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Neural Stem Cells Redefined

A FACS Perspective

Dragan Maric* and Jeffery L. Barker

Laboratory of Neurophysiology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892

Abstract

Using the generally accepted ontogenetic definition, neural stem cells (NSCs) are characterized as undifferentiated cells originating from the neuroectoderm that have the capacity both to perpetually self-renew without differentiating and to generate multiple types of lineage-restricted progenitors (LRP). LRPs can themselves undergo limited self-renewal, then ultimately differentiate into highly specialized cells that compose the nervous system. However, this physiologically delimited definition of NSCs has been increasingly blurred in the current state of the field, as the great majority of studies have retrospectively inferred the existence of NSCs based on their deferred functional capability rather than prospectively identifying the actual cells that created the outcome. Further complicating the matter is the use of a wide variety of neuroepithelial or neurosphere preparations as a source of putative NSCs, without due consideration that these preparations are themselves composed of heterogeneous populations of both NSCs and LRPs. This article focuses on recent attempts using FACS strategies to prospectively isolate NSCs from different types of LRPs as they appear in vivo and reveals the contrasting differences among these populations at molecular, phenotypic, and functional levels. Thus, the strategies presented here provide a framework for more precise studies of NSC and LRP cell biology in the future.

Index Entries: central nervous system; development; cortex; neural stem cells; neural progenitors; fluorescence-activated cell sorting; cell fate.

Introduction

Neural stem cells (NSCs) have recently received a great deal of attention due to their inherent capability to generate all major classes of cells of the nervous system. NSCs have therefore been purported as a useful resource for potentially repairing and restoring the physiological functions to damaged, diseased, or aging neural tissues (for recent reviews covering many aspects of neural stem cell biology, see refs. *1–20*). However, with the accelerated interest in

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* Author to whom all correspondence and reprint requests should be addressed. E-mail: maricd@ninds.nih.gov

and growth of the NSC field, there has been an increasing ambiguity in the interpretation of what cell phenotype actually constitutes a neural stem cell. Using ontogeny as a reference, NSCs are defined as undifferentiated cells that developmentally originate from the neuroectodermal layer during early embryogenesis. After neural tube closure, these undifferentiated precursor cells and their immediate progeny compose the neuroepithelial layer that encloses the lateral, third, and fourth ventricles in the midbrain and forebrain and the central canal in the spinal cord. They are the principal source of cells that later form all major structures of the brain and spinal cord. NSCs have the inherent capacity to self-renew over virtually unlimited generations of undifferentiated progeny that are thought to be morphologically, phenotypically, and functionally indistinguishable from their founders. Depending on spatiotemporal and microenvironmental conditions, these cells also have the capability to commit, either reversibly or irreversibly, to different types of lineage-restricted progenitors (LRPs). LRPs have also been shown to either self-renew, by undergoing limited proliferation without further differentiation, or continue lineage-restricted differentiation, ultimately creating highly specialized neuronal and glial phenotypes that make up the central nervous system (CNS) and the peripheral nervous system (PNS). Naturally, since both neural stem and progenitor cells have been purported to proliferate in order to generate more of themselves or to undergo multilineage differentiation, there has been an increasing tendency to merge these cells into one functional group. As a result, the current state of the field contends with multiple populations and subpopulations of NSCs (reviewed in refs. *4*,*17*). Adding to the profusion of NSC biological properties is the recent controversy that NSCs can transdifferentiate into other non-neural cell phenotypes *(21–24).*

Furthermore, a wide variety of experimental preparations have been utilized to study NSC biology in vitro, ranging from embryonic or adult NSC-derived progeny expanding in suspension as neurospheres to variably mixed populations of NSCs and LRPs isolated from neuroepithelial and/or subventricular zone tissues of developing and adult CNS regions. It may be concluded from this research that NSCs are highly plastic cells and that their properties, including growth factor requirements for these cells to self-renew and generate multiple neural cell phenotypes, vary from region to region and do not remain constant during CNS and PNS development. However, the major limitation of these studies is that the cellular preparations used as a source of NSCs are themselves inherently heterogeneous and composed both of NSCs and self-renewing, but more lineage-restricted, progenitors, thus making the retrospective studies of NSC biology skewed to an unknown, but variable, degree. Adding to this is the growing evidence that suggests clear functional differences between neural stem and progenitor cells (reviewed in ref. *19*). Therefore, there is a crucial need to employ novel strategies that aim to identify and separate pure populations of NSCs and LRPs in order to resolve their shared or unique biological properties with respect to cell-fate determination and lineage progression. In this brief and eclectic review we summarize the current state of the field in achieving this goal, focusing primarily on recently devised fluorescence-activated cell sorting (FACS) strategies, and propose useful methods for future studies of NSC and LRP cell biology.

Neuroepithelium is Composed of Heterogeneous Cell Populations

With few exceptions, the starting material for studies of NSC biology in vitro is generally derived from neuroepithelial tissues dissociated from different embryonic (E) CNS regions using enzymatic or mechanical dispersion protocols that provide variable yields of vital cells *(25–27).* Typically, these cells are expanded in serum-free media in the presence of one or more mitogenic growth factors and are propagated as clonal clusters that, over a matter of days, detach from the noncationic plating surface to form neurospheres. The neurospheres themselves are

periodically dissociated by mechanical dispersion and the cells recultured under the same growth conditions used for the primary cells to generate more "self-renewing" neurospheres. In order to induce multilineage differentiation into neuronal and glial phenotypes, neurospheres are plated either intact or dissociated on adherent surfaces coated with positively charged substrates (poly-D-lysine, laminin, or fibronectin). The preparations are then cultured in a wide variety of media that may include fetal calf serum (FCS), chick embryo extract (CEE), a combination of different growth factors, or that may lack the mitogenic growth factors altogether.

However, the major limitation of this strategy to elucidate the biological properties of NSCs is the heterogeneity of the starting preparation. In this regard, even during the earliest period of CNS development, when a substantial proportion of the cytoarchitecture throughout the CNS is composed of the proliferating neuroepithelium or germinal zone, NSCs coexist in close proximity to their immediate progeny (neuronal, neuroglial, and glial progenitors) and newly postmitotic cells. Immunohistochemical analysis of the neuroepithelium at the onset of neurogenesis using multiple lineage-specific antibodies reveals the complex distributions of proliferating precursors and progenitors that reside physically apposed to each other and are therefore not amenable to microdissection methods (Fig. 1). Furthermore, since there are no distinguishing and specific markers of NSCs in mixed-cell cultures of dissociated neuroepithelial cells (NECs), their actual presence has necessarily been inferred. At an early stage of the field, Davis and Temple *(28)* retrospectively identified the NSCs from the embryonic rat cerebral cortex by observing that some cortical NECs in culture could both proliferate—albeit not indefinitely—and then differentiate into the three major CNS phenotypes (neurons, astrocytes, and oligodendrocytes). However, in this study only a small fraction of the initially plated NECs proliferated and underwent the self-renewal or multilineage differentiation characteristic of NSCs. In fact, the vast majority of cultured NECs primarily differentiated, implying that these cells were composed mostly of LRPs and postmitotic phenotypes. Similar results were obtained when dissociated NECs were not plated, but instead allowed to proliferate in suspension as cellular aggregates or neurospheres *(29).* In these and many subsequent studies the number of neurospheres formed was taken as a quantitative index of the number of initially plated NSCs. However, the number of neurospheres derived from individual NECs ranged 100-fold and the size of the individual neurosphere itself was quite variable, leading to significant interexperiment variability. As such, molecular phenotyping of human clonal neurospheres revealed a heterogeneity that was related to the relative proportions of NSCs and LRPs composing neurospheres of different sizes *(30).* Each neurosphere expressed a unique combination of eight selected transcripts considered to be discrimination markers. These wide-ranging results likely reflected the heterogeneity of the founder cells generating primary neurospheres. Furthermore, Reynolds and Weiss *(29)* conceded that in their model the formation of secondary neurospheres subcloned from the primary colony could have involved a multipotent progenitor rather than a true NSC. With this experimental strategy, such a possibility would be difficult, if not impossible, to eliminate. In retrospect then, both monolayers and neurospheres derived from individual NECs in these studies included NSCs that composed only a minor or unknown fraction of the cells investigated.

The inherent heterogeneity of NECs has also precipitated some controversy regarding the growth factor requirements for NSC proliferation and differentiation in vitro. Mitogenic growth factors, like basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), have been most extensively studied *(25,31).* However, a great deal of confusion has accumulated over the years in identifying the precise role of each of these and other growth factors in NSC biology. Even studies on the role of bFGF on NSCs isolated from the same CNS region have produced highly contrasting results *(25,31).* Qian et al. *(32)* have shown that low concentrations of bFGF stimulate cortical NECs to become neurons,

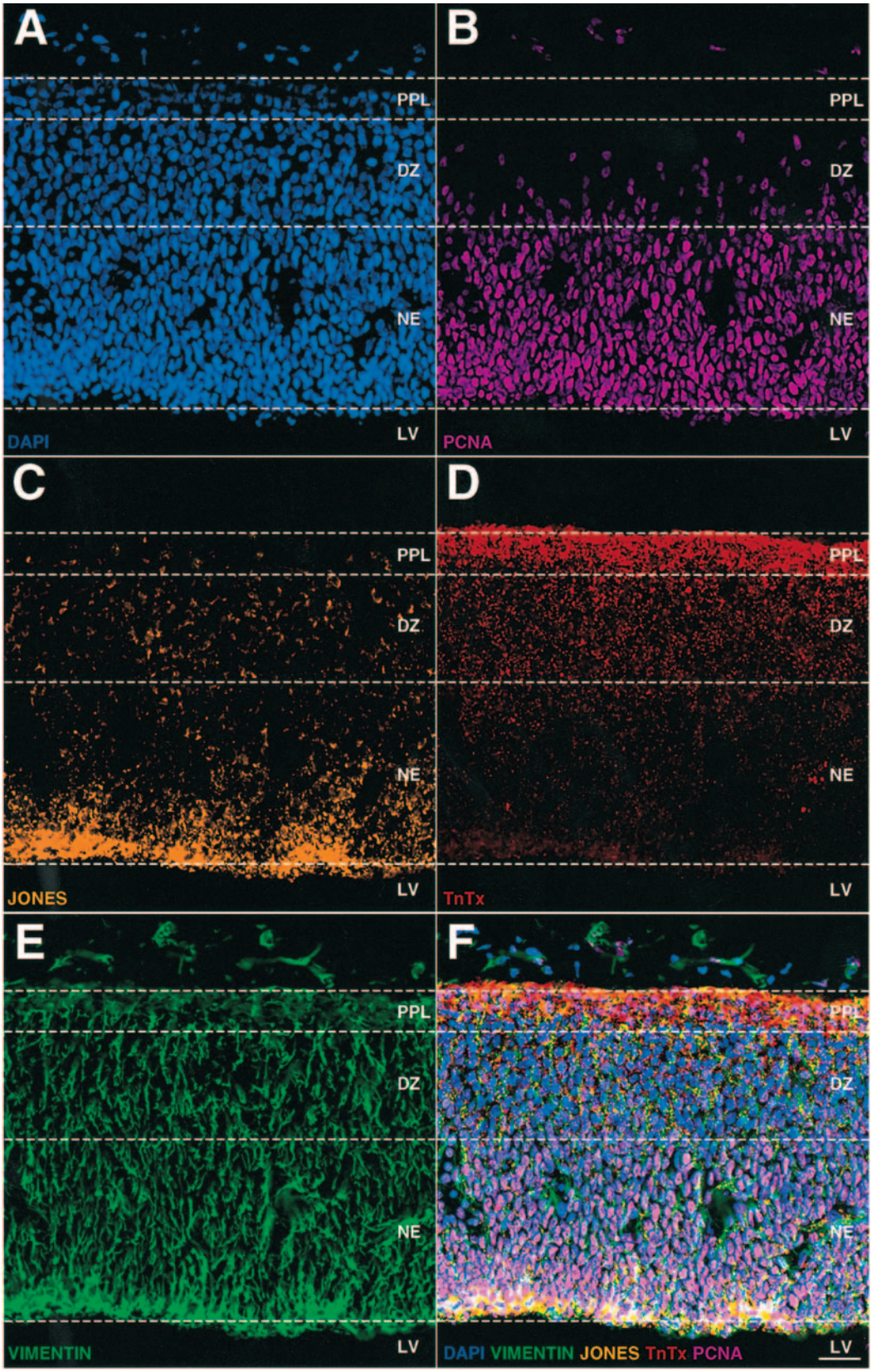
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Fig. 1. Multicolor labeling reveals the complex distribution of neural phenotypes in the E13 rat telencephalon. **(A)** DAPI staining discloses cell nuclei. **(B)** Anti-proliferating cell nuclear antigen (PCNA) antibody marks actively proliferating cells largely confined to the neuroepithelium (NE) adjacent to the lateral ventricle (LV). **(C)** JONES antibody identifies neuroglial progenitors residing primarily in the NE. **(D)** Tetanus toxin (TnTx) binding reveals neuronal progenitors in the NE and differentiating zone (DZ) and postmitotic neurons in the primordial plexiform layer (PPL). **(E)** Vimentin immunostaining outlines fibers of neural precursors and immature progenitors throughout the tissue. **(F)** Merged image of the five labeling reactions reveals the complex cytoarchitecture and heterogeneity of neural populations composing the telencephalic tissue at the beginning of neurogenesis. Calibration bar: 50 μm.

while at higher concentrations both neurons and glia are generated. However, in this study the self-renewing NSCs, which generate progeny that remain undifferentiated, were conspicuously absent regardless of the bFGF concentration used. On the other hand, Tropepe et al. *(25)* have shown that the cortical neuroepithelium produces different populations of EGF- and FGF-responsive NSCs that have the capacity to self-renew in the presence of these growth factors. Although this experimental variability may well be related to the developmental age, it demonstrates the difficulties inherent in studying NSC biology in a retrospective manner using heterogeneous populations isolated from the neuroepithelial tissues, where only a fraction of the cells are true NSCs. Ideally, the study of the underlying mechanisms that regulate self-renewal and differentiation of NSCs will be facilitated by prospectively isolating NSCs from their lineage-restricted progenitor and postmitotic progeny using different fluorescence-activated cell sorting strategies that target the unique biological properties of these cells (Fig. 2A). Direct FACS isolation of NSCs and LRPs from the CNS and PNS would also permit transplantation of specific subpopulations without their expansion and putative alteration or transformation in vitro.

Prospective FACS Isolation of NSCs From PNS

Isolation of specific subpopulations of hematopoietic cells including hematopoietic stem cells using fluorescently tagged immunoreagents targeting clusters of differentiation markers in conjunction with FACS has revolutionized the field of immunology. FACS analysis has elucidated the complexity of immune cell lineage progressions and FACS sorting has enabled direct access to hematopoietic stem and progenitor cell biologies. FACS strategies in conjunction with surface labeling have recently been developed by several laboratories in attempts to isolate NSCs directly from the PNS and the CNS for prospective studies.

In the first study of its kind, NSCs were isolated by FACS from the embryonic rat sciatic nerve in the PNS using surface expressions of the low-affinity neurotrophin receptor p75 and the peripheral myelin protein P_o *(33).* FACS analysis of dual immunostaining with p75 and P_o revealed a complex and continuous distribution in the signal intensity of both markers that did not clearly identify specific clusters of putative NSCs and LRP populations based on the relative expressions of these epitopes. Thus, the FACS profile of the entire bivariate p75 and P_o distribution signal was empirically subdivided into five subpopulations based on the relative intensities of the two markers. Sorting of each subpopulation by positive selection followed by long-term culture of single cells and immunophenotyping revealed different developmental potentials of each sorted subpopulation. Putative NSCs sorted from the sciatic nerve dissociate were identified retrospectively by their ability to both self-renew and generate all three lineages of the PNS (neurons, astrocytes, and smooth-muscle

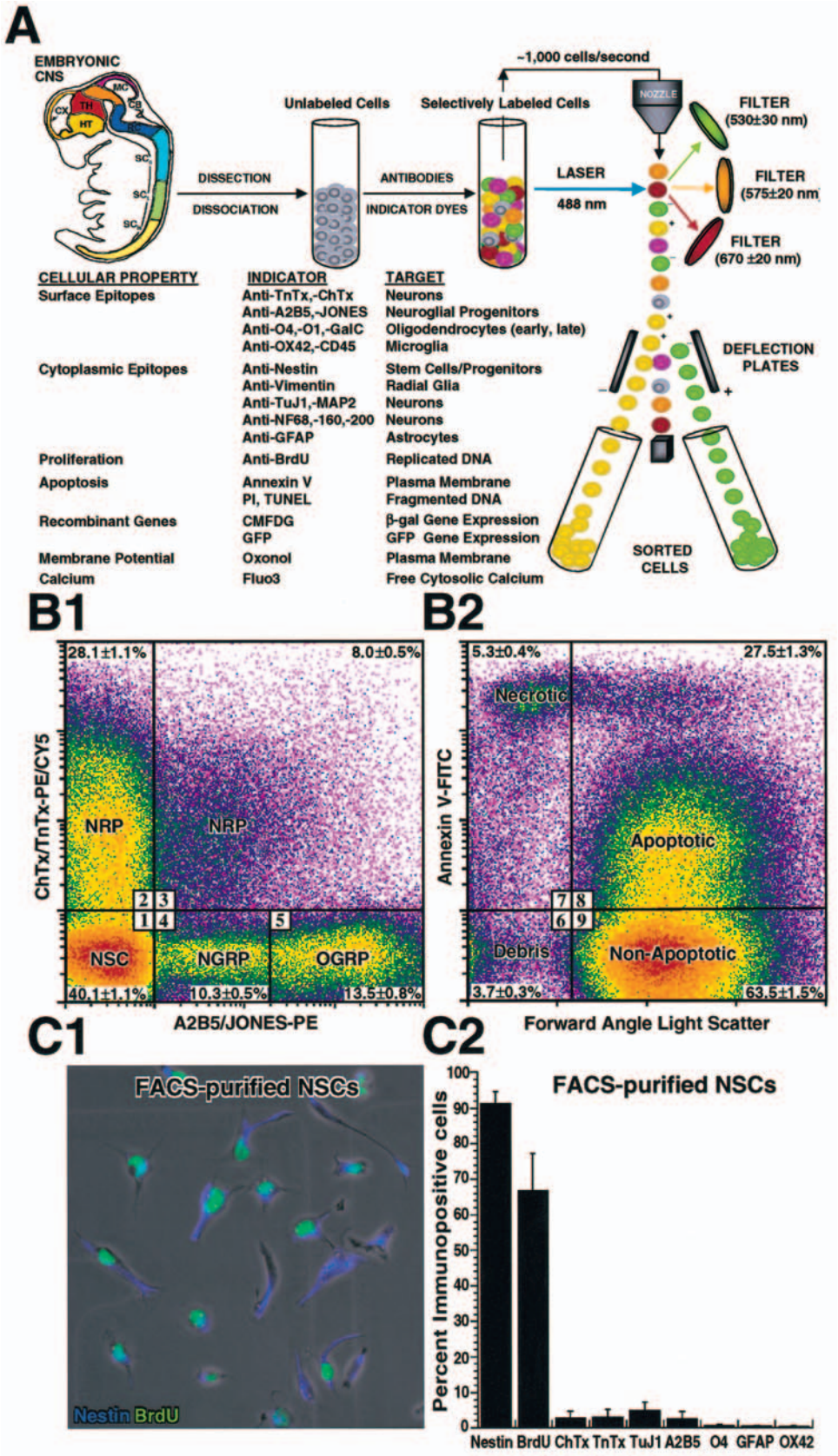

A
EMBRYONIC CNS
MC
CBL
CX
TH
HT
SC
DISSECTION
DISSOCIATION
Unlabeled Cells
ANTIBODIES
INDICATOR DYES
Selectively Labeled Cells
~1,000 cells/second
NOZZLE
LASER
488 nm
FILTER (530±30 nm)
FILTER (575±20 nm)
FILTER (670 ±20 nm)
DEFLECTION PLATES
SORTED CELLS
CELLULAR PROPERTY
INDICATOR
TARGET
Surface Epitopes
Anti-TnTx,-ChTx
Neurons
Anti-A2B5,-JONES
Neuroglial Progenitors
Anti-O4,-O1,-GalC
Oligodendrocytes (early, late)
Anti-OX42,-CD45
Microglia
Cytoplasmic Epitopes
Anti-Nestin
Stem Cells/Progenitors
Anti-Vimentin
Radial Glia
Anti-TuJ1,-MAP2
Neurons
Anti-NF68,-160,-200
Neurons
Anti-GFAP
Astrocytes
Proliferation
Anti-BrdU
Replicated DNA
Apoptosis
Annexin V
Plasma Membrane
PI, TUNEL
Fragmented DNA
Recombinant Genes
CMFDG
β-gal Gene Expression
GFP
GFP Gene Expression
Membrane Potential
Oxonol
Plasma Membrane
Calcium
Fluo3
Free Cytosolic Calcium
B1
28.1±1.1%
8.0±0.5%
NRP
NRP
NSC
NGRP
OGRP
40.1±1.1%
10.3±0.5%
13.5±0.8%
ChTx/TnTx-PE/CY5
A2B5/JONES-PE
B2
5.3±0.4%
27.5±1.3%
Necrotic
Apoptotic
Debris
Non-Apoptotic
3.7±0.3%
63.5±1.5%
Annexin V-FITC
Forward Angle Light Scatter
C1
FACS-purified NSCs
Nestin BrdU
C2
FACS-purified NSCs
Percent Immunopositive cells
Nestin BrdU ChTx TnTx TuJ1 A2B5 O4 GFAP OX42

Fig. 2. Fluorescence-activated cell sorting is a crucial strategy to prospectively isolate NSCs from LRPs. **(A)** Different embryonic CNS regions can be microdissected and optimally dissociated into uniform single-cell suspensions. Specific populations of cells composing each CNS region can be identified using multiple combinations of fluorochrome-labeled surface, cytoplasmic, nuclear, and protein markers and/or indicator dyes that target unique or shared cellular properties (*see* Table 1). Selectively labeled cells are then pressured through a 70-µm nozzle of the fluorescence-activated cell sorter (FACS) and excited one at a time using laser(s) tuned to specific wavelength(s). The resulting light scattering and fluorescence emission signals of each cell are collected using appropriate filters, which are amplified by photomultiplier tubes and acquired for computational analysis. By vibrating the nozzle at high frequencies (typically 31 kHz) and electronically charging the individual droplets of saline in which each cell is suspended, it is possible to sort-purify specific viable populations of cells based on a distinct combination of their light scattering and fluorescence emission properties. **(B1,B2)** A representative example in which a surface-epitope selection strategy is used in conjunction with flow cytometry to identify and directly isolate vital neural stem and progenitor cells from the embryonic rat telencephalon. **(B1)** Dissociates of E13 rat cortical neuroepithelium are simultaneously labeled using neuron-specific (ChTx and TnTx) and neuroglial progenitor-selective (A2B5 and JONES) markers that target specific gangliosides present at the cell surface (see "Ganglioside Expression Patterns Identify CNS Progenitors from NSCs"). The cellular distribution of these differentiating markers is quantified in a bivariate density plot in pseudocolor and identifies five predominant cell populations that compose the E13 cortical neuroepithelium. These include neural stem cells (NSC), neuroglial-restricted progenitors (NGRP), oligoglial-restricted progenitors (OGRP), and two subpopulations ($ChTx^+TnTx^+A2B5^-JONES^-$ and $ChTx^+TnTx^+A2B5^+JONES^+$) of neuron-restricted progenitors (NRP). **(B2)** Annexin V binding is used as an additional surface label together with forward angle light scatter, a measure of particle size, to identify cell debris from necrotic, apoptotic, and nonapoptotic (vital) cells. Annotated regions *(1–9)* enclosing the individual populations in **B1** and **B2** represent electronic windows used to define multiparameter logical gates for cell characterization, enumeration, and sorting, as previously described *(67).* The percentages of cells (means ± SEM) in each subpopulation are shown as insets in their respective delineated regions. A vital subpopulation of NSCs lacking all five of the surface epitopes (i.e., $ChTx^-TnTx^-A2B5^-JONES^-$ annexin V^-) is sorted by negative selection using a logical gating strategy, which involves the inclusion of cells in regions *1* and *9* and exclusion of cells in regions *2–8.* Viable/nonapoptotic subpopulations of NGRP, NRP, and OGRP are sorted using a positive-selection strategy, which involves the inclusion of cells delineated in regions *2–5* and the exclusions of cell debris in region *6* and cells with necrotic and apoptotic attributes in regions *7* and *8.* **(C1,C2)** Reanalysis of NSCs after sorting reveals that they are immature precursors ($nestin^+$, blue) and actively proliferating ($BrdU^+$, green) and lack the markers of differentiating neurons (ChTx, TnTx, TuJ1), neuroglial progenitors (A2B5), oligodendrocytes (O4), astrocytes (GFAP), and microglia (OX42). The bar graph represents the percentage (mean ± SEM) of immunopositive cells in FACS-purified NSC population. The self-renewing and multipotential properties of NSCs and lineage-restricted potential of NGRP, NRP, and OGRP cells have been previously described *(67).* BrdU, bromodeoxyuridine; ChTx, cholera toxin; CMFDG, 5-chloromethyl-fluorescein di-β-D-galactopyranoside; GFAP, glial fiblirary acidic protein; GFP, green fluorescent protein; MAP2, microtubule-associated protein 2; NF, neurofilament; PE, phycoerythrin; PE/CY5, phycoerythrin/carbocyanine dye 5; PE/TR, phycoerythrin/Texas red; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; TnTx, tetanus toxin; TuJ1, tubulin III. Panels **B1, B2, C1,** and **C2** are adapted from Maric et al. *(67).*

myofibroblast cells). These NSC properties could also be preserved by subcloning the primary clones.

Although three of the five subpopulations included NSCs, as defined above, one contained proportionally more NSCs compared to the others. This subpopulation, whose cells were $p75^+P_o^-$, composed 12% of the sciatic nerve dissociate with 60% of the cells being considered NSCs. However, since only 25% of the initially plated $p75^+P_o^-$ population formed colonies (of all types), the actual proportion of

NSCs was only about 2% of the original preparation. Despite the very small fraction of $p75^+P_o^-$ cells with NSC properties isolated from the embryonic sciatic nerve, follow-up studies with interspecies transplants and cell fate determination in vitro have been insightful and revealing. One study showed that NSCs can undergo intrinsic changes in their sensitivity to instructive signals while maintaining their self-renewing and differentiating capabilities *(34).* This study also demonstrated that the concentration of the neurogenic signal bone morphogenic protein 2 (BMP2) determined the neuronal phenotype of FACS-isolated NSCs in vitro. Low concentrations of BMP2 instructed NSCs to progress along a cholinergic lineage, while high concentrations of BMP2 stimulated noradrenergic lineage progression.

The success of these initial studies in isolating NSCs from the embryonic sciatic nerve led to a similar FACS-based strategy to sort NSCs from the embryonic gut *(35).* Triple immunostaining with p75, P_o, and α_4 integrin (α_4) revealed that $p75^+P_o^-$ cells from the sciatic nerve and gut also expressed α_4 on their surface. However, the expression of P_o was not as effective as that of α_4 in discriminating NSCs from LRPs in the gut. Thus, α_4 was used in combination with p75 to isolate $p75^+\alpha_4^+$ NSCs from this tissue. Cells with the highest expression levels of surface p75 and α_4, which composed only 1–2% of the dissociate, generated the greatest percentage of the colonies containing all three differentiating phenotypes of the PNS. About half of the $p75^+\alpha_4^+$ population generated colonies, 70% of which were multipotential. Thus, NSCs actually accounted for less than 1% of the initial preparation. Transplantation of the NSCs isolated from the gut reproduced the results obtained with NSCs sorted from the sciatic nerve by showing self-renewing and differentiating capabilities of these cells in vivo. The results of these studies established that NSCs isolated from the PNS can progress along neuronal or glial lineages depending on the local microenvironment and the presence of extrinsic signals. Phenotypic diversity as revealed by transplantation of NSCs was derived from interactions between the different types of NSCs isolated from the sciatic nerve and gut and the different microenvironments into which they were transplanted *(35).*

Clearly, the prospective isolation of NSCs from the PNS by flow cytometry using positive selection demonstrates not only the feasibility of using such a strategy to directly access these cells, but also its utility in disclosing insights into NSC biology that could not otherwise have been obtained. However, even the modest yield of NSCs from the PNS, which were not entirely pure, requires exceptional efforts. These considerations may challenge studies of NSCs ex vivo, immediately dissociated from the tissue rather than expanded in vitro. Furthermore, the same strategy cannot be applied to CNS regions like the telencephalon/cortex, which could potentially provide a sizable source of NSCs for prospective studies, since cortical cells do not express p75 *(33).*

Prospective FACS Isolation of NSCs From CNS

Several FACS strategies have recently been applied to sort NSCs from the CNS using positive and/or negative selection. Uchida and colleagues *(36)* systematically screened 50 monoclonal antibodies directed against known surface epitopes using human fetal brain cells. The survey led to the discovery that one cluster differentiation (CD) marker (CD 133), which is known to identify hematopoietic stem cells, is also expressed by a small fraction (1–6%) of fetal brain cells. Sorting of the $CD133^+$ population by positive selection revealed that these cells have the capacity to form neurospheres, initiate secondary sphere formation, and differentiate into neurons and astrocytes. In contrast, $CD133^-$ cells did not express these properties. However, sorting on CD133 expression only modestly enriched the neurosphere-initiating activity from approx 0.1% among unsorted cells to approx 3% in $CD133^+$ cells. Further enrichment was accomplished by sorting $CD133^+$ cells, which were low in their surface expression of

CD24 (heat-stable antigen). Quantitation of $CD133^{+}CD24^{-/lo}$ cells plated at clonal density showed that 5–10% generated neurospheres when cultured in a defined medium together with a cocktail of growth factors (bFGF, EGF, lymphocyte inhibitory factor and neural survival factor-1). However, only 10% of these cells could generate a secondary neurosphere after subcloning, implying that the majority of $CD133^{+}CD24^{-/lo}$ cells did not exhibit the salient properties characteristic of self-renewing NSCs. Differentiation of neurosphere cell progeny was induced by culturing these cells in defined medium with brain-derived and glial-derived neurotrophic factors instead of the aforementioned growth factors. Under these conditions, individual $CD133^{+}CD24^{-/lo}$ cells differentiated into only two of the three CNS phenotypes (neurons and astrocytes, but not oligodendrocytes). Transplantation of sorted or expanded $CD133^{+}$ population into brains of immunodeficient mice revealed that these cells proliferate and differentiate into neural phenotypes. Using this model, however, it was not directly shown that the modestly enriched fraction of $CD133^{+}$ cells that exhibited putative NSC properties in vitro was the same one generating these outcomes in vivo.

A similar positive-selection FACS strategy involving surface expression of the mouse homolog of CD24 (mCD24) and peanut agglutinin (PNA) has been applied to isolate putative NSCs from periventricular regions of the adult mouse brain *(37).* Cells expressing low levels of both PNA and mCD24, which represented only 0.3% of the unsorted population, were highly enriched in neurosphere-initiating activity. About 80% of PNA^{lo} $mCD24^{lo}$ cells isolated from periventricular zones generated neurospheres in defined medium containing two growth factors (bFGF and EGF). Secondary neurospheres derived from subcloning could be differentiated by plating them on a surface coated with a positively charged substrate (polyornithine), withdrawing the growth factors and including FCS. Under these culture conditions, neurospheres originally derived from single $PNA^{lo}mCD24^{lo}$ founders differentiated so that some of their progeny became neurons, astrocytes, and oligodendrocytes. Neurosphere cells also engrafted in vivo, differentiating into neurons and astrocytes. In addition, about half of the initially plated $PNA^{lo}mCD24^{lo}$ cells differentiated into myocytes or myotubes without proliferating when grown in the presence of C2C12 myogenic cells. Thus, a subpopulation of these cells was actually pluripotent, generating both neural and non-neural phenotypes. Unfortunately, the minute fraction of NSCs that could be derived using this model may limit studies of NSC biology, since it would require lengthy propagation of these cells in vitro, which itself may alter their physiological properties, to generate sufficient quantities of NSCs for prospective molecular and cellular studies.

In contrast to the above studies that used positive selection to access NSCs, Cai et al. *(38)* applied a negative selection strategy to directly isolate NSCs from LRPs in dissociates of the embryonic rat spinal cord. In this study, surface expressions of embryonic neural cell adhesion molecule (E-NCAM) and A2B5 were used to sort $E\text{-}NCAM^{-}$ $A2B5^{-}$ putative NSCs from $E\text{-}CAM^{+}$ $A2B5^{+}$ neuronal and glial restricted precursors (NRPs and GRPs). $E\text{-}NCAM^{-}$ $A2B5^{-}$ cells proliferated in vitro in the presence of 10% CEE. After switching to a defined medium containing bFGF together with platelet-derived growth factor BB, retinoic acid, neurotrophin-3, and triiodothyronine, most of these cells differentiated into astrocytes and neurons, while 20% were multipotential and also included oligodendrocytes. Positively selected $E\text{-}NCAM^{+}$ $A2B5^{+}$ cells did not generate multipotent clones, although what did differentiate was not reported. These results establish the feasibility of using negative and positive selection and flow cytometry to sort NSCs from LRPs in the spinal cord for prospective study in vitro. However, the use of uncharacterized CEE instead of defined media in order to propagate NSCs may have altered their biology to an unknown extent. Furthermore, the authors combined E-NCAM together with A2B5 in order to label NRP and GRP populations from the spinal cord, then sorted them as a single $E\text{-}CAM^{+}$

A2B5$^+$ population. This mixed population of progenitor cells was then used to stringently investigate the differences between NSC and LRP cell biology. Presumably, selective labeling and separation of NRP from GRP using appropriate lineage-selective surface markers conjugated to different fluorochromes would have provided a more precise insight into the biological properties of these distinct populations of cells that compose quite different lineage compartments in vivo.

Cai and colleagues *(38)* also examined the utility of using Hoechst 33342 staining properties in conjunction with FACS to distinguish NSCs from progenitor populations. Differential Hoechst staining has been previously used to isolate self-renewing/multipotential hematopoietic stem cells from other hematopoietic cells *(39,40).* The stem cells in these preparations typically exhibited the greatest efflux of the Hoechst dye, thus creating a "side population" that could be isolated by FACS. However, FACS analysis of Hoechst-loaded NECs at early and later embryonic ages did not reveal a population of vital cells with the staining characteristics of the side population. Rather, this population consisted entirely of cell debris, demonstrating that the Hoechst staining strategy is not useful for isolating NSCs from the spinal cord.

A completely different FACS strategy was applied to isolate NSCs from the embryonic and adult CNS using a genetic model *(41).* The authors developed a transgenic mouse line in which nestin enhancer-derived transcriptional activation can be visualized by the fluorescence associated with enhanced green fluorescent protein (EGFP) expression. Although nestin is not exclusively expressed by NSCs, the level of EGFP fluorescence signal intensity was found to vary fivefold among EGFP$^+$ embryonic cortical cells, all of which expressed nestin. FACS was used to sort highly fluorescent EGFP^{2+} cells from EGFP$^+$ and EGFP$^-$ cells. EGFP^{2+} cells, which were devoid of intracellular epitopes characteristic of differentiating neural phenotypes, gave rise to clones containing neurons and astrocytes, while only approx 3% generated neurospheres. However, FACS sorting of EGFP2^{2+} cells was found to be deleterious to the ability of these cells to generate neurospheres compared to unsorted cells. One explanation would be that these cells were the most susceptible to laser-induced phototoxic damage after excitation of EGFP. Although many of the sorted cells initially appeared viable, they died within a day or two. The great majority of the neurospheres derived from surviving EGFP^{2+} cells exhibited multipotential differentiating capability when cultured sequentially in defined medium with bFGF and EGF and neurosphere-conditioned medium and then switched to medium with fetal calf serum and retinoic acid. EGFP^{2+} cell-derived neurospheres could be subcloned and passaged repeatedly, indicating that the sorted population exhibited both self-renewal and multipotentiality. EGFP$^+$ cells were also sorted from periventricular regions of the adult transgenic mouse CNS. These studies revealed that 0.3% of EGFP$^+$ and 0.07% of EGFP^{2+} cells formed neurospheres, which could be subcloned and exhibited multipotentiality. Thus, adult CNS yielded a much lower number of EGFP-expressing cells that had the salient properties of NSCs compared to those isolated from the embryonic CNS. The authors concluded that this FACS strategy based on nestin enhancer-driven EGFP intensity is an effective method for sorting NSCs, but that nestin-derived EGFP signals are not an exclusive marker of NSCs. They recommended a sorting strategy combining EGFP intensity with surface markers. However, the possible phototoxic damage inherent in the positive selection of NSCs based on EGFP expression may necessitate the use of alternate FACS strategies using negative selection in order to maximize the viability of sorted NSCs and minimize deleterious effects on NSC biology observed in vitro.

Collectively, the above FACS strategies used to identify and to isolate NSCs from the PNS and CNS represent important initial steps to access these cells for prospective studies. These studies demonstrate that FACS technology coupled with positive and/or negative selection can be applied with varying degrees of

efficacy to sort NSCs. The initial FACS studies also revealed the complex and variable conditions required in supporting the proliferation of NSCs and their differentiation into neural phenotypes. In this regard, different growth factors, growth factor concentrations, growth factor schedule protocols, FCS, CEE, neurosphere-conditioned medium, and astrocyte-conditioned medium have been employed to sustain and study NSC biology. Although these inclusions vary from study to study and have been employed in a relatively empirical manner, it is evident that they are critically required to investigate self-renewal and differentiation of NSCs. Some of the variable requirements are likely related to the different biologies of NSCs isolated from different CNS regions at different ages. However, it should be evident that pure populations of FACS-sorted NSCs can both reduce some of the experimental variability and also facilitate investigation into the cellular and molecular basis underlying their self-renewal and multipotential differentiation. Unfortunately, none of the aforementioned FACS strategies have provided abundant sources of highly pure populations of NSCs that exhibit either long-term self-renewal without differentiation or the ability to undergo multilineage progression, when exposed to instructive differentiating conditions. Furthermore, comprehensive FACS strategies to effectively separate NSCs from different types of LRPs, such as NRP and GRP, in order to study their shared and unique biological properties, have been conspicuously absent. The development of such strategies is pivotal to better understanding of the cellular and molecular mechanisms that mediate the initial NSC commitment to specific lineages.

Ganglioside Expression Patterns Identify CNS Progenitors From NSCs

We have recently developed a novel surface-staining strategy to phenotype living neuroepithelial cells in terms of their precursor/progenitor and differentiating state, which is readily applicable to all developing CNS regions in vertebrates including humans. We targeted several complex gangliosides, which become ubiquitously expressed during phylogenetic evolution of vertebrate nervous systems and are recapitulated during ontogeny of the mammalian CNS *(42).* The structurally related di- and tri-sialogangliosides GD3 and GT1b are especially abundant during the embryonic period *(43,44).* GT1b binds tetanus toxin (TnTx) *(45–48),* while GD3, as well as several other gangliosides including GT3 and GQ1c, can be labeled with the monoclonal antibody A2B5 *(49–52).* TnTx has been previously used to identify differentiating and postmitotic neurons *(53,54),* while A2B5 was used to label O-2A progenitor cells, type 2 astrocytes and certain types of neurons *(55–59).* We also targeted the ganglioside GM1 using cholera toxin *(46)* to identify neuronal progenitors and differentiating neurons *(60)* and the 9-*O*-acetylated form of GD3, which can be identified with the JONES antibody *(61),* to label O-2A progenitors and migrating neurons *(52,62–64).*

In previous studies, we found that specific gangliosides such as GD3 and GT1b and other epitopes appear on the cell surface of proliferating LRPs during the earliest phases of neuronal and glial differentiation throughout the entire embryonic rat CNS, including the spinal cord *(65,66).* We reasoned that neural precursor cells, which do not yet express these surface gangliosides, indicative of differentiating LRPs, might be a source of undifferentiated NSCs (*67;* also *see* Fig. 2B1). Since proliferating precursors and immature progenitors compose most of the cortical neuroepithelium at the onset of neurogenesis (*66;* also *see* Fig. 1), we selected E13 rat telencephalon as an ideal CNS region to prospectively isolate large numbers of NSCs and different types of LRPs. In order to identify the vital populations of NSCs and LRPs, we stained the cells with annexin V, an anticoagulant protein that preferentially binds to phosphatidylserine phospholipids exposed on the surface of cells undergoing programmed cell

death *(68,69).* When used in conjunction with forward-angle light scatter, a flow-cytometric property related to cell size, annexin V labeling can additionally discriminate among apoptotic, necrotic, and nonapoptotic cells (*67;* also *see* Fig. 2B2). Differential staining of E13 rat telencephalic dissociates, using a combination of neuron-specific (ChTx, TnTx) and neuroglial progenitor-selective (A2B5, JONES) markers and annexin V revealed five discrete populations of vital cells that included putative NSCs, neuroglial-restricted progenitors (NGRP), oligoglial-restricted progenitors (OGRP), and two subpopulations of neuron-restricted progenitors (NRP) (Fig. 2B1,B2). In order to isolate NSCs, we used a negative-selection FACS strategy to sort a quintuple epitope-negative ($ChTx^-$ $TnTx^-$ $A2B5^-$ $JONES^-$ annexin V^-) population of undifferentiated and nonapoptotic precursor cells. In contrast, a positive-selection strategy was employed to isolate vital NRP, NGRP, and OGRP populations based on their expression of specific patterns of differentiating markers at various levels of intensity (Fig. 2B1,2B2).

Sorted NSC and LRP populations were reanalyzed by flow cytometry to determine the level of purity in the isolates. More than 98% of each LRP population exhibited the surface markers characteristic of their lineage-restricted phenotype, while NSCs did not express any of the five surface markers used for sorting. Multiepitope staining of acutely plated NSCs further demonstrated that the great majority were $nestin^+$ and proliferative, as revealed using a 2-h pulse labeling in vivo with bromodeoxyuridine prior to cell preparation, and >95% lacked differentiating epitopes characteristic of neuronal, neuroglial, oligodendroglial, astroglial or microglial phenotypes (Fig. 2C1,C2). In comparison, NRP, NGRP, and OGRP cells expressed differentiating intracellular and/or nuclear markers characteristic of early neuronal and/or glial lineage progressions. In addition, like the NSC population, a great majority of NGRP, OGRP, and NRP cells isolated from E13 rat telencephalon also expressed nestin *(67),* implying that most of these cells are immature and that nestin cannot be used as a sole marker in distinguishing NSCs from LRPs.

Clonal analysis of sorted NSC, NGRP, OGRP, and NRP cells in defined culture media containing bFGF *(67)* further revealed that each population exhibited the potential to expand from a single founder cell. Under this growth condition, 73% of primary NSC clones rapidly proliferated over 7 d in culture, producing up to six generations of undifferentiated $nestin^+$ progeny that lacked epitopes characteristic of differentiating neuronal or glial progenitors (Fig. 3A1,A2). The remaining putative NSCs either expanded, generating differentiating progeny (<5% of total cells plated), or remained solitary and either died (12% of total cells plated), remained undifferentiated (~5%), or differentiated into postmitotic neurons (~2%), radial glia (~1%), or astrocytes (~2%). The presence of rare differentiating phenotypes in the NSC population may reflect the low probability that either NSC precursors can spontaneously differentiate in bFGF medium or that few newly committed LRPs, which do not yet express differentiating surface ganglioside epitopes, were included in the NSC population after sorting. However, subcloning the predominantly undifferentiated NSC progeny from primary cultures in the presence of 10 ng/mL bFGF produced an increasing percentage of clones with exclusively self-renewing progeny, so that by the third passage >96% of clones were self-renewing. Furthermore, we have successfully subcloned and expanded the self-renewing progeny of NSCs in bFGF-containing growth medium for at least 10 passages, without overt changes in their immature phenotype, thus demonstrating the uncompromised potential of these cells to perpetually self-renew and generate virtually unlimited numbers of NSCs.

Acidic FGF mimicked the effect of bFGF in supporting self-renewal of E13 cortical NSCs (unpublished observations). However, substitution of bFGF with EGF induced only a fraction (~12%) of these cells to undergo self-renewal in primary cultures *(67),* while the addition of other growth factors commonly

used in studies of NSC biology was ineffective. The relative percentages of bFGF- and EGF-responsive NSCs paralleled the relative number of primary founders expressing FGFR-1, a dominant receptor for bFGF, and EGFR *(67)*, implying that negative FACS selection based on surface ganglioside markers, as described here, includes at least two subpopulations of NSCs. The data are also consistent with the previous findings of Tropepe and colleagues *(25)* that bFGF-responsive and EGF-responsive NSCs do coexist in vivo, while being phenotypically indistinguishable.

Interestingly, addition of EGF together with bFGF to the culture medium permitted both self-renewal of NSCs and a 10-fold increase in differentiation of their progeny into neuronal, astroglial and oligodendroglial phenotypes (Fig. 3B1,B2), revealing that both growth factors are required to induce multipotential lineage progression from these cells. Based on these findings, we hypothesize a dual role of EGF in cortical NSC development. EGF promotes self-renewal of EGF-responsive NSCs, and induces the emergence of EGFR on bFGF-responsive NSCs, which in turn primes these cells to undergo multilineage differentiation, rather than self-renewal. It remains to be determined whether bFGF-responsive and EGF-responsive NSC subpopulations constitute independent ontogenetic compartments or if these cells are derived from the same precursor population.

It should be mentioned that bFGF is also crucial in promoting initial clonal expansion and differentiation of NGRP (Fig. 3C1,C2), OGRP (Fig. 3D1,D2) and NRP (Fig. 3E1,E2) cells along their expected lineages *(67)*, implying that commonly used growth conditions with bFGF would not be an effective method to selectively enrich unsorted NSCs from heterogeneous preparations of NECs. In fact, we ascertained the utility of using sort-purified NSC and LRP populations to study differential effects of bFGF and EGF on self-renewal and differentiation, by culturing the nonsorted NECs isolated from E13 rat telencephalon, under the same growth conditions used for sorted cells *(67)*. This starting preparation contained a heterogeneous population of both NSCs and LRPs (Fig. 2B1,B2). The cells in the unsorted cultures proliferated in the presence of bFGF, but not EGF, similar to the results obtained with sorted NSC and LRP populations. However, the composition of the proliferating cell progeny consisted primarily of cells progressing along neuronal and glial lineage phenotypes, rather than of self-renewing, undifferentiated NSCs. This is consistent with the fact that at this stage of cortical development many NSCs and LRPs express FGFR-1 *(67)*, a primary receptor for bFGF, and have the potential to proliferate in the presence of bFGF, rather than EGF. However, since the starting population of viable/nonapoptotic NSCs in E13 cortical dissociates before culture is only approx 22% of unsorted cells *(67)*, the proliferation in bFGF-containing medium is biased toward expanding neuronal and glial progenitors, rather than NSCs. We have also observed that after 7 d in culture in defined medium with bFGF, the expanding neuronal and glial progenitor populations of nonsorted NECs greatly outnumbered the NSC population, which at that point represented less than 5% of the total cells. These findings reinforce the critical importance, the utility, and the efficacy of the sorting strategy using multiple surface ganglioside expressions to isolate NSCs from different LRP populations for more precise and prospective studies into their biological properties ex vivo and in vitro. Furthermore, the unique use of negative selection to isolate NSCs, as described here, provides an additional advantage by eliminating any concerns about possible adverse biological or laser-based phototoxic effects involving certain labeling reactions used in positive selection of these cells, as reported by others. It should be noted, however, that positive selection of different types of LRPs using surface ganglioside expressions, as detailed above, did not produce overt effects either on cell viability or their lineage-restricted potentials in vitro *(67)*, demonstrating that this strategy is effective in accessing the biology of these cells.

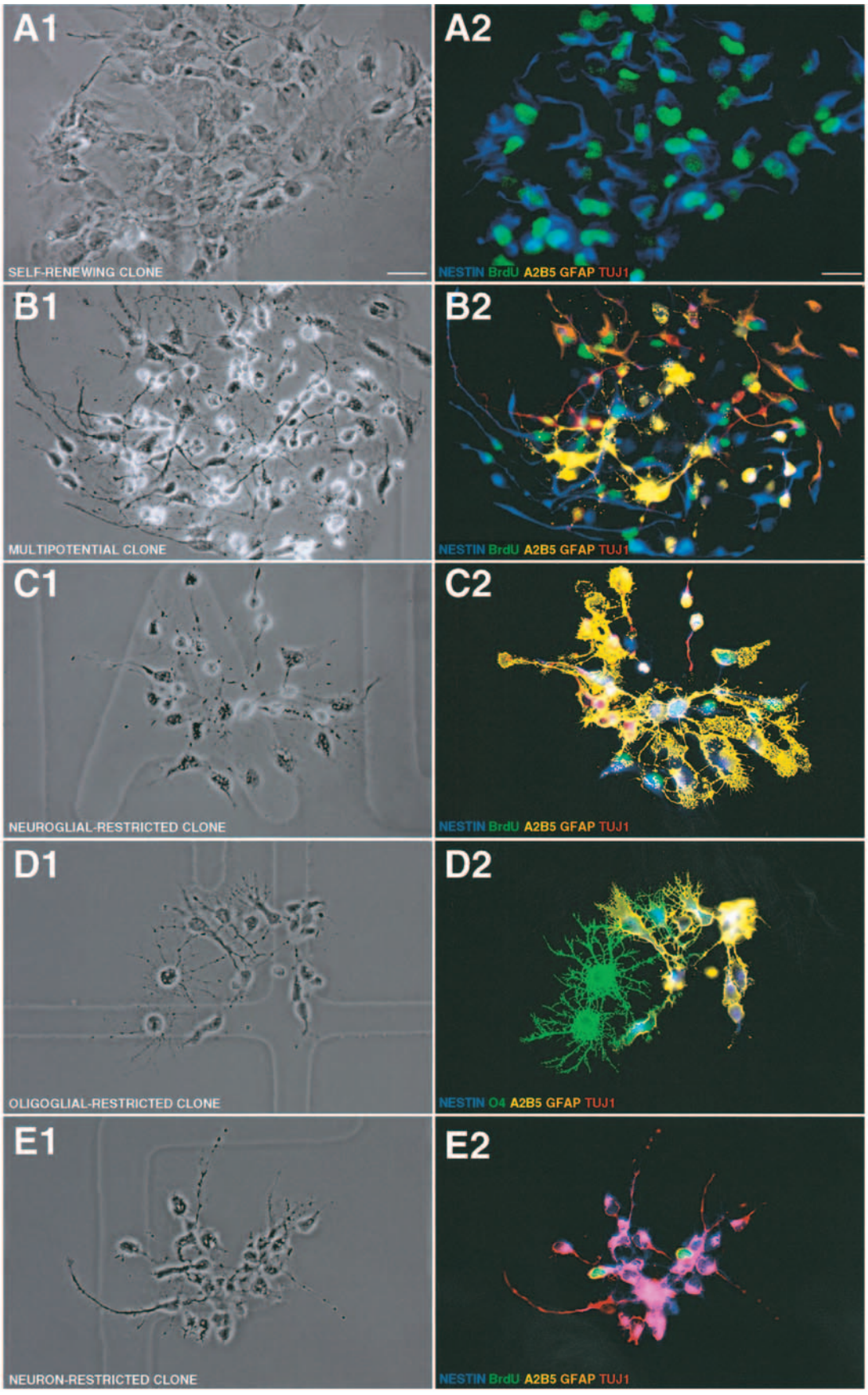

A1
SELF-RENEWING CLONE
A2
NESTIN BrdU A2B5 GFAP TUJ1
B1
MULTIPOTENTIAL CLONE
B2
NESTIN BrdU A2B5 GFAP TUJ1
C1
NEUROGLIAL-RESTRICTED CLONE
C2
NESTIN BrdU A2B5 GFAP TUJ1
D1
OLIGOGLIAL-RESTRICTED CLONE
D2
NESTIN O4 A2B5 GFAP TUJ1
E1
NEURON-RESTRICTED CLONE
E2
NESTIN BrdU A2B5 GFAP TUJ1

Fig. 3. Stereotypical phenotypes of clonal progeny identify NSCs from LRPs. FACS-purified NSC, NGRP, NRP, and OGRP isolated from the E13 rat telencephalon were clonally expanded for 7 d in defined culture media (Neurobasal/B27) supplemented with 10 ng/mL bFGF **(A,C–E).** NSCs were also clonally expanded in 10 ng/mL bFGF and 10 ng/mL EGF to promote multipotential differentiation **(B).** Quantitative phenotypic analysis was carried out by counting the number of undifferentiated precursors and differentiating progenitors in each expanded clone using both morphological appearance under phase-contrast optics **(A1–E1)** and expression patterns of different stage- and/or lineage-specific markers (nestin, BrdU, A2B5, GFAP, TUJ1, O4), as previously described *(67).* The distribution of each epitope was visualized separately under appropriate fluorescence optics and is shown here as merged images color-coded to the epitope identified in each panel **(A2–E2).** Based on the dominant phenotype of their expanded progeny, the clones were classified into five categories: self-renewing, multipotential, neuroglial-restricted, neuron-restricted, and oligodendroglial-restricted. **A1,A2** Progeny of a self-renewing NSC clone expanded in Neurobasal/B27/bFGF culture medium are entirely composed of morphologically epithelioid, immature (nestin$^+$, blue) precursors, which actively proliferate (BrdU$^+$, green) but do not express differentiating epitopes (TUJ1$^-$A2B5$^-$GFAP$^-$). **B1,B2** Clonally expanded differentiating progeny of a NSC cultured in Neurobasal/B27/bFGF+EGF are typically composed of proliferating nestin$^+$ immature precursors and nestin$^+$ process-bearing progenitors (blue) differentiating along neuronal (TUJ1$^+$, red), neuroglial (A2B5$^+$, yellow) and astroglial (GFAP$^+$, orange) lineages. **(C1,C2)** Expanding progeny of the NGRP clone are process-bearing nestin$^+$A2B5$^+$TUJ1$^-$ (blue and yellow, but not red) non-neuronal progenitors and nestin$^+$A2B5$^+$TUJ1$^+$ (blue, yellow, and red) immature neurons, but not GFAP$^+$ astrocytes. **(D1,D2)** Clonally-expanded progeny of an OGRP cell typically exhibit markers of immature nestin$^+$A2B5$^+$O4$^-$ (blue and yellow, but not green) and differentiating nestin$^+$ A2B5$^+$ O4$^+$ (blue, yellow, and green) oligoglial progenitors and transitional nestin$^-$ A2B5$^-$O4$^+$ (green, but not blue or yellow) oligodendrocytes. *(E1, E2)* Progeny of the NRP clone are phase-bright and process-bearing nestin$^+$TUJ1$^+$ (blue, red, and pink, at sites where these two epitopes co-localize) neuronal progenitors and nestin$^-$TUJ1$^+$ (red) postmitotic neurons. Calibration bar: 20 µm. Panels **A1, A2, B1,** and **B2** are adapted from Maric et al. *(67).*

It should further be noted that the utility of this and all FACS analysis and cell-sorting strategies depends critically on the quality of cell preparation and dissociation. The importance of dissociation and reproducible recovery of CNS cells becomes clear when commonly used enzymatic and mechanical protocols are compared and the results quantitatively analyzed by flow cytometry *(70,71).* In this regard, optimized dissociation protocols using papain digestion followed by gentle trituration consistently generate the highest and most reproducible yields of vital cells when compared with all other methods, particularly trypsin digestion or mechanical dissociation, which lead to far fewer cells recovered and significant numbers of dead cells. Without an optimal preparative strategy, the ex vivo and in vitro studies of NSC and LRP biological properties during CNS development will be inherently compromised due to the initial loss of the very cells that are intended for study. Therefore, the optimized cell preparation protocol using papain dissociation has the potential to provide an unparalleled and virtually complete perspective on the dynamically changing cell populations composing the embryonic vertebrate CNS and on their evolving physiological properties.

One should also consider the spatiotemporal events occurring during CNS development in designing the optimal FACS strategy to prospectively isolate large numbers of NSCs and different types of LPRs. In this regard, a multiepitope staining strategy similar to that outlined in Fig. 2 has been used to quantify the abundance of undifferentiated precursor and differentiating progenitor populations throughout the rodent CNS from the beginning of neurogenesis to the end of gliogenesis *(65).* As expected, the highest

abundance of undifferentiated precursors without surface-detectable neuronal or glial markers emerges at the beginning of neurogenesis (E11–E13), when the cells expanded logarithmically in all CNS regions *(70).* However, these cells precipitously decline thereafter, as the number of differentiating LRPs and postmitotic phenotypes progressively increases. Thus, the mid-embryonic period at the onset of neurogenesis provides the best opportunity to isolate large numbers of NSCs from the CNS by FACS, whereas later developmental ages are more suitable for sorting large numbers of LRPs. Nevertheless, NSCs can still be readily sorted throughout CNS development, albeit with progressively lower yields of recovery during the late embryonic and early postnatal periods, from all CNS regions since vital quintuple epitope-negative cells can consistently be identified in each region and isolated by negative selection as a pure NSC population. Direct isolation of NSCs and LRPs at different periods of CNS development and from different CNS regions will permit comparative studies of NSC and LRP biology in a prospective manner. The insights from these studies could, in turn, be used to better understand how NSCs and different LRPs regionally evolve as the biology of the CNS changes, as has been suggested by previous retrospective studies.

Additionally, it would be of interest to relate surface ganglioside markers (TnTx, ChTx, A2B5, and JONES) used in our FACS strategy with those used by others in the NSC field (CD133, SSEA1, CD24, PNA, E-NCAM). We have recently carried out such comparisons on E13 rat telencephalic NSCs using the commercially available rat-specific markers CD24, PNA and E-NCAM. The results revealed that <2% of the sorted quintuple-negative ($TnTx^-ChTx^-A2B5^-JONES^-$ annexin V^-) NSCs expressed CD24, PNA, or E-NCAM, thus making them octuple-negative ($CD24^-PNA^-E\text{-}NCAM^-TnTx^-ChTx^-A2B5^-JONES^-$annexin V^-). Although these findings are in agreement with those previously described by others, they do not provide a significant improvement in the quintuple-negative FACS strategy described in this review. With regard to CD133 and SSEA1 markers, there are currently no commercially available antibodies that are also reactive for rat cells, thus making the utility of these markers for characterization of rat NSCs unclear at present. Future studies utilizing surface ganglioside markers in conjunction with CD133 and SSEA1 labeling in murine and human NSC preparations will provide additional insight regarding the utility of these markers in purifying NSCs.

Molecular Investigations of NSCs and LRPs

The inaccessibility of pure populations of NSCs and LRPs from heterogeneous neurosphere and neuroepithelial preparations did not discourage systematic studies into gene-expression patterns in attempting to elucidate the underlying molecular mechanisms that dictate the biology of these cells. This has led to some innovative methods toward analyses of contrasting gene expression profiles of mixed populations of NSCs and LRPs under self-renewing and differentiating culture conditions. In this regard, Geschwind and colleagues *(72)* used representational difference analysis (RDA) in which cDNA derived from differentiating neurospheres was subtracted from neurospheres maintained under growth conditions and vice versa. Several rounds of RDA subtraction were used, followed by cloning of the subtracted products, then screening of subtracted product arrays with cDNAs derived from neurospheres before and after inducing differentiation and subsequent sequencing of the differentially expressed clones. An important final step included *in situ* hybridization screening of embryonic and adult CNS sections to locate the differentially expressed genes. The authors reported that virtually all of the growth-related genes were restricted in their anatomical expressions to the germinal zones of the developing mouse brain and the rostral part of the adult forebrain, while genes enriched in differentiating neurospheres were located outside the

germinal zones. In addition, a number of neurosphere growth-related genes in this and a subsequent study *(73)* were also found to be expressed in FACS-sorted hematopoietic stem cells, indicating that certain genes may be active in stem cells giving rise to cells in different tissues with quite different functions. However, in both these studies neurospheres remained composed of heterogeneous cell populations even after multiple passages under nondifferentiating growth conditions, with NSCs estimated to comprise only 3–4% of total cells and different types of LRPs and differentiating cells presumably representing the rest. Thus, the RDA subtraction would not be conducive to resolving differential gene expression as NSCs initially commit to neuronal and/or glial LRPs, since the undifferentiated neurosphere preparations used in microarray analyses contained all these types of cells, the least of which were true NSCs.

Molecular phenotyping of NSC and LRP biology was also carried out by Suslov et al. *(30),* who generated neurospheres of varying size from the developing human hippocampus and subependymal zone. The results revealed interclonal/interneurosphere heterogeneity with unique patterns of gene expression associated with neurospheres of different sizes, leading the authors to hypothesize that at least four types of clone-forming neural stem/progenitor cells are present in the starting preparation. However, no direct evidence was presented that these types of clone-forming cells are true NSCs, rather than different LRPs, that can also clonally expand.

In another study, select growth factors, including bFGF, EGF, and leukemia inhibitory factor (LIF), were used to propagate neurosphere cultures derived from human fetal cortical cells prior to molecular phenotyping *(74).* In this model, approx 90% of the cells in the neurospheres were nestin$^+$ and GFAP$^+$. Plating of whole spheres under differentiating conditions without growth factors showed that about 30% of these cells differentiated into neurons. This suggests that the nestin$^+$GFAP$^+$ cells could give rise to neuronal progeny and thus may represent neurogenic glia or radial glial cells, that have previously been shown to generate neurons during neurogenesis *(75–78).* Affymetrix chip microarray analysis of long-term neurospheres revealed that more than 33,000 known genes and expressed sequence tags (ESTs) could be detected. Since LIF was found to extend neurosphere self-renewal, microarray analysis was carried out following LIF withdrawal to identify LIF-dependent changes in gene expression that supported self-renewal. More than 500 genes (~4%) decreased and nearly 200 genes (1.5%) increased in relative abundance following LIF withdrawal, implying complex changes of gene expression as NSCs commit to differentiate. However, most of the putative NSCs in this study exhibited a nestin$^+$GFAP$^+$ phenotype, and thus presumably represented a specific type of LRP, with the bipotential capacity to generate neurons and astrocytes. Thus, it remains unclear how this population of cells relates to nestin$^+$ self-renewing and multipotential NSCs that emerge at the beginning of CNS development, which do not express any differentiating markers, including GFAP.

Recently, Luo et al. *(79)* used microarray analyses on acutely prepared NECs as well as FACS-sorted neuronal and glial restricted precursors isolated from the embryonic rat neural tube. Such analyses are indeed useful in revealing changing gene expression patterns occurring during development in vivo and provide a natural comparison with results obtained in vitro using neurosphere strategies. In this study, seven different focused microarrays covering approx 500 genes of interest were surveyed. About 30% of the probed genes were detected in unsorted NECs isolated from the neural tube at embryonic day 10.5 (E10.5), while 43% were detected at E14.5 when most of the cells in the neural tube have begun to differentiate. While 73% of the genes were expressed at both ages, only 11 were restricted to E10.5 NECs, and 68 encoding cytokines, chemokines, and their receptors and cell cycle regulators were restricted to E14.5 NECs. The authors also used single-labeling protocols for embryonic neural cell adhesion molecule (E-NCAM) to sort NRPs and

A2B5 to sort GRPs from E14.5 neural tube and then compared the gene expression patterns of each population. Nine genes were expressed by both subpopulations, 10 were restricted to E-NCAM$^+$ progenitors, and 5 to A2B5$^+$ progenitors. Restricted expressions of these genes were confirmed using *in situ* hybridization, immunocytochemistry, and immunoblots. The authors, however, did not take into consideration that the A2B5 labels both neuronal and glial restricted lineages *(53,56,66)* and therefore should be used in conjunction with other markers, such as TnTx or ChTx, to distinguish NRP from GRP.

In contrast to these studies, we have simultaneously used multiple cell surface markers reporting lineage differentiation or death in conjunction with FACS to isolate NSCs and NGRP, OGRP, and NRP populations directly from the embryonic rat telencephalon at the beginning of neurogenesis (Fig. 2B1,B2). These purified populations were then immediately processed for microarray analyses in order to avoid the unknown effects of short- or long-term cultures on gene expression patterns of these cells. An initial study was carried out using microarrays with Human Known Gene Filters (GF211, Invitrogen, Carlsbad, CA). Comparative analysis of molecular profiles showed that virtually all of the 4,324 genes surveyed (see ftp://ftp.resgen.com/pub/ genefilters for the list of genes) could be detected in the directly-sorted populations. Comparison of NSCs to NGRPs showed that approx 80% of these genes were equally expressed in both populations, while almost all of the remaining 20% were overexpressed in NGRPs. Only approx 1% of the surveyed genes were underexpressed in the latter population. Thus, with this particular molecular analysis, the transition from NSC to NGRP in vivo is accompanied by an overexpression in a relatively modest number of genes unique to NGRP. By comparison, the transition from NSC to NRP was accompanied by an overexpression in 7% of the genes, with 33% of the genes becoming underexpressed. The greatest contrast, however, was observed in the comparison between NSCs and OGRPs, where 52% of the genes were overexpressed and 14% became underexpressed. Although the identity and the functional role of these genes are still under investigation, the data demonstrate a proof of principle in using purified populations of NSCs and LPRs to determine the gene expression patterns at the earliest stages of NSC commitment to different cell fates.

In sum, recent attempts to apply molecular profiling using microarrays focused on genes of interest represent important initial steps in understanding the changing gene expression patterns as NSCs undergo commitment to differentiating cell phenotypes. However, prospective ex vivo sorting of pure populations of NSCs and different types of LRPs will clearly provide greater resolution and insight into gene expression patterns underlying cell fate determination in vivo rather than inferring such interpretations based on aggregate signals from heterogeneous cell populations expanded as neurospheres in vitro.

Neurotransmitter-Gated Receptors/Ion Channels Differentiate NSCs From LRPs

There has been increasing evidence that different neurotransmitters acting at specific ligand-gated receptors/ion channels can modulate the proliferation and differentiation of NECs and LRPs *(80–88).* With regard to NSCs, it has been reported that at the earliest stages of CNS development approx 50% of NECs isolated from E10.5 rat spinal cord *(38)* exhibit functional acetylcholine (ACh) receptors, which, when activated, regulate cytosolic Ca^{2+} (Ca_c^{2+}). These cells did not respond to other transmitters tested, including γ-aminobutyric acid (GABA), glutamate (GLUT), glycine (GLY), or dopamine (DA), nor did they exhibit detectable voltage-sensitive Ca^{2+} channels (VSSC) activated by elevated extracellular K^+ (K_o^+). The authors inferred that ACh-responding cells were NSCs, but did not provide direct evidence confirming this conclusion, nor did they identify the phenotype(s) of

the nonresponding population of NECs that constituted the other 50% of the preparation. Presumably, these other cells could have equally represented the true NSCs as the ACh-responding cells inferred by the authors.

By comparison, similar experiments carried out on E-NCAM$^+$ NRPs isolated from E12 mouse or E13.5 rat neural tube preparations revealed changing patterns of neurotransmitter responses as NPRs differentiated in culture *(89,90).* At the beginning of culture, many E-NCAM$^+$ NRPs responded to ACh, GABA, GLUT, and elevated K_o^+, while few responded to GLY or DA. After differentiation, the Ca_c^{2+} responses to GABA and GLY disappeared, while those to GLUT, ACh, and DA increased. However, the progeny of clonally expanded E-NCAM$^+$ precursors exhibited a significant degree of clone-to-clone heterogeneity in transmitter-induced Ca_c^{2+} responses. In contrast, E-NCAM$^+$ NRPs immunopanned from embryonic human brain tissues did not show robust Ca_c^{2+} signals to ACh *(91)* at the beginning of culture. This response did emerge, however, along with Ca_c^{2+} responses to GABA, GLUT, DA, and norepinephrine, as NPRs differentiated into neurons. Additionally, a similar investigation carried out on two cell lines of human neural precursor cells showed that maturing neuronal precursors all responded to ACh with a Ca_c^{2+} signal and about 50% responded to GLUT *(92).* Responses to GABA, GLY, DA, and elevated K_o^+ were either absent or found in fewer than 5% of these cells. Altogether, these investigations revealed an early and widespread appearance of cholinergic receptors during neuronal differentiation, while other transmitter receptors/ion channels coupled to Ca_c^{2+} regulation emerged after cholinergic receptors have appeared. However, these studies did not definitively show that the cholinergic receptors are expressed by true NSCs before their commitment to NRPs. Such a determination would require an effective separation of NSC from NRPs before characterizing their functional signaling properties.

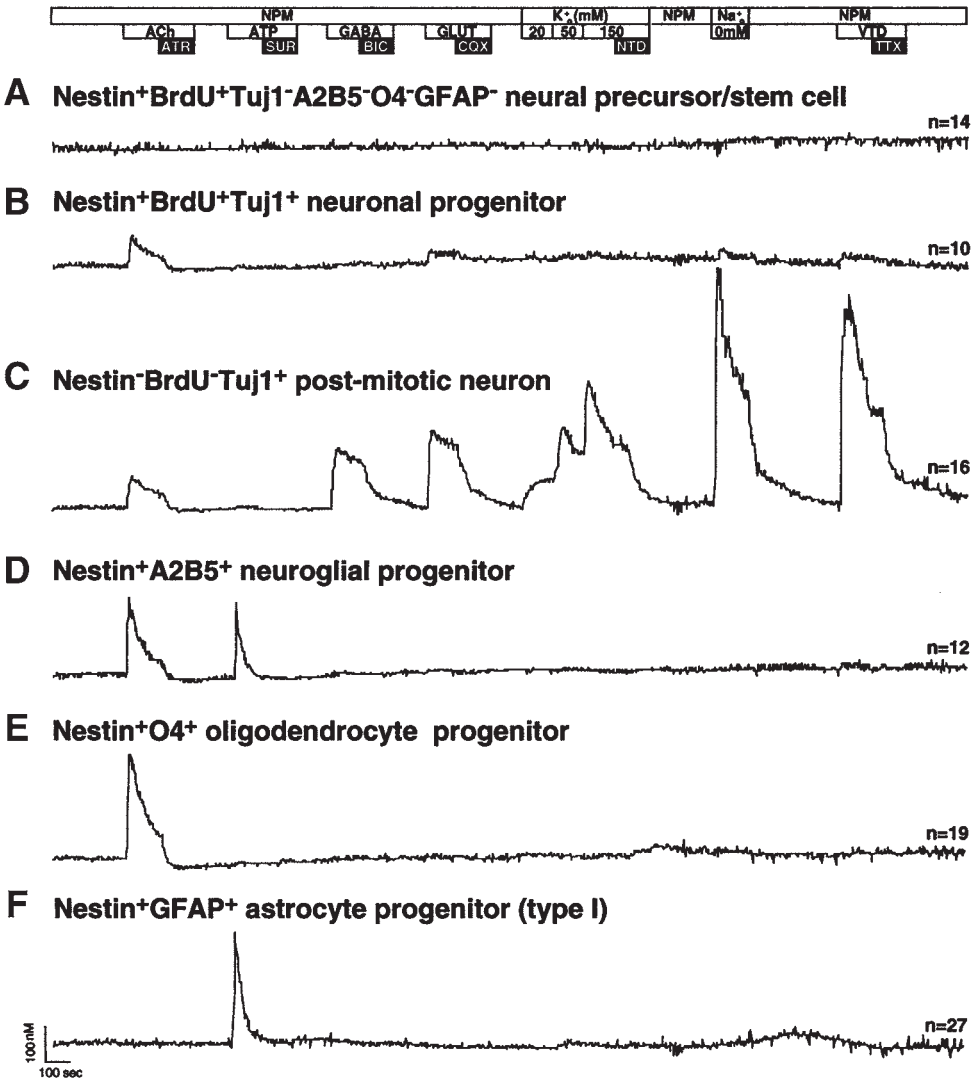
The possible developmental roles of cholinergic receptors during early neurogenesis have recently been studied in some detail using a heterogeneous population of bFGF-expanded NECs isolated from the embryonic rat telencephalon *(93).* The authors demonstrated that muscarinic, but not nicotinic, agonists stimulated an increased production of neuronal progenitors from NECs. These effects involved cholinergic signaling via three muscarinic receptor subtypes (m2, m3, m4) whose activation leads to Ca_c^{2+} responses with transient and sustained components. Muscarinic receptor-coupled regulation of Ca_c^{2+} levels correlated with the proliferation of neuronal progenitors since blocking changes in Ca_c^{2+} attenuated both proliferation and the production of more neurons. Similar cholinergic control of NEC progression along the neuronal lineage may occur in vivo since cholinergic neurons become differentiated at the beginning of neurogenesis in the forebrain *(94).* However, it remains to be determined whether cholinergic signaling underlies the mechanisms involved in the transformation of NSC to NRPs or, alternatively, in the transition of NRPs to differentiating neurons. Sorting both populations ex vivo followed by systematic analysis of their neurotransmitter receptor/ion channel properties under self-renewing and differentiating culture conditions will be a necessary first step in answering this crucial question.

We have previously characterized stereotypical neurotransmitter receptor/ion channel properties emerging on neural precursor, neuronal and glial progenitor, and differentiating phenotypes isolated from embryonic rat cortex at the end of neurogenesis *(66).* In order to determine at what stage these mechanisms become functional as NSCs initially commit to different LRPs at the beginning of neurogenesis, we isolated NSCs by FACS from E13 rat telencephalon, then instructed these cells to undergo self-renewal or multilineage differentiation under defined culture conditions *(67).* The membrane properties of the resulting undifferentiated progeny and progeny differentiating along neuronal, neuroglial, astroglial, and oligodendroglial lineages were characterized using Ca^{2+} imaging in conjunction with a test array of

Fig. 4. Diverse and stereotypical patterns of neurotransmitter-gated receptor and ion channel mechanisms regulating cell Ca^{2+} levels emerge among the differentiating NSC-derived progeny, but not self-renewing NSCs. FACS-purified NSCs were cultured at clonal density for 7 d in bFGF/EGF-containing Neurobasal/B27 medium and their self-renewing and differentiating progeny imaged for cytosolic Ca^{2+} (Ca_c^{2+}) responses to an array of neurotransmitters (NT) including acetylcholine (ACh), adenosine triphosphate (ATP), γ-aminobutyric acid (GABA), and L-glutamate (GLUT). The specificity of the NT responses was evaluated using selective antagonists to muscarinic receptors (atropine, ATRP), P2 purinoreceptors (suramin, SUR), $GABA_A/Cl^-$ channels (bicuculline, BIC) and kainate receptors (6-cyano-7-nitroquinoxaline-2,3-dione, CQX). The cells were also tested for expression of functional voltage-sensitive Ca^{2+} channels (VSCC) activated by elevated extracellular K^+ (K^+_o) and blocked by L-type Ca^{2+} channel blocker nitrendipine (NTD), voltage-sensitive Na^+ channels (VSSC) activated by veratridine (VTD) and blocked by tetrodotoxin (TTX), and Na^+-Ca^{2+}-exchanger activity revealed by perfusion in Na^+_o-free saline. After imaging, the cells were immunostained for epitope expression patterns (*see* Fig. 3) to precisely correlate their Ca_c^{2+} regulatory mechanisms with their precursor or differentiation state ***(A–F). (A)*** Self-renewing NSCs, which are by definition nestin $^+$BrdU$^+$Tuj1$^-$A2B5$^-$O4$^-$GFAP$^-$, do not respond to the test array of NT, VSCC, VSSC, and Na^+-Ca^{2+}-exchanger activity. **(B)** Proliferating nestin$^+$BrdU$^+$TUJ1$^+$ neuronal progenitors typically exhibit low amplitude Ca_c^{2+} responses to ACh and GLUT and just-detectable responses to Na_o^+-free saline and VTD. **(C)** Postmitotic nestin$^-$BrdU$^-$Tuj1$^+$ neurons show large-amplitude Ca_c^{2+} responses to ACh, GABA, and GLUT, which are sensitive to their respective antagonists. These cells also exhibit NTD-sensitive VSCC, TTX-sensitive VSSC, and robust Na^+-Ca^{2+}-exchanger activity. **(D)** In contrast, emerging nestin $^+$A2B5$^+$ neuroglial progenitors exhibit Ca_c^{2+} responses to ACh and ATP, but not to GABA or GLUT. These cells also do not exhibit functional VSCC, VSSC, or Na^+-Ca^{2+}-exchanger activity. **(E)** Differentiating nestin$^+$O4$^+$ oligodendrocyte progenitors manifest Ca_c2^+ responses to ACh alone. **(F)** In contrast, early nestin$^+$GFAP$^+$ astrocyte progenitors are responsive only to ATP. The data show a stereotypical lineage-restricted distribution of excitable NT receptors and ion channels in the differentiating progeny of NSCs and the conspicuous absence of these mechanisms in self-renewing undifferentiated NSCs. The numbers at the end of the traces represent the total number of cells surveyed in this particular experiment.

questions. These included responses to four classical neurotransmitters (ACh, ATP, GABA, and GLUT), elevated K_o^+ to reveal Ca^{2+} entry via VSCC, exclusion of extracellular Na^+ ions (Na_o^+) to identify a constitutive Na_o^{2+}-Ca_c^{2+} exchange mechanism, and stimulation with veratridine (VTD) to show functional voltage-sensitive Na^+ channels (VSSC). After imaging, each recorded cell was phenotyped using multiple immunoreactions reporting their precursor, progenitor, or lineage-restricted differentiation state, as previously described *(67).*

The results revealed that self-renewing NSC-derived progeny, which were nestin$^+$ but devoid of differentiating markers, predominantly remained unresponsive to the test array (Fig. 4A). These findings suggest that uncommitted NSCs do not express functional cholinergic, purinergic, GABAergic, or glutamatergic receptors, or VSCCs, Na_o^{2+}-Ca_c^{2+} exchanger activity, and VSSCs that are coupled to Ca_c^{2+} signaling of these cells. In contrast, NSC-derived NRPs exhibiting a nestin$^+$BrdU$^+$TUJ1$^+$ phenotype responded to ACh and GLUT, but not to ATP or GABA, while also expressing just detectable Na_o^{2+}-Ca_c^{2+} exchanger activity and VSSC-mediated Ca^{2+} entry (Fig. 4B). Further differentiation of NRPs into nestin$^-$BrdU$^-$TUJ1$^+$ postmitotic neurons was accompanied with robust Ca_c^{2+} responses to ACh, GABA, and GLUT, as well as to elevated K_o^+, exclusion of Na_o^+, and stimulation with VTD (Fig. 4C). Inclusion of specific antagonists respectively identified the neurotransmitter receptors as muscarinic cholinergic, ionotropic GABA, and glutamate/kainate subtypes, while nitrendipine-sensitive L-type Ca^{2+} channels and tetrodotoxin-sensitive Na^+ channels mediated Ca^{2+} influx into these cells. By



comparison, NSC-derived progeny differentiating into neuroglial, oligoglial, and astroglial (type 1) progenitors responded to ATP and/or ACh but to no other conditions of the test array. Neuroglial progenitors responded to both neurotransmitters (Fig. 4D), while oligoglial progenitors responded only to ACh (Fig. 4E) and astroglial progenitors only to ATP (Fig. 4F). These results in vitro reveal clear differences in the expression of neurotransmitter receptor/ion channel/ion transporter mechanisms capable of regulating Ca_c^{2+} levels as different types of LRPs begin to differentiate from NSCs.

The stereotypical nature of these mechanisms observed during *de novo* differentiation of LRPs from NSCs at the beginning of cortical neurogenesis are also recapitulated ex vivo at the end of cortical neurogenesis *(66)* and during early postnatal cortical development (unpublished observations). Together, these

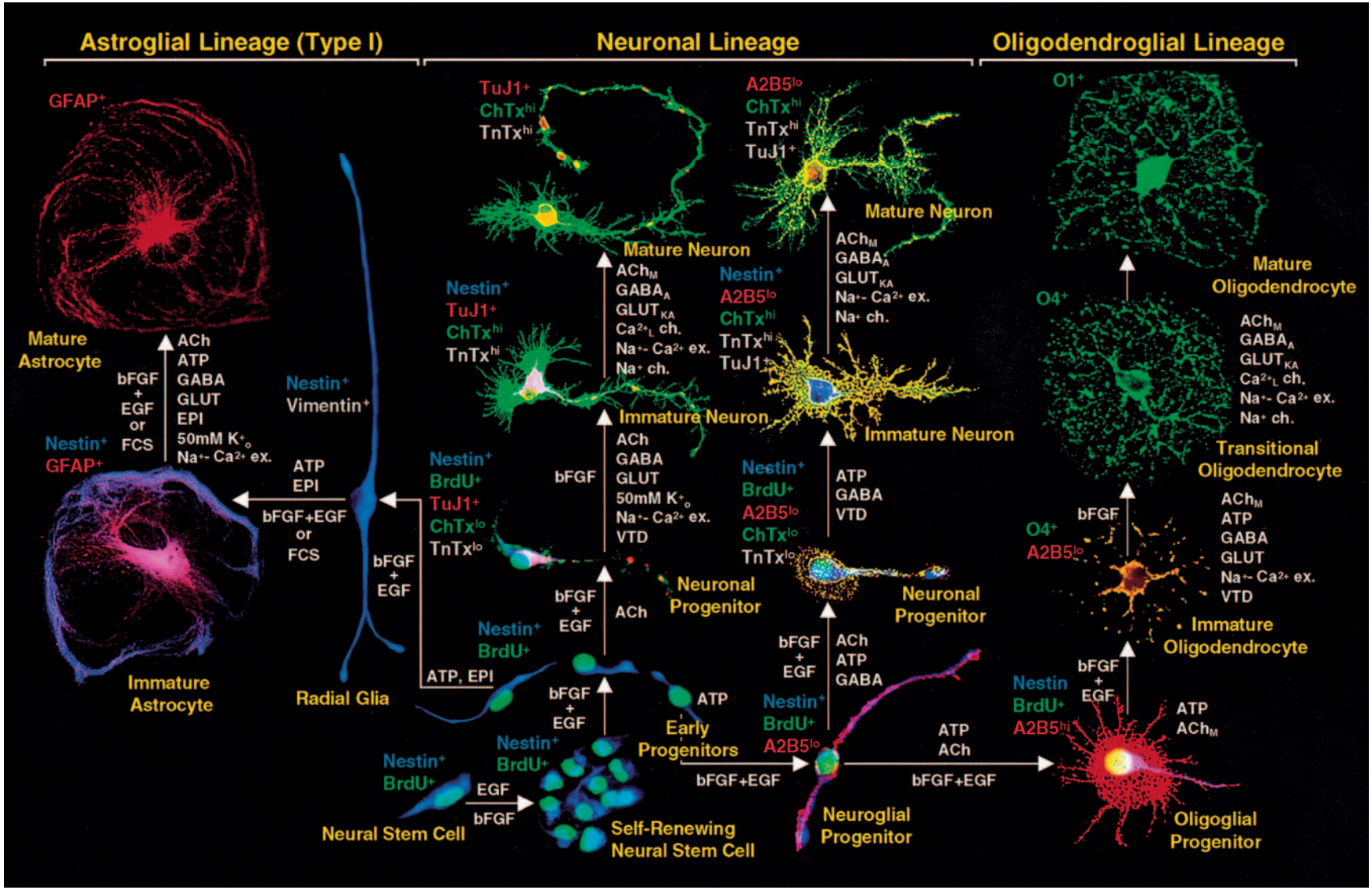

Astroglial Lineage (Type I)
Neuronal Lineage
Oligodendroglial Lineage
GFAP+
Mature Astrocyte
bFGF + EGF or FCS
ACh ATP GABA GLUT EPI 50mM K+o Na+- Ca2+ ex.
Nestin+ GFAP+
Immature Astrocyte
ATP EPI
bFGF+EGF or FCS
Nestin+ Vimentin+
Radial Glia
bFGF + EGF
ATP, EPI
TuJ1+ ChTxhi TnTxhi
Mature Neuron
AChM GABAA GLUTKA Ca2+L ch. Na+- Ca2+ ex. Na+ ch.
Nestin+ TuJ1+ ChTxhi TnTxhi
Immature Neuron
ACh GABA GLUT 50mM K+o Na+- Ca2+ ex. VTD
Nestin+ BrdU+ TuJ1+ ChTxlo TnTxlo
Neuronal Progenitor
bFGF + EGF
ACh
Nestin+ BrdU+
Early Progenitors
ATP
bFGF + EGF
Nestin+ BrdU+
Self-Renewing Neural Stem Cell
EGF bFGF
Nestin+ BrdU+
Neural Stem Cell
A2B5lo ChTxhi TnTxhi TuJ1+
Mature Neuron
AChM GABAA GLUTKA Na+- Ca2+ ex. Na+ ch.
Nestin+ A2B5lo ChTxhi TnTxhi TuJ1+
Immature Neuron
ATP GABA VTD
Nestin+ BrdU+ A2B5lo ChTxlo TnTxlo
Neuronal Progenitor
bFGF + EGF
ACh ATP GABA
Nestin+ BrdU+ A2B5lo
Neuroglial Progenitor
ATP ACh
bFGF+EGF
O1+
Mature Oligodendrocyte
O4+
AChM GABAA GLUTKA Ca2+L ch. Na+- Ca2+ ex. Na+ ch.
Transitional Oligodendrocyte
bFGF
O4+ A2B5lo
AChM ATP GABA GLUT Na+- Ca2+ ex. VTD
Immature Oligodendrocyte
bFGF + EGF
Nestin BrdU+ A2B5hi
ATP AChM
Oligoglial Progenitor

Fig. 5. Mapping of unique and shared properties characterizing astroglial, neuronal, and oligodendroglial lineage progression during cortical development in the rat. The figure provides a general summary of the diversity of cortical cell phenotypes identified using surface-labeling strategies with ganglioside markers described in this review. The figure also outlines the stereotypical properties (lineage-restricted markers, growth factor requirements, and expression of functional neurotransmitter receptors/ion channels that regulate Ca_c^{2+}) emerging on predominant cortical populations which could be distinguished using surface ganglioside labeling at different stages of cell lineage progression. The outline is a composite of the results from *de novo* development of NSC-derived progeny in vitro together with findings on differentiating lineage-restricted cortical cells identified ex vivo at the beginning (E13) and end of neurogenesis (E19) and at the onset of gliogenesis (P3) (*see* refs. *66,67,95,96,97*). Ca_c^{2+} responses to ATP are more prominent among proliferating glial progenitors than neuronal progenitors, which express a diverse pattern of neurotransmitter receptors, ion channels, and ion exchange mechanisms. However, later stages of both astroglial and oligodendroglial lineage progressions are associated with excitability patterns similar to those found in neurons. Pharmacological antagonisms of neurotransmitter receptors and ion channels become more pronounced at later stages of neuronal and oligodendroglial lineage progression. Thus, the responses to agonists have been identified as containing a pharmacologically identifiable component. ACh response becomes ACh_m, indicating atropine block of muscarinic receptors; GABA response becomes $GABA_A$, reflecting bicuculline block at $GABA_A/Cl^-$ channels that subsequently activate voltage-sensitive Ca^{2+} channels; glutamate response becomes $GLUT_{KA}$, identifying kainic acid-type receptors; 50 m*M* K_o^+ becomes $Ca^{2+}{}_L$ch; identifying nitrendipine-sensitive L-type Ca^{2+} channels; veratridine (VTD) becomes Na^+ ch., indicating a TTX-sensitive Na^+ channel component to the VTD-induced Ca_c2^+ response.

results suggest that distinct and diverse membrane mechanisms capable of regulating Ca_c^{2+} levels (summarized in Fig. 5) may mediate or facilitate the early commitment of cortical NSCs toward neuronal and/or glial differentiation. This remains to be determined.

Conclusions

The rigorous definition of NSCs as undifferentiated cells originating from the neuroepithelium with the inherent capacity to perpetually self-renew without differentiating or to undergo multilineage restricted differentiation upon specific microenvironmental cues has increasingly been abandoned in favor of a more generalized functional concept rather than focusing on the cellular entity. This has precipitated the reliance of the great majority of in vitro studies to use retrospective analyses in order to reveal the existence and characterize the functional properties of NSCs isolated from the PNS and CNS. However, almost without exception, the investigations into the mechanisms underlying NSC biology and cell fate may have been inherently compromised to an unknown degree since the starting preparation typically included heterogeneous populations isolated from the proliferating neuroepithelium. These preparations are composed of NSCs as well as different types of LRPs, which can also undergo limited self-renewal, generate neurospheres, and differentiate into neuronal and glial phenotypes. In fact, only a minor population of NECs exhibits the properties of true NSCs, with the unique capability to undergo virtually unlimited self-renewal without differentiation. Complicating the matter further is the current lack of methods to prospectively isolate large numbers of pure NSCs for immediate studies of their biology ex vivo. In addition, attempts to selectively expand NSCs in vitro have not resulted in a specific enrichment of these cells since the preparations and the culture conditions used also permitted the proliferation and differentiation of LRPs as well as NSC-derived progeny. Thus, propagation of NSCs in vitro using monolayer and

neurosphere protocols has not actually increased the NSC compartment relative to other types of cells. Rather, NSCs remain an invisible and minor subpopulation even after many generations of cells have been produced.

The well-established success in identifying the hematopoietic stem cells by their surface expressions of cluster differentiation markers and their subsequent isolation using the FACS technology has prompted recent attempts to use FACS in order to isolate pure populations of NSCs. Thus far, these FACS strategies using different combinations of surface or cytoplasmic markers have led to varying degrees of success with variable levels of NSC enrichment, but none have provided a compelling source of a pure self- renewing population of NSCs. The most recently devised FACS strategy, as described here, has exploited the expressions of complex gangliosides emerging at the surface of NECs in the earliest phases of neuronal and glial differentiation. Negative selection of NECs, which do not express these surface markers, revealed that these cells could both perpetually proliferate without differentiating over many generations of progeny and differentiate into the principal neural phenotypes, depending on instructive culture conditions. Thus, negatively selected NECs exhibit the defining properties of true NSCs. In contrast, positive selection of NECs using combinations of neuronal and glial-selective surface markers enables ex vivo purification of different types of immature LRPs that undergo limited proliferation before differentiating along their expected lineages. Molecular and cellular studies of FACS-sorted NSCs and different LRPs further demonstrated clear differences in gene expression patterns and expressions of functional transmitter receptors, ion channels, and ion transporters among self-renewing and differentiating progeny of NSCs both in vitro and in vivo. The reliability of this FACS strategy and its applicability throughout the developing CNS in vertebrates, including humans, indicates that this method will be useful in future studies of NSC and LRP biology. Furthermore, using pure populations of NSCs and LRPs that exhibit stereotypical functional properties involved in their cell fate and lineage progression will open new avenues for possible therapeutic use of these cells in restoring normal function to injured, aging, or neurodegenerative brain tissue.

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