Repair of glutamate-induced excitotoxic neuronal damage mediated by intracerebroventricular transplantation of neural stem cells in adult mice

Juan MA, Li-Jian YU, Run-Di MA, Yong-Ping ZHANG, Juan-Zhi FANG, Xiao-Yu ZHANG, Ting-Xi YU

Key Laboratory of Marine Materia Medica, Guangdong Ocean University, Zhanjiang 524025, China

Abstract: Objective To investigate a possibility of repairing damaged brain by intracerebroventricular transplantation of neural stem cells (NSCs) in the adult mice subjected to glutamate-induced excitotoxic injury. **Methods** Mouse NSCs were isolated from the brains of embryos at 15-day postcoitum (dpc). The expression of nestin, a special antigen for NSC, was detected by immunocytochemistry. Immunofluorescence staining was carried out to observe the survival and location of transplanted NSCs. The animals in the MSG+NSCs group received intracerebroventricular transplantation of NSCs (approximately 1.0×10^5 cells) separately on day 1 and day 10 after 10-d MSG exposure (4.0 g/kg per day). The mice in control and MSG groups received intracerebroventricular injection of Dulbecco's minimum essential medium (DMEM) instead of NSCs. On day 11 after the last NSC transplantation, the test of Y-maze discrimination learning was performed, and then the histopathology of the animal brains was studied to analyze the MSG-induced functional and morphological changes of brain and the effects of intracerebroventricular transplantation of NSCs on the brain repair. **Results** The isolated cells were Nestin-positive. The grafted NSCs in the host brain were region-specifically survived at 10-d post-transplantation. Intracerebroventricular transplantation of NSCs obviously facilitated the brain recovery from glutamate-induced behavioral disturbances and histopathological impairs in adult mice. **Conclusion** Intracerebroventricular transplantation of NSCs may be feasible in repairing diseased or damaged brain tissue.

Keywords: brain repair; neural stem cells; transplantation; excitotoxic injury; mice

1 Introduction

Excitotoxicity, a pathological process by which neural cells are damaged by overstimulation of glutamate membrane receptors due to excessive exposure to the neurotransmitter glutamate, has been implicated as one of key factors contributing to neuronal injury and death in both acute and chronic neurodegenerative disorders. Neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's chorea, HIV-associated dementia, multiple sclerosis, amyotrophic lateral sclerosis and glaucoma, are caused by different mechanisms but may share a final common pathway to neuronal injury, i.e.

overstimulation of glutamate receptors, especially the *N*-methyl-D-aspartate (NMDA) subtype^[1]. Therefore, antagonizing excitotoxicity is a particularly attractive target for the purpose of neuroprotection or brain repair. Our previous studies demonstrated that monosodium glutamate (MSG) could cross placental barrier and distribute to embryonic tissues. Maternal intragastric (i.g.) administration of excessive MSG at a late stage of pregnancy resulted in a series of behavioral disturbance and histopathological brain lesions in the filial mice^[2,3]. Recently, we successfully established an experimental model of excitotoxic brain injury following glutamate exposure, in which i.g. administration of excessive MSG would result in a series of behavioral disorders and obvious histopathological brain lesion in adult mice.

Neural stem cells (NSCs) are currently being investigated for their potential use in cell replacement strategies for diseased or damaged central nervous system (CNS)^[4,5]. Cells for transplantation must be nontransformed, well characterized, able to migrate toward sites of diseased or

Corresponding author: Li-Jian YU; Ting-Xi YU
Tel: 86-759-2382041
Fax: 86-759-2382424
E-mail: ywyj9578@sohu.com; yutingxi@yahoo.com
Article ID: 1673-7067(2007)04-0209-06
CLC number: R741.05;
Document code: A
Received date: 2006-12-21

damaged CNS tissues, and able to differentiate into the appropriate cell types. Moreover, they must survive in the diseased or damaged CNS and integrate into the neural network after transplantation and thus to functionally improve the clinical outcome of patients. Cultures containing human neural stem and progenitor cells (neurospheres) have the capacity to proliferate and differentiate into the major phenotypes of the adult brain. These properties make them be candidates for therapeutic transplantation in those neurological diseases due to cell loss. Expandable human neural stem/progenitor cells can survive transplantation, and migrate, differentiate, and proliferate in the injured brain^[5,6]. Therefore, stem cell transplantation through cell replacement or as vector for gene delivery is a potential strategy for treatment of neurodegenerative diseases^[4]. In this study, we used an experimental model of excitotoxic brain injury following glutamate exposure to examine the effects of intracerebroventricular transplantation of NSCs^[7,8] on the repair of excitotoxic brain injury in adult mice.

2 Materials and methods

2.1 Chemicals MSG was purchased from Shanghai Bio Life Science and Technology Co., Ltd. (China). DMEM was from Gibco Co.; Fetal bovine serum was from Sijiqing Organism Engineering Materials Co. Ltd (Hangzhou, China); Rabbit anti-mouse nestin IgG and Goat anti-rabbit IgG-FITC were from Boster Biological Engineering Co. Ltd (Wuhan, China); and Hoechst 33342 was from Sigma (USA).

2.2 Animal Kunming (KM) mice of 8-week-old were purchased from the Experimental Animal Center of Guangdong Medical College, Zhanjiang, China. The mice accessed to standard mouse food and chlorinated water *ad libitum* and were kept on a reversed 12/12 h light-dark cycle at room temperature of (22±1)°C. All the animals were treated in compliance with relevant animal welfare policies.

2.3 Isolation of NSCs One male mouse and two female mice were housed in each cage. The pregnant females were separated. The embryos at 15-day postcoitum (dpc) were dissected from the uteri after the mice were sacrificed humanely. Intact brains were removed from the embryos by aseptic techniques and transferred to a 35-mm plate containing cold D-Hanks solution and rinsed twice. The meninges and blood capillaries were carefully removed, and the tissues were placed in a sterile plate containing D-Hanks solution. All the operations above were performed on ice. When dissections were completed, the tissue suspension

was transferred into a sterile centrifugation tube. Tissue suspension was allowed settle and removal of the supernatant and then resuspended in 3 mL DMEM. The tissues were gently triturated by using a fire-polished Pasteur pipette until a fine single-cell suspension was obtained. The suspension was filtered by a 250-mesh aperture sieve to get a better unicellular suspension. The cell density was adjusted to 1.0×10^5 cells/mL by addition of DMEM.

2.4 Identification of NSCs Cells were dissociated by gentle trituration with a fire-polished Pasteur pipette. The suspension was centrifuged at 800 r/min for 5 min and the supernatant was removed. Re-suspend the cells in the DMEM medium containing 15% fetal bovine serum until reaching a cell density of 5×10^5 cells/mL. The cell suspension was incubated on a poly-L-lysine-coated glass slide for 24 h, then fixed with 4 % polyoxymethylene solution for 30 min at room temperature. The expression of the special antigen nestin was detected by immunocytochemical technique, with the primary anti-nestin antibody diluted to 1:100 and the secondary IgG-FITC antibody diluted to 1:50. Finally the immunoreaction was observed under a DMIRB fluorescence microscope (Leica, Germany) and photographs were taken.

2.5 Labeling cells with Hoechst 33342 Cells were gently resuspended in DMEM medium containing 10 µg/L of Hoechst 33342, then the unicellular suspension was transferred into a 50-mL culture flask and cultured at 37 °C for 30 min in a humidified incubator containing 5 % CO₂. Cells were rinsed with PBS for 3 times before 1 mL DMEM was added. The volume was precisely measured and the number of the living cells was counted by using dilution in trypan blue and hemacytometer. The cell density was adjusted to 1.0×10^8 cells/mL.

2.6 Immunofluorescence staining On day 1 and day 10 after MSG treatment, the animals in MSG+NSCs group were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and each animal received an intracerebroventricular transplantation of 10 µL of Hoechst 33342-labeled cells (1.0×10^7 cells/mL), approximately 1.0×10^5 cells, in 5 min. A 5-min waiting interval allowed grafted NSCs to settle before the needle was removed. On day 11 after the last transplantation, animals were terminally anesthetized with 10% chloral hydrate (3.5 mL/100 g), and then perfused intracardially with 0.9% saline followed by paraformaldehyde (4% in PBS). The animals were sacrificed and the brains were dissected. Series of 10-µm thick serial coronal sections were cut on a cry-

ostat microtome. The label immunofluorescence staining was carried out on the coronal sections adjacent to those used for immunohistochemistry. After rinsing with PBS, sections were blocked with 0.1% BSA for 30 min and incubated with rabbit anti-nestin IgG (1:50, diluted in PBS with 0.5% Triton X-100) for 2 h at room temperature, and then rinsed with PBS again. Subsequently, sections were incubated in fluorescence-labeled secondary antibody FITC-IgG (1:50) for 30 min followed by PBS washes. Fluorescence-labeled sections were viewed under the confocal microscope and Fluorescence staining was observed under the DMIRB fluorescence microscope.

2.7 Protocols of animal tests On day 1 and day 10 after the 10-d MSG exposure (4.0 g/kg per day, i.g.), the animals in MSG+NSCs group received intracerebroventricular transplantation of 10 μ L of NSCs suspension, approximately 1.0×10^5 cells once. The control mice without MSG exposure and the mice in MSG groups were intracerebroventricularly administered with 10 μ L of DMEM instead of NSCs. The test of Y-maze discrimination learning was performed on day 11 after the last NSC transplantation, and then the histopathology of the animal brains was studied to analyze the MSG-induced functional and morphological changes and the effects of intracerebroventricular transplantation of NSCs on the repair of MSG-induced excitotoxic brain injury [3,9]. The experiments were repeated three times.

2.8 Test of Y-maze discrimination learning Test of Y-maze discrimination learning was carried out according to Xu's method [10]. Test of Y-maze discrimination learning started on day 11 after the last NSC transplantation and performed for 6 consecutive days.

2.9 Examination of histopathology The histopathology changes of the animal brains were examined for two times: on day 1 after the treatment of MSG and after the completion of the behavior tests. The mice were anaesthetized intraperitoneal (i.p.) with sodium pentobarbital (60 mg/kg), and sacrificed by perfusion with the fixation 10% formalin. The whole brain was excised carefully, and was further fixed in 10% formalin for a week. The hippocampal region of each animal was sectioned at 10- μ m and stained with hematoxylin and eosin. The sections across the hippocampus region were examined by light microscopy.

2.10 Statistical analyses The repeated-measures analysis with a compound symmetry variance-covariance structure model was used to analyze the data from Y-maze discrimination learning test. $P < 0.05$ was considered statistically significant.

cally significant.

3 Results

3.1 Identification of NSCs Nestin is a specific marker for NSC. We examined the expression of nestin in the isolated cells by using immunocytochemical technique. The results showed that the cells had been stained green, indicating that the cells were nestin-positive (Fig. 1).

Fig. 1 Identification of neural stem cells by immunocytochemistry. A: Nestin-positive cells. B: Proliferation of the nestin-positive cells. Scale bar, 20 μ m.

Fig. 2 Survival and location of NSCs in adult mouse brains 10 d after transplantation. A: Hoechst 33342-labeled nestin-positive NSCs in the cortex. B: Nestin-positive NSCs in the cortex. C: Hoechst 33342-labeled NSCs in the hippocampus. D: Nestin-positive NSCs in the hippocampus. Scale bar, 50 μ m.

Fig. 3 Effects of intracerebroventricular transplantation of NSCs on MSG-induced lesion of Y-maze discrimination learning capacities. Eleven to thirteen animals were used in each group. The animals in the MSG+NSCs groups received intracerebroventricular transplantation of 10 μ L of NSCs suspension (approximately 1.0×10^5 cells) separately on day 1 and day 10 after the 10-d of MSG exposure (4.0 g/kg per day, i.g.). The mice in control and MSG groups received intracerebroventricular injection of 10 μ L of DMEM instead of NSCs. On day 11 after the last NSC transplantation, the test of Y-maze discrimination learning was started to analyze the MSG-induced functional changes and the effects of intracerebroventricular transplantation of NSCs on the recovery of MSG-induced excitotoxic lesion of brain function. Vertical error bars represent SEM. The repeated-measures analysis with an unstructured variance-covariance structure model was used to analyze the data from Y-maze discrimination learning test.

3.2 Immunofluorescence staining Transplanted NSCs were detected in the mouse brains at 10-d post-transplantation. Most survival cells remained near the injection track but some migrated. In the transplantation (MSG+NSCs) group, the NSCs could be detected in all mouse brains. The Hoechst 33342-labeled cells and nestin-positive cells distributed near the injection location and migrated along, but the region-specific survival and location

of the cells could be observed (Fig. 2).

3.3 Effects of intracerebroventricular transplantation of NSCs on MSG-induced lesion by measuring Y-maze discrimination learning capacities As shown in Fig. 3, the memory retention and Y-maze discrimination learning capacities of the mice in MSG group were significantly less than those in control ($P = 0.0006$) and MSG+NSCs ($P = 0.0008$) groups, but the latter two seemed comparable ($P =$

Fig. 4 Histopathological examination of hippocampal injury following exposure to glutamate in adult mice. The histopathology of the animal hippocampi was studied on day 1 after 10-d of MSG (1.0, 2.0, 4.0 g/kg per day) treatment. A: Control; B: MSG (1.0 g/kg per day for 10 d); C: MSG (2.0 g/kg per day for 10 d); D: MSG (4.0 g/kg per day for 10 d). Arrows a, degeneration and necrosis of neurons; b, intracellular edema. Scale bar, 50 μ m.

Fig. 5 Effect of intracerebroventricular transplantation of NSCs on MSG-induced histological lesions. After the completion of Y-maze test, the histopathology of the animal hippocampi was studied to analyze the MSG-induced morphological changes and the effect of intracerebroventricular transplantation of NSCs on the repair of MSG-induced excitotoxic brain injury. Sections through hippocampal regions of the mice treated with MSG or MSG+NSCs showing morphological effects of MSG and brain repair following MSG-induced excitotoxic neuronal injury mediated by intracerebroventricular transplantation of NSCs in adult mice. A: Control; B: MSG (4.0 g/kg per day for 10 d); C: MSG (4.0 g/kg per day for 10 d) + NSCs. Arrow a, degeneration and necrosis of neurons; b, hyperplasia. Scale bar, 50 μ m.

0.7120). The results suggest that intracerebroventricular transplantation of NSCs obviously facilitates the recovery of brain function from glutamate-induced excitotoxic lesion in adult mice.

3.4 Effects of intracerebroventricular transplantation of NSCs on MSG-induced histological lesions MSG-induced hippocampal lesions were characterized by intracellular edema, degeneration and necrosis of neurons, and hyperplasia (Fig. 4, 5B). However, no significant neuronal damage was detected in the hippocampi of the mice treated with MSG+NSCs (Fig. 5C). The results suggest that intracerebroventricular transplantation of NSCs obviously facilitates the functional and structural repair of glutamate-induced excitotoxic brain injury in adult mice.

4 Discussion

Immunocytochemistry displayed that the isolated cells were nestin-positive, suggesting that they were the NSCs capable of differentiating into neurons. The Hoechst 33342-labeled NSCs were grafted into the ventricles of mice, and tracked by immunocytochemistry. The common used pre-labeling methods could provide the information of the survival, migration and differentiation of grafted NSCs in the host brain^[11]. In this report, the green staining of the cells derived from labeled NSCs provided clear evidence that these NSCs could differentiate into neurons^[12].

It has been demonstrated that excitotoxic glutamate exposure results in an induction of extended neuronal depolarization (END), as well as protracted elevations in free intracellular calcium ($[Ca^{2+}]_i$). Both END and the prolonged $[Ca^{2+}]_i$ elevations were shown to correlate with subsequent neuronal death. Calcium influx constitutes the ionic basis for maintenance of glutamate-induced extended neuronal depolarization in association with hippocampal neuronal death^[13]. Neuronal death is the end-point of glutamate-induced excitotoxic brain injury. In the present study, the results revealed that excitotoxic glutamate exposure reduced memory retention and Y-maze discrimination learning capacities and prolonged hippocampal injury in adult mice.

Most neurons in adult CNS are terminally differentiated, exist through the life of the organism, and cannot be replaced after death. However, evidence exists that small populations of neurons continue to be born in the adult ventricular zone, olfactory system, and hippocampus. In the adult hippocampus, newly born neu-

rons originate from the putative stem cells that exist in the subgranular zone of the dentate gyrus. Progeny of these putative stem cells differentiate into neurons in the granule cell layer within a month of cell birth, and this late neurogenesis continues throughout the adult life in the rodent^[14]. Neurogenesis in the adult brain can be divided into three phases in accordance with the sequence of neurogenesis during CNS development: (a) proliferation when new cells are generated; (b) migration toward target areas; and (c) terminal differentiation into distinct phenotypes. Neural stem cell migration toward sites of damaged CNS tissue and its differentiation into special cell types of CNS may represent adaptive responses for the purpose of limiting and/or repairing damage. Our results suggest that NSCs can survive in the diseased or damaged CNS and integrate into the neural network after transplantation and thus functionally and structurally improve the outcome of animals.

This study indicates that intracerebroventricular transplantation of NSCs may facilitate the recovering of neurological function from glutamate-induced excitotoxic brain injury in adult mice. The finding suggests that intracerebroventricular transplantation of NSCs (cell replacement) might be a cell-based therapeutic strategy to reconstruct CNS tissue in the diseased or damaged adult brains.

Acknowledgements: The authors thank Dr. De-Pu Yu for his great encouragement.

References:

- [1] Lipton SA, Rosenberg RA. Mechanisms of disease: excitatory amino acids as a final common pathway in neurologic disorders. *N Engl J Med* 1994, 330: 613-622.
- [2] Yu TX, Zhao Y, Shi WC, Ma RD, Yu LJ. Effects of maternal oral administration of monosodium glutamate at a late stage of pregnancy on developing mouse fetal brain. *Brain Res* 1997, 747: 195-206.
- [3] Yu LJ, Zhang YP, Ma RD, Bao L, Fang JZ, Yu TX. Potent protection of ferulic acid against excitotoxic effects of maternal intragastric administration of monosodium glutamate at a late stage of pregnancy on developing mouse fetal brain. *Eur Neuropsychopharmacol* 2006, 16: 170-177.
- [4] Corti S, Locatelli F, Strazzer S, Guglieri M, Comi GP. Neuronal generation from somatic stem cells: current knowledge and perspectives on the treatment of acquired and degenerative central nervous system disorders. *Curr Gene Ther* 2003, 3: 247-272.
- [5] Wennersten A, Meier X, Holmin S, Wahlberg L, Mathiesen T. Proliferation, migration, and differentiation of human neural stem/progenitor cells after transplantation into a rat model of traumatic brain injury. *J Neurosurg* 2004, 100: 88-96.

- [6] Wachs FP, Couillard-Despres S, Engelhardt M, Wilhelm D, Ploetz S, Vroemen M, *et al.* High efficacy of clonal growth and expansion of adult neural stem cells. *Lab Invest* 2003, 83: 949-962.
- [7] Ourednik V, Ourednik J, Park KI, Teng YD, Aboody KA, Auguste KI, *et al.* Neural stem cells are uniquely suited for cell replacement and gene therapy in the CNS. *Novartis Found Symp* 2000, 231: 242-269; 302-306.
- [8] Park KI, Himes BT, Stieg PE, Tessler A, Fischer I, Snyder EY. Neural stem cells may be uniquely suited for combined gene therapy and cell replacement: Evidence from engraftment of Neurotrophin-3-expressing stem cells in hypoxic-ischemic brain injury. *Exp Neurol* 2006, 199:179-190.
- [9] Podhorna J, Brown RE. Strain differences in activity and emotionality do not account for differences in learning and memory performance between C57BL/6 and DBA/2 mice. *Gen Brain Behav* 2002, 1: 96-110.
- [10] Xu BH, Duan HF, Liu RY. The effect on learning and memory of application of RNAase to hippocampus of rats. *Chin Sci Bull* 1979, 24: 182-185.
- [11] Lu WG, Chen H, Wang D, Li FG, Zhang SM. Region-specific survival and differentiation of mouse embryonic stem cell-derived implants in the adult rat brain. *Acta Physiol Sin* 2007, 59: 51-57.
- [12] Tao KX, Chen JB, Wang GB, Shu XG. Culture and Identification of Monoclonal Neural Stem Cells Derived from Cerebral Cortex. *J Huazhong Univ Sci Tech (Med Sci)* 2006, 26: 451-454.
- [13] Limbrick DD Jr, Sombati S, DeLorenzo RJ. Calcium influx constitutes the ionic basis for the maintenance of glutamate-induced extended neuronal depolarization associated with hippocampal neuronal death. *Cell Calcium* 2003, 33: 69-81.
- [14] Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, *et al.* Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci USA* 1995, 92: 11879-11883.

脑室内神经干细胞移植修复谷氨酸导致的成年小鼠神经损伤

马娟, 于立坚, 马润娣, 张永平, 房娟芝, 张霄瑜, 于廷曦

广东海洋大学海洋药物研究与开发重点实验室, 湛江 524025

摘要: 目的 探讨脑室内神经干细胞移植修复成年小鼠谷氨酸神经毒性损伤的可能性。方法 从15日小鼠胚胎脑组织分离神经干细胞, 采用免疫细胞化学技术检测细胞Nestin抗原表达; 通过免疫荧光染色观察所移植神经干细胞在体内的存活及定位。除对照组外, 所有小鼠均以谷氨酸单钠(每天4.0 g/kg)灌胃, 连续10天。灌胃后第1天和10天, 谷氨酸加神经干细胞移植组行神经干细胞脑室内移植(1×10^5 细胞/鼠), 对照组和谷氨酸组注射DMEM液。末次移植后第11天进行Y迷宫试验, 试验结束后进行小鼠脑病理检查, 以分析谷氨酸引起的脑功能和形态学上的改变。结果 所分离细胞呈Nestin阳性表达; 移植10天后所移植神经干细胞在小鼠脑中呈区域特异性存活; 脑室内移植神经干细胞能明显促进成年小鼠脑谷氨酸兴奋性毒性损伤的修复。结论 脑室内移植神经干细胞可用于疾病或损伤脑组织的修复。

关键词: 脑修复; 神经干细胞; 移植; 兴奋性损伤; 小鼠