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Central Nervous System Damage: *Crusade for Recovery*

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Summary

Central nervous system (CNS) cells have historically been characterized by low proliferation and neural differentiation, with an inability to repair or replace themselves following damage or degeneration. Widespread scientific efforts have therefore been made to outsmart the restrictive mechanism of the CNS and to address the devastation caused by this shortcoming. Although stem cells were initially thought to be tissue-specific in their ability to differentiate and proliferate, they have demonstrated the capacity to achieve functional neuronal identity when provided access to a permissive neural environment. Aguayo et al. (1980) proposed that the failure of CNS neurons to regenerate is not an intrinsic deficit of the neuron, but rather a characteristic feature of the environment that either does not support, or prevents regeneration[1]. My lab's success in isolating stem cells from CNS tissues and inducing them to adopt a neuronal fate, both *in vitro* and *in vivo* upon transplantation, supports this suggestion. Therefore, others and we have made significant progress in identifying diverse sources of stem cells, determining elements required for the generation of functional CNS neurons, and exploring the potential of these cells for therapeutic neuroregenerative strategies through stem cell and gene therapy.
[1]

The Stem Cell Frontier

Stem cells (SCs) can be loosely defined as cells having some capacity for self-renewal, which can generate or give rise to specialized cell types other than themselves. In vertebrates, they have traditionally been divided into two groups: ES (embryonic stem) cells or pluripotent SCs, capable of becoming any cell type in the body, and tissue-specific stem cells or mutipotent stem cells, which arise from ES cells and generate the cell types comprising a particular tissue [1].

It had long been believed that tissue-specific stem cells could only differentiate into cells of the tissue of origin. However, recent studies suggested otherwise by demonstrating the ability of bone marrow (BM) stem cells to contribute to and acquire properties of many tissue cell types, including midbrain neuronal cells[2].

Profound devastation is caused by the inability of central nervous system neurons to regenerate correct axonal and dendritic connections following injury or degeneration. Therefore, scientists like myself have been inspired to further investigate the sources and plasticity of stem cells for potential application in methods of regeneration or replacement of damaged CNS cells.

Efforts have since determined that neurogenesis actually continues throughout adulthood in discreet regions of the central nervous system, due to the presence of stem cells with proliferative abilities in these regions. These cells can be directed toward specific neuronal fates both *in vivo* and *in vitro* upon exposure to a permissive neural environment.

Insight into stem cell plasticity, along with the knowledge gained thus far of cues required to initiate neuronal differentiation of stem cells, has led us to realize the potential for regeneration of damaged neurons in the CNS. Thus, the ability to initiate neurogenesis in damaged CNS tissue through either stem cell or gene therapy could provide possible treatments for severe neurodegenerative diseases.

Mission 1: The Search for Stem Cells

The Adult Forebrain: *It's Alive!*

In order for stem cell therapy to exist as a realistic possibility for the replacement of degenerated neurons, usable and attainable sources, or "pools," of stem cells must be identified in the CNS. The hippocampus is the most thoroughly studied source of CNS stem cells, and is also one of the only areas where new nerve cells are generated in the adult brain. Hence, it makes sense to begin our discussion of stem cells and their potential with this structure.

To investigate the prospective utility of hippocampal neurogenesis in the adult brain, it is important to determine factors allowing for the enhanced generation of these cells and their potential functionality due to such factors. We therefore conducted experiments manipulating the living environment of mice. In a 1997 study, we found that significantly more new neurons arise in the hippocampus of mice living in an enriched environment, consisting of a large cage with tunnels, wheels, and toys, when compared to littermates housed in standard cages[3].

A subsequent study in 1999 determined that running in particular contributes significantly to neurogenesis[4]. This study also demonstrated a correlation between enhanced neurogeneration and behavioral functionality in that spatial learning and long-term potentiation improved in mice stimulated through voluntary activity, indicating that certain factors can contribute positively to the behavioral function of the mouse.

To address whether the enhanced neurogeneration precedes the increased functional ability of the cells, we investigated the functionality of the individual neurons in a 2002 study, using cues including expression of neuronal markers (NeuN), evidence for synaptic transmission (calbindin/synaptophysin expression), existence of spines in the dendrites and synaptic terminals, and the presence of electrophysiological properties (membrane potential, currents, firing rate)[5]. We found that these cells do have neuronal morphology and can display

[1]This paper was written for BIO324 Molecular Neuroscience. In this assignment, Tammy Hibler role-played a noted neuroscientist, Fred Gage, and wrote a state–of-the-art review article on Dr. Gage's research field, as is she were Dr. Gage herself. She then presented a PowerPoint seminar as Dr. Gage in an annual public student research conference "NeuroFrontiers" held at Lake Forest College.

passive membrane properties, action potentials, and functional synaptic inputs similar to those found in mature dentate granule cells.

The degree of pluripotency of these hippocampal progenitor cells had already been demonstrated by our lab in 1996, by grafting adult hippocampal neurons (AHPs) into the rostral migratory pathway and observing their ability to both migrate into the olfactory bulb and differentiate into tyrosine-hydroxylase positive neurons characteristic of this region[6]. The functionality of newly generated neurons in the hippocampus, and the degree of pluripotency of the cells, both demonstrated by our lab, support the application of AHP populations as endogeneous progenitors.

Is there more life out there?

To extend the valuable and exciting knowledge already gained concerning stem cell potential, we must identify additional sources of stem cells to determine what cell types may arise from them, and if they too have the ability to differentiate to a neuronal fate.

We therefore examined the proliferation, distribution, and phenotypic fate of dividing cells in the rat spinal cord in a 2000 study[7]. These proliferating CNS cells were able to divide *in situ*, rather than generating in and migrating from the ventricles in the brain already known to produce such cells. These cells demonstrated the ability to differentiate into mature oligodendrocytes and astrocytes, which is not surprising considering that periodic renewal of glial cells is required for continued to support and nourishment of these CNS neurons.

These results, however, proposed a higher level of cellular plasticity for the intact adult spinal cord than that previously observed, suggesting that the adult neuronal character of the spinal chord should be re-evaluated for potential neuronal generation. It was possible that perhaps these cells do not maintain their intrinsic restrictions when provided with differing cues. To investigate this idea, my lab and I used the knowledge we had gained from a 1999 study in which we demonstrated the ability of Fibroblast Growth Factor-2 (FGF-2) to induce precursors, native to regions of the adult brain that generate only glia, to adopt a neuronal identity[8]. Aside from confirming that neuron competent precursors exist naturally in widely divergent tissues of the adult brain, it also highlighted the applicability of this growth factor for the signaling of adult spinal progenitors.

Our subsequent application of FGF-2 in 2000, to the spinal cord-derived progenitors, did lead to the generation *in vitro* of neurons as well as the astrocytes and oligodendrocytes seen previously[9]. Our ability to employ FGF-2 to induce neuronal fate inspired us to experimentally manipulate this newfound adult spinal progenitor population *in vivo*. We demonstrated, in this same study, that transplantation of these clonal cells back into the adult rat spinal cord results in their differentiation into glial cells only, which was the *in vitro* fate originally observed for this cell population. However, when transplanted into the adult dentate gyrus (hippocampus), these cells integrated into the granular cell layer and differentiated into neural cells characteristic of this region[9]. This study therefore demonstrates the importance of environmental cues in directing cellular fates.

Are these Sources Realistic and Ethical?

In considering stem cell therapy and its practicality, we must not only address where applicable sources of stem cells exist, but also whether these sources are exploitable. We cannot simply obtain mass amounts of stem cells from healthy people to treat those with neurological ailments. In addition, there are ethical implications involved in utilizing cultured embryos as a significant source of stem cells.

Therefore, in 2001, my lab investigated the quality of stem cells isolated from human brain tissue following death, and in doing so, demonstrated the potential of postmortem brain tissue to be induced to achieve a neuronal fate[10]. Specifically, we looked at the difference in quality of neonatal (11-week old postmortem male) and adult (27-year-old postmortem male) brain tissue, observing a significantly higher proliferation capacity yielded by tissues from the young individuals. This is not surprising considering the eventual termination or retardation of cell division that occurs in conjunction with senisence, or aging of the cell. Hence, neonatal postmortem brain tissue may provide a more quality source of stem cells.

Although intrinsic differences in growth potential and/or lineage potential may affect the utility of cell transplantation for therapeutic applications, the potential use of post-mortem cells for therapy bypasses some of the ethical complications associated with using adult-derived and fetal progenitor cells, and should therefore be seriously considered.

Retrieval of a Method of Isolation Proves Crucial

Considering that there are sources of proliferating stem cells in the spinal cord and specific regions of the brain, the development of successful and effective methods for the isolation of these cells was crucial. In 2000, my lab introduced a method for the direct isolation of human neural stem cells from fresh CNS tissues, through the identification of cell surface markers and fluorescence-activated cell sorting (FACS)[11]. The prospective isolation of human CNS stem cells provides the opportunity to directly delineate lineages derived from cells at particular stages of development.

The fetal brain stem cells isolated in this study alone, using the new FACS technique, demonstrated self-renewal capacity and potent proliferation, migration, and differentiation into neurons and glia following engraftment into mouse brains[11]. The ability of this method to detect distinct subsets of human fetal brain based on a CNS stem cell protein profile, allows for specific sources of stem cells with self-renewal and multilineage differentiation properties to be isolated and evaluated for potential replacement or repair of damaged tissue.

Mission 2: Decoding the Regulatory Mechanisms of the Stem Cell

Contributions to Fate Specification

As can be gathered from just my lab's progress thus far in identifying promising pools of proliferating stem cells in the CNS, most of which have the ability to give rise to neurons both *in vitro* and *in vivo*, reports of new cells being generated throughout the central nervous system have been mounting. These findings have lead to an emerging view that neural stem cells may be widely distributed in the adult CNS and that the local environmental cues may dictate their fate choice (Figure 1). It is therefore necessary for us to investigate the contributions to fate specification of stem cells made by different cell types and environmental factors.

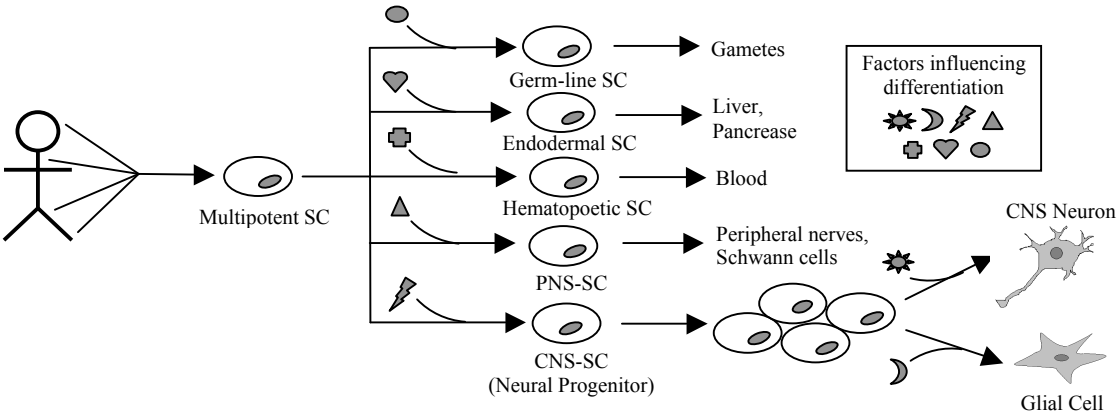

Figure 1. Schematic representation of the potential of stem cells to differentiate
Stem cells (SCs) residing in the embryo, brain, blood, or bone marrow may have many different fates, depending on the cues they receive from their environment. Hence, determination of the factors influencing their fate specification should prove to be very valuable in instructing these stem cells toward specific fates for therapeutic purposes. Such factors influencing differentiation may include, but are not limited to growth factors, contributions of different cells types (diffusible and or membrane-bound factors), and voluntary exercise.

Hence, in a 2002 study, my lab and I set out to determine which type of cells are necessary to promote neurogenesis—neurons, astrocytes, or both, and what factors these cell types contribute to neural promotion[12]. We found that while neurons have a role in inducingstem cells to become oligodendrocytes, astrocytes have a role in inducing stem cells to become neurons. Specifically, our results suggest that rat hippocampal astrocytes actively regulated neurogenesis of adult rat stem cells by promoting proliferation and increasing the rate of conversion from progenitors to neurons through both soluble and membrane-associated factors. This suggests that astrocytes play an active regulatory role by contributing neurogenerative-promoting cues, rather than the merely supportive role traditionally assigned to them. Logically, the next step would be to determine what these factors contributed by astrocytes are and whether they can be applied to other tissues in need of increased neural proliferation.

Long Live the Stem Cell!
While we want to devote significant attention to factors inducing stem cells to achieve neuronal identity, it is also essential to determine the factors which allow these stem cells to be maintained, or in other words to continue to produce more stem cells. Failure to gain this knowledge would render the transplantation of these cells into target tissues for therapeutic purposes useless.

In September of this year (2003), my lab and I demonstrated a dose-dependent increase in the proliferation of adult hippocampal progenitor cells upon treatment with Insulin-like Growth Factor-2 (IGF-2), following pretreatment with the already mentioned growth factor FGF-2[13]. Our study demonstrated that a combination of both of these factors is required for maximum proliferation of these hippocampal stem cells. In addition, my colleagues have shown the importance of another factor, BMP (bone morphogenic protein), on the continued survival and proliferation of stem cells[14].

Mission 3: Rescuing the Injured
Although substantial progress has been made in identifying sources of stem cells and in determining some of the factors responsible for promoting their differentiation to neuronal cell fate, it is necessary to extend our studies to determine the extent to which this gained knowledge can be applied to treatment of neurodegenerative diseases and neuronal damage.

Reviving Dopaminergic Neurons
Parkinson's disease (PD) is a severe neurological disease characterized by the progressive loss of dopaminergic neurons in the substantia nigra (SN), leading to debilitating motor dysfunction. Thus far, in our efforts to identify potential strategies for the replacement of these degenerated neurons, my lab and I (2002) have determined that progenitor cells actually reside in the adult SN[15]. We demonstrated that while these progenitors express a glial identity upon differentiation *in situ*, they are able to generate cells from all three neural lineages (neurons, astrocytes, and oligodendrocytes) *in vitro* and *in vivo* in the rat hippocampus, following removal from the SN and exposure to appropriate environmental signals. This progenitor population thus has the potential to achieve a neuronal fate, yet, considering their determined failure to achieve neuronal identity when grafted back into the SN, it is evident that factors that exist in the SN inhibit neuronal differentiation[15].

Therapeutic Potential
We have illustrated that the potential for Parkinson's treatment exists by way of stem cell therapy, provided we can identify and suppress the neural inhibitory mechanisms of the SN and identify the necessary proneural differentiation signals.

Despite our inability to induce a neuronal fate of SN-derived stem cells, my colleagues have had success in achieving such differentiation in rat models of PD using embryonic sources of stem cells[16], and also in human patients using fetal stem cells[17]. Kim, J. et al.

(2002) enriched the neuronal generation of midbrain neural stem cells, derived from mouse ES cells, through exposure to a combination of Shh, FGF-8, and Nurr1 signaling factors, and demonstrated the ability of these cells to functionally integrate into target tissue of the SN, leading to motor recovery in this rodent Parkinsonian model[16]. Scientists have also been utilizing fetal stem cells for trasnplantation into human PD patients for greater than a decade with actual motor function recovery[17]. Despite evident complications, this approach to therapy for neurodegenerative diseased provides great promise for the future.

Others in Need of Neuroregeneration

My lab is also interested in other neurodegenerative diseases affecting CNS structures. The identification of progenitor cells in the SN, where degeneration of neurons leads to PD, and illustration of the ability of non-native stem cells to differente into neurons in the SN, has lead us to wonder if progenitor cells exist in other CNS structures associated with severe neurodegenerative diseases, and if stem cell therapy can be utilized in these locations as well. For example, does the caudate nucleus, associated with PD and Huntington's disease, or the cerebellum, associated with Cerebellar Ataxia, also contain progenitor cells, and what can be done to induce these cells to differentiate into neurons?

Huntington's disease (HD) is a debilitating motor dysfunction disease characterized by the degeneration of caudate nucleus neurons, which leads to uncontrolled and involuntary movement. In October of this year (2003), we demonstrated the ability of adult rat-derived neural progenitor cells to survive and differentiate when transplanted into the striatum of a hemiparkinsonian rat[18]. Although we used a parkinsonian model, the dopaminergic neurons in the caudate nucleus and dentate gyrus that are degenerated in Parkinson's disease are also degenerated in HD. Thus, the research and findings of this study are therapeutically applicable to HD.

Although not yet tested, all progenitor cells derived from the various CNS sources, which I previously introduced, might also have the potential to be grafted into live human central nervous system tissues for therapeutic replacement of degenerated neurons. Recall that my lab demonstrated such potential in our ability to engraft human fetal brain tissue into the brains of mice[10].

This idea of providing the correct signals required for the differentiation of stem cells to specific neuronal fates, by supplying the cells with a proneuronal environment (Figure 1), has shown success in mouse models of spinal cord injury. Kim, J et al. (2003) for one, was able to successfully apply the known specific signals of
the motoneural differentiation pathway in mouse
spinal cord injury models to initiate axonal growth across the site of injury, which allowed function of the injured neurons to be partially regained[19].

Hence, the idea that progenitor cells simply need to be given the appropriate signals in order to coax them to a specific fate has gained a great deal of support, making it apparent that we must determine what these signals and cues are and how to apply them.

Gene Therapy: Another Potential Tactic

While the external environment of progenitor cells has been demonstrated to greatly influence the fate of the cell, the genetic components of the cell must also play a role. Hence, it is important to determine the extent to which genetic manipulation can direct cell fate.
Gene products known to aid in neurogenesis or to inhibit neurodegeneration can actually be applied to degenerated tissues in order to invoke neural growth, provided they have an effective mode of transport to the genome of the targeted cells. Previously available methods of gene delivery suffered from major limitations. Nonviral methods were inefficient and transient in their expression of the transgene, and the available viral vectors also demonstrated limitations (Figure 2).

Gene Therapy	**Vectors used in the Central Nervous System**				
Necessary Properties	Non-Viral	Adeno Viral Vectors	Retroviruses	Lenti viral Vectors	Adeno-associated Virus
Efficient Gene Transfer		√√	√	√√	√√
Long-Term Expression of Trasgene		√	√	√√	√√
Non-toxic (biosafety)	√√		√	√√	√√

Figure 2. Currently available methods of gene delivery and their relative value
Lenti-viral vectors and Adeno-associated viruses have proven to offer the most satisfactory combination of efficacy of gene transfer, sustained transgene expression, and biosafety. Therefore, these methods may be the most promising for *in vivo* gene delivery. √√ indicates high efficiency of the method, while √ indicated satisfactory efficiency.

While adenoviral vectors allow highly efficient delivery of the transgene *in vivo*, its expression is transient or short-lived mostly due to the immune response against the transduced cell[20]. Vectors derived from oncoretroviruses are able to integrate the transgene in the genome of the target cells and without transferring any viral gene, yet the cells they transduce die shortly after infection with the transgene[20]. In addition, transcription of the transgene is often shut off in the transduced cells[20]. For a vector to be acceptable for gene therapy, it must offer a satisfactory combination of efficient gene transfer to specific targets,

sustained expression of the transgene, and biosafety, by not inducing cell death (Figure 2)[20].

New and Improved Viral Vectors to the Rescue

Tools capable of delivering therapeutic gene products safely, efficiently, and in sufficient concentration to affected neurons had to first be developed in order to successfully deliver gene products known to aid in neurogenesis or in preventing neurodegeneration in specific regions of the CNS (growth factors). We have succeeded in developing vectors that will allow therapeutic gene products to be delivered to specifically targeted, deficient host cells *in vivo*, without killing the cells in the process (Figure 2).

In 1996, my lab and I developed a lentiviral vector (HIV-based vector) capable of efficiently transferring, integrating, and sustaining long-term expression of a transgene, β-galactosidase, injected into adult rat brains[20]. The ability of the lentiviral vector to sustain long-term expression of the transgene is due to its advantage of integrating into the genome of non-dividing cells. This study thus demonstrated the potential for sustained delivery of a therapeutic gene product, and hence may permit reversal of the genetic causes of several innate and acquired diseases in a wide variety of somatic tissues.

The promise of using the lentiviral vector was made apparent by Mazarakis, N.D. et al. (2001), who demonstrated the ability of a delivered rabies viral-based vector to transduce regions distal from but projecting to the injection site through retrograde axonal transport[21]. Delivery of the gene marker-containing vector the rat striatum led to the transduction of neurons expressing the marker to distal regions, including neurons of the SN, globus pallidus, and cerebral peduncle. In addition to the efficient transfer, the delivered gene product also exhibited long-term expression, thus opening up the possibility for the treatment of incurable human neurodegenerative diseases such as Alzheimer's, Parkinson's, and motoneuron diseases.

Parkinson's Disease and Amyotropic Lateral Sclerosis

Kordower, JH. et al. (2000) actually demonstrated the utility of the lentiviral approach in the delivery of glial derived neurotrophic factor (GDNF) to prevent the degeneration of nigrostriatal neurons in primate models of PD[22]. These effects were non-toxic and long lasting, and the primates demonstrated potent reversal of structural and functional effects of dopamine insufficiency upon delivery of GDNF into the striatum and SN.

In August of this year (2003), we did a similar experiment to that of Kordower et al. in an attempt to reverse the effects of another neurodegenerative disease, Amyotropic Lateral Sclerosis (ALS)[23]. However, we compared the effects of two growth factors, GDNF, used in Kordower's study, and IGF-1, and employed an alternative viral vector, the adeno-associated virus (AAV). This virus shows the ability to efficiently transport gene products from the sight of injection (muscle) to targeted motor neurons of the spinal cord by transporting the virus from presynaptic terminals of projecting neurons through the entire length of the axon. The product then enters the projecting cell nucleus, providing sustained gene delivery. The delivery of IGF-1 in our study prolonged life and delayed progression of the disease in mutant mice to a greater degree than GDNF. In addition, we showed that the effects of IGF-1 delivered by lentivirus are significantly reduced when compared to the effects of IGF-1 delivered by AAV.

What about Alzheimer's disease?

Alzheimer's disease (AD) is another neurodegenerative diesease associated with the breakdown of nervous tissue in the brain, which gives rise to dementia in the patient. Abnormal production and accumulation of Aβ protein is currently being investigated as one of the central mechanisms leading to (AD). Hence, in March of this year (2003), we looked at the ability to deliver nephrilysin, a human cell-surface associated peptidase implicated to be a major extracellular Amyloid-β (Aβ) degrading enzyme in the brain, into transgenic mouse models of amyloidosis, using a lentiviral vector[24]. This study demonstrated the inhibitory effect of nephrilysin on Aβ deposits in mice, either through increased degradation or reduced growth of already existing plaques, and its ability to reduce neurodegeneration. Our study therefore supports a role for nephrilysin in the regulation of amyloid deposition, and highlights the potential use of gene therapy approaches for the treatment of Alzheimer's disease.

Conclusion

Considering that this field of study has both an enormous potential impact on human health and quality of life, and that it is an exploding area in new research discovery, continued research efforts made on my part and on the part of my fellow colleagues is inevitable. We are currently striving to further expand our scope of knowledge concerning the ability to manipulate the fate of these self-renewing progenitor cells and to apply the knowledge we gain to the treatment of central nervous system damage.

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