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Human Neural Stem Cells and Brain Transplantation

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ABSTRACT

Existence of multipotent neural stem cells (NSCs) has been known in developing and adult mammalian central nervous system (CNS) tissues. These cells have capacity to grow indefinitely and multipotent potential to differentiate into three major cell types of CNS, neurons, astrocytes and oligodendrocytes. Recently we have generated continuously dividing immortalized cell lines of human NSCs by introduction of oncogenes and these immortalized NSC lines have been used in studies in basic science of neural development, and transplanted into brain of animal models of human neurological diseases to explore possibility of cell replacement therapy or gene therapy in human patients.

It is desirable to obtain a large number of selected subpopulation of neurons or glial cells from continuously growing human NSCs by controlling the differentiation steps more rigorously. One way to accomplish this goal is transfer of relevant regulatory genes into the NSC cells. When human NSCs were transduced with a full length coding region of NeuroD, a neurogenic bHLH transcription factor, NSCs differentiated into neurons expressing neurofilament protein and tetrodotoxin-sensitive Na+ currents. When human NSCs were similarly transduced with Olig2 gene, NSCs differentiated into galactocerebroside-positive, O4-positive oligodendrocytes.

When human NSCs were implanted into the brain of rat models of Parkinson disease, Huntington disease and stroke, transplanted human NSCs were found to migrate to the lesion side, differentiate into neurons and astrocytes, and restore functional deficits characteristic ofthe neurological diseases.These results demonstrate that intravenously transplanted human NSCs differentiate into various neural cell types and compensate for the lost functions in rats caused by the focal cerebral ischemia.

Continuously dividing immortalized cell lines of human NSCs have have emerged as highly effective source of cells for genetic manipulation and gene transfer into the CNS ex vivo and once transplanted into damaged brain they survive well, integrate into host tissues and differentiate into both neurons and glial cells.

By introducing relevant regulatory genes into the human NSC cell line, it is now possible to obtain a large number of selected populations of neurons or glial cells from continuously growing human NSCs. Further studies are needed in order to identify the signals for proliferation, differentiation and integration of NSCs and to determine favorable conditions of host brain environment for implanted NSCs to survive, prosper and restore the damaged brain.

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What are Neural Stem Cells?

Stem cells are defined as cells that have the ability to renew themselves continuously and possess pluripotent ability to differentiate into many cell types. Two types of mammalian pluripotent stem cells have been identified and they are embryonic stem (ES) cells derived from the inner cell mass of blastocysts and embryonic germ (EG) cells obtained from post-implantation embryos (McKay, 1997; Gage, 2000; Temple, 2001; Gottlieb, 2002). During the past several years, existence of multipotent neural stem cells (NSCs) has bee known in developing or adult rodent central nervous system (CNS) tissues (Snyder, 1994; Reynolds and Weiss, 1996; Whittemore and Snyder, 1996; Brustle et al., 1997; Martinez-Serrano and Bjorklund, 1997). These cells have capacity to grow indefinitely and multipotent potential to differentiate into three major cell types of CNS, neurons, astrocytes and oligodendrocytes.

Neurogenesis in the adult rat CNS was reported previously by Altman in early 1960s using tritiated thymidine autoradiography (Altman, 1962; Altman, 1966) and actively dividing cells are seen in adult brain subventricular zone of lateral ventricles (Lois and Alvarez-Buylla, 1993) and subgranular zone of hippocampus (Bayer et al., 1982; Doetsch et al., 1999). There is confusion related the identification of cell type from which NSCs arise. Earlier it was assumed that ependymal cells are stem cells from which neurons and glial cells are derived (Johansson et al., 1999). Others have reported that in the hippocampus, astrocytes cultured in the presence of basic FGF and EGF are able to form expanding neurospheres and differentiate into neurons (Doetsch et al., 1997; Laywell et al., 2000; Song et al., 2002). It is now clear that new neurons could be generated from both ependymal cells and astrocytes even in the adult CNS.

In human, existence of NSCs with multipotent differentiation capability has been reported in embryonic and adult human brain (Buc-Caron, 1995; Brustle and McKay, 1996; Sah et al., 1997; Eriksson et al., 1998; Flax et al., 1998; Villa et al., 2000). Among these studies, a study has demonstrated that in a group of cancer patients who had bromodeoxyuridine (BrdU) infusion for diagnostic purposes and later died, BrdU-labelled proliferating cells that colabeled with neuronal marker were found in granular layer of hippocampal dentate gyrus (Eriksson et al., 1998). It is evident that new neurons are continuously being generated in adult CNS. Why then there is only limited capacity to repair in adult CNS suffering from injury or neurological diseases? It appears that endogenous brain environment that is responsible for induction of NSC proliferation and consequent NSC differentiation into neurons is not adequate in most of cases.

Neural Stem Cells and Neurosphere Formation

In early days of NSC studies, striatum of adult mice was dissociated into single cells by enzyme treatment and cultured as monolayer in chemically defined medium with EGF (Reynolds and Weiss, 1992). A small percentage of cells formed multicellular spheres that quickly increased in size and contained large number of NSCs, and these were called neurospheres. When the neurospheres were again dissociated into single cells and subcultured, they would become new neurospheres owing to active cell division. A previous study has reported that when neurospheres were cultured in serum-free medium with supplementary basic FGF and leukemia inhibitory factor (LIF), they proliferated actively and the cell number increased to 100-fold. Upon plated unto substrates, cells in neurosphere differentiated into neurons, astrocytes and oligodendrocytes (Carpenter et al., 2001). Similarly an established cell line of human NSCs was obtained by repeated subculture of neurospheres derived from human fetal brain (Vescovi et al., 1999). In contrast to these studies, our culture studies of neurspheres derived from humn fatal brain indicate that continuous passage of human NSCs resulted in loss of ability to proliferate and loss of ability to differentiate into neurons. When human neurospheres were subcultured once a month by enzymatic or mechanical measures and grown in serum-free medium with added bFGF and EGF, they could be passaged up to 8 times, and then NSCs startd to degenrate and die (Kim, unpublished results). More recent study has shown an immunoselection method to isolate human NSCs. When dissociated hu-

man fetal brain cells were screened by a panel of antibodies directed against cell surface antigens, neurosphere- forming NSCs which constituted 4% of the total cell population were CD133-positive. These CD133-positive cells were expanded and differentiated into neurons and glial cells (Uchida et al., 1997). Several groups of investigators were also successful in isolating NSCs from adult brain tissues. Adult human hippocampal tissues obtained during the surgery for epilepsy was enzymatically dissociated and grown in serum-containing medium and resultant neurospheres yielded neurons and glial cells when plated unto substrates (Kukekov et al., 1999). Similarly dissociated cells from hippocampal surgery were transfected with a plasmid containing nestin promoter-driven green fluorescent protein (GFP), and the nestin/GFP plasmid was selectively expressed by NSCs (Roy et al., 2000). Recently we have obtained adult hippocampal tissues from epilepsy surgery, dissociated into single cells following enzyme treatment, processd via a Percoll gradient and oligodendrocyte-enriched cultures were generated as reported previously (Kim, 1990). When these cells were grown in serum containing medium with added bFGF for 2 weeks and processed for RT-PCR analysis, mRNA messages for nestin, neurofilament proteins (NF), glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) were all demonstrated in these culture samples. These results indicate that major cell types of the CNS including NSCs, neurons, astrocytes and oligodendrocytes other members of the CNS are all present in adult human hippocampus cultures (Kim, unpublished data). With the identification of NSCs in cultures derived from adult human brain tissues, more work is needed to determine what signals or factors are required to induce further proliferation and differentiation of adult human NSCs.

Immortalized NSC Cell Lines Via Oncogen Expression

Recently continuously dividing immortalized cell lines of NSCs have been generated by introduction of oncogenes and these immortalized NSC lines have advantageous characteristics for basic studies on neural development and cell replacement therapy or gene therapy studies: (a) NSC cell line can be expanded to large numbers in culture; (b) stable expression of therapeutic genes can be achieved readily; (c) the NSC cells are homogeneous since they were generated from a single clone (Snyder et al., 1992; Hoshimaru, 1996; Martinez-Serrano and Bjorklund, 1997; Uchida et al., 1997). Immortalized NSCs have emerged as highly effective source for genetic manipulation and gene transfer into the CNS ex vivo; immortalized NSCs were genetically manipulated in vitro, survive, integrate into host tissues and differentiate into both neurons and glial cells after transplantation to the intact or damaged brain (Renfranz et al., 1991; Snyder et al., 1992; Martinez-Serrano and Bjorklund, 1997; Uchida et al., 1997). In collaboration with a Harvard group, we have generated immortalized cell lines of human NSCs. Fetal human telencephalon cells were transformed with a retroviral vector carrying v-myc oncogene and clones with continuously dividing NSCs selected. Both in vivo and in vitro these cells were able to differentiate into neurons and glial cells and populate the developing or degenerating CNS (Flax et al., 1998). More recently we have generated new lines of immortalized human NSCs using a retroviral vector carrying v-myc as described above. HB1.F3, one of the newly generated human NSCs, is a clonally isolated, multipotent human neural stem cell line, and has the ability to self-renew, differentiate into cells of neuronal and glial lineages in both in vivo and in vitro (Cho et al., 2002; Ryu et al., 2003), and successfully integrated into host brain upon transplantation into the brain of animal models of focal ischemia (Chu et al., 2003), Huntington disease (Ryu, et al.) and lysosomal storage disease mucopolysaccharoidosis VII (Meng, et al., in press).

Transcription Factor-mediated Cell Fate Determination

Existence of multipotent neural stem cells (NSCs) has bee known in developing or adult mammalian central nervous system (CNS) tissues including human (Snyder, 1994; Reynolds and Weiss, 1996; Whittemore and Snyder, 1996; Brustle et al., 1997; Martinez-Serrano and Bjorklund, 1997). These cells have capacity to grow indefinitely and pluripotent

potential to differentiate into three major cell types of CNS, neurons, astrocytes and oligodendrocytes. However, differentiation of NSCs into neurons and glial cells occurs spontaneously when they are grown on top of permissive substrates. It is therefore desirable to obtain a large number of selected subpopulation of neurons or glial cells from continuously growing human NSCs by controlling the differentiation steps more rigorously. One way to accomplish this goal is transfer of relevant signal molecules or regulatory genes into the NSC cell line (Nieto et al., 2001). Differentiation of NSCs in neurosphere into neurons could be increased by treatment with NT3, NT4 and platelet derived growth factor (PDGF) (Caldwell et al., 2001). There was a large increase in TH-positive neurons when neurospheres derived from fetal rat midbrain were grown in the presence of bFGF and astrocyte-consditioned medium (Daadi and Weiss S, 1999). Another approach to induce specific subpopulation of neurons or glial cells is to introduce neurogenesis associated master genes into NSCs and induce cellular differentiation. Since NSCs are readily transfectable with DNAs and stable sublines expressing transgenes could be generated, NSCs could be transfected with a neurogenic transcription factor gene and consequently induced to differntiate into a specific neuronal cell type. It has been known previously that Notch signaling, a member of basic helix loop helix (bHLH) transcription factors, is an important pathway that controls a broad spectrum of cell fate and shown to induce glial cells in the CNS. Transient activation of Notch1 in rat NSC cell line induced commitment of these cells to astrocytes (Tanigaki et al., 2001). A recent study from my laboratory has demonstrated that following transfection of HB1.F3 human NSCs with a full length coding region of NeuroD, a neurogenic bHLH transcription factor, F3.NeuroD cells differentiated into neurons expressing two types of K+ current and tetrodotoxin- sensitive Na+ current (Cho, 2002). For the induction of oligodendrocytes from NSCs, recent studies have identified Olig1 and Olig2, novel bHLH transcription factors, as important intracellular signal to regulate the differentiation processes of oligodendrocytes (Lu et al., 2000; Zhou et al., 2000). We have recently transfected HB1.F3 human NSC cells transiently with Olig2 DNA and obtained an induction of oligodendrocytes as shown by expression of oligodendrocyte specific markers galactocerebroside and O4 antigens in these cells (Kim, unpublished data). It is well known that both extrinsic and heritable intrinsic signals play important roles in generating cellular diversity in the CNS. It is now possible to obtain a large number of selected populations of neurons or glial cells from continuously growing human NSCs by introducing relevant signal molecules or regulatory genes into the NSC cell line.

Cell Replacement Therapy for Parkinson Disease (PD)

Cell replacement and gene transfer to the diseased or injured CNS have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases including Parkinson disease, Huntington disease (HD), amyotrophic lateral sclerosis and Alzheimer disease (AD). PD is an attractive target for these therapeutic approaches because the degeneration and cell death of dopaminergic neurons in substantia nigra, is well characterized. Previous studies have reported that implantation of fetal ventral mesencephalic cells into the caudate and putamen of PD patients provided a marked improvement in clinical course in these patients (Lindvall et al., 1990; Olanow et al., 1996; Hagell et al., 1999). However, this therapeutic approach has faced with grave limitations because of ethical, religious and logistical problems in acquiring fetal tissues. To circumvent these difficulties, it is necessary to procure alternative source of cells producing dopamine or L-dihydroxyphenylalanine (L-DOPA), dopamine precursor. Successful application of in vivo gene transfer to the CNS will depend on the identification of suitable cells that can serve as carriers for a wide range of potentially therapeutic transgenes and provide platforms for functionally efficient expression and secretion of transgene products. Immortalized CNS-derived neural stem cells (NSCs) have recently been introduced as potentially interesting candidates for this purpose. NSC transplantation has the potential to prevent or restore anatomic of functional deficits associated with injury or disease in the CNS via

cell replacement, the release of specific neurotransmitters, and/or the production of factors that promote neuronal growth and regeneration.

Earlier studies have focused on the development of genetically engineered cell lines that overexpress tyrosine hydroxylase (TH), the rate-limiting step in catecholamine biosynthesis (Fisher et al., 1991; During et al., 1994) or neurotrophic factors that promote survival of dopaminergic cells (Levivier et al., 1995; Bilang-Bleuel et al., 1997). However, parkinsonian animal models grafted with genetically modified TH cells have shown only partial restoration of behavioral and biochemical deficits. The limited success of this approach may be related to the limited availability of cofactor, tetrahydrobiopterin (BH4), because neither the dopamine-depleted striatum nor cells used for gene transfer possess a sufficient amount of BH4 to support TH activity (Bencsics et al., 1996). Therefore, it is necessary to transfer GTP cyclohydrolase I (GTPCHI) gene that is the first and rate-limiting enzyme in the BH4 biosynthetic pathway (Bencsics et al., 1996). Recently in my laboratory, we have newly generated immortalized human NSC lines. HB1.F3, one of new human NSC line with doubling time of 26 hr, is a clonally isolated, carries normal human karyotype of 46 XX, and has the ability to self-renew, differentiate into cells of neuronal and glial lineages, and populate the developing or degenerating CNS upon transplantation into the brain of experimental animals (Wagner et al., 1999; Lee et al., 2002). In the present study, F3 human NSCs were genetically engineered to produce L-DOPA by double transduction with cDNAs for human TH and rat GTPCHI. Transplantation of these cells in the brain of PD rat model led to enhanced L-DOPA production in vivo and induced long-term functional recovery following intrastriatal transplantation (Kim et al., 2000).

It appears that differentiation of NSCs into neurons and glial cells occurs spontaneously without any apparent regulatory factor(s) controlling it when NSCs were plated onto substrates. It would be advantageous if one could control the differentiation steps more rigorously so that one can obtain a large number of desired neuronal or glial subpopulations from continuously growing population of human NSCs. One way to accomplish this goal is transfer of relevant signal molecules or regulatory genes into the NSC cell line. Recently subline of F3 human NSC cells expressing the dopaminergic neuron-associated nuclear receptor Nurr-1 was generated (Lee et al., 2002). The cell line did not produce dopamine or L-DOPA when they were grown in normal serum-containing medium, but they became dopaminergic when culture medium was supplemented with FGF-8, sonic hedgehog protein and astrocyte-conditioned medium (Wagner et al., 1999). It appears that F3.Nurr-1 human NSCs are capable of becoming dopaminergic neurons when given appropriate extracellular signals.

Cell Replacement Therapy for Huntington Disease (HD)

Huntington disease (HD) is an autosomal dominant neurodegenerative disorder characterized by involuntary choreiformic movements, cognitive impairment, and emotional disturbances (Greenamyre and Shoulson, 1994; Harper, 1996). Despite identification of the HD gene and associated protein, the mechanisms involved in the pathogenesis of HD remain largely unknown and thus hamper effective therapeutic interventions. Transplantation of fetal human brain tissue may serve as a useful strategy in reducing neuronal damage in HD brain and a recent study has documented improvements in motor and cognition performance in HD patients following fetal cell transplantation (Bachoud-Levi, 2000). This trial follows previous reports that positive effects of fetal striatal cell transplantation to ameliorate neuronal dysfunction in animal models of HD (Nakao and Itakura, 2000) and that striatal graft tissue could integrate and survive within the progressively degenerated striatum in transgenic HD mouse model (Dunnett, 1998). The latter study is consistent with results obtained from HD patients indicating survival and differentiation of implanted human fetal tissue in the affected regions (Freeman, 2000).

A major limiting factor in the transplantation of cells is the difficulty in supplying sufficient amounts of embryonic striatal tissue and the concomitant ethical issues associated with the use of human embryonic tissue. An ideal source of cell transplantation in HD would be neural stem cells (NSCs)

which could participate in normal central nervous system (CNS) development and differentiate into regionally-appropriate cell types in response to environmental factors (Brustle et al., 1997; Flax et al., 1998). In this regard previous studies have shown that NSCs isolated from embryonic or adult mammalian CNS can be propagated in vitro (Gage, 2000; Temple, 2001; Gottlieb, 2002) and subsequently implanted into the brain of animal models of human neurological disorders including HD (Bjorklund and Lindvall, 2000). Transplantation of NSCs to replace degenerated neurons or genetically modified NSCs producing neurotrophic factors have been used to protect striatal neurons against excitotoxic insults (Martinez-Serrano and Bjorklund, 1997). At present, little is known regarding whether implantation of NSCs prior to neuropathological damage could alter the progressive degeneration of striatal neurons and motor deficits that occur in HD. This question is important since genetic study of Huntington disease gene mutation (No authors listed The Huntington's Disease Collaborative Research Group, 1993) and neuroimaging can provide details on factors involved in the progression of HD (Harris et al., 1999; Thieben et al., 2002) suggesting early intervention using brain transplantation could be effective in "would-be" HD patients.

Systemic administration of 3-nitropropionic acid (3-NP) in rodents leads to metabolic impairment and gradual neurodegeneration of the basal ganglia with behavioral deficits similar to those associated with HD (Beal et al., 1993; Brouillet et al., 1995). The compound 3-NP is a toxin which inhibits the mitochondrial enzyme succinate dehydrogenase (SDH) and tricarboxylic acid (TCA) cycle thereby interfering with the synthesis of ATP (Alston et al., 1977). Recent studies have demonstrated that brain-derived neurotrophic factor (BDNF) could block neuronal injury under pathological conditions in animal models of HD (Bemelmans et al., 1999; Perez-Navarro et al., 2000).

We have investigated the effectiveness of transplantation of human NSCs in adult rat striatum prior to striatal damage induced by the mitochondrial toxin 3-nitropropionic acid (3-NP) (Ryu et al., in press). Systemic 3-NP administration caused widespread neuropathological deficits similar to ones found in HD including impairment in motor function and extensive degeneration of cresyl violet+ neurons, glutamic acid decarboxylase (GAD)+ neurons and calbindin+ striatal neurons. Animals receiving intrastriatal implantation of human NSCs one week prior to 3-NP treatments exhibited significantly improved motor performance and increased resistance to striatal neuron damage compared with control sham injections. In contrast, transplantation of human NSCs at 12 hr following 3-NP administration did not show any protective effects against 3-NP-induced behavioral impairment and striatal neuronal damage. The neuroprotection provided by the proactive transplantation of human NSCs in the rat model of HD appears to be contributed by brain-derived neurotrophic factor (BDNF) secreted by the transplanted human NSCs. Active production of BDNF by human NSCs in vivo and in vitro was firmly established by studies using RT-PCR, immunocytochemistry, dot-blot, and ELISA. These novel findings suggest that proactively transplanted human NSCs were well integrated in the striatum and supported the survival of host striatal neurons against neuronal injury induced by 3-NP toxicity.

Since genetic screening of HD gene and neuroimaging could provide the progression of HD signs and symptoms in "would be" HD patients, the results of our study indicate that early intervention using brain transplantation with human NSCs overexpressing neurotrophic factors should be effective in blocking the progrssion of clinical pathology in "would-be" HD patients.

Cell Replacemeent Therapy in Stroke

Ischemic stroke cuased by abrupt and near-total interruption of cerebral blood flow, produces ischemic changes in the striatum and cortex, leading to a long-term sensorimotor deficit. Once damage from a stroke occurred, little can be done to restore premorbid functions, and although numerous neuroprotective agents have been clinically tried, no specific agents replaced the lost neurons, improved the deteriorated functions, and reduced the long-term sequelae (Savitz et al., 2002). There have previously been several reports of cell transplantation in the brain of ischemia animal models. Various cellular sources such as rodent mesenchymal stem cells (Chen et al., 2001; 2002; Zhao et al.,

2002), human umbilical cord blood cells (Chen et al., 2001), mouse hippocampal cells (Onifer and Low, 1990), immortalized mouse neural precursor cells (Sinden et al., 1997; Veizovic et al., 2001; Modo et al., 2002), and human teratocarcinoma-derived neurons (Borlongan et al., 1998; Saporta et al., 1999) were grafted into the ischemic brain, and reduced the neurological deficits induced by experimental brain ischemia. A recent study has reported that in human with ischemic infarct, some functional improvement was found with implantation of human teratocarcinoma-derived neurons (Kondziolka, 2000).

We investigated if intravenously introduced human NSCs could selectively migrate into lesioned brain sites, differentiate into new neurons and/or glial cells, and improve the functional deficits in rat models of focal ischemia (Chu et al., 2003). NSCs can circumvent blood-brain barrier and migrate to the specific pathologic areas of brain with trophism. We introduced HB1.F3 immortalized human NSCs intravenously via tail veins and F3 NSCs migrated into the the adult rat brain with transient focal cerebral ischemia. Transplanted human NSCs migrate to the lesion side, differentiate into neurons and astrocytes, without immunosuppression. We observed an increase in number of the grafted human NSCs between 1 and 2 weeks. Three weeks post-transplantation, a functional improvement was observed in the tranplanted animals compared with ischemic controls on rotarod and turning-in-an-alley tests. Our results demonstrate that intravenously transplanted human NSCs differentiate into various neural cell types and compensate for the lost functions in rats caused by the focal cerebral ischemia.

Future Perspectives

There are a number of issues to be clarified before adoption of NSCs for cell replacement therapy and gene therapy is widely accepted in clinical medicine such as which type of stem cells are most suitable for cell replacement therapy in patients with neurological disorders or brain injury. Since neurons could be derived not only from NSCs, but also from embryonic stem (ES) cells, embryonic germinal (EG) cells, bone marrow mesenchymal stem cells and even from fat cells or skin cells, the most pressing question is which cells are best suited for cell replacement therapy. With the presence of NSCs in adult CNS is known, it is only a matter of time before neurons and glial cells are cultured from adult samples. There are ongoing debates as to why should one use embryonic or fetal materials to generate stem cells when stem cells could be isolated from adult tissues. However, most of research up to now indicates that embryonic or fetal stem cells are significantly more versatile and plastic than adult counterparts.

Continuously dividing immortalized cell lines of human NSCs as generated by introduction of oncogenes have advantageous features for cell replacement therapy and gene therapy and the features include that human NSCs are homogeneous since they were generated from a single clone, can be expanded to large numbers in vitro, and stable expression of therapeutic genes can be achieved readily. Immortalized human NSCs have emerged as highly effective source of cells for genetic manipulation and gene transfer into the CNS ex vivo and once transplanted into damaged brain they survive well, integrate into host tissues and differentiate into both neurons and glial cells.

It is known that both extrinsic and heritable intrinsic signals play important roles in generating cellular diversity in the CNS. By introducing relevant signal molecules or regulatory genes into the human NSC cell line, it is now possible to obtain a large number of selected populations of neurons or glial cells from continuously growing human NSCs. Further studies are needed in order to identify the signals for proliferation, differntiation and integration of NSCs and to detemine favorable conditions of host brain environment for implanted NSCs to survive, prosper and restore the damaged brain.

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