluated and quantified at 24, 48, and 72 h post transfection using fluorescence microscopy and flow cytometry, respectively. Fluorescence microscopy was performed with an Olympus microscope, model IX51 (Olympus, Shinjuku, Tokyo, Japan). In the flow cytometry analysis, 30,000 gated events (cells) were collected. A cell was considered positive for YFP expression when its fluorescence was higher than the threshold settings of the non-transfected control sample. The percentage of YFP-fluorescent cells was calculated with respect to the total number of cells. Flow cytometry analysis was performed with a FACSAria II analyzer (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed with the FACSDiva software program (BD Biosciences).

2.5. Immunostaining of EAAT2 in transfected MSCs and HEK cells

Cells were grown on glass coverslips, which were previously treated with 0.1 mg/mL poly-D-lysine hydrobromide (Sigma-Aldrich). Cells were rinsed with phosphate buffered saline (PBS) and fixed in 4% (w/v) paraformaldehyde (PFA) solution (Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at room temperature (RT), and then washed thrice with PBS. Non-specific binding sites were blocked with 0.5% (w/v) bovine serum albumin (BSA) (GE Healthcare, Chicago, IL, USA) solution for 30 min at RT. Cells were incubated with anti-EAAT2 antibodies (Millipore, Burlington, MA, USA) diluted 1:100 in 0.5% (w/v) BSA in PBS solution for 24 h at 4 °C. Cells were washed thrice with PBS at RT and incubated with a biotinylated horse anti-mouse IgG antibody, rat adsorbed (dilution 1:200; Vector Laboratories, Burlingame, CA, USA), for 1 h at RT, followed by a second incubation with a 1:100 dilution of avidin–biotin (Vector Laboratories) for 30 min. Once washed, the samples were incubated with DyLight 594 streptavidin (diluted 1:500 in PBS; Vector Laboratories) for 30 min at RT, followed by a last incubation step with Hoechst stain (diluted 1:6000; Invitrogen) for 5 min. Fluorescence was analyzed with a confocal microscope (LEICA AOBSP-SP5X; Leica, Wetzlar, Germany).

2.6. Characterization of transfected MSCs

Flow cytometry, tube formation assay, and growth factor release analysis were performed to verify that the transfected MSCs maintained their intrinsic phenotype properties.

Flow cytometry analysis: MSCs were harvested using trypsinization, washed, and resuspended in PBS. The typical phenotype of MSCs is CD90+/CD73−/CD45−. Cells were stained with CD90-PerCP-Cy5 (clone OX-7; 5 μL/1 × 10⁵ cells; Immunostep, Salamanca, Spain), CD73-APC (clone 5F/B9; 1 μL/1 × 10⁵ cells; Immunostep), and CD45-PE (clone OX-22; 2.5 μL/1 × 10⁵ cells; BD Biosciences) antibodies. After immunostaining, the cells were suspended in 200 μL PBS for subsequent analysis. The analysis was performed using a BD FACSAria II analyzer (BD Biosciences). Unlabeled MSCs were used as a negative control. Dead cells and debris were excluded based on side and forward scatter profiles (SSC and FSC, respectively). Appropriate isotype control antibodies, “fluorescence minus one” staining, and staining of negative populations were used to establish compensation parameters and sorting gates. Data were analyzed using FACSDiva software (BD Biosciences).

Tube formation assay: Matrigel-reduced growth factor (BD Biosciences) was used as a substrate to assess tube formation capacity as an in vitro measurement of functional angiogenesis in non-transfected MSCs (MSCs−) and transfected MSCs (MSCs+). Standard 24-well plates were coated with 200 μL cold matrigel. MSCs were added onto matrigel-coated wells at 1 × 10⁴ cells per well in growth medium. Cells were incubated for 12 h at 37 °C and 5% CO₂ in a humidified atmosphere. After medium removal, cells were fixed in 4% (w/v) PFA in PBS, and images were captured using an inverted microscope (Olympus IX51). Tube formation was assessed by counting the number of circular structures (rings) and branching points (tube joints). Five random fields were examined for each well.

Vascular endothelial growth factor (VEGF) analysis in cell culture supernatants: The levels of VEGF in MSCs− and MSCs+ were measured with a Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions. The detection limit was 31.2 pg/mL (VEGF ELISA kit), and values were corrected for the amount of total cell protein calculated by the Bradford assay (Bio-Rad, Hercules, CA, USA).

2.7. Analysis of interleukin-6 production in MSCs after transfection and lipopolysaccharide treatment

Non-electroporated and electroporated MSCs (without plasmid and with control and EAAT2-plasmid), were seeded in culture dishes pretreated with poly-D-lysine (Sigma-Aldrich). After 24 h, the medium was removed, and it was replaced with fresh medium containing lipopolysaccharide (LPS) (1 μg/mL; Sigma-Aldrich) [28]. The supernatants were harvested by centrifugation at 3000 rpm for 20 min and stored at −80 °C until further use. The levels of interleukin-6 (IL-6) were measured with an IL-6 ELISA kit (Abbexa, Cambridge, UK) following the manufacturer’s instructions. The detection limit was 3.3 pg/mL (IL-6 ELISA kit), and values were corrected for the amount of total cell protein calculated by the Bradford assay (Bio-Rad). The plasmid encoding the fluorescent protein eGFP (pmaxGFP) was used as the control (VPE 1001; Lonza, Basel, Switzerland).

2.8. Glutamate uptake assay

The uptake of radiolabeled glutamate was assessed using a competition assay. Transfected cells (HEK− cells and MSCs+) expressing EAAT2

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were plated in triplicate (1.5 × 10^2 cells per well) on 24-well tissue culture plates pre-treated with poly-d-lysine hydrobromide (Sigma-Aldrich). Non-transfected cells (HEK− and MSCs−) were used as the negative control, and astrocytes were employed as the positive control. After 24 h, the medium was aspirated, and cells were washed thrice with 250 μL PBS at RT. Then, 500 μL PBS supplemented with 13 nM l-[3]H glutamate (1 μCi/μL; Sigma-Aldrich; specific activity = 51.1 Ci/mmol) and increasing concentrations (10⁻⁸–10⁻³ M) of non-labeled glutamate were added. Non-labeled glutamate was added to each well, and the plate was incubated at 37 °C for 10 min. The plates were placed on ice, and cells were washed thrice with ice-cold PBS. Cells were lysed with 200 μL 0.2 M NaOH for 4 h at 4 °C under continuous shaking. From each lysate, 50 μL was transferred to a 96-well multiplex plate, and 150 μL Optiphase buffer was added to each well. The multiplex plate was shaken for 30 min at RT. Radioactivity was determined by scintillation counting (PerkinElmer, Waltham, MA, USA), and the total protein content in lysates was determined by the Bradford assay (Bio-Rad). The results were expressed in radioactive disintegrations per minute per microgram of cellular protein.

To determine the specificity of glutamate uptake, two inhibitors of EAAT2—dihydrotkainate (DHK) and DL-threo-beta-benzoxazoylaspartate (TBOA) (Tocris, Bioscience, Avonmouth, Bristol, UK)—were employed. Both inhibitors were used individually and jointly at concentrations of 300 μM (DHK) and 500 μM (TBOA) based on previous studies [8].

2.9. In vitro toxicity test

Cell death was evaluated by measuring the leakage of lactate dehydrogenase (LDH) into the medium, as well as the cell proliferation rate. Basal LDH and LDH release at 24, 48, and 72 h after transfection were measured in control and transfected cells. A negative control of lysed cells was also included (death control), according to the manufacturer’s protocol. After centrifugation (7 min, 3000 rpm) of the supernatants, 75 μL was transferred to a 96-well plate and mixed with 150 μL LDH reagents following the manufacturer’s protocol (Lactate Dehydrogenase Assay Kit; Sigma-Aldrich). After 20 min incubation at RT, the LDH activity was determined at 490 nm using a spectrophotometer (Synergy 2; BioTek, Winooski, VT, USA).

2.10. Proliferation rate

To determine the proliferation rate, the total cell number was examined using a Neubauer chamber. The number of viable cells was determined using the Trypan blue exclusion assay.

2.11. Experimental animals

Experimental protocols were approved by the local Animal Care Committee according to the European Union (EU) rules (86/609/CEE, 2003/65/CE, and 2010/63/EU). Male Wistar rats (Harlan Laboratories, Barcelona, Spain) weighing 300 ± 25 g were used. The animals were kept at controlled conditions of temperature (22 °C ± 1 °C) and humidity (60% ± 5%) with a 12/12 h light/dark cycle for a week prior to surgery. All excluded or deceased animals were replaced until the total number of animals indicated for each group was attained. Additional details of the surgical procedures are indicated in the Supporting Information.

2.12. Surgical procedures

Transient focal ischemia was induced in rats by transient middle cerebral artery occlusion (MCAO) following previously described surgical procedures [12,29]. According to our reported protocol [29], the following exclusion criteria were used: (i) <70% reduction in relative cerebral blood flow during artery occlusion; (ii) arterial malformations, as determined by magnetic resonance angiography; (iii) baseline lesion volume of <25% or >45% with respect to the ipsilateral hemisphere, as measured using apparent diffusion coefficient (ADC) maps; and (iv) absence of re-perfusion or prolonged reperfusion (>10 min until achievement of at least 50% of the baseline cerebral blood flow) after filament removal. All excluded or deceased animals were replaced until the total number of animals indicated for each group was attained. Additional details of the surgical procedures are indicated in the Supporting Information.

2.13. In vivo experimental protocol

This study was divided into two steps: a dose-response study and a protective study.

Dose-response study: different doses of MSCs+ and HEK+ cells were evaluated for blood glutamate reduction after i.v. administration and compared with the same dose of non-transfected cells (MSCs− and HEK−) with an oxaloacetate-treated group and a control group treated with saline.

Protective study: To evaluate the protective effect of MSCs+ and HEK+ cells against ischemic lesions, transfected cells were tested in ischemic animal models, and their effect on blood glutamate reduction, infarct volume, and functional recovery was compared with the effect of the administration of non-transfected cells (MSCs− and HEK−) with oxaloacetate and with that of a control group treated with saline.

The experimental groups, glutamate and IL-6 analysis in blood samples and functional test are summarized in the Supporting Information.

2.14. Magnetic resonance studies

All magnetic resonance imaging (MRI) studies were conducted on a 9.4 T horizontal bore magnet (Bruker BioSpin, Ettlingen, Germany) according to a previously described protocol [16,29,30]. MRI post-processing was performed using ImageJ software (https://imagej.nih.gov/ij/). Additional details about MRI sequences and data analysis are described in the Supporting Information.

2.15. Brain histological analysis

Animals were sacrificed 14 days after surgical treatment, and brain tissue was processed for histological analysis. Neuronal nuclear protein (NeuN) labeling was combined with labeling of the astrocyte marker; glial fibrillary acidic protein (GFAP), GFAP-positive and NeuN-positive cells and nuclei were counted manually in Image J software. Detailed protocols are described in the online Supporting Information.

2.16. Statistical analysis

All data are presented as the mean ± standard deviation of the mean (mean ± SD). One-way or two-way analysis of variance (ANOVA) followed by post-hoc Bonferroni evaluation was used for multiple groups to determine significant differences. Student’s t-test was used to test the differences between two groups. Statistical significance was set at *p < 0.05, **p < 0.01, and ***p < 0.001. The statistical analysis was conducted using GraphPad Prism v.6.05 for Windows (GraphPad, La Jolla, CA, USA).

3. Results

3.1. EAAT2 expression in MSCs and HEK 293 cells

EAAT2 was initially expressed in MSCs and HEK 293 cells by rAAV2 transduction. However, we did not achieve sufficient transduction rates (>30%) in MSCs (see supplemental results and Figs. S2 and S3),
which led us to change the heterologous expression strategy. For MSCs, three different transfection methods were consecutively tested: calcium phosphate co-precipitation, lipofectamine transfection, and electroporation (With electroporation, we obtained successful and reliable expression rates of ≥60%).

Since the construct coded for an N-terminally YFP-tagged EAAT2 protein, successful expression of the fusion protein in MSCs (MSCs⁺) could be monitored by confocal microscopy (Fig. 1A). Using flow cytometry analysis, maximum cells (≥60%) expressing the YFP-EAAT2 protein were observed 48 h after transfection (Fig. 1B). No YFP expression was detected in MSCs electroporated without the plasmid (MSCs⁻). To evaluate the potential cell damage induced by electroporation, MSC proliferation and toxicity rates were determined. Notably, electroporation caused a significant (p < 0.05) reduction in MSC⁺ proliferation 24 and 48 h after transfection compared to MSCs⁻. This effect, however, was not observed 72 h after electroporation (Fig. 1C). Toxicity analysis was performed by LDH assay. There were no significant differences between MSCs⁻ and MSCs⁺ (Fig. 1C, lower panel). Finally, confocal microscopy confirmed the expression of YFP-EAAT2 in MSCs⁺ (Fig. 1D, E), with a preferential localization in the cell membrane (Fig. 1D).

In addition to MSCs, HEK 293 cells were transfected with the same plasmid using the calcium phosphate co-precipitation technique [27]. Since the plasmid harbors a neomycin resistance gene, stably transfected cell clones constitutively expressing YFP-EAAT2 (HEK⁺) were selected and maintained in the presence of the antibiotic geneticin (G418). The expression of the construct in HEK⁺ cells was inspected by confocal microscopy (Fig. 2A). Flow cytometry showed that the initial transfection efficiency was ≥50% (Fig. 2B). In contrast to electroporation used for MSCs, calcium phosphate co-precipitation did not have an impact on the proliferation rate or toxicity of cells transfected without (HEK⁻) or with the plasmid (HEK⁺) (Fig. 2C). Selection with geneticin resulted in the isolation of cell clones that homogeneously (≥95%) expressed the construct (Fig. 2D, E).

Summary of the procedures used to induce the expression of EAAT2 in MSCs and in HEK cells is represented in Fig. 54.

3.2. Functional analysis of EAAT2 in MSCs⁺ and HEK⁺ cells

To evaluate the functionality of EAAT2 expressed in both cell types, uptake assays with [3H]glutamate were conducted in MSCs⁺ and HEK⁺ cells. For the control, assays were also performed in primary astrocytes that are known to express EAAT2 endogenously [3]. The uptake of [3H]glutamate (13 nM) in astrocytes was determined in the presence of increasing concentrations of unlabeled glutamate (10⁻⁸–10⁻³ M). The uptake of [3H]glutamate was significantly reduced (EC₅₀ = 19 μM), and the concentration-response curve saturated at glutamate concentrations of ≥5 × 10⁻⁴ M. The uptake of [3H]glutamate was also measured in the presence of specific inhibitors of glial EAAT2 activity [8,31]: TBOA and DHK. We found that 500 μM DHK and 300 μM TBOA efficiently inhibited the [3H]glutamate uptake in astrocytes (see Fig. S5).

The incubation of MSCs⁻ with 13 nM [3H]glutamate in the presence of increasing amounts of non-labeled glutamate (10⁻⁸–10⁻³ M) showed specific glutamate uptake. The competitive effect was saturated at concentrations of ≥5 × 10⁻⁴ M of non-labeled glutamate, and the EC₅₀ calculated from the concentration-response curve was 65 μM. In MSCs⁺, no specific uptake of [3H]glutamate was detected (Fig. 3A). Glutamate uptake in MSCs⁻ was abolished when TBOA (300 μM) and DHK (500 μM) were included in the assay either in combination (Fig. 3B) or individually (p < 0.001) (Fig. 3C). Glutamate uptake experiments were performed in a similar manner on HEK⁺ cells. As observed in MSCs⁻, HEK⁻ cells showed a specific [3H]glutamate uptake with a similar EC₅₀ (62 μM) as that determined for MSCs⁺. No specific [3H]glutamate uptake was observed in HEK⁻ cells (Fig. 3D). [3H]Glutamate uptake into HEK⁺ cells was blocked when EAAT2 inhibitors (TBOA and DHK) were added together (Fig. 3E) or separately (p < 0.001) (Fig. 3F).

3.3. Phenotype evaluation of MSCs⁺

Some of the most important characteristics contributing to the beneficial effects of MSCs in stroke are their angiogenic properties, as well as their capability to act as a source of growth factors and cytokines [32]. Here, we evaluated whether EAAT2 expression modified the intrinsic characteristics of MSCs⁺ and determined their angiogenic capacity, their capacity to secrete VEG, and the expression of membrane mesenchymal markers (CD90⁺, CD73⁺, and CD45⁻).

The angiogenic capacity of MSCs⁺ was examined by an in vitro matrigel tube formation assay, confirming that the number of rings identified was similar to those obtained with MSCs⁻ (Fig. 4A–C). The release of VEGF by MSCs⁺ and MSCs⁻ was also similar (Fig. 4D). The analysis of membrane mesenchymal phenotype markers (CD90⁺, CD73⁺, and CD45⁻) was performed by flow cytometry, which showed that MSCs⁺ and MSCs⁻ did not differ in surface marker expression (Fig. 4E).

3.4. Animals included in the study

Once the expression and functionality of EAAT2 was evaluated in MSCs⁺ and HEK⁺ cells, their blood glutamate-grabbing activity was analyzed first in healthy animals and then in ischemic animal models. A total of 179 experimental animals (male Wistar rats) were used in this study. Forty-eight rats were used to study the effect of MSCs⁺ and HEK⁺ cells on blood glutamate-grabbing, and the remaining 131 were used in the ischemic study. From these, 72 rats were excluded because they did not meet the inclusion criteria and 11 died; finally, 48 ischemic rats were examined (Fig. S6).

3.5. Analysis of blood glutamate concentration in healthy animals treated with MSCs⁺ and HEK⁺ cells

To evaluate the effect of MSCs⁺ and HEK⁺ cells on blood glutamate reduction in healthy animals, a cell dose/response study was performed with i.v. administered 1 × 10⁶, 2 × 10⁶, and 3 × 10⁶ cells/animal. A dose of 3 × 10⁶ cells/animal was necessary to obtain a reduction in glutamate levels (Fig. 5A). Then, eight experimental groups were designed to compare the efficacy of the cells’ glutamate-grabbing therapy on glutamate reduction with respect to oxaloacetate treatment. These groups were as follows: (i) a control group treated with saline (vehicle), (ii) a group treated with oxaloacetate (35 mg/kg) [13] used as a positive control for blood glutamate reduction, (iii) a group treated with 3 × 10⁶ HEK⁺ cells, (iv) a group treated with 3 × 10⁶ MSCs⁺ cells, (v) two groups treated with 3 × and 9 × 10⁶ MSCs⁺, and (vii, viii) two groups treated with 3× and 9 × 10⁶ MSCs⁻. Glutamate levels were determined before cell administration (i.e., basal levels) and 1, 3, and 4 h after cell administration. All treatments were administered by i.v. bolus in 1 mL of saline.

MSCs⁺ for animal treatment were taken 48 h after transfection (see Fig. 1B). HEK⁺ cells were taken from the stably transfected cell clone (see Fig. 2D). Blood glutamate concentrations in animals treated with saline did not change significantly within the first 4 h after treatment. Similarly, the groups of animals treated with non-transfected cells—HEK⁻ and MSCs⁻—did not show a significant reduction in blood glutamate levels after treatment administration either. In line with our previous results [13], oxaloacetate led to a reduction in glutamate at 1 h (p < 0.05) and 4 h (p < 0.05) after the administration of the compound. The administration of 3 × 10⁶ HEK⁺ cells resulted in a reduction in blood glutamate levels 1 and 3 h after injection. With 3 × 10⁶ MSCs⁺, a significant glutamate reduction was measured 1 and 2 h after cell administration (p < 0.05, n = 6/group) (Fig. 5B). A higher dose of MSCs⁺ (9 × 10⁶) did not increase the reduction in blood glutamate levels compared to the values achieved with 3 × 10⁶ MSCs⁺; however, the effect lasted longer and was detectable even 4 h after cell administration. Notably, the treatment of rats with MSCs⁺ reduced the blood glutamate to a level similar to that measured 4 h after oxaloacetate administration.
**Fig. 1.** Analysis of YFP-EAAT2 expression in MSCs. (A) Confocal image of control (MSCs−) and YFP-EAAT2-expressing MSCs (MSCs+) after electroporation (scale bar, 25 μm). (B) YFP fluorescence in MSCs analyzed by flow cytometry: non-transfected cells (FITC) and transfected cells (FITC 24 h, FITC 48 h, and FITC 72 h) after transfection. (C) Analysis of the proliferation rate and toxicity (measured by an LDH assay) of electroporated cells without plasmid (MSCs−) and with the YFP-EAAT2-encoding construct (MSCs+). Data shown are normalized to MSC− levels (*p < 0.05, n = 3). (D) Immunocolocalization of YFP-EAAT2 in MSCs+ (scale bar, 10 μm). (E) Immunofluorescent labeling of nuclei (Hoechst, blue), YFP (green), and EAAT2 (red) in control (MSCs−) and transfected MSCs+ (scale bar, 25 μm).
Fig. 2. Analysis of YFP-EAAT2 expression in HEK cells. (A) Confocal image of YFP-EAAT2-expressing HEK cells after calcium phosphate transfection (scale bar, 25 μm). (B) YFP fluorescence in HEK cells analyzed by flow cytometry: before and 48 h after transfection. (C) Analysis of proliferation rate and toxicity (measured by an LDH assay). Data are normalized to HEK− levels (*p < 0.05, n = 3). (D) An HEK+ colony constitutively expressing YFP-EAAT2 (scale bar, 100 μm). (E) Immunofluorescent labeling of nuclei (Hoechst, blue), YFP (green), and EAAT2 (red). Scale bar, 25 μm. Non-transfected HEK cells (HEK−); transfected HEK cells (HEK+).
3.6. Comparative protective effect of MSCs+ and HEK+ cells against oxaloacetate treatment in ischemic animal models

Once the blood glutamate-grabbing efficacy in healthy rats was evaluated, the protective effect of MSCs+ and HEK+ cells was tested in ischemic rat models. No effect on blood glutamate reduction was observed in the rats treated with saline and non-transfected cells (3 × 10^6 HEK−, 3 × 10^6 MSCs−, and 9 × 10^6 MSCs−). Ischemic rats treated with oxaloacetate and 3 × 10^6 HEK+ cells showed significant reductions (p < 0.05, n = 6/group) in blood glutamate levels 1 h after treatment. The effect lasted even longer (3–4 h) when 3 × 10^6 or 9 × 10^6 MSCs+ were administered (Fig. 6A).

MRI analysis during the occlusion revealed that all animals showed similar ischemic lesions before the administration of cells or oxaloacetate (average ischemic lesion, 42% ± 4%, adjusted to the ipsilateral hemisphere). Inspection of the infarct volume on day 14 showed that oxaloacetate, 3 × 10^6 MSCs+, and interestingly, 3 × 10^6 and 9 × 10^6 of both MSC−-treated groups showed reductions in infarct volume (30.9% ± 5% vs. 24.3% ± 4%; 30.9% ± 5% vs. 25.4% ± 3%; 30.9% ± 5% vs. 22.7% ± 3%, and 30.9% ± 5% vs. 13.5% ± 11%, respectively, p < 0.05, n = 6/group). A trend of reduced infarct volume was also observed with 9 × 10^6 MSCs+, but this effect was not statistically significant (Fig. 6B and C). Associated with the regression of the infarct volume, a functional recovery of the rats occurred, which was examined by the cylinder test. Notably, the administration of 3 × 10^6 HEK+ cells also resulted in functional improvement (Fig. 6D).

Histological analysis of a neuronal marker (NeuN) and a glial marker (GFAP) performed 14 days after lesioning did not reveal expression...
Fig. 4. Examination of MSC characteristics. Phase-contrast image of matrigel angiogenic assay in MSCs⁻ (A) and MSCs⁺ (B), (scale bar, 100 μm). (C) Analysis of ring formation of MSCs⁻ and MSCs⁺. Data are shown as mean ± SD (n = 3). (D) VEGF release determined at different time-points in MSCs⁻ and MSCs⁺. Data are shown as mean ± SD (n = 3). (E) Analysis of mesenchymal phenotype markers (CD90⁺, CD73⁺, and CD45⁻) in MSCs⁻ (upper panel) and MSCs⁺ (lower panel) by flow cytometry (n = 3). Non-transfected MSCs (MSC⁻), transfected MSCs (MSC⁺), fluorescein isothiocyanate (FITC), allophycocyanin (APC), peridinin chlorophyll protein c complex (PerCP), phycoerythrin (PE), yellow fluorescent protein (YFP).
Fig. 5. Analysis of cell glutamate in healthy rats. (A) Dose/response analyses of $1 \times 10^6$, $2 \times 10^6$, and $3 \times 10^6$ HEK+ cells and MSC−/animal on blood glutamate levels 4 h after cell administration. (B) Effect of the different treatments on blood glutamate concentration at distinct time-points in healthy animals. Data are normalized to basal glutamate levels ($^*p < 0.05$, $^{**}p < 0.01$, n = 6/group). The dashed line represents the 100% value of basal glutamate levels. Non-transfected MSCs (MSC−); transfected MSCs (MSC+); non-transfected HEK cells (HEK−); transfected HEK cells (HEK+).

4. Discussion

Blood/brain glutamate-grabbing is an emerging protective strategy to reduce the excitotoxic effect of the elevated extracellular glutamate that accumulates in the brain during the acute phase of ischemic stroke [9,18]. This novel protective strategy has been tested by several laboratories in different ischemic animal models [13,16,35-38], using the two most common pharmacological approaches, i.e., oxaloacetate and rGOTI application. The protective mechanism has also been validated by non-pharmacological approaches like peritoneal dialysis, used to filter the blood and remove excess glutamate [19,39,40]. All these studies imply that blood glutamate reduction holds great potential as an effective protective strategy to reduce the neurotoxic increase in glutamate in the brain. On the basis of the high specificity of excitatory amino acid transporters, e.g., EAAT2, for glutamate uptake, we developed a novel cell-based blood/glutamate-scavenging approach by introducing the coding sequence of EAAT2 into MSCs, combining the intrinsic properties of these cells with excitotoxic protection. Here, we compared the blood glutamate-grabbing efficacy of this cell-based strategy with the “gold standard,” i.e., oxaloacetate treatment.

The transporter was successfully expressed in MSCs and HEK 293 cells. In both cell types, the expression of YFP-EAAT2 was confirmed by immunological staining and flow cytometry. The functionality of the transporter was determined by $[^{3}H]$glutamate uptake assays, and the blood glutamate-grabbing activity was validated in healthy rats. The results demonstrated that systemic administration of cells expressing EAAT2 significantly reduces blood glutamate levels. The effect on blood glutamate reduction produced by MSC− and HEK− was very similar to that resulting from oxaloacetate administration. The highest dose of MSC+ (9 $\times$ 10^6 cells) reduced the glutamate concentration for up to...
4 h; yet, the absolute reduction was not higher than that with \(3 \times 10^6\) cells or oxaloacetate. It is tempting to speculate that a higher expression level of EAAT2 or higher cell doses per animal could increase the glutamate-grabbing efficacy. Here, as well as in previous studies [26,27], the expression of EAAT2 in MSCs+ and HEK+ cells was technically optimized to gain high transporter levels per cell and simultaneously keep the cells healthy. We are aware that alternative strategies, e.g., invoking lentivirus infection, may result in even higher transduction rates, and this aspect should be addressed in forthcoming studies. Regarding the cell dose used, we established \(3 \times 10^6\) cells per animal as a threshold necessary to induce a significant reduction in blood glutamate levels. The risk of cell aggregation and pulmonary embolism has been described in other studies [41]; therefore, to avoid the death of the animals, doses higher than \(9 \times 10^6\) MSCs+ were not used. Owing to the lack of experience and literature on HEK cells for i.v. administration and to reduce the risk of systemic embolisms, doses higher than \(3 \times 10^6\) cells were not tested.

The therapeutic effect of MSCs+ and HEK+ cells was thoroughly examined in ischemic rats and compared with that of oxaloacetate treatment. In line with the results in healthy rats, the administration of transfected cells and oxaloacetate significantly reduced blood glutamate levels, which was associated with the functional recovery of the animals. However, oxaloacetate, \(3 \times 10^6\) MSCs+, and surprisingly, MSCs− (\(3 \times 10^6\) and \(9 \times 10^6\) cell doses) were the only treatments that dually caused functional recovery and a reduction in infarct volume. HEK 293 cells are widely used in vitro cell studies because they are easy to handle, grow rapidly, are resistant to stressful conditions, and can be readily transfected [42]. Therefore, this cell line represented an excellent model to test the effect of EAAT2 cell expression on blood glutamate reduction. The functional improvement observed in ischemic animals treated with HEK+ cells confirmed that this effect was indeed mediated by the glutamate-grabbing activity associated with EAAT2 functionality.

The administration of HEK+ cells and oxaloacetate in ischemic animals resulted in almost similar glutamate reductions. However, the protective effect was quite different. This suggests that, in addition to the obvious blood glutamate-grabbing effect, oxaloacetate may induce a pleiotropic mechanism protecting the animal against ischemic stroke. Oxaloacetate has been shown to serve as an antioxidant when cells are subjected to stressful stimuli, such as hydrogen peroxide, thiobarbituric acid reactive species, or excitotoxic damage [15].

MSCs are currently the most promising candidates for stem cell therapy against ischemic stroke owing to their intrinsic capability to secrete growth factors and immunomodulatory cytokines, which equip these cells with neuroprotective and neurorecovery properties [32]. Therefore, we hypothesized that the addition of a glutamate-grabbing property should increase the protective effect of MSCs against cerebral ischemia. The expression of EAAT2 in MSCs effectively conferred the expected glutamate-grabbing activity validated in healthy and ischemic animals. Unexpectedly, the protective effect observed on infarct volume reduction and functional recovery after MSC+ administration was not pronounced as with MSC−. This finding suggests that the transfection procedure most likely interferes with some of the intrinsic protective mechanisms of mesenchymal cells. We could exclude the effect of the transfection procedure on the release of VEGF and membrane phenotype. The quantification of IL-6 secretion, which is considered one of the most representative cytokines [34], in MSC supernatants revealed that the electroporation procedure rather than plasmid expression induced the release of this cytokine. This might explain the loss of the beneficial effect with MSCs+ that we observed with MSCs. In fact, MSCs electroporated with control plasmids (sham MSCs) were not included as an additional group in the animal study mainly because we wanted to compare the behavior of MSCs+ with that of healthy MSCs.

That MSCs release IL-6 upon LPS stimulation or other stressful stimuli like \(\text{H}_2\text{O}_2\) [43] is well-documented [44]. However, there are conflicting reports on the potential effects of this cytokine in stroke. Some studies have found that the external administration of IL-6 in ischemic animal models leads to an improvement in the animal outcome [45], while we recently reported IL-6 to be associated with poor outcome in a study on 4112 stroke patients [46], a finding that is corroborated by the present findings. We cannot exclude that an alteration in the release of other mesenchymal factors or cytokines could contribute to reduce the beneficial effect of transfected MSCs in ischemic animals. From our study, we can deduce that the experimental manipulation of mesenchymal cells induces an alteration of their intrinsic properties and reduces their beneficial properties, which is an important issue for studies focusing on the therapeutic effects of MSCs.

We previously evaluated the fate and biodistribution of MSCs after i.v. administration in the same ischemic animal model as we have used in this study [30]. We observed that the benefits observed after i.v. delivery of MSCs were not attributable to the cell engraftment in the brain as MSCs did not reach the brain tissue, and positively labeled cells were found to be distributed mainly along the lungs. Therefore, we can assume that the effect of MSCs on glutamate and infarct reduction was mediated by a systemic effect and not the engraftment and dedifferentiation into cerebral EAAT2-expressing cells, as astrocytes or endothelial cells.

This study had some limitations that should be taken into consideration. One of the main limitations of protective drugs against glutamate excitotoxicity after stroke is the narrow therapeutic time window, mainly because the increase in glutamate release appears immediately after stroke; therefore, treatments against glutamate excitotoxicity should be administered as soon as possible but within a clinically relevant time window. In this study, cell infusions were applied upon reperfusion, only 75 min after occlusion onset. This could be considered an early treatment regimen, which might be impractical in the clinic setting. However, although the metabolic rate in rats is faster than that in humans (~6.4 times faster [47]), making the time window clinically acceptable, further studies should determine the time window of these cell therapies.

In summary, our results show that cell-based therapies using the administration of EAAT2-expressing cells to ischemia-lesioned animal models is a novel alternative to reduce glutamate neuronal excitotoxicity. Although further studies are necessary to optimize the EAAT2 expression in mesenchymal cells, this study opens up avenues for combining a protective strategy with a cell therapy for cerebral ischemia.

**Fig. 6.** EAAT2−cell-based glutamate-grabbing therapy in ischemic rat models. (A) Effect of the different treatments on blood glutamate concentration at different time-points in ischemic animals. Data are shown as \(\pm\) SD relative to the basal levels (*p < 0.05, **p < 0.01 compared with the control group at the same time-point; n = 6/group). The dashed line represents the 100% value of basal glutamate levels. (B) Representative MRI of each experimental group at different time-points. ADC maps were determined during ischemia induction and T2-weighted imaging at 1, 7, and 14 days. ADC maps were used to determine the basal ischemic lesion before treatment administration. (C) Effect of the different treatments on infarct size at different time-points in ischemic animals. Data are shown as \(\pm\) SD relative to the ischemic hemisphere (*p < 0.05, **p < 0.01 compared with the control group at the same time-point; n = 6/group). The dashed line represents the functional deficit of the control group (vehicle) at 14 days after stroke. Non-transfected MSCs (MSCs−), transfected MSCs (MSCs+), non-transfected HEK cells (HEK−), transfected HEK cells (HEK+), middle cerebral artery occlusion (MCAO), oxaloacetate (Oxal), neurons (NeuN), glial fibrillary acidic protein (GFAP).
Fig. 7. In vitro and in vivo determination of the release of IL-6. (A) In vitro IL-6 quantities in the supernatants of MSC− and MSC+ cultures under basal MSCs is associated with a release of IL-6 conditions 24 h after transfection (with and without plasmid), and 48 h after LPS treatment for 24 h. Data are shown as mean ± SD (***p < 0.001, n = 9). The plasmid encoding the fluorescent protein eGFP (pmaxGFP) was used as the control. pEAAT2 represents the YFP-EAAT2-encoding construct (B) Serum levels of IL-6 in ischemic rats treated with vehicle, 9 × 10^6 MSCs−, and 9 × 10^6 MSCs+. Data are shown as mean ± SD (**p < 0.01, n = 3). Non-transfected MSCs (MSCs−); transfected MSCs (MSCs+); lipopolysaccharide (LPS).

Author contributions

Conception and design of the experiments: M.P.-M., J.C., T.S., and F.C. Collection, analysis, and interpretation of data: M.P.-M., R.I.-R., A.V.-P., A. D.-L., B.A., H.-F.-S., A.-S.-C., A.-P.-D., C.-C.-P., A.G., P.A.-G., M.I.L., A.B., J.C., T.S., and F.C. Drafting the article: A.B., J.C., T.S., and F.C. Supervision of the project: J.C. and F.C.

Conflict of interest

The authors declare that no conflict of interest exists.

Acknowledgement

This study was partially supported by grants from Instituto de Salud Carlos III (PI13/00292 and PI17/0054), Spanish Research Network on Cerebrovascular Diseases RETICS-INVICTUS (RD12/0014), Fundación Mutua Madrileña; the Ministry of Economy and Competitiveness of Spain (SAF2014-56336-R), Xunta de Galicia (Programa de Desarrollo Precomercial de los resultados de investigación del Sistema Público de Salud de Galicia_ PRIS); and the European Union program FEDER. Furthermore, F. Campos (CP14/00154) and T. Sobrino (CP12/03121 and CPI17/00027) are recipients of a research contract from Miguel Servet Program of Instituto de Salud Carlos III. Funders had not any role in study design, data collection, data analysis, interpretation, writing of the report.

We thank Prof. C. Fahlike (Institute of Complex Systems – Cellular Biophysics (ICS-4), Forschungszentrum Jülich, Germany) for assistance and for providing the pRcCMVmYFPEAAT2 plasmid.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.11.024.

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