Original Paper

Transplantation of N-Acetyl Aspartyl-Glutamate Synthetase-Activated Neural Stem Cells after Experimental Traumatic Brain Injury Significantly Improves Neurological Recovery

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Key Words

NAAGS • Neural stem cells • Traumatic brain injury • Neurological recovery

Abstract

Background/Aims: Neural stem cells (NSCs) hold considerable potential as a therapeutic tool for repair of the damaged nervous system. In the current study, we examined whether transplanted N-acetyl aspartyl-glutamate synthetase (NAAGS)-activated NSCs (NAAGS/NSCs) further improve neurological recovery following traumatic brain injury (TBI) in Sprague-Dawley rats. **Methods:** Animals received TBI and stereotactic injection of NSCs, NAAGS/NSCs or phosphate buffered saline without cells (control) into the injured cortex. NAAGS protein expression was detected through western blot analysis. Dialysate NAAG levels were analyzed with radioimmunoassay. Cell apoptosis was detected via TUNEL staining. The expression levels of specific pro-inflammatory cytokines were detected with enzyme-linked immunosorbent assay. **Results:** Groups with transplanted NSCs and NAAGS/NSCs displayed significant recovery of the motor behavior, compared to the control group. At 14 and 21 days post-transplantation, the motor behavior in NAAGS/NSC group was significantly improved than that in NSC group ($p < 0.05$). Additionally, transplanted NAAGS/NSCs inhibited cell apoptosis and the expression levels of specific pro-inflammatory cytokines, including interleukin-1 β , interleukin-6 and tumor necrosis factor- α . **Conclusion:** Our results collectively demonstrate that NAAGS/NSCs provide a more powerful autoplasmic therapy for the injured nervous system.

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Introduction

Neural stem cell (NSC) transplantation has considerable potential as a therapeutic intervention in several central nervous system (CNS) disorders, including Parkinson's disease, Huntington's disease, spinal cord injury, and ischemic and traumatic brain injury (TBI) [1-4]. NSCs display the ability to self-renew and differentiate into cells of broad neuronal and glial lineages. The isolation and characterization of NSCs has opened up new possibilities for direct transplantation into the injured brain, owing to their multipotential ability to self-renew and produce neurons, astrocytes and oligodendrocytes. Moreover, the inherent ability of NSCs to adapt to signals from host cells and the extracellular environment is necessary for interactions with host tissues and regeneration of injured tissues [5]. Previous studies on animal models of human neurodegenerative disease have demonstrated recovery of lost neurological functions and partial reconstruction of neural circuitry upon NSC transplantation in damaged adult brains [6]. Transplanted NSCs differentiate into various phenotypes that promote restoration of function after injury or disease in the CNS, including TBI, depending on transplant location.

Glutamate excitotoxicity is an important mechanism causing neuronal damage through excessive receptor activation. For example, extracellular glutamate elevated immediately after TBI contributes significantly to delayed neuronal cell death [7-9]. Pharmacological blockade of glutamate receptors early after TBI improves outcomes in laboratory animals [7, 10, 11]. N-acetyl aspartyl-glutamate (NAAG) is an abundant peptide neurotransmitter found in millimolar concentrations in the mammalian CNS [12, 13]. NAAG selectively activates the group II metabotropic glutamate receptor subtype 3 (mGluR3), with about 15-fold more efficacy than mGluR2 [14, 15]. NAAG is inactivated by at least two peptidases, glutamate carboxypeptidase II and III (GCP II and GCP III). Elevation of NAAG levels through GCP II-mediated inhibition or direct administration has a neuroprotective effect in both *in vivo* and *in vitro* model systems [16-19].

An alternative strategy involves increasing NAAG levels following TBI by enhancing NAAG synthetase (NAAGS) expression. The enzyme(s) synthesizing NAAG have been well characterized. Expression of the NAAGS gene is sufficient to induce NAAG synthesis in primary astrocytes or CHO-K1 and HEK-293T cells [20]. However, no reports are available on methods of elevating NAAGS levels with transplanted NAAGS-activated NSCs (NAAGS/NSCs) to facilitate recovery of rat brain from TBI. In the present study, we evaluated the effects of transplanted NAAGS/NSCs on neurological function, nerve cell apoptosis, and inflammatory cytokine levels in a model of experimental TBI in rats.

Materials and Methods

Construction of a PcDNA3.1-NAAGS expression vector

The PcDNA3.1-NAAGS expression vector was generated as follows: the cDNA sequence encompassing the complete reading frame of NAAGS was obtained from rat brain tissue using polymerase chain reaction (PCR). The following primers were used for amplification: forward: 5'-AAA GTG GGC GAG TGG GTG AGG ACG-3', and reverse: 5'-AAT GAG AAA AAG AGG AAG GCA GGG-3'. Following digestion with *Hind*III and *Xho*I, cDNA was inserted into PcDNA3.1 to obtain the PcDNA-NAAGS expression vector.

Culture and establishment of stably transfected NAAGS/NSCs and control NSC lines

The methods of isolation and culture of NSCs were similar to those described previously [21]. Briefly, forebrain tissue of embryonic 14 day-old Sprague-Dawley rats (Experimental Animal Center of Fudan University, China) was harvested under a dissecting microscope. The shredded tissue was incubated in a tube with ~1 ml PBS, followed by aspiration. Next, 1 ml of Accutase (Sigma) was added and incubated for 10 min at room temperature. A single cell suspension was generated by gentle pipetting and washed once with culture medium. Subsequently, cells were plated into 10-cm cell dishes at a density of 0.8×10^5 cells/ml, and maintained at 37°C in a humidified atmosphere with 5% CO₂. Expansion medium (DMEM and F12,

1:1; L-glutamine, 2.92 g/100 ml; N'-a-hydroxythylpiperazine-N'-ethanesulfonic acid solution (HEPES), 5 mM; NaHCO₃, 7.5%; glucose, 0.915 g/100 ml; and heparin, 50 mg/100 ml; Sigma) contained N₂ supplement (1%; Invitrogen), epidermal growth factor (EGF, 10 ng/ml; Sigma), and basic fibroblast growth factor (bFGF, 10 ng/ml; Invitrogen). Sphere formation was observed after 4–7 days. NSCs cultured in suspension were detached every 5–7 days with Accutase. Neurospheres were expanded for an additional 3–10 weeks in suspension in standard medium containing DMEM (Gibco), 10% fetal bovine serum, 5% horse serum, 2 mM glutamine, and antibiotic [22].

Stable NSC line overexpressing NAAGS have been produced through retroviruses harboring NAAGS expression vectors (Hanbio, Shanghai, China) or mock as a control. After infection, cells were subjected to Puromycin for selection. Western blotting was carried out to verify overexpression of NAAGS. The stable cells were then used to transplant into TBI rats.

Preparation of cells for transplantation

On the day of transplantation, NAAGS/NSCs or naive NSCs were harvested, dissociated into a single cell suspension with Accutase, counted on a hemocytometer, and rewashed with 3 ml of phosphate buffered saline (PBS). Following centrifugation (400×g) for 5 min, the loose cell pellet was resuspended in sterile PBS at a concentration of 2×10^7 /ml. Cells were maintained on ice during surgery, and the remaining cells assayed for viability using trypan blue dye exclusion. An aliquot (25 µl) of the cell suspension was transplanted into each rat.

Surgical procedures and transplantation

Twenty-two adult male Sprague-Dawley rats (about 300 g, provided by the Animal Experiment Center, Shanghai Medical School, Fudan University, China) were divided into four groups (Normal group: no surgery and injection, Model group: surgery and injection with PBS, NSC group: surgery and injection with NSCs, NAAGS/NSC group: surgery and injection with NAAGS/NSCs). All animal experiments were approved by the Animal Research Committee at Tongji University, Shanghai. The details of surgery and brain injury tissue preparation are similar to those described previously [23, 24]. Experimental TBI was produced using a fluid percussion device (VCU Biomedical Engineering, USA). At 72 h after TBI, animals were re-anesthetized, and a 10 µl Hamilton syringe slowly advanced through the dura and cortex until the desired depth was reached. With controlled use of the Stoelting quintessential injector (Stoelting Co., USA), the cell suspension was injected over a period of 3 min (1 µl/min). The needle was left in place for an additional 5 min and withdrawn slowly. No immunosuppressants were used in any of the animals subjected to cell transplantation. At 1, 2, and 4 weeks, animals were administered a lethal dose of sodium pentobarbital (200 mg/kg intraperitoneally), transcardially perfused with cold PBS, and prepared for western blot analysis.

Western blot analysis

Three rats per group were sacrificed at each time-point. Brain tissue encompassing the transplant site was obtained immediately and dissected into two parts on a bed of ice along the center of the injury cavity for western blot analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot with chemiluminescence detection were performed as described previously [25]. Blots were incubated overnight at 4°C with mouse polyclonal anti-NAAGS (1:1000; Sigma) or anti-mGluR3, anti-nestin, anti-NeuN, anti-GFAP (1:1000; Abcam) antibody. Following primary antibody incubation, blots were washed four times with TBST (10 mM Tris, 150 mM NaCl, pH 7.4, with 0.1% Tween-20), incubated for 1 h with horseradish peroxidase (HRP)-linked secondary antibodies, washed as above, and developed using the enhanced chemiluminescence western blotting detection kit (ECL Plus).

Quantitative real-time PCR (qPCR) analysis

Total RNA was isolated from cells with a purified RNA Extraction kit (BioTeke, China). Single-stranded cDNA synthesis was performed using the Superscript III qPCR RT Kit (Invitrogen). qPCR was performed using SybrGreenERTM qPCR SuperMix Universal (Invitrogen) on a Rotor-Gene 6000 (Corbett Life Science). The DeltaCT method was employed for analysis, and data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primer pairs were obtained from Invitrogen, and are specified below: NAAGS Forward (5'-3'): AGA TCT TAC GAG CGT TGA AGG CCA, Reverse (5'-3'): TCC GAA GAC CCA GAT TTC CTT GCT; mGluR3 Forward (5'-3'): TAG GCT GTT AGA CAA AGT GCT CA, Reverse (5'-3'): GAA GGG GCT GTT AAT TAG

GGC A; GAPDH Forward (5'-3'), TGT GAT GGG TGT GAA CCA CGA GA, Reverse (5'-3'): GAG CCC TTC CAC AAT GCC AAA GTT.

Behavioral testing

Rats were placed on a plate, and the Neurological Severity Scores (NSS) were recorded. All rats were trained prior to surgery, and reached a stable baseline level of performance within 3 days. NSS were measured at 1 day after injury and 7, 14, and 21 days post transplantation (n=6 in each group at different time-points). The standards of NSS have been described previously [26]. Details are presented in Table 1. Higher scores represent more severe injury.

The rotarod test (Columbus Instruments, Columbus, OH) was performed to further assess sensorimotor coordination and balance. Rats were pretrained over two consecutive days on the rotarod consisting of a rotating spindle with 8.5 cm in width and 7 cm in diameter, and had to continuously walk forward to avoid falling. The spindle was gradually accelerated from 0 to 30 rpm over the course of 1 min and continued at 30 rpm for an additional 2 min. Rats were given as many trials as required to reach this performance criterion, after which they were returned to their home cage until testing the following day. Rats that did not reach this criterion were eliminated from the present study. The rotarod test was conducted at 1 day after injury and 7, 14, and 21 days post transplantation with 3 trials for each rat, and the average latency to fall was used as the result.

NAAG level analysis

1.5 h prior to TBI, the microdialysis probe was inserted into the striatum region through stereotaxic apparatus (Narishige SN-2; BAS Co., Japanese), positioned at the following coordinates: anterior-posterior -0.2 mm; lateral 4.0 mm; vertical -4.5 mm. The probe was removed 1 min prior to TBI and replaced immediately after TBI. Then continuous perfusion was operated with Ringer's solution (140 mM NaCl, 1.0 mM MgCl₂, 1.2 mM CaCl₂, 5.0 mM NaHCO₃, pH 7.4) at a constant flow rate of 1.0 µl/min prior to injection. 90 min after perfusion when it reached a balance in brain, dialysis sample aliquots were collected from the TBI area before and after transplantation at the required time-points.

NAAG levels were analyzed with radioimmunoassay using protein A-agarose (Roche Applied Science, USA) [23]. Briefly, NAAG antiserum was produced in rabbits by repeated immunizations with NAAG coupled to colloidal gold, and the specificity was detected by the combination rate of anti-sera in different dilution and [³H]NAAG. Then protein A-agarose suspensions were sedimented, washed twice with PBS, resuspended in NAAG antiserum, and incubated for 4 h on a rocking platform at 4°C. The PBS wash was repeated as above, and pellets collected for resuspension with [³H]NAAG and sampled. The mixture was incubated overnight at 4°C with rotation. After sedimentation, [³H]-bound radioactivities of samples were detected via liquid scintillation and normalized to standard NAAG.

Immunostaining

Brain tissue encompassing the transplant site was obtained 24 h after transplantation, and immunofluorescence for detection of NAAGS was performed on paraffin sections of rat brain samples. Slides were incubated with polyclonal mouse anti-NAAGS (1:1000; Sigma) followed by incubation with FITC-conjugated secondary antibody IgG (1:200; Invitrogen), and then mounted with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories). Images were obtained under confocal microscope (Olympus). To observe the neuron cells and astroglia in brain following transplantation, slides were incubated with mouse anti-NeuN or anti-GFAP antibodies (1:100; Chemicon). NeuN-labeled or GFAP-labeled sections were also incubated in Alex Fluor 488 or Alex Fluor 647 (1:200, Chemicon) and examined under a fluorescence microscope (Olympus). To quantify the images, NeuN-positive or GFAP-positive cells were counted using fluorescence microscope in five separate fields.

At 1, 7, 14 and 21 days after transplantation, five animals from each group were killed. Brain tissues around transplantation sites with a depth of about 2 mm were removed and postfixed in 4% paraformaldehyde for 24 h. Cell apoptosis was detected via TUNEL staining (Millipore). To quantify the rate of apoptosis, TUNEL-positive cells were counted using standard light microscopy (Zeiss, Axioskop 40) on paraffin sections in five separate fields.

Fig. 1. NAAGS is highly expressed in stable NAAGS/NSC cells. (A) Expression profiles of NSCs and NAAGS/NSCs harvested at week 10 after culture were determined using western blot. (B) PcDNA3.1-NAAGS was digested with *Hind*III and *Xho*I, and the products run on a 1% agarose gel. 1: marker; 2: NAAGS. (C) NAAGS protein expression was determined using western blot in normal NSC and NAAGS/NSC groups at different time-points, using GAPDH as an endogenous control. (D) qPCR shows mRNA levels of mGluR3 in normal NSC and NAAGS/NSC groups. Values were normalized with internal control GAPDH. Data are expressed as means $\pm$ SD (n=3). (E) mGluR3 protein expression was determined using western blot in normal NSC and NAAGS/NSC groups, using GAPDH as an endogenous control. * $p < 0.05$.

Brain interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α detection

Transplanted NSCs may participate in the production of several proinflammatory cytokines, including IL-1 β , IL-6, and TNF- α . For detection of cytokine content, five animals from each group were killed at 24 h after transplantation. IL-1 β , IL-6, and TNF- α levels were detected with enzyme-linked immunosorbent assay (ELISA) using commercial kits (R&D), and all protocols were followed according to the manufacturer's instructions.

Glutamate measurement

Cerebrospinal fluid (CSF) dialysates were collected from rats at different time-points. Glutamate was measured by High Performance Liquid Chromatography (HPLC).

Statistical analysis

Differences between groups were analyzed with the Student's *t*-test using SAS statistical software. A *p*-value of less than 0.05 was considered statistically significant.

Results

Overexpression of NAAGS in NAAGS/NSCs *in vitro*

To determine the expression profiles of NSCs, the NSC-specific marker nestin, neuron-specific marker NeuN, and astroglia-specific marker GFAP were studied. As shown in Fig. 1A, NSCs expressed higher levels of nestin and decreased levels of NeuN and GFAP, indicating that NSCs sustained the stemness *in vitro*. To examine whether NAAGS-modified NSCs can be utilized in autoplasmic therapy for the injured nervous system, we initially constructed a

Fig. 2. Sustained expression of NAAGS mRNA and protein in brain at different time-points post-transplantation. (A) qPCR shows a significant increase in NAAGS gene expression in NAAGS/NSC-transplanted rat brains, compared to that in NSC-transplanted rats at 1 and 2 weeks post-transplantation. Values were normalized with internal control GAPDH. (B) Western blots disclose higher expression of NAAGS in NAAGS/NSC-transplanted rats, compared to NSCs-transplanted rats at 1, 2 and 4 weeks post-transplantation. GAPDH served as an internal control. Data are expressed as means $\pm$ SD ($n=6$ in each group). (C) Immunofluorescent detection of NAAGS after transplantation. NAAGS immunoreactivity is shown in green. Sections were stained with DAPI (blue) to show all nuclei. The scale bar is 50 μ m. * $p<0.05$.

PcDNA3.1-NAAGS expression plasmid. Following double digestion with *Hind*III and *Xho*I, a 4 kb fragment was obtained (Fig. 1B). NAAGS in the newly constructed plasmid was confirmed by sequencing (ABI PRISM310, USA). After obtaining stable transfectants, NAAGS protein expression was detected in the NSC control and NAAGS/NSC groups using western blot analysis. As shown in Fig. 1C, the NAAGS protein was constantly expressed in the NAAGS/NSCs 2 weeks after transfection, but not the NSC group. Notably, NAAGS/NSCs also showed elevated nestin expression and downregulated NeuN and GFAP levels, the expression of which was similar with NSCs control (Fig. 1A), suggesting that overexpression of NAAGS did not change their expression profiles. In addition, the expression of mGluR3 was also elevated in NAAGS/NSCs at both mRNA and protein levels as compared to NSCs (Fig. 1D and E), further confirming the successful overexpression of NAAGS in NSCs.

Sustained expression of NAAGS post-transplantation following TBI

To ensure that NAAGS/NSCs were successfully transplanted into the TBI area, expression of NAAGS was examined at the mRNA and protein levels with qPCR and western blot analysis, respectively. Five rats per group were sacrificed for analysis at 1, 2 and 4 weeks post-transplantation. Notably, at these post-transplantation time-points, NAAGS mRNA expression in the NAAGS/NSC group was significantly higher than that in the control group (Fig. 2A). Western blot analysis further disclosed high expression of NAAGS protein in the NAAGS/NSC group, compared to the control group (Fig. 2B). Immunofluorescence for detection of NAAGS also showed high expression level of NAAGS in NAAGS/NSC group (Fig. 2C).

Table 1. Neurological Severity Scores (NSS) Points

Motor tests	
Raising rat by the tail	3
1 Flexion of forelimb	1
1 Flexion of hindlimb	1
1 Head moved.10° to vertical axis within 30 s	1
Placing rat on the floor (normal50; maximum53)	3
0 Normal walk	0
1 Inability to walk straight	1
2 Circling toward the paretic side	2
3 Fall down to the paretic side	3
Sensory test	2
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles)	2
Beam balance tests (normal50; maximum56)	6
Balances with steady posture	0
1 Grasps side of beam	1
Hugs the beam and one limb falls down from the beam	2
Hugs the beam and two limbs fall down from the beam, or spins on beam (.60 s)	3
Attempts to balance on the beam but falls off (.40 s)	4
Attempts to balance on the beam but falls off (.20 s)	5
Falls off: No attempt to balance or hang on to the beam (.20 s)	6
Reflexes absent and abnormal movements	4
Pinna reflex (head shake when touching the auditory meatus)	1
1 Corneal reflex (eye blink when lightly touching the cornea with cotton)	1
1 Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
1 Seizures, myoclonus, myodystony	1
Maximum points	18

Fig. 3. Behavioral functional tests in each group at different time-points. (A) Six animals from each group were tested according to the NSS system (Table 1) at 1, 7, 14 and 21 days post-TBI. (B) Rotarod test was performed to assess sensorimotor coordination and balance of rats in different groups (n=6). Data are presented as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

Functional recovery of motor behavior mediated by NAAGS/NSC and NSC transplantation

Six rats per group were selected for the NSS test at 1, 7, 14 and 21 days after TBI. All injury groups displayed significant motor impairment at 1 day following TBI. The test system was performed according to a previously described procedure (Ref. Table 1). Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). In terms of severity scores of injury, a score of 1 point was awarded for inability to perform the test or lack of tested reflexes. Thus, higher scores represent more severe injury. Transplantation of both NAAGS/NSCs and NSCs after TBI led to a decrease in the NSS index, compared to the model group (Fig. 3A), indicating that NSC transplantation is beneficial for brain recovery after TBI. At 14 and 21 days, the NSS index in the NAAGS/NSC group was significantly lower than that of the NSC group ($p < 0.01$).

Fig. 4. Detection of dialysate NAAG and glutamate levels. The dialysis sampling was performed after transplantation of NSCs or NAAGS/NSCs. (A) Dialysate NAAG levels in the first post-TBI sample increase ~15-fold in the control group, compared with pre-transplantation baseline values (0 h). In contrast, dialysate NAAG levels increase ~25-fold in the NSC group and ~35-fold in the NAAGS/NSC group, compared with baseline. Differences between groups are significant during post-transplantation periods, starting at 0.5 h. Dialysate NAAG levels in NAAGS/NSC group are significantly higher than those in the NSC group, while NAAG levels in the control and NSC groups return to near baseline by 72 h post-transplantation. Data are presented as means $\pm$ SD. (B) Log-logit transformation of special radiomunoassay standard curve of NAAG. The NAAG standard curve ranged from 0.25 to 25 pM. (C) Glutamate release in CSF dialysates was measured by HPLC. The percentage of NSC or NAAGS/NSC group to control were shown. Data are presented as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

To further assess sensorimotor coordination and balance, the rotarod test was performed. As shown in Fig. 3B, transplantation of both NAAGS/NSCs and NSCs after TBI had a significantly longer latency to fall, compared to the model group. At 14 and 21 days, the latency to fall in the NAAGS/NSC group was significantly longer than that of the NSC group, clearly suggesting that NAAGS/NSCs promote recovery from injury more effectively.

Dialysate NAAG levels in the TBI area

Six rats per group were selected for analysis of dialysate NAAG levels at 0.5, 1.0, 1.5 h, and 24, 48, 72 h after transplantation. As shown in Fig. 4A and B, data analysis revealed a significant effect of transplanted NAAGS/NSCs or NSCs, compared to the control group ($p < 0.05$). Baseline NAAG levels in the control group did not differ from those in the NAAGS/NSC and NSC groups. NAAG levels in 0.5 h post-transplantation dialysate samples of the control, NSC and NAAGS/NSC groups increased ~15-, ~25- and ~35-fold, respectively, relative to pre-transplantation baseline values. The differences between groups were significant during post-transplantation sample periods, starting at 0.5 h ($p < 0.01$). Dialysate NAAG

Fig. 5. Transplantation of NAAGS/NSCs suppresses brain cell death. (A) Images of NSCs from each group acquired by TUNEL on a microscopy reader (A: normal group; B: model group; C: NSC group; D: NAAGS/NSCs group). (B) Quantification of apoptotic cells via TUNEL staining. At 1, 7, 14 and 21 days after transplantation, five animals from each group were killed. The number of apoptotic cells, which were related to the brain tissues around transplantation sites, was detected using TUNEL, and TUNEL-positive cells on paraffin sections in five separate fields were counted using standard light microscopy. Data are presented as means $\pm$ SD. (C) Neuron cells and astroglia in brain following transplantation were detected by immunofluorescence labeling. Expression of NeuN or GFAP was shown in green or red. The scale bar is 50 μ m. (D) Quantitative analyses are used to describe NeuN-positive and GFAP-positive cells. Five separate fields were counted and data are presented as means $\pm$ SD. * $p < 0.05$.

levels in the NAAGS/NSC group were markedly higher than those in NSC group ($p < 0.01$). Dialysate NAAG levels in both NSC and NAAGS/NSC groups remained elevated (~15 to 30-fold) within 0.5–1.5 h post-transplantation. In contrast, NAAG levels in the control and NSC groups returned to near baseline levels in 72 h post-transplantation. Thus, transplantation of NAAGS/NSCs appears significantly associated with a more robust increase in dialysate NAAG levels, compared to that of NSCs. In addition, the glutamate release in NAAGS/NSC-treated CSF was significantly decreased as compared with NSC group (Fig. 4C) at each time-point after transplantation, suggesting that NAAGS/NSC transplantation sustained the endogenous glutamate levels.

Transplantation of NSCs and NAAGS/NSCs after TBI inhibits brain cell apoptosis

Six rats per group were sacrificed for immunohistochemistry analysis. In uninjured control rats, almost no apoptosis in brain tissue was detected (Fig. 5A). Conversely, significantly higher levels of apoptotic cells were observed in TBI animals, indicating that the injury induces considerable cell death accounting for neurological diseases. As shown in Fig. 5A, images of cell death were detected with the TUNEL method. Transplantation of NSCs

Fig. 6. Brain IL-1 β , IL-6, and TNF- α levels from each group. (A-C) Levels of IL-1 β , IL-6, and TNF- α from each group were detected by ELISA. Data are presented as means $\pm$ SD. * $p < 0.05$.

decreased cell death, compared to that observed in the model group, which was consistent with previous findings [1, 27, 28]. Notably, transplantation of NAAGS/NSCs suppressed cell death more significantly at 7 and 14 days after TBI, compared to NSC transplantation (Fig. 5B). Immunolabeling of NeuN and GFAP showed that NAAGS/NSC group has more neuron cells and astroglia than NSC group, which confirmed that transplantation of NAAGS/NSCs decreased cell death and promoted tissue repair (Fig. 5C and D).

Effects of transplantation of NAAGS/NSCs on proinflammatory cytokines

Transplantation of NAAGS/NSCs led to a significant decrease in the IL-1 β level in brain, while NSCs had no significant effect, as shown in Fig. 6. However, transplantation of both NAAGS/NSCs and NSCs markedly inhibited the levels of IL-6 and TNF- α , compared to the model group.

Discussion

In the current study, we successfully established a NSC line expressing high levels of NAAGS (Fig. 1). To our knowledge, this study demonstrates for the first time that transplantation of NAAGS/NSCs following experimental TBI significantly attenuates impairment of neurological functions.

Expression of the NAAGS protein was sustained around the trauma area for at least 4 weeks post-transplantation (Fig. 2). Clearly, NAAGS/NSC transplantation improves neurological motor function in selected behavioral tests more significantly than naive NSC transplantation after experimental TBI.

The NAAG level in the trauma area was elevated in the NAAGS/NSC transplantation group by 40%, compared to the NSC group (Fig. 4). Moreover, elevated NAAG protein levels following transplantation are consistent with the previous finding where increased NAAGS enhances the NAAG level in the TBI area, in turn, potentially decreasing the glutamine concentration.

Glutamate excitotoxicity is a significant determinant of TBI pathophysiology. Numerous laboratory [7, 9] and clinical [29-31] studies have documented excessive glutamate release following TBI. Experimental cerebral ischemia is also associated with a significant and rapid increase in extracellular glutamate [32-34].

Excess glutamate release has been implicated in these models of human pathological conditions. These findings support the theory that NAAG peptidase is a significant therapeutic target. A strategy involving inhibition of NAAG peptidase and subsequent NAAG hydrolysis may afford protection of the brain from TBI in several ways. Firstly, inhibition of NAAG peptidase activity, resulting in increased endogenous NAAG, may provide neuroprotection through activation of group II metabotropic receptors and subsequent reduction in glutamate release [17]. Secondly, blockade of NAAG peptidases may exert a neuroprotective effect by reducing a secondary source of glutamate as a by-product of NAAG hydrolysis [35]. Thus, NAAG peptidase inhibition offers multifold potential neuroprotection against glutamate excitotoxicity, and subsequently reduces both astrocyte and neuronal damage under pathological conditions. This strategy represents a novel means of reducing damaging glutamate release associated with TBI by augmenting the brain's natural endogenous system of controlling glutamate release via presynaptic feedback on mGluR3 by the NAAG peptide.

An alternative strategy involves augmentation of NAAG levels following TBI via overexpression of NAAGS. Expression of the NAAGS gene encoding NAAGS was sufficient to induce NAAG synthesis in primary astrocytes or CHO-K1 and HEK-293T cells [20]. Our results showed for the first time that transplantation of NSCs genetically modified to encode the NAAGS gene provides a better therapeutic effect than naive NSCs in a rat model of TBI. Moreover, elevated NAAG was observed in the trauma area.

The mechanisms underlying promotion of recovery upon NSC transplantation after TBI are currently unknown. TBI leads to cell death via release of glutamate and a combination of apoptosis and necrosis over a 24 h interval following injury. Administration of NAAG has been reported to improve striatal neuroprotection in the endothlin-1 rat model [16]. In the current study, transplantation of both NSCs and NAAGS/NSCs inhibited cell apoptosis in the TBI model. Notably, the NAAGS/NSC group promoted inhibition more significantly at 7 and 14 days after injury, compared to the NSC group. Inhibition of cell apoptosis may thus account for better recovery in a TBI brain rat model.

It is well-known that after TBI inflammatory processes occur, with proinflammatory cytokines released (Fig. 6) and immune cell activated. These early responses may be as deleterious to transplanted stem cells [36]. Molcanyi et al. demonstrated that in a model of rat TBI, transplanted embryonic stem cells could be phagocytized by inflammation-activated macrophages 7 weeks after transplantation [37]. Therefore, transplanted cells may gradually lose their function and process apoptosis mediated via phagocytosis by macrophages in inflammatory environment [38]. In the present study, although we could not fully exclude the possibility that activated macrophage might uptake transplanted NSCs and NAAG might come from other types of cells, the therapeutic effects of NAAGS/NSCs were beneficial. Transplanted NAAGS/NSCs significantly inhibited the secretion of proinflammatory cytokines as compared with transplanted NSCs in the early stage after transplantation (Fig. 6), which might subsequently inhibit the activation of macrophages. This may partly explain why transplanted NAAGS/NSCs were not uptaken by macrophages and NAAG was really synthesized from the grafts.

Neural transplantation is potentially useful as a therapeutic intervention in several CNS diseases, including Parkinson's disease, Huntington's disease, ischemic brain injury, and spinal cord injury [27, 39-41]. The most important finding of this study is that NAAGS-activated NSCs delivered to TBI tissue through an intravenous route provide more significant

therapeutic benefits. Our data support the utility of NAAGS/NSC transplantation as an effective therapy to improve neurological outcomes after TBI. However, further studies on this novel neuroprotective strategy are warranted. It has been reported that transplanted NSCs in mammalian brain following TBI have the potential to generate the differentiated cell types of the central nervous system after prolonged periods following transplantation [42]. Shindo et al. demonstrated that surviving transplanted NSCs could be identified and mainly differentiated into β -III-tubulin⁺ neurons at 12 weeks after TBI, but GFAP⁺ astrocytes were barely found within the grafts [43]. In contrast, Sun et al. reported that transplanted NSCs could migrate from the injection site into surrounding area, with the majority of which differentiating into astrocytes or oligodendrocytes, and very few cells located in the cortical region expressing neuronal marker [44]. The discrepancy between these studies may result from different sites for cell deposition, different stem cell sources or different injury regimens. In the present study, we found that transplanted NAAGS/NSCs effectively promoted tissue repair early after transplantation (Fig. 5C and D), clearly suggesting that NAAGS was protective for TBI. However, it remains unknown whether NAAGS also plays a critical role in differentiated cell types derived from NAAGS/NSCs, or whether other neuroprotective transmitters are involved, such as GABA, serotonin, norepinephrine, dopamine, and acetylcholine as described previously [13]. This is quite important and we will further study it in the future.

Our findings are consistent with previous brain injury studies reporting recovery of function following cell transplantation [1, 22, 28, 45]. In our experiments, injection of NAAGS/NSCs after TBI decreased the NSS index to a greater extent, compared to NSCs, suggesting that NAAGS/NSCs provide higher recovery from TBI in rats.

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