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Repair of Excitotoxic Neuronal Damage Mediated by Neural Stem Cell Lysates in Adult Mice

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Abstract

The present study aimed to investigate a possibility of brain repair from diseased or damaged disorders mediated by cell-free filtrate of neural stem cell (NSC) lysates (FNSCL). Mouse NSCs were isolated from the brains of embryos at 15 day postcoitum (dpc). The expression of the Nestin was examined by immunocytochemical technique. Sonication of NSCs cultured and nestin-positive was performed in a bath-type sonicator, and the cell-free filtrate was recovered from a filtrative step of the lysates. The animals in the monosodium glutamate MSG group received intragastric (ig) administration of MSG (2.0 g/kg per day) for 10 days the animals in the MSG+NSCs and MSG+FNSCL groups received intracerebroventricular transplantation of 10 μ l of NSC suspension, approximately 1.0×10^5 cells, or intracerebroventricular injection of 10 μ l of cell-free filtrate of lysates of approximately 1.0×10^5 NSCs on day 1 and day 11 after 10-d MSG exposure, respectively. The mice in control and MSG group were intracerebroventricularly administered with 10 μ l of DMEM instead of NSCs or FNSCL. On 11 day after intracerebroventricular transplantation of NSCs or intracerebroventricular injection of FNSCL, the test of Y-maze discrimination learning were performed, and then the histopathology of the animal brains was studied, to analyze MSG-induced functional and morphological changes and the effects of intracerebroventricular transplantation of NSCs or intracerebroventricular injection of FNSCL on the brain repair from MSG-induced excitotoxic injury. Both intracerebroventricular transplantation of NSCs and intracerebroventricular injection of FNSCL facilitated the brain repair following glutamate-induced excitotoxic injury in adult mice, suggesting that there are certain NSC factors inside NSCs which are effective in repairing glutamate-induced excitotoxic brain injury. Administration of FNSCL might be a cell-free-based therapeutic strategy to repair diseased or damaged central nervous system CNS tissue.

Keywords: Brain repair; Filtrate of neural stem cell lysates; Neural stem cell factors; Neural stem cells; Excitotoxic brain injury; Adult mice

Introduction

Excitotoxicity, a pathological process by which neural cells are damaged by overstimulation of its membrane receptors due to excessive exposure to the neurotransmitter glutamate, has been implicated as one of the key factors contributing to neuronal injury and death in a wide range of 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 due to overstimulation of glutamate receptors, especially of the N-methyl-D-aspartate (NMDA) subtype [1]. Therefore, antagonizing excitotoxicity is a particularly attractive target for neuroprotective or brain repair.

Our previous studies demonstrated that monosodium glutamate (MSG) crosses placental barrier and distribute to embryonic tissues. Maternal intragastric (ig) 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 exposure to glutamate, in which ig excessive administration of MSG results in a series of behavioral disorders, and obvious histopathological brain lesion in adult mice [4]. Neural stem cells (NSCs) are currently being investigated for their potential use in cell replacement strategies for the diseased or damaged CNS [5,6]. Cells for transplantation must be nontransformed, well characterized, able to migrate toward sites of diseased or 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 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 optimal candidates for therapeutic transplantation in cases of neurological diseases that involve cell loss. Expandable human neural stem/progenitor cells survive transplantation, and migrate, differentiate, and proliferate in the injured brain [6,7]. Therefore, stem cell transplantation through cell replacement or as vector for gene delivery is a potential strategy for the treatment of neurodegenerative diseases [5].

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On the other hand, neuronally expressed stem cell factor induces neural stem cell migration to areas of brain injury [8]. NSCs migrate through the parenchyma along nonstereotypical routes in a precise directed manner across great distances to injury sites in the CNS, where they might engage niches harboring local transiently expressed reparative signals. Migration in vitro of NSCs from subventricular zone toward explants from ischemic brain is mediated by interaction of stromal cell-derived factor 1 α and CXC chemokine receptor 4SDF-1 α /CXCR4 [9]. NSCs transplanted into ischemic rats can differentiate into astrocytes and neurons during the process of migration [10]. In vitro studies show that the neural precursor cells isolated from rat and human being have the capacity for in vitro neurogenesis when stimulated with epidermal growth factor (EGF) and/or basic fibroblast growth factor (β FGF) [11,12].

Using an experimental model of excitotoxic brain injury following exposure to glutamate, we demonstrated that both intracerebroventricular transplantation of NSCs and intracerebroventricular injection of FNSCL facilitated the brain repair following glutamate-induced excitotoxic injury in adult mice.

Materials and Methods

Chemicals

Monosodium glutamate (MSG) was purchased from Shanghai Bio Life Science & Technology Co., Ltd. (China); Dulbecco's modified Eagle medium (DMEM) was from Gibco Co.; fetal bovine serum (FBS) 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).

Animal

Kunming (KM) mice, 8 weeks old, were purchased from the Experimental Animal Center of Guangdong Medical College (Zhanjiang, China). The mice were provided with standard mouse food and chlorinated water ad libitum and kept on a reversed 12: 12 h light-dark cycle at room temperature of 22 \pm 2 $^{\circ}$ C. All the animals were treated in compliance with "Guidance Suggestion for the Care and Use of Laboratory Animals" issued by The Ministry of Science and Technology of People's Republic of China.

Isolation of NSCs

One male mouse and two female mice were housed to each cage. The pregnant females were separated. The embryos at 15-day postcoitum (dpc) were dissected from the uterus 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 to settle and was removed of 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 \times 10⁵ cells/mL by addition of DMEM.

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. Resuspend the cells in the DMEM medium containing 15% fetal bovine serum until reaching a cell density of 5.0 \times 10⁵ 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.

Preparation of FNSCL

Sonication of NSCs cultured and nestin-positive was performed in a bath-type laboratory sonicator for 5 min at 4. The lysates of NSCs were inspected under a light microscope, and cell-free filtrate was recovered from a filtrative step of the lysates. Protein concentration was determined by a modification of Lowry's method [13].

Protocols of animal tests

Eleven to thirteen animals were used in each group. On day 1 and day 10 after the 10-day MSG exposure (2.0 g/kg per day, ig), the animals in MSG+NSCs group received intracerebroventricular transplantation of 10 μ l of NSC suspension, approximately 1.0 \times 10⁵ cells, and the animals in MSG+FNSCL group received intracerebroventricular injection of 10 μ l of cell-free filtrate of lysates of approximately 1.0 \times 10⁵ NSCs, respectively. The control mice without MSG exposure and the mice in MSG group were intracerebroventricularly administered with 10 μ l of DMEM instead of NSCs or FNSCL. The test of Y-maze discrimination learning was performed on day 12 after the last NSC transplantation or injection of FNSCL, and then the histopathology of the animal brains was studied to analyze MSG-induced functional and morphological changes and the effects of intracerebroventricular transplantation of NSCs and injection of FNSCL on the repair of MSG-induced excitotoxic brain injury (Figure 1). The experiments were repeated three times.

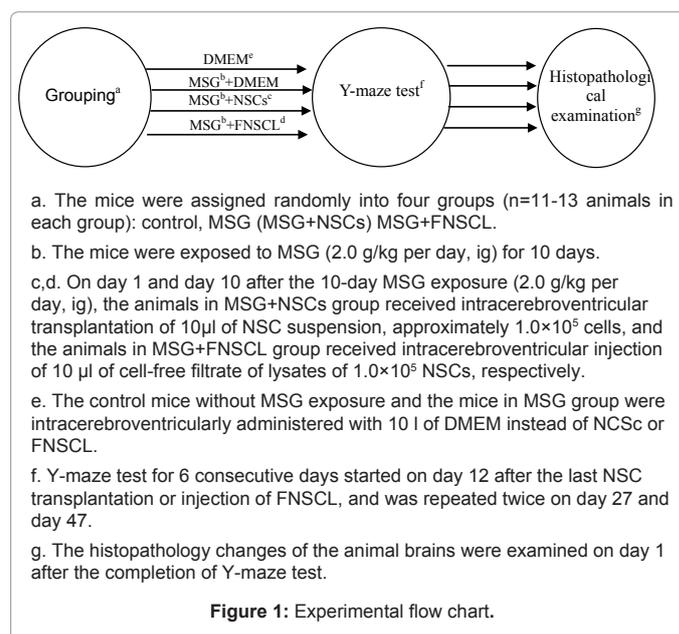


Figure 1: Experimental flow chart.

Test of Y-maze discrimination learning

Test of Y-maze discrimination learning was carried out by Xu's method [14]. Test of Y-maze discrimination learning for 6 consecutive days started on day 12 after the last NSC transplantation or injection of FNSCL, and was repeated twice on days 27 and 47.

Examination of Histopathology

The histopathologic changes of the animal brains were examined for two times: on day 1 after the treatment of MSG and on day 2 after the last NSC transplantation or injection of FNSCL. The mice were anaesthetized intraperitoneal (ip) with sodium pentobarbital (60 mg/kg), and sacrificed by perfusion fixation of CNS with 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-µm and stained with hematoxylin and eosin. The sections across hippocampal region were examined by light microscopy.

Statistical analyses

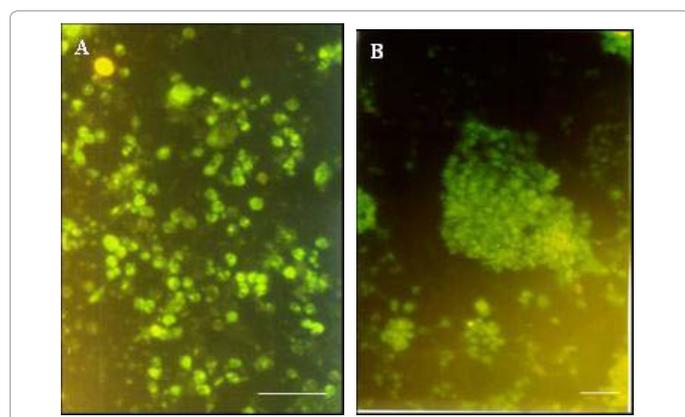
The repeated-measures analysis with a compound symmetry variance-covariance structure model was used to analyze the data from Y-maze discrimination learning test. P<0.05 was considered statistically significant.

Results

Identification of NSCs

Nestin is a special marker for NSC. We examined the expression of nestin in the isolated cells using immunocytochemical technique. The results showed that the cells had been stained green, indicating that the cells were nestin positively (Figure 2).

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



A: Nestin-expressed cells (100×),
B: Cultured nestin-expressed cells (100×), Scale bar, 20 µm

Figure 2: of neural stem cells with immunocytochemical technique. 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. Immunoreaction was observed under DMIRB fluorescence microscope (Leica,Germany) and photographs were taken.

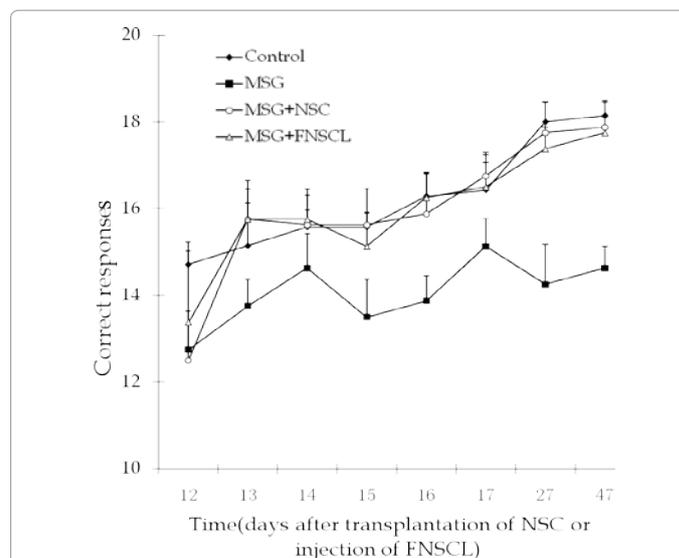


Figure 3: Effects of intracerebroventricular NSC transplantation and injection of FNSCL on glutamate-induced brain lesion by measuring Y-maze discrimination learning capacities. On day 12 after the last NSC transplantation or injection of FNSCL, the test of Y-maze discrimination learning was performed to analyze the glutamate-induced functional changes and the effects of intracerebroventricular transplantation of NSCs and injection of FNSCL on the recovery of glutamate-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.

Comparisons of P Values	P Values
MSG vs. Ctrl	0.0002 ^a
MSG+NSCs vs. Ctrl	0.6020
MSG+FNSCL vs. Ctrl	0.6236
MSG+NSCs vs. MSG	0.0005 ^a
MSG+FNSCL vs. MSG	0.0005 ^a
MSG+NSCs vs. MSG+FNSCL	0.9744

^aIndicates the significant result at alpha = 0.05 level.

Table 1:

antibody diluted to 1:50. Immunoreaction was observed under DMIRB fluorescence microscope (Leica,Germany) and photographs were taken.

Effects of intracerebroventricular transplantation of NSCs and injection of FNSCL on MSG-induced lesion by measuring Y-maze discrimination learning capacities

As shown in (Figure 3, Table 1), the memory retention and Y-maze discrimination learning capacities of the mice in MSG group were significantly less than those in control (P=0.0002), MSG+NSCs (P=0.0005) and MSG+FNSCL (P=0.0005) groups, but those of the mice in control, MSG+NSCs, and MSG+FNSCL (P=0.6020, P=0.6236 vs control, respectively) groups seemed comparable. It was also noted that the difference of Y-maze discrimination learning capacities between MSG+NSCs and MSG+FNSCL groups was insignificant (P=0.9744). The results suggest that both intracerebroventricular NSC transplantation and intracerebroventricular injection of FNSCL facilitated nearly equally the recovery of brain function from glutamate-induced excitotoxic lesion in adult mice, suggesting that there are certain NSC factors inside NSCs which are effective in recovering glutamate-induced excitotoxic brain lesion.

Effects of intracerebroventricular transplantation of NSCs and injection of FNSCL on glutamate-induced histological lesions

It is well known that the area most sensitive to brain damage is the hippocampus, which plays an important role in learning and memory. After the completion of Y-maze test, the histopathology of the animal hippocampi was studied to analyze the MSG-induced morphological changes and the effects of intracerebroventricular transplantation of NSCs or injection of FNSCL on the repair of MSG-induced excitotoxic injury of brain structure. MSG-induced hippocampal lesions was characterized by intracellular edema, degeneration and necrosis of neurons, and hyperplasia (Figure 4, 5B). However, no significant neuronal damage was detected in the hippocampi of the mice treated with MSG+NSCs or MSG+FNSCL (Figure 5C,D). The results suggest that both intracerebroventricular NSC transplantation and intracerebroventricular injection of FNSCL facilitated nearly equally the repair of brain structure following glutamate-induced excitotoxic injury in adult mice, suggesting that there are certain NSC factors inside NSCs which are effective in repairing glutamate-induced excitotoxic brain injury.

Discussion

Immunocytochemical staining displayed that the isolated cell were nestin-positive, suggesting that both intracerebroventricular transplantation of NSCs and intracerebroventricular injection of FNSCL facilitated the brain repair following glutamate-induced excitotoxic injury in adult mice, suggesting that there are certain NSC factors inside NSCs which are effective in repairing glutamate-induced excitotoxic brain injury.

It has been demonstrated that excitotoxic glutamate exposure results in the induction of an 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 the maintenance of glutamate-induced extended neuronal depolarization associated with hippocampal neuronal death

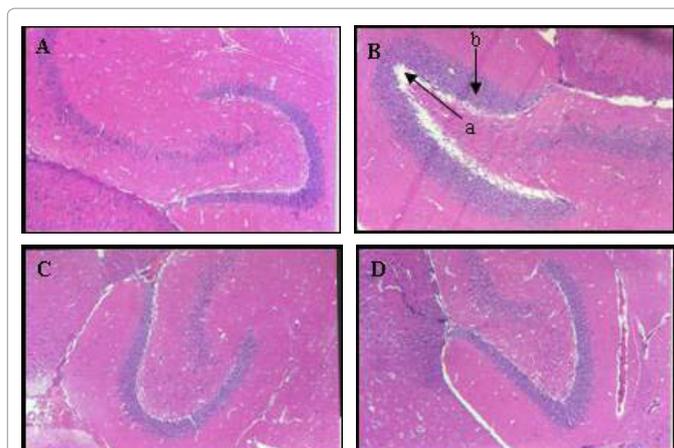


Figure 5: Effects of intracerebroventricular transplantation of NSCs and intracerebroventricular injection of FNSCL on MSG-induced histological lesions. Sections through hippocampal regions of the mice treated with or without MSG (MSG+NSCs, MSG+FNSCL) showing morphological effects of MSG and brain repair following glutamate-induced excitotoxic neuronal injury mediated by intracerebroventricular transplantation of NSCs or injection of FNSCL in adult mice. On day 1 and day 10 after the 10-day MSG exposure (2.0 g/kg per day, ig), the animals in MSG+NSCs group received intracerebroventricular transplantation and the animals in MSG+FNSCL group received intracerebroventricular injection of FNSCL respectively. The histopathology of the animal hippocampi was studied on day 2 after the last NSC transplantation or injection of FNSCL.

A. Control, $\times 20$; B. MSG, $\times 20$; C. MSG+NSCs, $\times 20$; D. MSG+FNSCL, $\times 20$. Arrows: a. degeneration and necrosis of neurons; b. intracellular edema and hyperplasia.

[15]. Neuronal death is the cellular end-point of glutamate-induced excitotoxic brain injury. The results revealed that excitotoxic glutamate exposure resulted in reduction of memory retention and Y-maze discrimination learning capacities and prolonged hippocampal injury in adult mice.

Most neurons in the adult CNS are terminally differentiated, exist through the life of the organism, and are not replaced when they die. However, evidence exists that small populations of neurons continue to renew slowly in the adult ventricular zone, olfactory system, and hippocampus. In the adult hippocampus, newly born neurons originate from 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 the cells' birth, and this late neurogenesis continues throughout the adult life of the rodent [16]. 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. The results suggest that NSCs can survive in the diseased or damaged CNS and integrate into the neural network after transplantation to functionally and structurally improve the outcome of animals.

It is understandable that intracerebroventricular transplantation of NSCs facilitates the repair following glutamate-induced excitotoxic brain injury in adult mice. Intracerebroventricular transplantation of NSCs (cell replacement) might be a stem cell-based neuroprotective and neurorestorative therapeutic strategies to reconstruct CNS

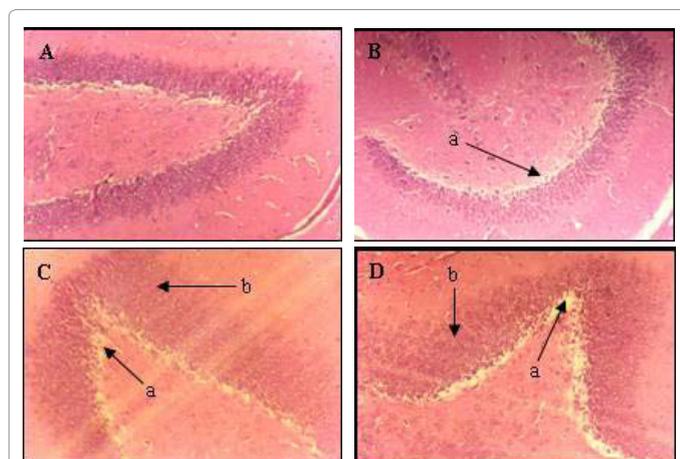


Figure 4: Histopathological examination of hippocampal injury following exposure to glutamate in adult mice. The histopathology of the animal hippocampi was studied on day 1 after the exposure of 10-day MSG (1.0, 2.0, 4.0 g/kg/d ig). A. Control, $\times 50$; B. MSG (1.0 g/kg per day), $\times 50$; C.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

tissue in the diseased or damaged adult brains. However, the novel experimental fact obtained from our study that intracerebroventricular injection of cell-free FNSC also facilitates the repair from glutamate-induced excitotoxic brain injury in adult mice suggests that there are certain NSC factors inside NSCs which are effective in repairing glutamate-induced excitotoxic brain injury. It is putative that cell-free FNSCL which contains a wide variety of, and an appropriate proportion of NSC factors unknown and known, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), EGF and bFGF, etc, which together constitute the microenvironment of CNS cells for survival and regeneration, stimulate and repair injured neural cells, and confer the capacities for proliferation, migratory, and differentiation responses on NSCs in the adult ventricular zone, olfactory system, and hippocampus. Actually, the finding suggests that not only intracerebroventricular transplantation of NSCs but also intracerebroventricular administration of FNSCL may be applied to repairing brain injury.

Taken together, the observation has pointed out the perspective of using an ethical and accessible cell-free material to “repair” diseased or damaged CNS. Administration of FNSCL (NSC factors) might be a cell-free-based therapeutic strategy to reconstruct CNS tissue and to avoid potential side effects of NSC transplantation in diseased or damaged CNS.

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