Sharon A. Louis, PhD, Senior Scientist • **Brent A. Reynolds**, PhD, Consultant

The Central Nervous System

The mature mammalian central nervous system (CNS) is composed primarily of three differentiated cell types: neurons, astrocytes and oligodendrocytes. Neurons, the functional unit of the nervous system, transmit information to other neurons, muscle cells or gland cells. Astrocytes and oligodendrocytes provide a critical support role for optimal neuronal functioning and survival. Very early on in mammalian development, the CNS begins to develop with the induction of the neuroectoderm, which forms the neural plate and then folds to give rise to the neural tube. Within these primitive neural structures there exists a complex and heterogeneous population of neuroepithelial progenitor cells (NEPs), the earliest neural stem cell type to form.[1,2] As CNS development proceeds and continues on to adulthood, NEPs give rise to temporally and spatially distinct neural stem/progenitor populations. During the first stage of neural development, NEPs undergo symmetrical divisions to expand neural stem cell pools. In the second stage of neural development, neural stem cells switch to asymmetrical division cycles and give rise to lineage restricted progenitors. Intermediate progenitor cells that generate neurons are first formed, which subsequently differentiate to give rise to neurons. Following this neurogenic phase, neural stem/progenitor cells undergo asymmetric divisions to produce glial-restricted progenitors, which give rise to astrocytes and oligodendrocytes. The final stage of CNS development involves a period of axonal pruning and neuronal cell death which fine tunes the circuitry of the CNS. After this final stage of CNS development, a previously long-held dogma maintained that cell genesis in the adult mammalian CNS was complete, rendering it incapable of mitotic divisions to generate new brain cells and therefore lacking in the ability to repair damaged tissue due to disease (e.g. neurodegenerative disease, Parkinson's disease, multiple sclerosis) or injury (e.g. spinal cord and brain ischemic injury). However, over the past 20 years, there is now well-established evidence that multipotent neural stem cells (NSCs) do exist, albeit in only specialized microenvironments of the mature mammalian CNS. This discovery has fuelled a new era of research into understanding the tremendous potential these cells hold for therapy of CNS neurodegenerative diseases and injury.

Identification of Neural Stem Cells

In the simplest definition, multipotent stem cells are characterized as undifferentiated cells with the capacity for extensive proliferation that gives rise to more stem cells (exhibit self-renewal) as well as progeny that will terminally differentiate into cell types of the tissue from which they are obtained. Prior to 1992, numerous reports demonstrated only limited in vitro proliferation of neural progenitor cells isolated from embryonic tissue in the presence of growth factors.[3-5] While several types of neural progenitor cells had been identified in the embryonic CNS, none exhibited the characteristic features of a stem cell which included self-renewal, extended proliferative potential and ability to retain multi-lineage potential. In vivo studies supported the notion that proliferation occurred early in life and the adult CNS was mitotically inactive, unable to generate new cells following injury. Notable exceptions included several studies in the 1960s that clearly identified a region of the adult brain that exhibited proliferation (the forebrain subependyma),[6] but this was believed to be species-specific and was not thought to occur in higher mammals. However, in the early 1990s, cells which responded in culture to specific growth factors and exhibited stem cell features in vitro were isolated from the embryonic and adult CNS.[7-9] With these studies Reynolds and Weiss demonstrated that a rare population of cells isolated in vitro exhibited the defining characteristics of a stem cell: self-renewal, capacity to produce a large number of progeny and multi-lineage potential. The location of stem cells in the adult brain was therefore identified to be within the striatum[10] and researchers began to show that cells isolated from this region and in the dorsolateral region of the lateral ventricle of the adult brain were capable of differentiating into both neurons and glia.[11]

The Function of Neural Stem Cells In Vivo

During mammalian development, neural precursor cells (defined here to include neural stem cells and neural progenitor cells) which arise from the neural tube function to produce pools of multipotent and more restricted neural precursors cells which then proliferate, migrate and further differentiate into neurons and glial cells within distinct regions of the developing and adult CNS. Neural stem cells have been identified in nearly all regions of the embryonic mouse,

TOLL-FREE T. 1 800 667 0322 • T. +1 604 877 0713 • TOLL-FREE F. 1 800 567 2899 • F. +1 604 877 0704
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rat and human CNS, including the septum, cortex, thalamus, ventral mesencephalon and spinal cord. NSCs isolated from these regions have a distinct spatial identity and differentiation potential. Unlike the developing nervous system, where NSCs are fairly ubiquitous, cells with neural stem cell characteristics are localized to two regions of the mature CNS: the subventricular zone (SVZ) lining the lateral ventricles of the forebrain and the subgranular layer of the dentate gyrus of the hippocampal formation. In the adult mouse brain, the SVZ is comprised of a heterogeneous population of proliferating cells, however, it is believed that the type B cells (activated $GFAP^+$/$Pax6^+$ astrocytes or astroglial-like NSC) are the cells that exhibit stem cell properties and these cells may be derived directly from radial glial cells, the predominant neural precursor cell in the early developing brain and thought to convert in the postnatal brain into astrocytic-like NSCs. SVZ cells under normal physiological conditions are relatively quiescent, but can be induced to proliferate and to repopulate the subventricular zone following irradiation.[10] SVZ NSCs maintain neurogenesis throughout adult life and they do so through the production of fast-dividing transit amplifying progenitors (TAPs) which then differentiate and give rise to neuroblasts. TAPs and neuroblasts migrate through the rostral migratory stream (RMS) and further differentiate into new interneurons in the olfactory bulb. This ongoing neurogenesis, which is supported by the NSCs in the SVZ, is essential for maintenance of the olfactory bulb by providing a source of new neurons in the olfactory bulb of rodents and in the association cortex of non-human primates.[12] Although the RMS in the adult human brain has been more elusive, a similar migration of neuroblasts through the RMS has now also been observed.[13] Neurogenesis which leads to the production of new granule cells also persists in the subgranular zone of the hippocampus, a region important for learning and memory. Location studies have mapped the neural progenitor cells to the dorsal region of the hippocampus, in a collapsed ventricle within the dentate gyrus.[10] Studies have demonstrated that the neurogenic cells from the subgranular layer may have a more limited proliferative potential than the SVZ NSCs and are more likely to be progenitor cells than stem cells.[14] Recent evidence also suggests that neurogenesis plays a different role in the hippocampus than in the olfactory bulb. Where the SVZ NSCs play a maintenance role, it is thought that hippocampal neurogenesis serves to increase the number of new neurons and contributes to hippocampal growth throughout the adult's lifespan.[12]

Neural Stem Cell Culture Systems

In vitro methodologies designed to isolate, expand and functionally characterize NSC populations have revolutionized our understanding of neural stem cell biology and increased our knowledge of the genetic and epigenetic regulation of NSCs.[15] Two well-described culture systems commonly used to isolate and expand neural stem cells include the neurosphere culture system where cells are cultured as free-floating aggregates and the adherent monolayer culture system.

Neurosphere culture: The neurosphere culture system, the method originally used to identify neural stem cells, has been widely used in the study of NSCs.[16-19] A specific region of the CNS is microdissected, mechanically dissociated and plated in a defined serum-free medium in the presence of a mitogenic factor such as EGF and/or bFGF. In the neurosphere culture system, NSCs as well as some neural progenitors begin to proliferate after about 24 hours in culture in response to the mitogens, forming small clusters of cells by 2 - 3 days. The clusters continue to grow in size, and by day 3 - 5, the majority of the clusters detach from the surface and float in suspension. By approximately day 7, depending on cell source, the cell clusters, called neurospheres, typically measure 100 - 200 µm in diameter and are composed of approximately 10,000 - 100,000 cells. At this point, the neurospheres should be passaged, to prevent the cell clusters from growing too large, which can lead to necrosis as a result of a lack of oxygen and nutrient exchange at the centre of the neurospheres. To passage the cultures, neurospheres are individually, or as a population, mechanically or enzymatically dissociated into a single cell suspension and replated under the same conditions as the primary culture. Neural stem and progenitor cells again begin to proliferate within 24 hours of replating to form new cell clusters that are ready to be passaged approximately 5 - 7 days later, depending on the cell source. By repeating the above procedures for multiple passages, NSCs present in the culture will self-renew and produce a large number of progeny resulting in a relatively consistent arithmetic increase in total cell number over time. Neurospheres derived from embryonic mouse CNS tissue treated in this manner have been passaged for up to 10 weeks with no loss in their proliferative ability, resulting in a 10^7-fold increase in total cell number. Neural stem/progenitor cells can be induced to differentiate by removing the mitogens and plating either intact neurospheres or dissociated cells on an adhesive substrate or extracellular matrix (ECM) in the presence of a low serum-containing medium. After several days, virtually all of the stem cell progeny will differentiate into the three primary cell types found in the CNS: neurons, astrocytes and oligodendrocytes. While the culture medium, growth factor requirements and culture protocols may vary, the neurosphere culture system has been successfully used to isolate NSCs and progenitors from different regions of the embryonic and adult CNS of many species including mouse, rat and human.

Adherent monolayer culture: Alternatively, cells obtained from CNS tissues have also been cultured in defined, serum-free medium with EGF and/or bFGF but with the addition of a substrate such as poly-ornithine, laminin, or fibronectin coating on the surface of the cultureware. When plated under these conditions, the neural stem and progenitor cells will adhere to the substrate-coated

cultureware, as opposed to each other, which results in the formation of neurospheres. The reported success of expanding NSCs in long-term adherent monolayer cultures is variable, and may be due to differences in the substrates, serum-free media and growth factors used.[15] Recently, protocols that have incorporated laminin as the substrate along with an appropriate serum-free culture medium containing both EGF and FGF have been able to support long-term cultures of neural precursors from mouse and human CNS tissues.[20-22] These adherent cells will continue to proliferate and become confluent over the course of 5 - 10 days. To passage the cultures, cells are detached from the surface by enzymatic treatment and replated under the same conditions as the primary culture. It has been reported that NSCs cultured under adherent monolayer conditions undergo symmetrical divisions in long term cultures.[20,23] Similar to the neurosphere culture system, adherently-cultured cells can be subcultured for multiple passages and can be induced to differentiate into neurons, astrocytes and oligodendrocytes upon mitogen removal and plating the dissociated cells on the same substrate or another appropriate substrate in the presence of a low serum-containing medium.

Recently, several reports have suggested that culturing CNS cells in neurosphere cultures does not efficiently maintain NSCs and produces a heterogeneous cell population.[15] In contrast, it is reported that culturing cells in serum-free adherent culture conditions containing both EGF and FGF in the presence of laminin does maintain NSCs. While these reports have not directly compared neurosphere and adherent monolayer culture methods using the same medium, growth factors or ECM to evaluate NSC numbers, proliferation and differentiation potential, they emphasize the fact that culture systems influences the in vitro functional properties of neural stem and progenitor cells. Therefore, in vitro methodologies designed to study NSCs must ultimately be robust and meaningful so the user clearly understands what the methodologies are purported to measure.[24-25]

Isolation Strategies for Neural Stem Cells

Immunomagnetic cell separation strategies using antibodies directed against cell surface markers present on stem cells, progenitors and mature CNS cells have been applied to the study of CNS stem cells. Like stem cells in other systems, the phenotype of CNS stem cells has not been completely determined. Expression, or lack of expression, of the CD34, CD133 and CD45 antigens has been used as a strategy for the preliminary characterization of potential CNS stem cell subsets in mammalian cells. A distinct subset of human fetal CNS cells with the phenotype $CD133^{+}5E12^{+}CD34^{-}CD45^{-}CD24^{-/lo}$ has demonstrated the ability to form neurospheres in culture, initiate secondary neurosphere formation and differentiate into neurons and astrocytes.[26] Using a similar approach, isolating $Nestin^{+}PNA^{-}CD24^{-}(HSA^{-})$ from the adult mouse periventricular region enabled ~80% of NSCs to be purified (100-fold increase in NSCs frequency) by FACS.[27] However, the purity of NSCs was lower when NSC frequency was recently reevaluated using the same FACS sorting strategy and the more rigorous NCFC assay.[28-29] NSC subsets detected at different stages of CNS development have been shown to express markers such as: Nestin, GFAP, CD15, Sox2, Musashi, CD133, EGFR[30-31] (SVZ cells) and Pax6, BLBP and GLAST[32-33](radial glial cells), however, none of these markers are uniquely expressed on NSCs, and many of these markers are also expressed on neural progenitor cells and other non-neural cell types.

Recent research has demonstrated that stem cells in a variety of tissues, including bone marrow, skeletal muscle and fetal liver can be identified by their ability to efflux fluorescent dyes such as Hoechst 33342. Such a population, called the “side population”, or SP, based on its profile on the flow cytometer, has also been identified in both mouse primary CNS cells and cultured neurospheres.[34] Other non-immunological methods have been used to identify populations of cells which demonstrate some in vitro properties of stem cells from normal and tumorigenic CNS cells, including high aldehyde dehydrogenase (ALDH) enzyme activity. $ALDH^{br}$ cells from embryonic rat and mouse CNS have been isolated and shown to have the ability to generate neurospheres, neurons, astrocytes and oligodendrocytes in vitro, as well as neurons in vivo when transplanted into the adult mouse cortex.[35-38]

Brain Tumor Cells

Multipotent neural stem-like cells, known as brain tumor stem cells (BTSCs) or cancer stem cells (CSCs), have been identified and isolated from different grades (low and high) and types of brain cancers, including gliomas, medulloblastomas, astrocytomas, and ependymomas.[39-40] Similar to neural stem cells, these brain tumor stem cells exhibit self-renewal, high proliferative capacity and multi-lineage differentiation potential in vitro. Importantly, they also initiate tumors in immunocompromised mice that phenocopy the original tumor.[41] No unique marker of BTSCs has been identified, but recent work suggests that tumors contain a heterogenous population of cells with a subset of cells expressing the putative neural stem cell marker CD133.[41] When $CD133^{+}$ cells were purified from primary tumor samples, they formed primary tumors when injected into primary immunocompromised mice and secondary tumors upon serial transplantation into secondary recipient mice.[41] However, CD133 is also expressed in differentiated cells in different tissues and $CD133^{-}$ BTSCs can also initiate tumors in immunocompromised mice.[42-43] Therefore, it remains to be determined if CD133 alone or in combination with other markers can be used to discriminate between tumor initiating cells and non-tumor initiating cells in different grades and types of brain tumors.

Both the neurosphere and monolayer culture methods have been applied to the study of BTSCs. When culturing normal NSCs,

the mitogen(s) EGF (and/or FGF) are required to maintain NSC proliferation, however, there is some indication these mitogens are not required when culturing BTSCs.[44] Interestingly, the neurosphere assay may be a clinically relevant functional read-out for the study of BTSCs, with emerging evidence suggesting that renewable neurosphere formation is a significant predictor of increased risk of patient death and rapid tumor progression in cultured human glioma samples.[45-46] Alternatively, adherent monolayer culture has been shown to enable pure populations of glioma-derived BTSCs to be expanded in vitro and cell lines derived from malignant high-grade gliomas which show stem cell properties in vitro produce gliomas following xeno-transplantation.[47]

Summary

Research in neural stem cell biology has made a significant leap forward over the past ~30 years. In contrary to the beliefs of the past century, the adult mammalian brain does retain a small number of cells located in specific CNS regions that satisfy the criteria of being neural stem cells. The identification of neural stem cells and the discovery that adult somatic cells from mouse and human can be reprogrammed to a pluripotent state (iPSCs)[48-54] has opened the door to new therapeutic avenues aimed at replacing lost or damaged CNS cells. This may include transplantation of neural progenitors derived from fetal or adult CNS tissue, as well as pluripotent (hESC or hiPSC) sources. Recent work has shown that appropriate transcriptional factors can be used to directly induce adult somatic cells to defined mature cell fates, such as neurons, bypassing the need for an iPSC intermediate.[55] Astroglia from the early postnatal cerebral cortex can be reprogrammed in vitro to neurons capable of action potential firing by a single transcription factor, such as Pax6 or the pro-neural transcription factor neurogenin-2 (Neurog2).[56] To develop cell therapies to treat CNS injury and disease, a greater understanding of the cellular and molecular properties of neural stem and progenitor cells and the discovery of epigenetic and genetic methods to influence stem cell behavior is required. Towards this end, STEMCELL Technologies has developed NeuroCult™ Proliferation Kits (Human, Mouse, Rat), including the xeno-free medium NeuroCult™-XF, to reduce variability and remove components derived from non-human sources in culture systems, with the goal of facilitating attempts to evaluate potential clinical applications of NSCs. The NeuroCult™ NCFC Assay provides a simple and more accurate assay to enumerate NSCs compared to the Neurosphere Assay. Combined with the research tools for neural stem cell research, new specialized serum-free medium formulations (NeuroCult™ SM Neuronal Supplements) for culturing primary neurons will go a long way in moving the field of neuroscience from the experimental to the therapeutic phase.

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