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How the discovery of neuronal stem cells have changed neuroscience and perspective for the therapy for central nervous system illnesses

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List of nonstandard abbreviations:

AD = Alzheimer's disease
ALS = Amyotrophic lateral sclerosis
ASIA = American Spinal Injury Association
BrdU = bromodeoxyuridine
CNS = central nervous system
FDA = Food-and-drug-administration
FGF-2 = Fibroblast growth factor -2
GABA = gamma aminobutyric acid
hEMCs = human embryonic stem cells
iPSCs = induced pluripotent stem cells
NSCs = neuronal stem cells
NSPCs = neural stem/progenitor cells
PMD = Pelizaeus-Merzbacher disease
SCI = spinal cord injury
SGZ = subgranular zone
SVZ = subventrical zone

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Abstract

Mostly-incurable central nervous system diseases and disorders, such as neurodegenerative diseases, stroke, brain and spinal cord injuries and psychiatric illnesses, represent one of the most difficult health problems today, in terms of mortality, disability, productivity loss and health-care costs. After disappointing results regarding the translational value of neuroprotective molecules and protocols from preclinical research on animals to clinic, a new hope for the developing effective treatments for brain and spinal cord disorders came with the discovery of neuronal stem and progenitor cells, which have the potential to differentiate into a myriad of different glial and neuronal cell types. The basic biology behind the neuronal stem cells is becoming discovered, paving the way to possibilities for their manipulation and reprograming and for their clinical applications. Some of those protocols and clinical trials are described in this paper, with the emphasis on spinal cord injury treatments.

CENTRAL NERVOUS SYSTEM (CNS) DISEASES AND DISORDERS – THE SCALE OF THE PROBLEM

Central nervous system diseases and disorders, such as stroke, brain or spinal cord injuries and neurodegenerative diseases like Alzheimer's or Parkinson's disease, represent the major disease burden worldwide in terms of mortality, disability, productivity loss and health-care costs, with still deficient and delayed treatment rates *(74, 88)*. In Europe, disorders of the brain contribute to 26.6% of the total burden, thus a greater proportion as compared to other regions of the world *(87)*. It has been estimated that the susceptibility and morbidity to acute disorders such as stroke as well as chronic neurodegenerative diseases, will increase almost exponentially as the lifespan of individuals increases globally *(43)*. For example, it is supposed that mortality due to stroke (now the second leading cause of death and the leading cause of long term disability worldwide, for which there is limited treatment beyond the primary insult) will double by 2020 worldwide, owing to an aging population and an increasing incidence in developing countries *(47)*.

The core issue regarding the inexistent or inadequate therapy for most of CNS illnesses is the fact that, in contrast to some other tissues like skin or bones, the CNS, thus brain and spinal cord, has very limited capacity for self-recovery and regeneration in adults. Because of this limitation, brain or spinal cord injuries and diseases represent one of the

most important scientific and therapeutic challenges. The basic biological problem common to the most of the CNS injuries and diseases is the dysfunction and/or death of neuronal cells which are postmitotic cells unable to divide, and therefore incapable of replacing neighboring dead or injured cells. Moreover, those neurons that survive the injury but have their axons damaged, show only limited capacity to regrow their neuronal fibers and to re-establish functional connections within specific neuronal networks *(71, 78)*.

In recent years, important advances have been made in our understanding of the molecular pathways that are implicated in the pathogenesis of CNS diseases and that lead to cell dysfunction and cell death in Parkinson's and Huntington's diseases *(33, 65, 66)* or stroke *(57)*. Those studies have identified molecular targets for candidate interventions designed to slow or reverse neurodegenerative course and have pointed the way to develop mechanisms-based neuroprotective strategies. Unfortunately, despite the discovery and development of many pre-clinically promising neuroprotective molecules and procedures and a large number of clinical trials, currently there is not even one neuroprotective drug shown to be successful in functional recovery of patients after stroke or spinal cord injury or able to stop the progressive neurodegenerative scenario in neurodegenerative diseases *(16, 21, 40, 76)*. Why the neuroprotective strategies that work in animals have either failed or have shown only a minimal effect when translated to humans is the matter of great debate *(16)*. Factors like the complexity of the molecular and cellular pathways involved and the difficulty to comprehend and manipulate them are probably the leading cause. In recent years, the strategies focusing on the endogenous brain and spinal cord immune cells are intensively studied for the treatment of the CNS diseases, since it became clear that the immune cells are very important players in the pathology of different brain and spinal cord pathologies *(68)*.

However, even if the neuroprotective drugs would be available, they would solve the problem of the acute and progressive conditions when neurons are rapidly dying, but they would not be useful for the chronic states when damage repair rather that neuroprotection becomes a crucial goal.

The improvements in neural stem cell biology in the last 25 years, which are considered as the most extraordinarily productive quarter century in developmental neuroscience *(22)*, have opened up new strategies for the treatment of the brain and spinal cord diseases.

DISCOVERY OF NEURAL STEM/ PROGENITOR CELLS

For a long time it has been accepted that the adult mammalian central nervous system completely lacks regenerative capability and that this state is fixed and immutable. This dogma was removed with the discovery of neuronal stem and progenitor cells as a lifelong source of glia and neurons, changing dramatically our way of thinking about the CNS structure, development and plasticity, and giving new hope for the treatment of CNS diseases and disorders *(22)*. Neuronal stem cells are self-renewing, multipotent cells that repeatedly produce more restricted neuronal progenitors, that divide just few times to produce a small number of differentiated progeny, giving finally birth to myriad types of neurons and glia *(22)*. The neuronal stem cells (NSCs) and their progenitors are commonly referred as neural precursor cells *(7)* or, in this review, as neural stem/progenitor cells (NSPCs).

The discovery of NSPCs has introduced new basic biological concepts regarding how individual transcription factors can direct and reprogram cell fate, control cell proliferation and differentiation and also how extracellular molecular cues can guide the complex but precise processes of NSPCs migration during development. In the 1960s the first reports appeared, based on autoradiography, showing the existence of dividing cells in certain zones of the brain: in subependymal zone, later named subventrical zone (SVZ; frontal horns of the lateral ventricles in the forebrain) and in the antero-lateral subgranular zone (SGZ) of the hippocampal dentate gyrus *(1, 2, 4, 73)*. Later, these results were confirmed using advanced immuno-histological techniques combined with confocal microscopy and stereological techniques *(19)*. Interestingly, adult neurogenesis in humans has been also demonstrated in cancer patients who were given BrdU for diagnostic purposes (Eriksson et al., 1989) and recently in the post-mortem hippocampal tissue of individuals born during the Cold War (1955-1963), taking advantage of the elevated atmospheric ^{14}C levels caused by above-ground nuclear bomb testings *(75)*.

The discoveries of the heterogeneous progenitor cells, with large proliferative potential that were able to produce both neuronal and glial progeny and had capacity of self-renewal, a cardinal property of stem cells *(10, 15, 32, 82)*, led to the concept that the nervous system develops from multipotent neuronal stem cells. Interestingly, even though neurogenesis is observed in the SGZ throughout the life, playing an essential role in memory functions *(9)*, in the SVZ neurogenesis does not actually take place. Instead, the SVZ precursor cells have to migrate, in an ordinate pattern formation called rostral migratory stream, to the olfactory bulb to become neurons *(3)*. It is also interesting that NSCs in SVZ stop generating neurons in humans at about 2 years of age *(62)* and that their number declines with normal aging, and is dramatically reduced in Alzheimer's disease (AD) patients *(27)*. How the progressive, temporal restriction of the stem potential, when early progenitors with wide multipotency are changing to late progenitors, is controlled is not yet clear. Multiple cues, such as cytokine cardiotropin, transcrip-

tion factors and epigenomic changes such as DNA methylation and chromatin modifications have been proposed *(22)*.

In the 1990s new results demonstrated that stress levels, alcohol or antidepressant drugs negatively affect proliferation of progenitors in the SGZ *(24, 25)*. Conversely, adult neurogenesis is substantially increased by motor activity like running *(84),* signals from an enriched environment *(31)* or learning processes *(18)*. Recent evidence show that endogenous NPCs might also have non-neurogenic homeostatic functions via the release of different types of neuroprotective molecules important to health and disease conditions *(9)*.

Furthermore, new progress has been made in our understanding of the location and constituents of stem cell "niches", where neuronal stem cells are born and maintained *(22)*. A neuronal stem cell niche is defined as a limited and specialized anatomical region in which stem cells reside, and includes astrocytes, endothelial cells, microglia and blood vessels that integrate local and systemic factors to allow stem cell proliferation, survival and differentiation *(7)*. As neuronal stem cell niches turn out to be more complex than other cell niches in our body, our understanding of the molecules and events that regulate them needs to be largely amended.

NEURONAL STEM CELL MANIPULATION AND REPROGRAMMING

Further progress in the field has come with the development of the techniques to isolate, propagate, differentiate, transplant and genetically modify CNS stem cells. Importantly, it has been shown that neural progenitor cells retain their neurogenic potential when transplanted in vitro and that their neuronal differentiation can be stimulated by certain molecules such as Fibroblast Growth Factor -2, retinoic acid or forskolin *(54)*, indicating that extrinsic factors play a major role in stimulation of neurogenesis. Moreover, it has been shown that NSPCs isolated from SVZ and transplanted into ectopic regions of the adult brain, differentiate into glial cells (oligodendrocytes and astrocytes) *(67),* but that the NSPCs isolated from the spinal cord, which is considered to be non-neurogenic region, when grafted into SGZ of the dentate gyrus can differentiate into neurons, supporting the idea that external cues from the local microenvironment promote neuronal differentiation of NSPCs *(69)*.

It has been initially shown that somatic cells can be reprogrammed by transferring their nucleus into enucleated unfertilized oocytes *(85)* or by in vitro fusion with embryonic stem cells *(12, 80)*, indicating that embryonic stem cells contain factors that can recover totipotency or pluripotency of somatic cells. These discoveries were followed by one of the most striking invention in the field of stem cell biology: the development of a protocol to allow molecular reprograming of somatic cells (mouse fibroblasts) into embryonic-like induced pluripotent stem cells (iPSCs), which have the capacity to differentiate in any type of cell (81). Somatic cells were reprogramed by retroviral introduction of four transcription factors: Oct3/4, Sox2, c-Myc, and Klf4. The obtained iPSCs exhibited cell marker genes, morphology and growth properties of embryonic stem cells, and their subcutaneous transplantation into nude mice resulted in formation of teratoma tumors *(81)*. The authors won in 2012 Nobel Prize in Physiology and Medicine for these discoveries, which had a dramatic impact on modern biology, as a proof of principle that essentially all cells in our body maintain intrinsic plasticity for differentiating into diverse types of cell. Since then, many types of human iPSCs have been produced, giving hope for the clinical translation of these technical improvements to treat neurodegenerative diseases *(83)*, spinal cord injury *(36)*, autism *(6)*, epilepsy *(55)* or other diseases *(72)*. Some of these clinical trials will be described and discussed below. Nevertheless, before these trials actually become routine clinical procedure, further advances in basic understanding of neural stem biology is needed.

Developments in the iPSCs and human fetal and embryonic stem cells (hEMCs) technology are used not only for therapeutic interventions of CNS illnesses, but also to study human development and to model early-onset neurodegenerative and neurodevelopmental diseases *(22)*. In fact, many of these disorders, such as Huntington's disease, familial dysautonomia, Rett's syndrome or schizophrenia, turn out to be related to inadequate neuronal maturation, synaptic deficits and failed neuronal connectivity, all of which can be mimicked by targeted genome modifications of the iPSCs or hEMCs, which are then transplanted into in vivo animal models *(8, 11, 35, 45, 58)*. These novel interdisciplinary methods, bringing together basic cell and molecular biologists with bioengineers and clinicians, are gradually complementing traditional studies on postmortem brain tissues or rodent models *(46)*. One example of studying human CNS development using stem cells is the transplantation of human NSPCs into the rodent brain, where they produce physiologically functional GABAergic interneurons, mimicking the human neuronal development *(52)*. In another study, it was possible to produce chimeric mice with murine gray matter and predominantly human white matter, after neonatal transplantation of human glial progenitors into the brain of the shiverer demyelinated mouse *(86)*. The implanted human glial progenitor cells have produced functional oligodendrocytes that spread through the murine nervous system, suggesting the possibility that the neonatal transplantation of human glial progenitor cells can effectively treat disorders of congenital and perinatal hypomyelination *(86)*. Another important aim of these studies is to clarify if the pathology of neurodegenerative and neurodevelopmental diseases is

restricted to neuronal cells only, or it may be also dependent on the dysfunction of other neighboring cells present in the neuronal stem cell niche *(22)*.

TRANSPLANTATIONS OF NSPCS AS NOVEL THERAPEUTIC STRATEGIES FOR CNS ILLNESS

The discovery of the neuronal stem cells has led to rapid recognition of their therapeutic potential, which was tested already in numerous clinical trials. Most of them are controlled trials, based on the use of defined human NSPCs products and on procedures previously tested in appropriate animal models which have provided proof of concept and safety data. The results have in general shown that neuronal stem cells might supply trophic and immunomodulatory factors to the injured nervous tissue, and may, thus, enhance axonal growth and remyelination of spared axons, and contrast neuroinflammation *(23),* with the possibility of generation of new neurons and their functional integration into existing neuronal circuits *(13)*. The common problems related to transplantation-based strategies are the need for embryonal or fetal cells with related ethical concerns, the risk of immune rejection and the oncogenic potential of autologous pluripotent stem cells. An intense debate on the challenges to successful translation of promising experimental preclinical studies to clinically efficacious treatments for patients is in progress *(34)*.

The initial neuronal stem cell transplantations have been conducted using human embryonal and fetal neuronal stem cells. Encouraging preliminary results are coming for example from the Phase I clinical trial for demyelinating Pelizaeus-Merzbacher disease (PMD), started in 2010 at the University of California, San Francisco, in which banked human neural stem cells HuCNS-SCs (StemCell Inc.) were transplanted into children (four males, between 6 months and five years of age, with genetic confirmation of PMD). The 2-year follow up report indicates safety of the procedure and the preliminary results show a small, positive outcome with progressive myelination in all four patients (Stem Cell press release: http://investor.stemcellsinc.com/phoenix.zhtml?c=86230&p=irol-newsArticle&ID=1678880&highlight=). The HuCNS-SCs have been also used in phase I/II clinical trial for the Dry Age-Related Macular Degeneration (the leading cause of blindness in elderly) and for spinal cord injury (SCI). In Italy a Phase I clinical trial, started in 2011, has shown safety of a transplantation of human neural stem cells into spinal cord of 18 Amyotrophic lateral sclerosis (ALS) patients (age between 20 and 75 years). In the USA the first FDA-approved stem cell Phase I safety trial for ALS, based on the transplantation of human spinal cord stem cells directly into the gray matter of the spinal cord of 18 patients, is in progress. In preclinical animal work, these neural stem cells have been shown to make synaptic contacts with the host motor neurons and to express neurotrophic growth factors, which are thought to be neuroprotective *(91, 92, 89)*.

NEURAL STEM CELLS FOR SCI THERAPY

In contrast to neurodegenerative diseases such as Parkinson's disease, where a specific population of neurons degenerate, spinal cord injury, as well as stroke or traumatic brain injury, affects a heterogeneous population of cell types over large regions, so that cell therapies should replace multiple cell populations, with their integration into appropriate and functional neuronal circuits, and reconstruction of the disrupted vascular system *(39)*.

Clinical studies in which SCI patients received different types of stem cell have been recently described *(38, 42, 70)*, attracting much public attention. Unfortunately, there is no fully documented functional benefit for the majority of the SCI patients recruited in such trials. The first clinical trial for SCI, started in 2009, run by Geron Corporation, USA, was approved by FDA despite controversies regarding the ethical and safety concerns (formation of teratoma). The trial was based on the transplantation of human embryonic-derived oligodendrocyte progenitor cells (GRNOPC1) in four SCI patients between the ages of 18 and 65, with neurologically complete American Spinal Injury Association (ASIA) Impairment Scale grade A thoracic injuries. GRNOPC1 cells (dose of two million cells) were delivered by injection into the lesion site, between 7 and 14 days after injury. The patients were temporary immune-suppressed by low-dose tacrolimus. The results have shown the safety of the procedure, with no neurological recovery of the patients (Geron press release: ir.geron.com/phoenix.zhtml?c=67323&p=irol-newsArticle&ID=1635760).

Similar results have been obtained in clinical trials based on the transplantation of bone marrow derived mesenchymal stem cells, which have shown promising results in preclinical studies on different animals (rats, pigs, non-human primates) with improved locomotor performance. Nonetheless, the procedure has failed to show functional benefit to SCI patients in several studies *(48)*.

The beneficial effects of autologous bulbar olfactory ensheating glial cells transplanted 21 months after injury to a 38-year-old male patient with complete traumatic SCI at upper vertebral level (ASIA A scale), have been reported, with motor improvement to ASIA C, improved trunk stability and partial recovery of voluntary movements of lower extremities *(79)*.

The clinical use of the iPSCs to cure SCI is still restricted due to limited preclinical data *(59)* and the safety problems regarding their potential formation of teratomas or other neoplasms *(14, 37)*. However, iPSCs are the sub-

ject of intensive studies that have already shown interesting, long-distance axonal growth of human iPSCs (isolated from a healthy 86-year-old male and differentiated into neural stem cells) grafted into adult immunodeficient rats after experimental SCI *(41)*.

For other types of stem cell, like for example umbilical cord and adipose derived mesenchymal stem cells, more pre-clinical studies are required before safety is established for controlled clinical trials, to avoid undesired results such as development of the tumors in treated patients *(5)*. Thus, it is of the great importance to perform well-defined clinical trials and to educate patients to reduce their attraction to unproven therapies *(22)*.

A valid alternative to transplantation of exogenous cells at the site of lesion after SCI, could be the activation of the endogenous stem cells normally present in the mammalian spinal cord *(49)*, since this is a non-invasive method that does not require immune suppression. Indeed, in lower vertebrates, which can completely and spontaneously recover after SCI, endogenous spinal stem cells play a major role in the regeneration process *(50)*

The neural stem cell potential in the adult mammalian caudal nervous system resides mainly within the population of ependymal cells lining the spinal central canal *(30)*. This region contains a pool of stem and progenitor cells that are readily activated and recruited after experimental spinal damage *(28)*. Even though their sustained adult neurogenesis was not observed, the adult spinal neural stem cells are recruited and proliferate after SCI *(90)* to produce scar-forming astrocytes and myelinating oligodendrocytes *(49)*. They can also be pharmacologically (with growth factors) and genetically manipulated to stimulate neurogenesis and oligodendrogenesis *(53)*. Recent reports have indicated an important role of spinal endogenous stem cells in restricting the tissue damage and neural loss after injury through the formation of glial scar and exerting the neurotrophic effect required for survival of neurons adjacent to the lesion *(61)*.

Although spinal cord endogenous NSPCs cells represent a potential source for future SCI treatments, there is the immediate need to fully identify them prior to any manipulation for their clinical recruitment. Useful data may come from an in vitro SCI models, in which novel molecules, such as transcription factors, were recently discovered to be involved in activation and migration of endogenous spinal stem cells after injury *(51)*.

NEURAL STEM CELLS FOR STROKE THERAPY

Current preclinical evidence and clinical experience using various donor cell types show great promise for cell transplantation as a new therapeutic modality for stroke *(29, 39)*. Multiple stem/progenitor cells have been tested in preclinical studies on animals as potential sources for cell based therapy for stroke and have demonstrated the ability to survive, mature, migrate to the lesion, and decrease neurological sequelae induced by stroke *(39)*. The scientific community, conscious of the importance of defining fundamental criteria related to the cell characterization, dosage, fate, safety, outcome measures, patient selection, therapeutic timing, etc., has prepared detailed guidelines for stroke clinical trials (STEPS Participants, 2009), further revised more recently *(64)*. The guidelines contain recommendations for both experimental and early-stage clinical studies on the cell therapy for stroke, with the goal to increase the reliability of the data *(39)*.

Two recent reports have shown that transplantation of bone marrow mononuclear stem cells *(56)* and allogeneic mesenchymal stem cells from adipose tissue *(17)* into patients with acute ischemic stroke is safe, yet of no benefit to stroke outcome.

OTHER APPLICATIONS OF NSCS TO TREAT CNS ILLNESSES

Not only transplantation of neuronal stem cells, but other approaches like the so-called PharmaNutri-Neurogenesis *(22)* can be therapeutically pursued. Namely, since adult NSPCs are responsive to environmental stimuli, it has been proposed to protect or induce them with an appropriate diet, behavior or with drugs *(44, 60)*. Furthermore, various molecules and biomaterials with the potential to influence stem cell proliferation, differentiation and migration are explored as eventual supplements *(22, 26, 63)*.

CONCLUSIONS

Former disappointing results from clinical studies exploring neuroprotective and neurorepair strategies to treat neurodegenerative diseases and brain and spinal cord injuries, should stimulate novel approaches aimed at exploiting the transplantation of exogenous neuronal stem cells, as well as the endogenous rewiring potential of the brain and spinal cord stem/progenitor cells. Even though ongoing clinical trials already indicate the feasibility of such therapeutic strategies, much more basic knowledge about the maintenance, quiescence, mobilization, migration, proliferation, differentiation and guidance of the neuronal stem cells is necessary to fully control future stem cell-based clinical interventions.

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