

NEURAL STEM CELLS IN THE EMBRYONIC AND ADULT
MOUSE BRAIN

Maria Alexandra Nunes Capela

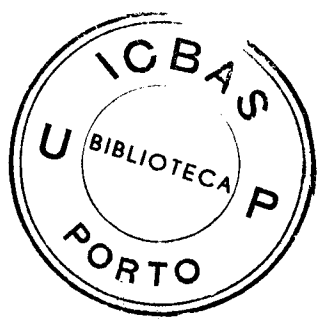
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The research described in this thesis was performed in the Center for Neuropharmacology and Neuroscience, Albany Medical College, Albany, NY, USA, and was financially supported by PRAXIS XXI through the Gulbenkian PhD Program in Biology and Medicine and by the NIH grant R01 NS33529.

*“When does the butterfly read
what flies written on its wings?”*

*“Cúando lee la mariposa
lo que vuela escrito en sus alas?”*

Pablo Neruda

Acknowledgements

Throughout my “scientific life”, I have had the privilege of working with many talented and generous individuals. In one way or another, all of them contributed to this thesis and I would like to acknowledge their help and support.

In Albany

First of all I would like to express my sincere gratitude to **Dr Sally Temple**, my mentor, for accepting me as a student in her lab, for her trust, support and for being such a never-ending source of inspiration and help. Thank you in particular for allowing a “to taste” scientific freedom in the laboratory that ultimately instilled in me the pleasure for creative and independent thinking.

Thank you...

Susan Goderie, the “Cell Culture Goddess”, the manager, the friend and the clown, for all your help, the troubleshooting, for the “I can do that, sure I can”, your love for the work, the laughs and the jokes (“and they won’t die!!!”).

David Corliss, for your friendship, for being my lunch buddy and for your encouragement.

Qin Shen and **Wenlei He**, for all the discussions, help and good moments in the lab. Thank you **Qin** for all your help with the manuscripts and computer work.

Yu Sun (big thank you), for your incredible and most generous help with the *in vivo* studies; we are an example of how team work can be so pleasurable and successful – even when the experiments don’t work!!!

Natasha Abramova for your generous help with molecular techniques and the lovely dinners.

It was truly a pleasure to share laboratory equipment and space with **Dr Kimmelberg’s** group. Thank you **HKK, Gary Schools, Alexander Mongin, Min Zhou** and **Carol Charniga**, for all the scientific and non-scientific discussions and the great parties!

I would also like to acknowledge the friendship and support of **Dr Frank Rice, Dr Chuck Lowry, Marilyn Dockum, Kim Musser, Bob Dilwith, Teresa Passaretti, Lynn** and **Weiguo Liu**.

Thank you so much **Tej** for being such a good friend. Your encouragement and support made the last months in Albany much easier. A big thank-you to **Jane Ramee** for the company, laughs, advice on the “many aspects of life”, and for making me feel at home.

In Portugal

I would like to express my gratitude to **Carlos Bandeira Duarte (Cajó), Armando Cristovão, Graça Baltazar, Ildete Luisa Araujo** and **Prof Dr Arsélio Pato de Carvalho** at the Coimbra Neuroscience Institute for teaching me the methodology of science and for all the good times in the lab. Thank you **João Ramalho-Santos** for the great classes that made me want so badly to be part of this crazy group of people.

I am very grateful and proud for being part of the Gulbenkian PhD Program in Biology and Medicine sponsored by the Gulbenkian Foundation through its Science Institute. To **Prof. Dr. António Coutinho** and **Prof. Dr. Alexandre Quintanilha**, my sincere appreciation for creating such a wonderful environment that made possible for so many of us to do what we love best. Thank you **Domingos Henrique**, my Portuguese co-supervisor for the technical help, reagents and advice. To **Carla, Elio, Maria, Marta, Mónica and Victor**, my 4th PGDBM colleagues, thank you and good luck.

To my friends **Lena, Isabel, Jónatas, Ana Maria, Lino, Dora, Sara & João Paulo, Pedro Seabra, Vitorino, Pedro Cenoura, George & Susana**, thanks for the good times.

To my parents **António** and **Dorinda Capela** and my sister **Sofia**, thank you for your support and love.

To **Shasha** go my deepest words of thanks for your constant support, patience and love. This thesis is also yours.

SUMMARY

Neural stem cells are defined as multipotent, self-renewing progenitor cells that can generate specialized cell types such as neurons, astrocytes and oligodendrocytes.

These important cells were first identified in the embryonic rodent brain by in vitro sub cloning experiments and were later identified in the human brain. The realization that neural stem cells also reside in the adult brain has opened a whole new avenue in research, mainly encouraged by the need to develop alternative therapies for neurodegenerative diseases like Parkinson's, Alzheimer's and Huntington's and for brain injury caused by stroke. Ultimately, transplantation and/or mobilization of endogenous neural stem cells will be the strategy of choice for treatment of CNS cell loss. Understanding the biology of neural stem cells will help us unravel how such elusive cells can be used to save our most precious possession: cognitive function. The work presented in this thesis adds to the ever-growing effort of "getting to know our stem cells".

Neural stem cells generate neurons and glia in a stereotyped order both in vitro and in vivo: neurons are born first and glia later. The switch from neuron to glia production can be pinpointed to a single asymmetric division. In **chapter two**, the molecular nature of the "neuron-glia switch" is explored by testing the hypothesis that murine homologues of the *Drosophila glial cells missing (gcm)* gene such as *mgcma* and *mgcmb* are responsible for such switch. In the first part of the chapter, we showed that *mgcmb* is expressed in the mouse cortex and that there is a correlation between its expression and onset of gliogenesis. However, due to the fact that *mgcmb* is expressed at very low levels in the mouse embryonic cortex and is likely to be expressed in the stem cells, a protocol was designed with the objective of enriching the test population in neural stem cells. The second part of **chapter two** describes the development of such methodology. We demonstrate that highly proliferative cortical cells isolated from embryos at thirteen days of gestation (E13) can be purified using antibodies against a carbohydrate epitope called LewisX (LeX) that is present in pluripotent stem cells like embryonic stem (ES) cells and primordial germ cells (PGCs). Our studies show that LeX⁺ cells include restricted neuroblasts and most importantly, stem cells. Moreover, we show that *mgcmb* is expressed by such highly proliferative cells and possibly also by stem cells and is excluded from differentiated glial cells and committed neuroblasts (LeX⁻ E13 cells).

Interestingly, *mgcmb* is only expressed in LeX⁺ cells, in agreement with our initial hypothesis that *mgcmb* is expressed in stem cells. The difficulty of addressing single cell gene expression within a stem cell lineage makes it hard to pinpoint exactly when *mgcmb* is expressed and in which cell. Such analysis can only be accomplished when transgenic mice carrying a reporter gene under the control of *mgcmb* promoter are made available.

Neural stem cells are rare and little is known about their unique characteristics, leaving their *in vivo* identity enigmatic. In **chapter two** we describe the development of the methodology to prospectively identify and purify neural stem cells based on a surface marker (LeX). In **chapter three** we show that LeX is made by adult mouse subventricular zone (SVZ) stem cells and shed into their environment. Only 4% of acutely isolated SVZ cells are LeX⁺; this sub-population, purified by fluorescence-activated cell sorting or FACS, contains the SVZ stem cells. Ependymal cells are LeX⁻ and purified ependymal cells do not make neurospheres (the *in vitro* diagnose of stem "cellness") resolving the controversial claim that these are stem cells. Preliminary data from experiments designed to address the behavior of LeX⁺ stem cells when transplanted into an *in vivo* embryonic environment is also presented. In **chapter four** we show that in the developing nervous system, stem cells and highly prolific progenitor cells express LeX. Moreover, LeX is highly expressed in regions with prolonged growth and neurogenesis, including basal forebrain, hippocampus, mid/hindbrain isthmus and olfactory epithelium, regions which express the growth inducers FGF8 and Wnts. LeX is known to bind FGFs, and we show it binds Wnt-1. LeX-containing vesicles reminiscent of argosomes, a means of *Drosophila* Wg dispersal, are found within neuroepithelia.

Taken together, our findings suggest that LeX is critical for stem cell function, binding and distributing factors that regulate their proliferation and self-renewal.

SUMÁRIO

As células estaminais neuronais são definidas por duas características fundamentais: são multipotentes, isto é, geram os dois tipos básicos de células do sistema nervoso – neurónios e células da glia – e têm capacidade de se renovar, isto é, de produzir mais células estaminais.

Estas células, inicialmente descobertas no cérebro de embriões de rato usando culturas clonais, foram mais tarde identificadas no cérebro humano. A descoberta subsequente de que células estaminais neuronais residem também no cérebro adulto abriu novas perspectivas para o tratamento, não só de doenças neurodegenerativas como Parkinson, Alzheimer e Huntington, mas também para o tratamento de lesões neuronais. O transplante e/ou a mobilização de células estaminais neuronais endógenas tornar-se-á a estratégia terapêutica de escolha para o tratamento de doenças nas quais se verifica perda de células nervosas. O estudo da biologia das células estaminais neuronais ajudar-nos-á a perceber como é que estas células podem ser utilizadas para recuperar a função cognitiva. O trabalho apresentado nesta tese tem como objectivo elucidar alguns aspectos básicos da biologia das células estaminais neuronais do sistema nervoso central de ratinho.

As células estaminais neuronais geram neurónios e glia de um modo estereotipado, tanto "in vitro" como "in vivo": os neurónios "nascem" primeiro e só depois as células da glia. A transição de neurogénese (geração de neurónios) para gliogénese (geração de glia) ocorre numa única divisão celular. No **capítulo dois**, a natureza molecular da transição "neurónio-glia" é estudada com base na hipótese de que os genes de ratinho (*mgcma* e *mgcmb*) homologos do gene de *Drosophila* "glial cells missing (*gcm*)" são responsáveis por esta transição. Na primeira parte deste capítulo, mostramos que *mgcmb* é expresso no cortex cerebral embrionário de ratinho e que existe uma correlação entre a sua expressão e o período de gliogénese. No entanto, o nível de expressão de *mgcmb* é muito baixo e é, provavelmente, exclusivo das células estaminais. Com o objectivo de enriquecer a nossa população inicial em células estaminais neuronais para assim mais facilmente estudar a expressão de *mgcmb*, desenhamos um método de imunopurificação de células estaminais neuronais. A segunda parte do **capítulo dois** descreve o desenvolvimento dessa metodologia. Demonstramos que as células com elevada capacidade proliferativa isoladas de embriões com 13 dias de gestação (E13) podem ser purificadas usando anticorpos contra um epítopo constituído por três açúcares (fucose-L-acetil lactosamina) chamado LewisX (LeX) que também é expresso em células pluripotentes embrionárias ("embryonic stem cells" ou ES) e células germinais ("Primordial germ cells", PGCs). Os nossos estudos mostram que células LeX⁺ incluem neuroblastos (células progenitoras unipotentes que geram apenas neurónios) e células estaminais neuronais. Neurónios e células

da glia diferenciadas são LeX⁻. O estudo da expressão de *mgmb* em células LeX⁺ e LeX⁻ demonstrou que *mgmb* é expresso somente por células LeX⁺, o que está de acordo com a nossa hipótese inicial de que células estaminais neuronais expressam este gene. Dada a dificuldade em analisar a expressão de genes em células individuais, não é fácil demonstrar exactamente quando é iniciada a expressão de *mgmb* e em que células é expresso. Este tipo de análise só será possível quando ratinhos transgênicos que expressam um gene-reporter sob o controlo do promotor de *mgmb* forem criados.

As células estaminais neuronais são células raras e muito pouco se conhece acerca das suas características, o que torna a sua identidade "in vivo" enigmática. No **capítulo dois** descrevemos o desenvolvimento da metodologia que nos permite identificar e purificar células estaminais neuronais com base na expressão de um marcador de superfície (LeX). No **capítulo três** demonstramos que LeX é sintetizado por células estaminais neuronais presentes na zona subventricular (SVZ) do ratinho adulto e é libertado por estas células para a matriz extra-celular. Apenas 4% das células isoladas da SVZ são LeX⁺; esta subpopulação, purificada por FACS ("fluorescence-activated cell sorting"), contem a maioria das células estaminais neuronais da SVZ. As células endimais são LeX⁻ e quando purificadas por FACS, não geram neurosféricas (o diagnóstico "in vitro" de células estaminais neuronais), resolvendo a conclusão controversa de outros autores de que células endimais são estaminais. Dados preliminares de experiências desenhadas para estudar o comportamento de células estaminais neuronais LeX⁺ após transplante em embriões de ratinho são também apresentados. No **capítulo quatro** demonstramos que durante o desenvolvimento embrionário do sistema nervoso, as células estaminais neuronais e os progenitores com alta capacidade proliferativa também expressam LeX. O epítipo LeX é expresso em regiões com crescimento e neurogénese prolongados, como o hipocampo, o istmo (a constricção entre o metencéfalo e o mesencéfalo) e o epitélio olfactivo, onde os factores de crescimento FGF8 e Wnt também são expressos. Sabe-se que LeX liga FGF, e neste trabalho mostramos que também se associa com Wnt-1. Vesículas intracelulares contendo LeX estão presentes no epitélio neuronal, parecendo reminiscentes de argosomas, um modo de dispersão de Wg em *Drosophila*.

Em conclusão, o nosso estudo sugere que a presença de LeX é fundamental na fisiologia das células estaminais neuronais, em particular na ligação e distribuição de factores que regulam a proliferação e auto-renovação das mesmas.

SOMMAIRE

Les cellules souches neurales sont définies comme des cellules multipotentes et progeneritrices auto-renouvelantes qui peuvent produire des types spécialisés de cellules tels que des neurones, des astrocytes et des oligodendrocytes. Ces cellules importantes ont été identifiées pour la première fois dans le cerveau embryonnaire de rongeur, par des expériences secondaires in vitro de clonage et ont été plus tard identifiées dans le cerveau humain. La découverte que les cellules souches neurales résident également dans le cerveau d'adulte a ouvert une nouvelle avenue entière dans la recherche, principalement encouragée par la nécessité de développer des thérapies alternatives pour les maladies neurodegeneratives comme Parkinson's, Alzheimer's et Huntington's et pour des endommagements de cerveau provoqués par l'apoplexie. De ce fait, la transplantation et/ou la mobilisation des cellules souches neurales endogènes seront la stratégie du choix pour le traitement de la perte de cellules de CNS. La compréhension de la biologie des cellules souches neurales nous aidera à découvrir comment de telles cellules évanescentes peuvent être employées pour sauver notre possession la plus précieuse: la fonction cognitive. Le travail présenté dans cette thèse s'ajoute à l'effort toujours croissant du parvenir à connaître nos cellules souches.

Les cellules souches neurales produisent des neurones et du glia dans un ordre stéréotypé in vitro et in vivo: des neurones sont produits d'abord et le glia ensuite. Le passage du neurone à la production de glia peut être indiqué exactement par une division asymétrique simple. Dans le **chapitre deux**, la nature moléculaire du commutateur de neurone-glia est exploré en évaluant l'hypothèse que les homologues murins du gène "glial cells missing -gcm" tel que le *mgcma* et le *mgcmb* sont responsables d'un tel commutateur. Dans la première partie du chapitre, nous avons prouvé que le *mgcmb* est exprimé en cortex de souris et qu'il y a une corrélation entre son expression et le début de gliogenesis. Cependant, étant donné que le *mgcmb* est exprimé en niveau très bas dans le cortex embryonnaire de souris, et est susceptible d'être exprimé en cellules souches, un protocole a été conçu avec l'objectif d'enrichir la population d'essai en cellules souches neurales. La deuxième partie du **chapitre deux** décrit l'élaboration d'une telle méthodologie. Nous démontrons que des cellules corticales fortement prolifériques d'isolement dans des embryons à treize jours de gestation (E13) peuvent être épurées en utilisant des anticorps contre un epitope d'hydrate de carbone appelé LewisX (LeX) qui est présent dans les cellules souches pluripotentes telles que les cellules embryonnaires souches (ES) et les cellules germinales primordiales (PGCs). Nos recherches prouvent que

les cellules de LeX⁺ contiennent des neuroblasts restreints et d'une manière primordiale, des cellules souches. D'ailleurs, nous démontrons que le *mgcmb* est exprimé par de telles cellules fortement prolifératives et probablement aussi par des cellules souches et est exclu des cellules gliales différenciées et des neuroblasts commises (cellules de LeX⁻ E13). En fait, *mgcmb* est seulement exprimé en cellules de LeX⁺, en accord avec notre hypothèse d'origine que le *mgcmb* est exprimé en cellules souches. La difficulté d'adresser l'expression unicellulaire de gène dans une lignée de cellules souches rend difficile l'identification de l'expression du *mgcmb* et dans quelle cellule. Une telle analyse peut seulement être accomplie quand des souris transgéniques, portant un gène "reporter" sous la commande de l'instigateur de *mgcmb*, sont rendues disponibles.

Les cellules souches neurales sont rares et peu est connu au sujet de leurs caractéristiques uniques, laissant leur identité *in vivo* énigmatique. Dans le chapitre deux nous décrivons l'élaboration de la méthodologie pour l'avenir pour identifier et épurer les cellules souches neurales basées sur un marqueur extérieur (LeX). Dans le **troisième chapitre**, nous prouvons que LeX est fait par les cellules souches de la zone subventriculaire (SVZ) de souris d'adulte et est excrété dans leur environnement. Seulement 4% de cellules de SVZ isolées sont LeX⁺; cette sous-population, épurée par la cellule fluorescence-activée assortissant ou FACS, contient les cellules souches de SVZ. Les cellules d'ependymal sont LeX⁻ et les cellules ependymal épurées ne font pas des "neurosphères" (diagnostique *in vitro* des "cellness" souches) donnent résolution à la réclamation contestée que ce sont des cellules souches. Les données préliminaires des expériences conçues pour adresser le comportement des cellules souches de LeX⁺ une fois transplantées dans un environnement embryonnaire *in vivo* sont également présentées. Dans le **chapitre quatre** nous prouvons qu'en système nerveux se développant, les cellules souches et les cellules fortement prolifériques de progenitor expriment LeX. En plus, LeX est fortement exprimé en régions de croissance prolongée et de neurogenèse, y compris le "forebrain" basique, l'hippocampe, l'isthme de "mid/hindbrain" et l'épithélium olfactif, les régions ce qui expriment les inducteurs de croissance FGF8 et Wnts. LeX est connu pour lier FGFs, et nous montrons qu'il lie également Wnt-1. Des vésicules réminiscentes des "argosomes" qui contiennent LeX, des moyens de dispersion de Wg de *Drosophila*, sont trouvés dans le neuroepithelia.

Pris ensemble, nos résultats suggèrent que LeX est critique pour la fonction de cellules souches, lie et distribue des facteurs qui règlent leur prolifération et renouvellement automatique.

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Chapter One

INTRODUCTION

Despite all the great discoveries of modern science, the generation of a multicellular organism from a single fertilized oocyte remains an amazing mystery, a true miracle of nature.

The development of metazoan organisms requires the generation of multiple cells types, the building blocks of organs that ultimately define their specific functions. Fusion of the gametes to generate the totipotent zygote (the primordial stem cell) initiates the program of embryonic development, leading to the formation of somatic stem cells for the construction of adult tissues and of germline stem cells, which insures the next generation of germ cells. Stem cells are generally defined as cells that are capable of self-renewal (creation of more stem cells) and multilineage differentiation (generation of an array of differentiated cells characteristic of a particular organ or system) (reviewed by Potten and Loeffler, 1990; Morrison et al., 1997; Fuchs and Segre, 2000).

During mammalian embryonic development, cell proliferation occurs at an incredible pace. It is critical that the right numbers of cells are made at the right time and in the right place. The immense proliferative and differentiative potential of stem cells is used to fuel the need for growth and diversification of a developing organism. Stem cells in the adult animal also contribute to the normal cycle of regeneration of high turnover tissues like the skin, hair, lining of the small intestine and blood cells (Fuchs and Segre, 2000). Some adult mammalian stem cells can even respond to injury by regenerating tissue like the liver and skin, a modest contribution if one takes as example the capacity of newts to regenerate the tail, limbs and even heart tissue (reviewed by Brockes et al., 2001).

Thus stem cells are the most fundamental cell type in the generation and maintenance of most tissues. The nervous system, with its wealth of diverse, intricately connected cells was initially thought to be hard-wired and to be built from precursors with little plasticity. Moreover, the irreversibility of tissue loss in the adult led to the dogma that the mammalian nervous system had little regenerative potential. Yet over the course of the last decade it has become clear that the nervous system is built using stem cells, and even that the adult nervous system contains stem cells. Along with the

description of neural stem cells came an outpouring of excitement for the possibility of neural regeneration, which swept from science into the public domain. The discovery of neural stem cells opened up a new set of questions in developmental biology - what is the role of stem cells in normal development, how do these cells "choose" to make one type of progeny versus another, what are the characteristics of stem cells from embryos and adults. And it raised a critical question with clinical implications - can these cells be used to make new tissue for neural repair. This is still a relatively new field, and many of these fundamental questions remain unanswered. In this introduction I will provide background information on stem cells in the nervous system, and indicate the questions that I addressed in my studies.

Neural stem cells in the embryonic and adult brain

Stem cells actively participate in the construction of both the central and peripheral nervous system (CNS and PNS). They do so by generating transit amplifying cells that in turn divide and originate restricted progenitors that differentiate further into appropriate cell types, a strategy common amongst other stem cells in the body (Potten and Loeffler, 1990). The two major classes of cells in the nervous system are neurons and glia. They have fundamentally different functions: neurons receive, process and transmit information while glial cells have a metabolic and structural support role. However different their functions, neurons and glia can be generated by a common progenitor cell, the neural stem cell (figure 1.1).

There are two main neural stem cells in mammals: CNS stem cells and neural crest stem cells (NCSCs) that contribute to the construction of the CNS and PNS respectively. Much more is known about the lineage determination of NCSCs and CNS stem cells than in other stem cells like the hematopoietic or small intestine stem cells. This is because neural stem cells undergo self-renewal and multilineage differentiation in culture while the others do not. Such capacity provides a unique opportunity to study how different growth factors instruct or select different cell fates.

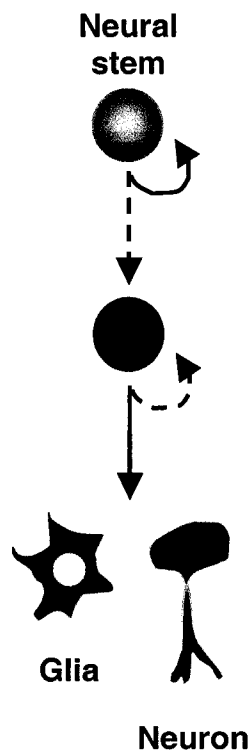


Figure 1.1. Neural stem cells generate differentiated progeny.

The CNS and PNS relies on neural stem cells as building blocks for the generation of appropriate cells types. Like other somatic stem cells, neural stem cells self-renew and generate transit amplifying cells that further differentiate into neurons and glia.

Neural crest stem cells

Neural crest stem cells delaminate from the dorsolateral margins of the neural tube and migrate extensively to specified sites throughout the developing embryo, where they differentiate into neurons and glia of the PNS, as well as melanocytes, smooth muscle cells, facial bones, and cartilage (Le Douarin, 1980, 1982, 1986). The particular fate of a neural crest cell is influenced by local cues provided by neighboring cells in the dorsal neural tube and also present along the migration routes (reviewed by Le Douarin et al., 1993; Edlund and Jessell, 1999).

Work conducted by David Anderson and colleagues led to the identification and later isolation of rat NCSCs that can generate most neural crest cell types *in vitro* while undergoing self-renewal (Stemple and Anderson, 1992; Morrison et al., 1999). *In vitro*

studies have determined that BMPs instruct neuronal differentiation, while neuregulin (Nrg-1 or glial growth factor) instructs glial differentiation, and transforming growth factor β (TGF- β) instructs myofibroblast differentiation of NCSCs (Shah et al., 1994, 1996). Recently, Notch activation in NCSCs has been shown to instruct Schwann cell differentiation in a way that is dominant over the neurogenic influence of BMP2/4 (Morrison et al., 2000). Expression studies suggest that this collection of instructive molecules are most likely involved in fate decisions *in vivo* (Reissman et al., 1996; Schneider et al., 1999).

CNS stem cells

Embryonic CNS stem cells capable of multilineage differentiation and self-renewal have been identified in cultures of murine septum, cortex, striatum and spinal cord (Temple, 1989; Davis and Temple, 1994; Reynolds and Weiss, 1996; Mayer-Proschel et al., 1997). A number of soluble factors like FGF2 and EGF have been shown to stimulate division of embryonic stem/progenitor cells *in vitro* (Gensburger et al., 1987; Cattaneo and McKay, 1990; Lillien and Cepko, 1992; Reynolds et al., 1992; Kilpatrick and Bartlett, 1993). As in NCSCs, instructive signals also influence lineage commitment of CNS stem cells *in vitro*. BMPs can both instruct neurogenesis and astrocytic differentiation in CNS stem cells (Gross et al., 1996; Li et al., 1998), PDGF (platelet derived growth factor), BDNF (brain derived neurotrophic factor) and NT-3 (neurotrophin -3) promote neural fates (Ahmed et al., 1995; Ghosh and Greenberg, 1995; Johe et al., 1996), leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) are potent inducers of astrocyte production (Johe et al., 1996; Bonni et al., 1997; Rajan and McKay, 1998) while thyroid hormone instructs oligodendrocyte differentiation (Barres et al., 1994). FGF2 has also been shown to promote glia differentiation *in vitro* in a concentration dependent manner (Qian et al., 1997). As for NCSCs, an important challenge is to understand if/how these lineage determination factors operate *in vivo* so that the neural stem cells can generate correct types of differentiated progeny at the appropriate times.

Stem cells in the mouse cortex

The mammalian cortex is composed of diverse and highly differentiated neuronal and glial cell types. Early in development, the cerebral cortical epithelium consists of a single

cell sheet of morphologically indistinguishable columnar neuroectoderm cells that constitute the germinal layer or ventricular zone (VZ). Although all cells contact both the ventricle and the pial surface, their nuclei appear in different apical-basal positions depending on the cell cycle stage. For instance, mitosis occurs near the ventricle while G1 and S occur progressively closer to the pial surface. The loop closes when the nucleus comes down in G2 for another mitosis (figure 1.2) (Jacobson, 1991). The VZ is believed to be the source of the vast majority of neurons and macroglia in the cerebral cortex. It proliferates extensively generating immature neurons that migrate away towards the pial surface and complete their differentiation in the cortical plate from which the six cortical layers derive (figure 1.3). Layer I is generated first. The remaining layers form in an inside-out manner, like an "active volcano": early born neurons fill the deepest layers (VI and V), and later born neurons migrate past these to make more superficial layers (IV-II) (Caviness, 1982; Takahashi et al., 1994, 1995).

Neuronal migration to appropriate locations relies on the establishment of a radial scaffolding system made by a type of elongated glial cells that span the entire thickness of the cortex. Radial glia have their cell body in the VZ, a well developed foot in the ventricular surface and extend a long process whose end feet touch the pia (Levitt and Rakic, 1980; Edwards et al., 1990). Curiously, the radial glial cell is the first morphologically distinguishable cell in the early VZ. When neuronal migration is complete, radial glia transform into astrocytes (Schmechel and Rakic, 1979; Voigt, 1989).

During the course of development, neurons are generally produced before glia; murine cortical neurogenesis starts at embryonic day 12 or E12 and is complete before birth (which occurs around day 20-21), whereas gliogenesis starts modestly at midgestation and only peaks after birth.

A small percentage of cells in the early cortex of the mouse embryo are stem cells that can generate hundreds of progeny including neurons, astrocytes and oligodendrocytes (Davis and Temple, 1994; Qian et al., 1997). The majority of the cells in the early cortex are restricted progenitors: more than 80% are neuroblasts (only generate neurons) while only around 1% are glioblasts (which only generate glial cells). Therefore, the majority of glia must be generated by stem cells. Interestingly,

generation of neurons and glia by cortical stem cells isolated from early mouse embryos is temporally regulated: neurons are generated first, usually via asymmetric divisions and glia arise later in the lineage, via symmetric divisions (Qian et al., 2000, figure 1.4), a recapitulation of the *in vivo* sequence of cell genesis. Incredibly, the generation of specific neuronal cell types *in vitro* also seems to follow the *in vivo* order rule. For instance, layer I neurons (the first neurons to be born on the early cortex) are the *in vitro* initial progeny of the stem cell; later born neurons (layer V) are also made later in the stem cell lineage (Qin Shen and Sally Temple, unpublished observations, figure 1.4). Taken together, these observations show that the study of cortical stem cell development in isolation provide a great model to address how neuronal and glial cell types are generated and also how neuronal diversity is accomplished.

It is not known how the stem cell switches from making neurons to making glia. The separation of the lineages occurs at a special asymmetric division that generates one restricted neuroblast and a restricted glioblast instead of a stem cell and a neuroblast. What is the molecular mechanism involved in this switch? In *Drosophila* neuro-glioblast lineages, this fate switch is also observed and involves the activity of the *glial cells missing (gcm)* gene. In *Drosophila* CNS and PNS, *gcm* acts as a master regulatory gene in the neuron vs glia decision (Hosoya et al., 1995; Jones et al., 1995; comment in Anderson, 1995). One possibility is that *gcm* mouse homologues are involved in the neuron-glia switch observed during the development of cortical neural stem cells. Chapter 2 addresses this question.

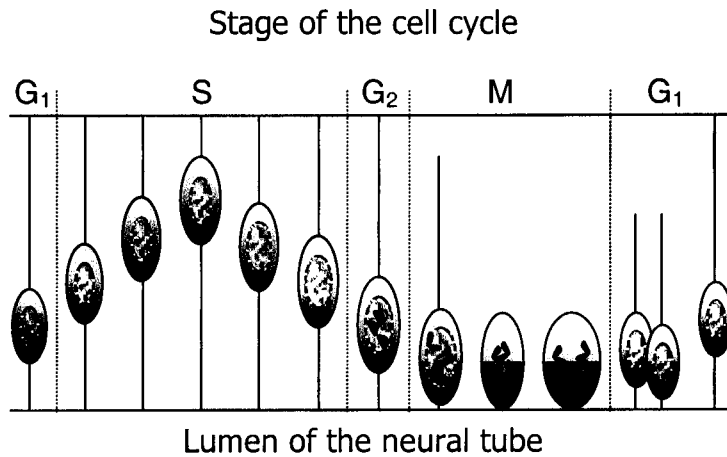


Figure 1.2. Schematic section of the chick embryo neural tube illustrating the position of the nucleus in a neuroepithelial cell as a function of the cell cycle. Mitotic cells are found near the lumen of the neural tube. Adapted from Sauer, 1935.

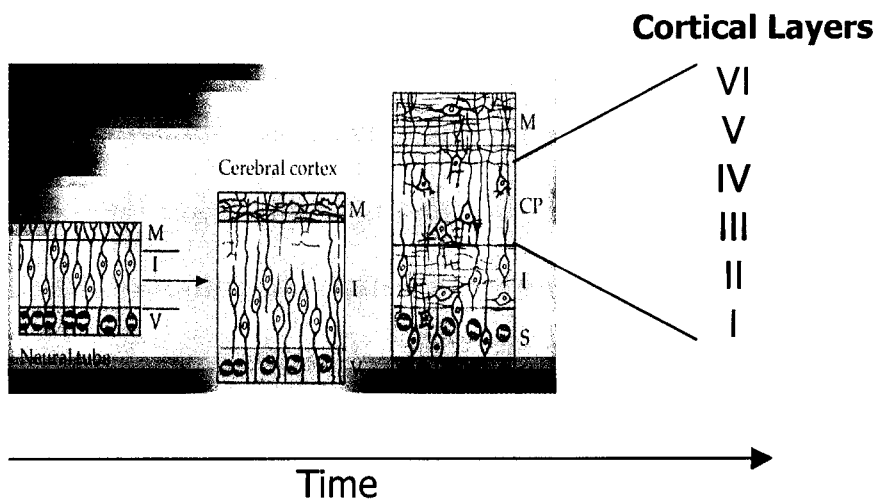


Figure 1.3. Development of the mammalian cortex.

Early in development, the cerebral cortical epithelium consists of a thin sheet of columnar neuroectoderm cells. This germinal layer or ventricular zone (V in figure) proliferates extensively and generate immature neurons that migrate away towards the pial surface and complete their differentiation in the cortical plate (CP) from which the cortical layers derive in a inside-out manner. (Adapted from Gilbert, 2001).

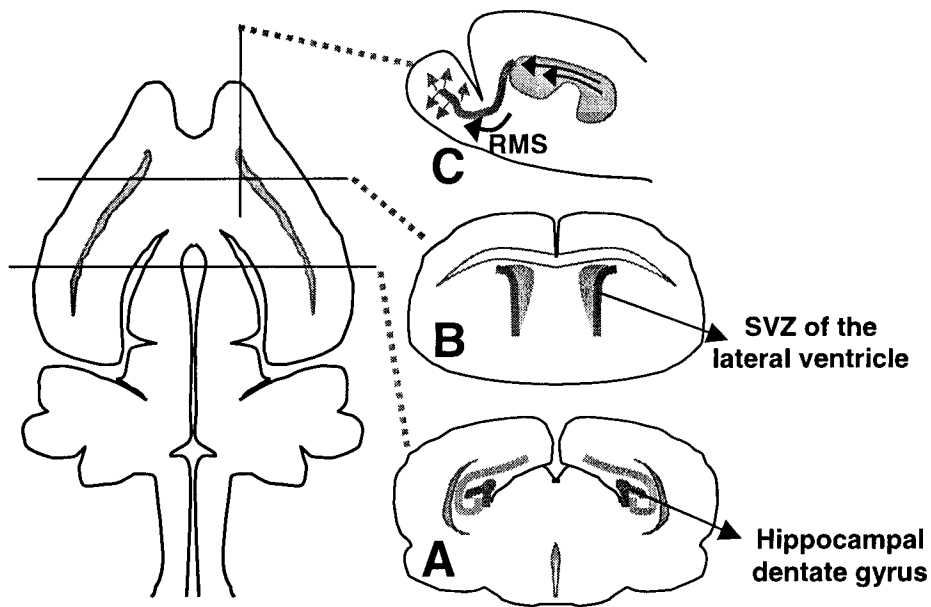


Figure 1.5. Schematics showing where neurogenesis occurs in the adult mouse brain.

The hippocampus and the olfactory bulb continue to acquire new neurons in adult animals. Lines on the left panel represent the anterior/posterior level of the coronal sections represented in A and B and the medial/lateral level of the sagittal section in C.

(A). The dentate gyrus is the site of origin of new hippocampal granule cells.

(B). Olfactory bulb neurons originate in the SVZ surrounding the lateral ventricles.

(C). SVZ-born neuroblasts proliferate while migrating towards the olfactory bulbs using the rostral migratory stream (RMS) and differentiate into interneurons that disperse within the bulbs.

These observations suggested that a neural stem cell might be the cellular source of such new neurons and predicted that cells with stem characteristics ought to be isolated from those areas. Indeed, various studies later demonstrated that cells derived from adult murine SVZ and DG can grow *in vitro* in the presence of defined factors such as FGF2 and EGF (Reynolds and Weiss 1992; Morshead et al., 1994; Gritti et al. 1996; Palmer et al., 1997). These cells grow as adherent monolayers or as neurospheres in suspension, can self-renew and undergo multilineage differentiation and thus have the characteristics of neural stem cells.

Interestingly, *in vitro* expanded stem cells have a surprisingly broad developmental capacity when transplanted into different parts of the brain. Adult hippocampal stem cells can generate granule hippocampal and olfactory neurons when injected in the hippocampal DG (Gage et al., 1995) and RMS respectively (Suhonen et al., 1996), but not when injected in adult cerebellum (Suhonen et al., 1996). Also, injections of adult hippocampal stem cells into the developing retina generate cells with appropriate neuronal and glial morphology. However, they never express mature retinal differentiation markers, suggesting that differentiation is incomplete (Takahashi et al., 1998).

Surprisingly, neurospheres can also be obtained from adult brain regions that do not produce neurons *in vivo* like the spinal cord or the peripheral retina (Weiss et al., 1996; Tropepe et al., 2000), suggesting that cells may acquire stem-like properties *in vitro* due to reprogramming or dedifferentiation (Palmer et al., 1999; Kondo and Raff, 2000). Interestingly, spinal cord-derived FGF2 responsive stem cells do not generate neurons if transplanted back into the adult spinal cord, although they do so if transplanted into the hippocampal DG (Shihabuddin et al., 2000). These results highlight the importance of niches/local cues in adult neurogenesis: cultured cells with neural stem characteristics can only generate neurons if transplanted into a neurogenic region that provides the appropriate niche.

The notion of a specialized neurogenic niche is also supported by transplantation experiments of uncultured cells. For instance, SVZ cells transplanted into the SVZ or olfactory bulb of another animal generate many new neurons (Lois and Alvarez-Buylla, 1994; Doestch and Alvarez-Buylla, 1996; Herrera et al., 1999); however, transplantation of SVZ cells into the striatum generates mainly astrocytes and very few neurons

(Herrera et al., 1999). Interestingly, BMPs and noggin, besides being involved in neural induction, participate in the establishment of the neurogenic niche in the SVZ. Noggin protein derived from extra-SVZ cells that line the ventricles provides the BMP inhibitory signal necessary for neurogenesis. Ectopic expression of noggin in the striatum creates a neural niche which allows the generation of neurons by grafted SVZ cells (Lim et al., 2000).

In contrast to the relatively broad potential exhibited by cultured stem cells, transplantation studies indicate that uncultured cells are more restricted in the neuron types that they can generate. For instance, neonatal SVZ cells transplanted into the embryonic brain do not generate projection neurons even though the host tissue is producing such neurons (Lim et al., 1997). A comparison of cultured versus freshly isolated postnatal mouse SVZ tissue was made by transplanting these cells into the chick embryo in routes of neural crest migration (Durbec and Rougon, 2001). This study also indicated that cultured SVZ cells are more plastic, able to integrate into neural crest lineages in a way that freshly isolated stem cells could not. These studies highlight the importance of exogenous factors on stem cell behavior.

Stem cell identity: location *in vivo* and markers

The SVZ is a germinal layer of embryonic origin (Altman and Bayer, 1990) that forms adjacent to the telencephalic VZ. It is most prominent in the lateral wall of the ventricle, facing the developing ganglionic eminences. Most importantly, and contrary to the VZ, the SVZ is maintained into adulthood (Altman, 1969; Sturrock and Smart, 1980).

The SVZ cytoarchitecture has been studied by electron microscopy (Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1997). The SVZ is separated from the lateral ventricles by a layer of ciliated ependymal cells and contains PSA-NCAM⁺ neuroblasts (type A cells) that migrate "in chains" encircled by slowly dividing GFAP⁺ astrocytes (type B cells). Clusters of rapidly dividing immature precursors (type C cells) are scattered along the network of migrating chains (figure 1.6).

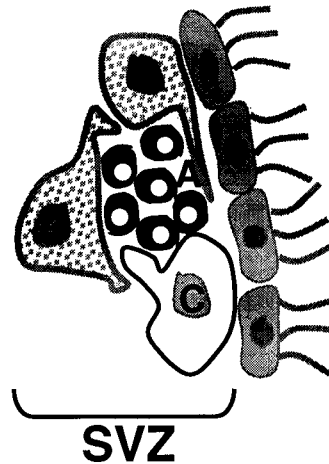


Figure 1.6. Organization of the SVZ in the lateral ventricles of adult mice.

The SVZ is separated from the ventricular space by a layer of ciliated endodermal cells. Chains of neuroblasts (type A cells) are ensheathed by SVZ astrocytes (type B cells) and are adjacent to clusters of immature precursors (type C cells). Adapted from Doetsch et al., 1997.

Recently two types of cells have been identified as being the SVZ neural stem cells: Notch⁺ ciliated endodermal cells (Johansson et al., 1999) and GFAP⁺ astrocytes or type B cell (Doetsch et al., 1999a). Both cell types proliferate *in vivo*, are capable of generating olfactory neurons and generate neurospheres in culture.

There are some pitfalls in Johansson's study: the claim that endodermal cells are stem cells *in vivo* results from the observation that DiI (a lipophilic dye) injected into the ventricles to specifically label endodermal cells is later found in neurons of the olfactory bulb. However, DiI can transfer to other cells intimately close to endodermal cells; moreover, electron microscopy studies show that some astrocytes possess a cilium that can reach the ventricle and thus easily pick up dye (Doetsch et al., 1999b). Moreover, the demonstration that endodermal cells self-renew and have multilineage capacity *in vitro* is controversial because other groups have not been able to reproduce this finding. Chiasson et al., 1999 and Laywell et al., 2000 instead show that endodermal derived spheres are unipotent and do not self-renew *in vitro*.

In Doestch's study, targeting of GFAP⁺ astrocytes was accomplished in a more specific, "dye-free" way. In this study, an adenovirus carrying alkaline phosphatase (AP) as reporter gene was used that only infects cells expressing a specific receptor on their surface. When such adenovirus were injected in the ventricles of transgenic mice expressing that specific receptor under the control of the GFAP promoter, only GFAP astrocytes were shown to express AP. Moreover, AP positive neurons were found in the olfactory bulb thus demonstrating that GFAP⁺ astrocytes are neurogenic cells in the adult SVZ. Furthermore, virus infected GFAP⁺ astrocytes generate neurospheres with self-renewal and multilineage differentiation capacity. These results were later augmented by another elegant study. Ependymal cells and astrocytes are the only cells that survive long-term treatment with cytosine arabinoside (AraC), a drug that kills actively dividing cells. However, only astrocytes divide after removal of the drug and thus are responsible for the complete regeneration of the SVZ, including the neuroblast chain network (Doestch et al., 1999b).

In the hippocampal dentate gyrus, the subgranular layer (SGL) generates neurons that migrate a short distance and differentiate into granule neurons that project into the CA3 area (Markakis and Gage, 1999). Using an approach similar to that used to identify astrocytes as stem cells in the SVZ, Seri et al., 2001 reported that the SGL neurogenic stem cell is also a GFAP⁺ astrocyte (type B cell). Interestingly, a very recent *in vitro* study suggests that new DG neurons are the progeny of a restricted neural precursor cell and not of a stem cell (Seaberg and Van der Kooy, 2002). By careful microdissection of the hippocampus versus the adjacent periventricular (PV) region, these authors were able to show that neurospheres were only generated from the PV region and not from the DG region. The nature of the stem cells in the PV region - whether astrocytic or not - remains unclear at this point.

Why is it important to identify markers for stem cells?

Currently, stem cells are identified largely retrospectively, based on their behavior *in vitro*. A cell grown in isolation in a culture dish that proliferates extensively and generates multiple neural progeny is defined as a neural stem cell. However by the time we recognize it as such, the original stem cell is lost after days of proliferation and differentiation. This *in vitro*, retrospective identification method also does not allow us to

identify stem cells *in vivo*. If we had a means to identify stem cells with specific markers, we could identify them, study their endogenous niches and behavior *in vivo*. Such specific markers would also enable us to isolate fresh stem cells, study them acutely and use them before they are treated extensively with growth factors. Identification of stem cell markers is a critical step towards making progress in understanding stem cell biology.

A breakthrough study by Morrison et al., 1999 showed that NCSCs can be prospectively isolated using the surface marker p75 (the low affinity neurotrophin receptor). The capacity to directly isolate NCSCs by flow cytometry provides, for the first time, the unique opportunity to test the potential of neural crest stem cells *in vivo* upon transplantation, bypassing culture periods that might irreversibly change the properties of such cells.

Some progress has been made in the prospective isolation of human embryonic CNS stem cells (Uchida et al., 2000). In this study, it was established that the prospective neural stem cell has the CD133⁺CD24^{-/lo} phenotype and generates multilineage self-renewing neurospheres in culture. Moreover, upon transplantation into the ventricles of neonate NOD-SCID mice, the *in vitro* expanded CD133⁺CD24^{-/lo} cells generate neurons and astrocytes in the SVZ, olfactory bulb and hippocampal dentate gyrus. It would be of great importance to test if freshly isolated CD133⁺CD24^{-/lo} stem cells have the same engraftment and differentiation capacity as their cultured counterparts.

A recent study used sequential sorts based on cell size, differential binding of peanut agglutinin lectin (PNA) and expression of the heat stable antigen (HAS or CD24) to separate a highly enriched population (80%) of adult mouse SVZ neural stem cells (Rietze et al., 2001). The prospective stem cell has a diameter bigger than 12 μ m and the PNA^{lo}HSA^{lo} phenotype. However, given that only 63% of the total SVZ stem cells were selected, stem cells with a different phenotype do exist. Contrary to the previous two studies, stem cell purification was done by negative selection, or lack of marker expression, which does not permit their localization *in vivo*.

The work described in this thesis provides first time evidence for the prospective isolation and purification of freshly isolated adult (chapter 3) and embryonic (chapter 4) neural stem cells using a positive surface marker. Our study establishes LewisX (LeX) as a surface marker present on the surface of forebrain neural stem cells throughout their development. Antibodies against LeX can be used not only to purify neural stem cells (by FACS) but also to localize these rare cells *in vivo*. Moreover, we describe LewisX expression in embryonic regions with high growth potential, correlating with the expression of the secreted molecules FGF8 and Wnt-1. Recent studies have implicated Wnts and FGFs (including FGF8) as important players in the early events of neural induction (reviewed by Wilson and Edlund, 2001). Moreover, FGF8 is expressed in the anterior neural ridge (ANR), an important telencephalic organizer and in the isthmus (the midbrain-hindbrain organizer). In both locations, FGF8 can mimic organizer activity (reviewed by Wilson and Rubenstein, 2000; Martinez, 2001; Rhinn and Brand, 2001). The co-localization of such powerful inductive molecules within neural stem cells LeX-containing niches provides yet another important piece of information regarding neural stem cell biology (chapter 4).

Thesis outline and main objectives

This thesis is a report of my research experience while working in the laboratory of Dr Sally Temple at the Albany Medical College in Albany, New York.

Chapter two describes the first experiments conducted to investigate how stem cells in the embryonic cortex generate the main types of cells in the nervous system: neurons and glia. Studies in the lab using time lapse video microscopy led to the observation that neural stem cells generate neurons and glia in a stereotyped order: neurons are born first and glia later. The switch from neuron to glia production can be pinpointed to a single asymmetric division. One main aim of our work is to understand the molecular nature of the "switch". We examined the hypothesis that murine homologues of the *Drosophila glial cells missing (gcm)* gene are responsible for such switch. In the first part of the chapter, the expression of one murine *gcm* gene is studied in the mouse cortex. Due to the fact that *gcm* mouse homologues are expressed at very low levels in the mouse embryonic cortex and are likely to be expressed in the stem cells, a protocol was designed with the objective of enriching the test population in neural stem cells. The second part of this chapter describes the development of such methodology.

In **Chapter three** we sought to determine if the protocol designed for the enrichment of embryonic cortical neural stem cells could be used for the same purpose in the adult mouse SVZ, a region of the brain that harbors stem cells. We show that LewisX/SSEA-1 is a surface marker that can be used to enrich for adult mouse SVZ neural stem cells and prospectively identify such cells *in vivo*. The results are discussed in the context of the actual controversy regarding the identity of the stem cell within the SVZ. We also provide preliminary data from experiments designed to address the behavior of LewisX positive stem cells when transplanted into an *in vivo* embryonic environment.

In **Chapter four** we expand on the study of LewisX/SSEA-1 surface marker to demonstrate that neural stem cells isolated from the cortex of mouse embryos at

different ages are also LewisX positive. We discuss the possible biological roles of LewisX/SSEA-1 in stem cell development and also address the implications for neural induction and patterning of its co-expression with growth factors in highly proliferative brain regions.

Chapter five consists of a summarizing discussion of the work as a whole in the context of the stem cell field and draws some main conclusions. Future directions are indicated in this final chapter, marking the beginning of another journey.

Chapter Two



PART I GLIAL CELLS MISSING IN THE MOUSE EMBRYO.

Introduction

The analysis of the reconstructed lineage trees for cortical stem cells reveals that neurogenesis and gliogenesis diverge at a critical point in the lineage where a special asymmetric division occurs: it generates a neuroblast (neuronal progenitor cell) and a glioblast (glia progenitor cell) instead of a stem cell and a neuroblast (figure 2.1). This bifurcating point of neuronal and glial lineages marks the most fundamental cell fate decision in the nervous system and therefore should be under tight control. What determines this switch?

The identification, in *Drosophila*, of *gcm* - *glial cells missing*- as a master regulatory gene involved in the neuron-to-glia production marked an important turning point in our understanding of how a major alteration in cell fate can be accomplished by a seemingly "simple" molecular switch (Anderson, 1995; Jones et al., 1995a; Hosoya et al., 1995; Vincent et al., 1996; reviewed by Wegner and Reithmacher, 2001). In *Drosophila*, the presence or absence of *gcm* determines the choice between neuronal and glial cell fates. In loss-of-function *gcm* mutants, almost all CNS and PNS glia fail to differentiate; conversely, when GCM is over-expressed in early neuronal precursors, the number of glia increases dramatically. For instance, in one PNS lineage that gives rise to the dorsal bipolar dendritic (BD) neuron, the sensory organ precursor cell (SOP) divides once to generate 2 cells: the BD neuron and its support glial cell (figure 2.2 A). In *gcm* loss-of-function mutants, both daughters of the progenitor cell become neurons (Figure 2.2 B) whereas in gain-of-function mutants (where *gcm* expression is driven by a neuronal promoter), both cells become glial cells and express REPO (Figure 2.2 C), a ubiquitous glia marker (Xiong et al., 1994; Campbell et al., 1994). These results show that *gcm* acts as a binary genetic switch for neuron vs. glial in multipotent CNS and PNS precursors. How is *gcm* expression regulated in the multipotent lineages?

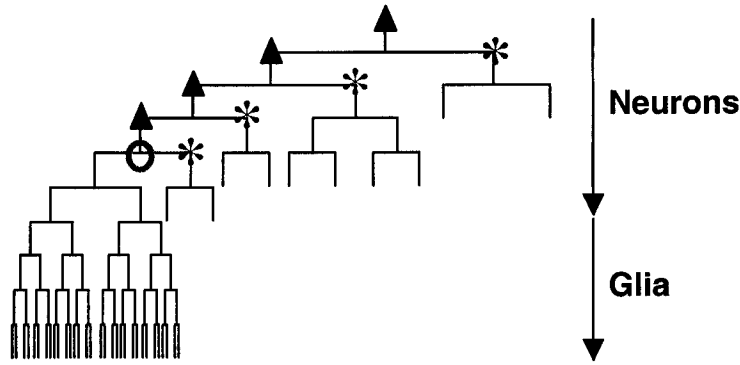


Figure 2.1. Model of Neuron-Glia Generation from multipotent cortical stem cells. Embryonic stem cells (black triangles) divide asymmetrically to generate more stem cells and restricted neuroblasts (green asterisks). Later, a special asymmetric division occurs (the "switch point", orange ring) in which the stem cell generates a glioblast and a neuroblast. Glioblasts divide symmetrically to produce lots of progeny. The switch point thus separates an early neurogenic phase from a later gliogenic phase. Adapted from Qian et al., 2000.

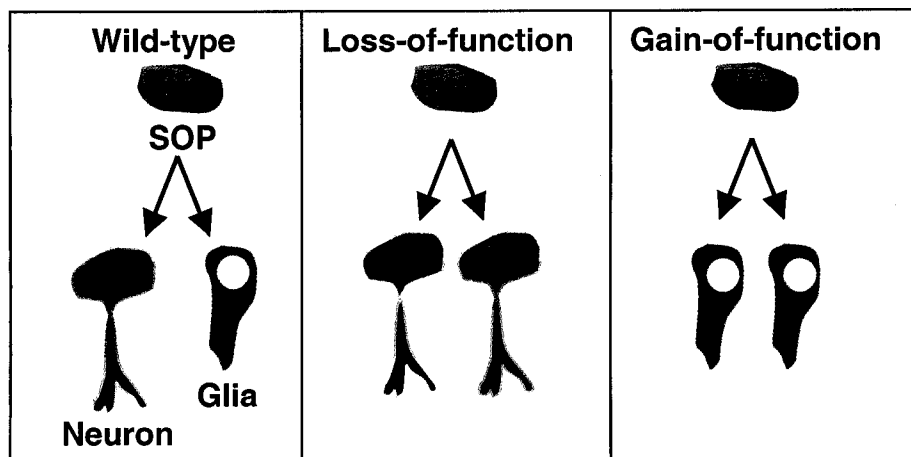


Figure 2.2. Function of *gcm* in *Drosophila*. In the lineage that generates the dendritic bipolar neuron, the SOP divides to generate one neuron and one glial cell (left panel). In loss-of-function mutants, the SOP generates two neurons (middle panel) while in flies over-expressing *gcm* (gain-of-function) it generates two glial cells (right panel).

What is *gcm* and how does it direct cell fate?

The GCM protein is a novel DNA binding protein with a nuclear localization signal (NLS) that localizes to the nucleus and causes changes in transcription. Such characteristics suggest that GCM acts as a transcription factor or a transcription regulatory molecule. It binds with high specificity to the DNA target sequence (A/G)CCCGCAT, a binding site found in the *repo* gene (Xiong et al., 1994; Campbell et al., 1994) whose expression is dependent on *gcm* (Jones et al., 1995a; Hosoya et al., 1995). Gcm activates other transcription activators and repressors required for normal gliogenesis, like *pointed*, and *tramtrack* (Giesen et al., 1997), which together with *repo*, execute the glial differentiation program initiated by *gcm* (reviewed in Granderath and Klämbt, 1999; figure 2.3).



Figure 2.3. Function of *gcm* in lateral glial cell development.

Gcm activates the transcription of *repo*, *pointed* (*pnt*) and *tramtrack* (*ttk*). *Repo* and *pnt* propagate glial differentiation downstream of *gcm* while *ttk* represses neuronal gene expression in glial cells. GCM also positively regulates its own transcription (Miller et al., 1998).

Studies by Akiyama-Oda et al., 1999 and Bernardoni et al., 1999 describe the lineage of the neuroglioblast NB6-4T, in which the neuronal and glial cell lineages bifurcate in the first cell division. When NB6-4T divides, it generates one medial (M) and one lateral daughter cell (L). The L daughter does not express GCM and produces ganglion mother cells, which are neuronal precursors. On the other hand, the M daughter expresses GCM and originates glial cells. Both studies propose the *Gcm* mRNA is present evenly throughout the cytoplasm of NB6-4T before division but becomes asymmetrically localized to the M

daughter cell during the first division. The asymmetric localization of *gcm* mRNA triggers glial commitment in M and its absence in L leads to the generation of neuronal progeny (figure 2.4 A).

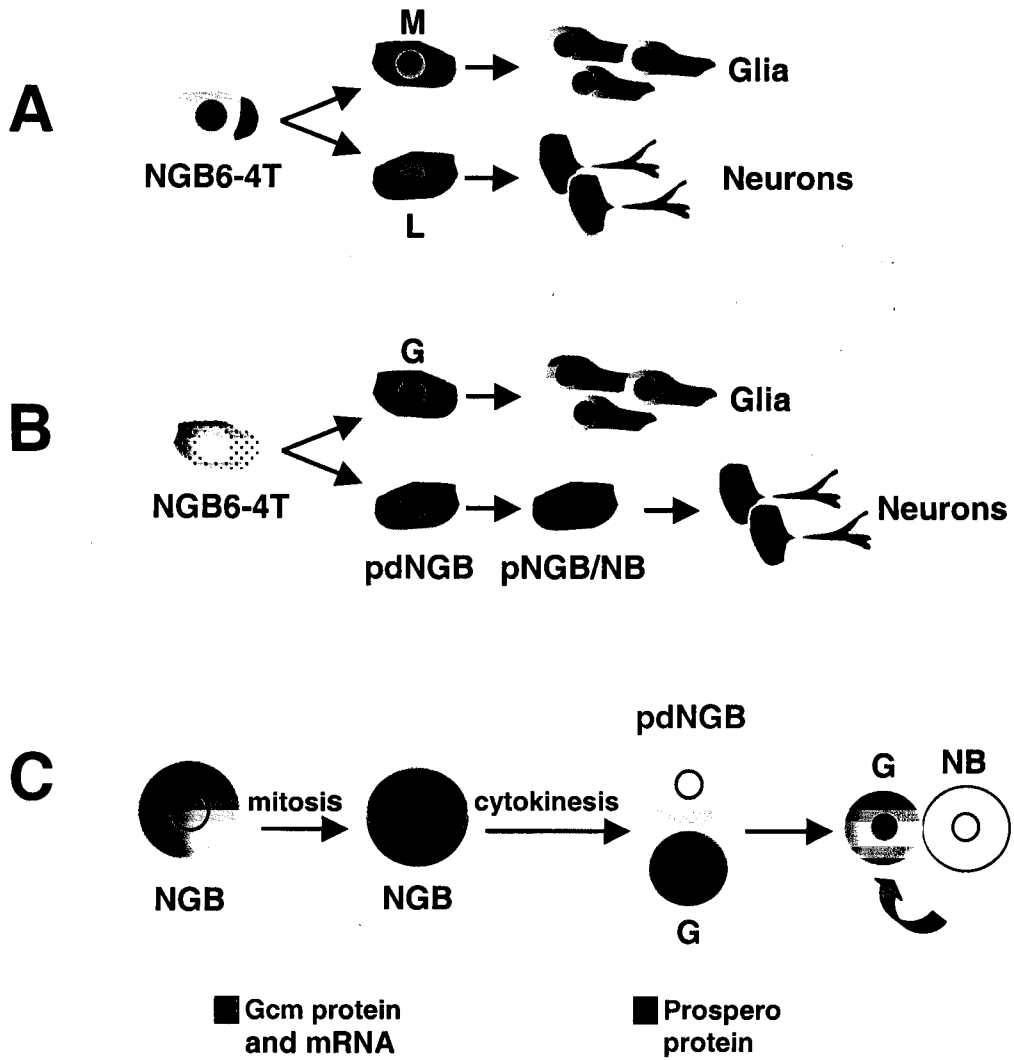
A recent study intended at understanding the molecular mechanisms of *gcm* mRNA localization, revealed different results. Freeman and Doe, 2001, found no evidence for asymmetric localization of *gcm* protein or mRNA in NB6-4T (or NGB). Instead, *gcm* mRNA is uniformly distributed in the mitotic NGB and equally distributed in the post-divisional NGB (pdNGB) and G (same as M in Akiyama-Oda et al., 1999). Later, *gcm* mRNA and protein are up-regulated in G (which migrates from an apical to a more medial position relative to pdNGB) and its progeny but down-regulated in the pdNGB (figure 2.4 B). This is accomplished by the asymmetric localization of *prospero* to the M (glia producing) daughter, which is crucial for the maintenance of *gcm* expression and glial fate induction (figure 2.4 C) (*prospero* is expressed in longitudinal glial cells which become spatially disorganized in *prospero* mutants- Doe et al., 1991). Mislocalization of *prospero* mRNA and protein in *miranda* mutants (resulting in equal distribution of Prospero in the NGB and G) leads to ectopic expression of *gcm* and production of extra glia. Prospero thus acts as a potent activator of *gcm* expression.

Figure 2.4. Expression of *gcm* in *Drosophila* NB6-4T CNS neuroglioblast lineage.

A. Transcripts of *gcm* (orange) become asymmetrically localized in the neuroglioblast and end up preferentially in the medial daughter (M), which generates glial cells. The lateral daughter (L) lacks *gcm* expression and generates neurons. After Akiyama-Oda et al., 1996 and Vincent et al., 1996.

B. Gcm protein (black dots) and low levels of *gcm* mRNA (solid orange) are uniformly expressed in the neuroglioblast. Both daughters (G and pdNGB – post divisional NGB) inherit the mRNA and the protein which immediately enters the nucleus. The G daughter maintains high levels of Gcm protein and mRNA and produces 3 glial cells. Gcm protein and mRNA are downregulated in the pdNGB which continues to divide and produces neurons.

Figure 2.4



C. *Gcm* expression in G is regulated by Prospero asymmetric distribution in the NGB before division. Prospero protein is only expressed in the G daughter. Upregulation of *gcm* in G is depicted as intensification of the orange color.

Adapted from Freeman and Doe, 2001.

Gcm in mammals

The novel DNA binding specificity indicates that GCM contains a new type of DNA binding domain, defined as the "gcm motif" (Akiyama et al., 1996; Schreiber et al., 1997). The evolutionary conserved motif allowed the identification of additional "gcm motif" genes present not only in other *Drosophila* genes but also in mouse and human genes (figure 2.5). Vertebrate homologues of *Drosophila gcm* include the mouse genes *mgcma/Gcm1* and *mgcmb/Gcm2* and the human *hgcm* gene (Akiyama et al., 1996; Altshuler et al., 1996; Kim et al., 1998). In mouse, the "a" allele was identified using placental mRNA while *mgcmb* was isolated from adult brain mRNA, suggesting that it might also be expressed early in brain development.

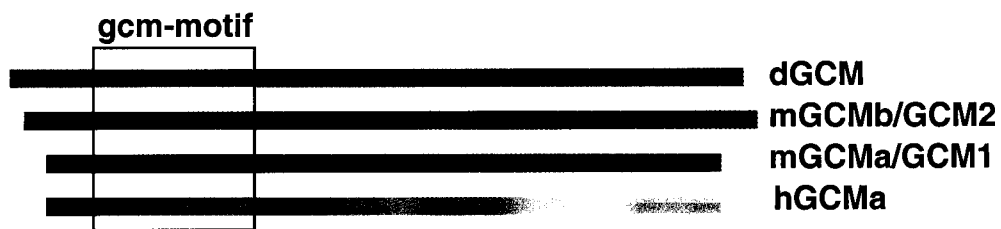


Figure 2.5. The *gcm* gene products. Scheme of the sequence alignment of the human (hGCMa), mouse (mGCMb/GCM2 and mGCMa/GCM1) and *Drosophila* GCM proteins, highlighting the conserved DNA-binding domain or gcm motif.

When I joined the laboratory of Dr Sally Temple, the expression pattern of *mgcma/Gcm1* and *mgcmb/Gcm2* in the murine nervous system was not known. Likewise, it was also uncertain if such genes could have a similar cell fate "genetic switch" function as in *Drosophila*. We were interested in the molecular mechanisms that governed the switch from neurogenesis to gliogenesis that could be pinpointed to a single cell division in the stem cell lineage tree. We thus hypothesized that expression of *mgcm* expression underlined the cell fate switch observed.

Results

MgcmB/Gcm2 is expressed in the mouse cortex from E10 to P8

Given that *mgcmA/Gcm1* was originally cloned from placenta and that it was undetected by *in situ* hybridization throughout mouse embryonic development, at postnatal day 4 and in adult mouse brain (Altshuller et al., 1996), we concentrated our studies on *mgcmB/Gcm2*. We studied the expression of *mgcmB/Gcm2* during embryonic development of the mouse cortex (from E10 on) and also during the first postnatal week (P0 to P8), a time window wide enough to include the onset and peak of neurogenesis and gliogenesis.

For that, a primer pair was designed that amplifies a 296bp sequence on the *gcm*-motif. RT-PCR analysis indicates that *MgcmB/Gcm2* is expressed in the mouse embryo cortex as early as E10 and is maintained after birth although its expression declines around P3-P8 (figure 2.6, first lane for each age). GAPDH cDNA was amplified as the control product (figure 2.6, second lane for each age). When reverse transcriptase was omitted from the reaction, no product was formed, confirming that contaminating DNA is not present in our RNA samples (not shown). Moreover, the PCR primers used span an exon-intron junction allowing the distinction between spliced messages from genomic DNA (the size of genomic DNA amplified fragment is 420bp). It would have been very interesting to confirm the developmental regulation of *mgcmB* expression suggested by our qualitative RT-PCR data using real-time PCR. Unfortunately, such technique was not available. It is likely that *mgcmB/Gcm2* expression in the cortex starts even earlier than E10, but such possibility was not tested.

The expression level of mgcmB/Gcm2 in the CNS is below the detection limit of *in situ* hybridization techniques

In order to study *mgcmB/Gcm2* expression pattern in mouse embryos, whole-mount *in situ* hybridization was performed using digoxigenin labeled RNA probes. Unfortunately, this approach proved unsuccessful so we opted for *in situ* hybridization in frozen sections of mouse embryos of different developmental ages. Yet again, we were unable to detect *mgcmB/Gcm2* transcripts in the CNS or PNS using this technique. The use of delta as control probe on sister slides consistently revealed their expected expression domains in the

forebrain and hindbrain at E10 (figure 2.7 A,B), demonstrating that the *in situ* hybridization technique worked. Strikingly, the only region in the entire embryo showing positive expression of *mgcmb/Gcm2* was the parathyroid (Figure 2.7 C), suggesting that this gene might play an important role in the development of this endocrine gland. Taken together, these results show that *mgcmb/Gcm2* is expressed very early in the developing cortex, and suggest that its expression is temporally regulated. However, further studies of expression pattern were hampered due to the low sensitivity of the *in situ* hybridization technique combined with the low abundance of the *mgcmb/Gcm2* transcript.

Figure 2.6. RT-PCR analysis of the expression of *mgcmb/Gcm2* genes in mouse cortex.

cDNAs derived from mouse tissues from early embryonic age until the first postnatal week were obtained by reverse transcription using Oligo(dT) primers and amplified with gene specific primers that generate a 296bp fragment. GAPDH cDNA was amplified as a control product (613bp). No product was formed when reverse transcriptase was omitted from the cDNA preparation step (not shown).

Figure 2.7. Expression of *mgcmb/Gcm2* and *delta* in sections of E11 mouse embryos.

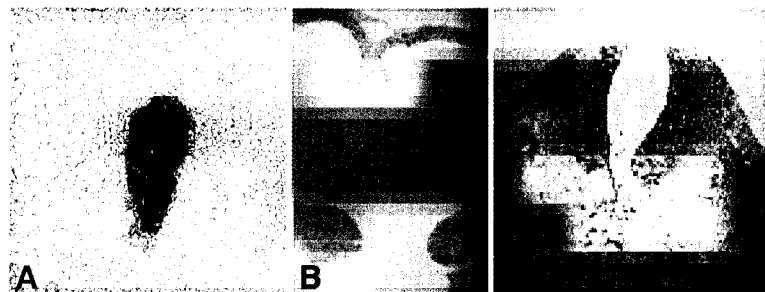
A. *mgcmb/Gcm2* is only expressed in the parathyroid.

B,C. as expected, *delta* is expressed in the embryonic forebrain (B) and spinal cord (C) at low levels in a characteristic "salt and pepper" pattern.

Figure 2.6



Figure 2.7



Discussion

The results described above were confirmed and further completed by others. Kim et al., 1998 reported that *Gcm2* is expressed (by RT-PCR) in the head region of E9.5 and E11.5 mouse embryos and in the cortex at E16.5. Our characterization of *mgcmb/Gcm2* expression in the cortex is more complete than that of Kim et al., 1998, given that it analyzes a wider developmental window. Moreover, it reveals a temporal regulation of *mgcmb/Gcm2* not previously appreciated. In agreement with our observations, Kim et al., 1998 report that the expression of *Gcm2* by *in situ* hybridization is restricted to the parathyroid and that, similarly to *Gcm2*, *Gcm1* is also mainly expressed in non-neuronal tissues, such as the placenta and the kidneys. Because *mgcmb/Gcm2* is expressed at very low levels in the brain, it is difficult to examine the expression of this cell in different cell populations to investigate its function. However, knock out data indicate that the function of the murine *gcm* genes is not conserved from that of the *Drosophila gcm*. Analysis of mouse *gcm* mutants revealed no apparent CNS or PNS phenotype exists. *Gcm1* null mutants are embryonic lethal due to placental failure (Anson-Cartwright et al., 2000; Schreiber et al., 2000); the embryo itself showed no obvious abnormalities, consistent with the extremely low expression of *gcm1* in embryo tissues (Akiyama et al., 1996; Altshuler et al., 1996; Kim et al., 1998). On the other hand, *gcm2* deficient mice lack parathyroid glands, implying that *gcm2* is a master regulatory gene of parathyroid development. Although presenting hypoparathyroidism and a mild abnormal bone phenotype, such mice are viable and fertile (Gunther et al., 2000). Unexpectedly, *Gcm2* deficient mice have parathyroid hormone (PTH) levels similar to wild-type animals. This is the result of the existence of an auxiliary PTH source, the thymus, uncovered by the *Gcm2* deletion.

Interestingly, although the development of the placental labyrinth and of the parathyroid glands are dramatically affected by *Gcm* genes, in neither case does cell fate transformation occur. Likewise, ectopic expression of *gcm* in the mouse retina failed to cause the neuron to glia transformations seen in the *Drosophila* nervous system (Hojo et al., 2000). Taken together, these observations demonstrate that murine *Gcm* function is not conserved from that of *Drosophila*. Nevertheless, *Gcm1* shares regulatory capacities with

gcm. Pan-neural expression of *Gcm1* in *Drosophila* leads to an increase in the number of REPO⁺ glial cells (similar to the *gcm* over-expression phenotype), but this does not occur when *Gcm2* is expressed instead of *Gcm1*. Moreover, *Gcm1* was able to rescue the *gcm* phenotype while *Gcm2* was not (Kim et al., 1998). This suggests that GCM1 shares with GCM important key features as a transcription factor, although it does not seem to be involved in mammalian glial cell specification. In this regard, it is important to point out a fundamental difference between vertebrates and invertebrates, as far as gliogenesis is concerned. In invertebrates, glial cells assume all the functions that in vertebrates are assured by two types of glial cells: astrocytes and oligodendrocytes. Perhaps the separation of duties into astrocytes and oligodendrocytes in vertebrates led to a different and/or more complex way to generate glial cells. It is also possible that in vertebrates, *mgcms* cooperate with other genes and thus may be part of a concerted mechanism to orchestrate the neuron to glia switch. Hence, we believe that *mgcms* should not be dismissed as players in this "switch hunt".

PART II CD15 IS A SURFACE MARKER FOR HIGHLY PROLIFERATIVE CELLS IN THE MOUSE EMBRYONIC CORTEX THAT EXPRESS MGCMB.

Introduction

Our working hypothesis for the function of *mgcmb/Gcm2* in the embryonic mouse cortex is that *mgcmb/Gcm2* is expressed in the bifurcating point of the neurogenic and gliogenic branches of the stem cell lineage. Given that *mgcmb/Gcm2* is expressed at very low levels in the mouse cortex, and that its expression might be transient (as in *Drosophila*), enrichment of our starting biological material in cortical stem cells should improve the chance of finding *mgcmb/Gcm2* expression.

In our and other's laboratories, identification of neural stem cells has been accomplished retrospectively, using *in vitro* assays in which "stem cellness" is attributed to the founder cell that either generates a neurosphere (when grown in suspension) or a big clone of many cells containing neurons and glia (when grown on a substrate). Such methods are thus based on performance (or physiology) rather than on characteristics that distinguish stem cells from other cells in the dish (phenotype).

What are then the morphological characteristics of stem cells? Can it be size, shape or another feature easily identifiable under the microscope? Unfortunately, dissociated early cortical cells are identical in shape, size and do not seem to have any distinctive characteristic. Do stem cells express unique proteins or carbohydrates that are can function as subpopulation markers? Maybe. The best example of such cell classification methodology is the hematopoietic system. A collection of monoclonal antibodies is used to define cell subpopulations based on the molecules expressed on their surface (reviewed by Morrison et al., 1994). Such method provides a very reliable way for the purification of live cells for further studies. In the case of neural stem cells, such purification methods are only just becoming available as specific surface markers are described. Mouse PNS and human embryonic CNS stem cells have been purified based on their expression of p75 and of CD133, respectively (Morrison et al., 1999; Uchida et al., 2000). Interestingly, CD133 is a

hematopoietic stem cell marker and is thus an example of conservation of a stem cell marker across different lineages. However, in the mouse, such parallelism has not been found.

Candidate molecules for neural stem cell markers include Nestin, and Musashi. But are they stem cell specific? Nestin is an intermediate filament protein present in early neural progenitor cells (Hockfield and McKay, 1985). It labels around 98% of the cells in the E10 mouse cortex and thus is not useful to distinguish stem cells from other early progenitors. Musashi is an RNA binding protein whose expression pattern in the early CNS is similar to that of nestin (Sakakibara et al., 1996) and therefore it is also not a good stem cell marker. Moreover, Nestin and Musashi are both intracellular antigens only accessible to antibody staining after permeabilization, and therefore do not permit live cell purification. An ideal marker should be a molecule present at the surface of stem cells that enabled their purification via fluorescence-activated cell sorting or FACS. So far, no such markers have been described that functionally sub-divide early cortical cells in subpopulations.

The work of Gray and Sanes, 1992 suggested that radial glial cells (the earliest cell type in the CNS to be morphologically identifiable) might provide more than structural support for neuronal migration in the vertebrate brain. Their *in vivo* study suggested that radial glia might have neurogenic capacity. Indeed, Alvarez-Buylla et al., 1990, demonstrated that radial glia in the canary high vocal center are stem cells and generate high numbers of neurons. Inspired by these results, we hypothesized that radial glia in the embryonic mouse cortex are stem cells and sought to identify markers that could be used to purify radial glia. Radial glia are early components of the neuroepithelium where they form palisades of narrow, elongated cells stretching from the ventricular to the pial surface (reviewed by Schmechel and Rakic, 1979). Such morphological characteristics enabled investigators to label radial glial by placing very small DiI crystals on the pial surface of the brain (Voigt, 1989; Hunter and Hatten, 1995). Radial glial cells can also be labeled with RC2 (Misson et al., 1988) an antibody that, similarly to nestin, recognizes an intermediate filament protein in most of the cells present in the early cortex (~90%). Due to its ubiquitous expression in the early cortex and to its intracellular nature, RC2 presents the same disadvantages as nestin and musashi for its use in the purification of stem cells.

A more recent study shows that CD15, an antibody produced by the hybridoma clone MMA, recognizes the LewisX (LeX) epitope at the surface of radial glial cells in the mouse embryo forebrain (Mai et al., 1998). To date, this is the only reference to the use of a surface molecule to identify radial glial cells.

We thus began this project by addressing the proliferative and differentiative potential of LeX positive cortical cells isolate from mouse embryos.

Results

LewisX positive cells generate large clones containing neurons, astrocytes and oligodendrocytes.

An E13 cortical cell suspension contains around 38% of LeX⁺ cells as judged by acute staining, which is a suitable percentage for sorting using magnetic beads (Dyna). To test the potential of LeX⁺ cortical cells, a single cell suspension of E13 cortical cells was generated, stained with anti-CD15 antibody and further incubated with magnetic beads coated with a suitable secondary antibody (scheme of the immunomagnetic sorting protocol in figure 2.8). LeX⁺ and LeX⁻ cells were separated using a magnet and cultured for up to 10 days in poly-L-lysine coated Terasaki plates in the presence of 10ng/ml FGF2. The purity of the cell subpopulations separated using this technique is around 95% (93-99%) for the LeX⁺ population and 82% (81-85%) for the LeX⁻ population. To further purify the LeX⁻ population, we performed a complement kill incubation that considerably increased its purity to 99.5%. E13 LeX⁺ and LeX⁻ cortical cells survive the immunomagnetic procedure and generate clones in culture. The presence of beads at the surface of LeX⁺ cells does not impair their capacity to generate large clones (figure 2.9 A, B). Interestingly, the LeX⁻ cells proliferate much less than LeX⁺ cells and generate very small clones (figure 2.9 C, D).

In order to study the proliferative capacity of the different cell subpopulations, BrdU (a thymidine homologue taken up by dividing cells) was added to the LeX⁺ and LeX⁻ cultures 2 hours after plating. The cells were fixed 12, 24 and 36 hours later and processed for BrdU immunolocalization. The graph in figure 2.10 shows that the percentage of cells that undergo cell division (and thus incorporate BrdU) is consistently higher in the LeX⁺ than in the LeX⁻ subpopulation, indicating that LeX⁺ cells have a higher proliferative potential than LeX⁻ cells.

In our culture conditions, cortical stem cells generate big clones containing neurons and glial cells (astrocytes and oligodendrocytes). To test whether LeX⁺ cells can generate those three cell types *in vitro*, purified cells were grown for up to 14 days. The cultures were fixed and incubated with cell-type specific antibodies: β -tubulin isoform III for neurons (β -tub), O4 for oligodendrocytes and GFAP for astrocytes. Strikingly, clone composition was very different in LeX⁺ and LeX⁻ cultures. LeX⁻ cells generated mainly neurons while LeX⁺

could generate neurons, oligodendrocytes and astrocytes, suggesting that stem cells were present only in the LeX⁺ fraction (figure 2.11). In addition to stem cells, the LeX⁺ population also contains restricted neuroblast progenitors that only generate neurons (not shown). Moreover, the number of neurons generated by stem cell clones varied suggesting that the LeX epitope is present in stem cells at different points in the lineage tree, perhaps also in the cell that bifurcates the neuronal and glial production. Taken together, these results indicate that LeX is a surface marker of highly proliferative cells, including stem cells. Hence, LeX is a useful marker for enriching a cell population in stem cells.

Figure 2.8. Immunomagnetic selection of E13 cortical cells expressing the Lewis epitope.

A single cell suspension of E13 cortical cells is incubated with anti-CD15 antibody (an IgM), followed by an incubation with magnetic beads coated with anti-IgM antibodies. The cells that express the LewisX antigen will bind the magnetic beads and can be separated from the LewisX negative cells with a magnet.

Figure 2.9. LeX⁺ and LeX⁻ survive the immunomagnetic sorting and generate clones in culture.

(A) LeX⁺ cells with magnetic beads attached.

(C) Bead-free LeX⁻ cells 2 hours after plating in PLL coated Terasaki plates.

(B) LeX⁺ cells generate big clones containing cells with varied morphology after 5 days in culture in the presence of 10ng/ml FGF2.

(D) LeX⁻ cells are less proliferative and seem to generate mainly neurons after 5 days in culture. Size bar 50µm.

Figure 2.8

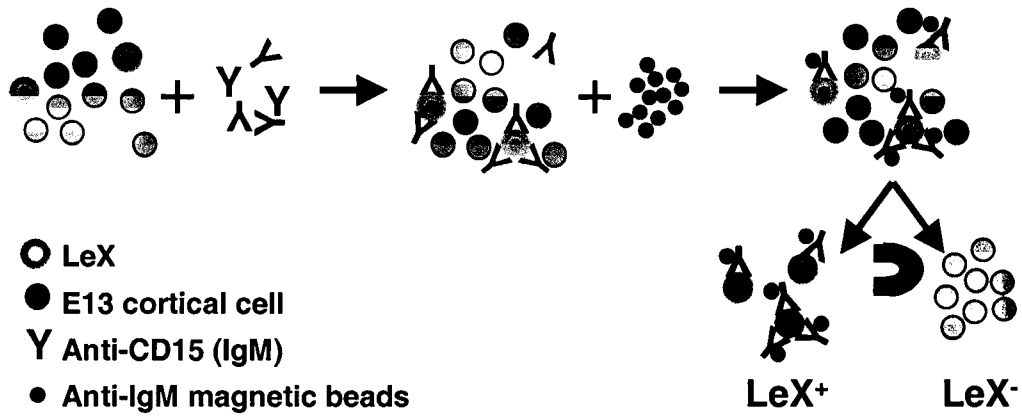
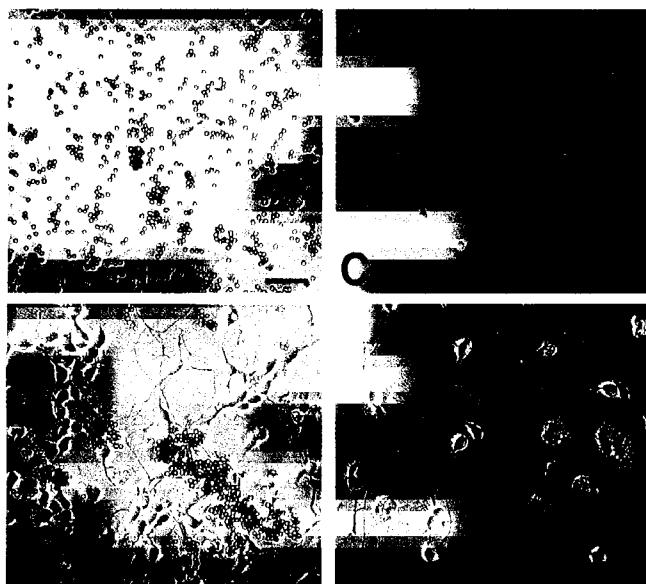


Figure 2.9



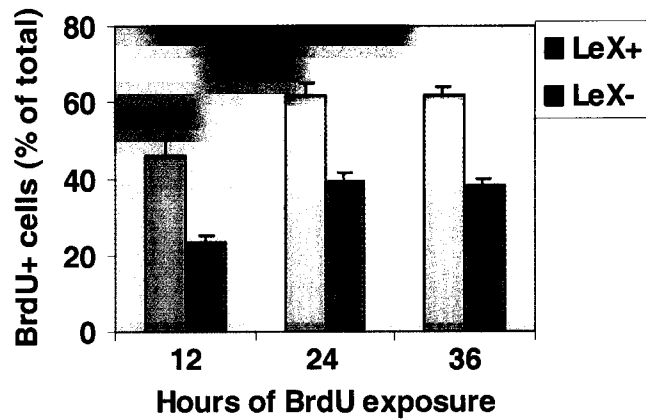


Figure 2.10. Proliferation of LeX⁺ and LeX⁻ E13 cortical cells in culture.

LeX⁺ and LeX⁻ were plated on PLL coated Terasaki plates and BrdU (100ng/ml) was added to the wells 2 hours later. The plates were fixed and processed for BrdU immunohistochemistry 12, 24 and 36 hours after beginning of incubation with BrdU. BrdU positive nuclei were counted and expressed as a percentage of the total cells as determined by DAPI staining. Values are averages±SE.

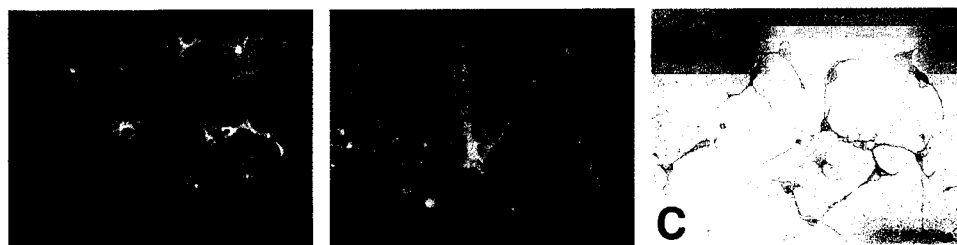


Figure 2.11. E13 LeX⁺ cells are highly proliferative and include stem cells. LeX⁺ E13 cortical cells growing on PLL in the presence of 10ng/ml FGF2 generate GFAP positive astrocytes (A), β -tub positive neurons (B) and O4 positive oligodendrocytes after 7-10 days in culture. Size bar 50 μ m.

Mgcm2/Gcm2 is expressed in LeX⁺ E13 cortical cells but not in differentiated cultures of astrocytes and oligodendrocytes.

Stem cells are the major source of glia in the cerebral cortex. Given that the stem cell lineage tree bifurcates neuronal and glial production, we ought to observe *mgcm2/Gcm2* expression in a LeX⁺ population enriched in stem cells. RT-PCR was used to test this prediction.

Pilot experiments were conducted in order to determine the optimal PCR conditions. We found that nested primers (scheme on figure 2.12) were necessary for the amplification of *mgcm2/Gcm2* cDNA from the total RNA of E10 cortical cells grown for 6 days on PLL (not shown).

The expression of *mgcm2/Gcm2* was studied in freshly purified LeX⁺ and LeX⁻ E13 cortical cells and in later cells of the glial lineage (purified cultures of cortical astrocytes and freshly purified O4⁺ oligodendrocytes – generously provided by Dr Ingraham at the Albany Medical College and obtained by immunopanning of cortical cells from P4 rat pups [Ingraham et al., 1999]). A positive control (cDNA derived from E10 cells used to optimize the technique) and a water negative control were also included.

Figure 2.13 A shows the results of the first round of PCR, and figure 2.13 B shows the outcome of the second round of PCR in which an aliquot of the first reaction is used as a template for the second PCR reaction. We were unable to obtain any signal while testing O4⁺ oligodendrocyte progenitors (lane 1) or astrocytes (lane 2). However, freshly isolated E13 LeX⁺ cells expressed *mgcm2/Gcm2* (lane 5) while LeX⁻ did not (lane 4). Lane 3 is a positive control (E10 cortical cells grown for 6 days on PLL) and lane 6 is a negative water control (no cDNA). This result demonstrates that similarly to *Drosophila*, *mgcm2/Gcm2* is not expressed in fully differentiated glia. Moreover it reveals a lineage segregation of the *mgcm2/Gcm2* gene expression that is very similar to that observed in the *Drosophila* NB6-4T: it is absent in a population of restricted neuroblasts and it is present in a population that contains multipotent cells.

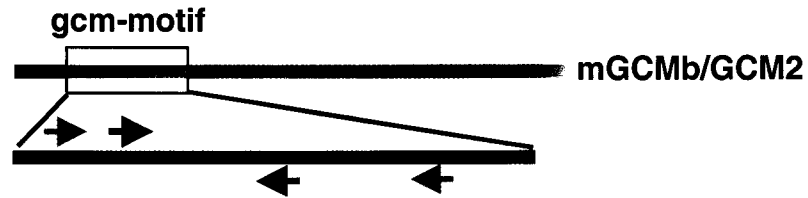


Figure 2.12. Nested PCR strategy for amplification of *mgcmb/Gcm2* cDNA from fresh and cultured cells. Two pairs of primers are used: the outside pair (orange) amplifies a 655bp fragment. An aliquot of the first reaction is used as a template for a second PCR using the inside pair of primers (black) which amplifies a 296bp fragment.

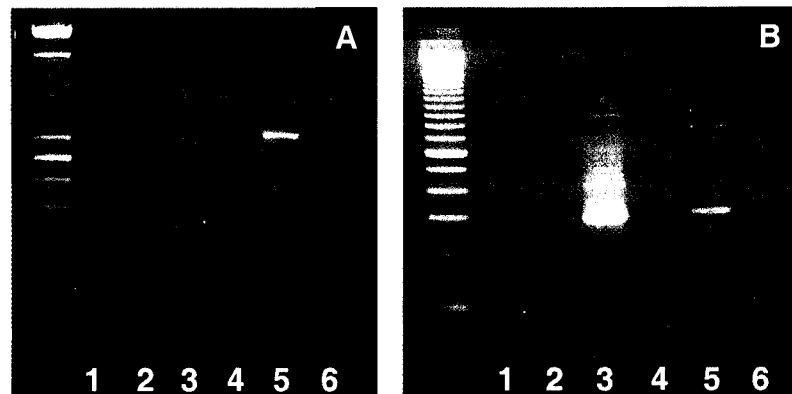


Figure 2.13. RT-PCR analysis of the expression of *mgcmb/Gcm2* in different cells. (A) First round of PCR; (B) second round of PCR. cDNAs were derived from freshly purified O4⁺ oligodendrocytes (lane 1), pure astrocyte cultures (lane 2), E10 cortical cells grown for 6 days on PLL (lane 3), freshly purified E13 LeX⁻ cells (lane 4) and LeX⁺ cells (lane 5), and a water negative control (no template). Only LeX⁺ cells and the positive control express *mgcmb/Gcm2*.

The next step in our project was to determine at which point in the stem cell lineage is *mgcmb/Gcm2* expressed. Our prediction was that it would be present in cell α and possibly in cell β (figure 2.14). The best approach to this study would be to follow the progeny of stem cells isolated from a transgenic mouse in which the green fluorescent protein gene (GFP) was "knocked in" in the *mgcmb/Gcm2* locus. Such experiments would allow time-lapse video microscopy analysis (using a fluorescence microscope) of cortical LeX⁺ cells isolated from the transgenic mouse. Unfortunately, this mouse is not available. Another possible approach was to follow the development of LeX⁺ cells by standard time-lapse video microscopy, fix the cultures at different time points and analyze the expression of *mgcmb/Gcm2* by *in situ* hybridization or *in situ* RT-PCR. Similarly to the results in tissue sections, we did not observe any cells expressing *mgcmb/Gcm2* by *in situ* hybridization in tissue culture, possibly again due to the low sensitivity of the technique. Likewise, attempts to implement direct and indirect *in situ* RT-PCR technique to the study of *mgcmb/Gcm2* expression in single cells proved unsuccessful.

Discussion

We have demonstrated that highly proliferative E13 cortical cells can be purified using antibodies against a carbohydrate epitope LeX. LeX⁺ cells include restricted neuroblasts and most importantly, stem cells. Moreover, we demonstrated that *mgcmb* is expressed by such highly proliferative cells and possibly also by stem cells. The difficulty of addressing single cell gene expression within a stem cell lineage makes it hard to pinpoint exactly when *mgcmb* is expressed and in which cell. Such analysis will be feasible when transgenic mice carrying a reporter gene under the control of *mgcmb* promoter are made available.

It is interesting that the expression of *mgcmb* was excluded from differentiated glial cells and committed neuroblasts (LeX⁻ E13 cells), and is present in LeX⁺ cells, which contain stem cells. These results hint towards a similar expression pattern of *mgcmb/Gcm2* and *Drosophila gcm* in multipotent lineages. However, given the discouraging neural phenotypes of *Gcm1* and *Gcm2* null mutants, it is likely that in the murine vertebrate nervous system, other players are involved in the neuron glia-switch decision. It should not be ruled out, however, the possibility that other *gcm*-like genes might be co-expressed in mammals. Functional degeneracy then would provide the answer for the inexistence of a neural phenotype of known *gcm* null mutations.

Which molecules have so far been shown to influence neuron-glia decision in mammals?

Basic helix loop helix (bHLH) proteins are transcriptional regulators of crucial importance in cell fate determination in the nervous system and in other tissues (reviewed by Lee, 1997). In the nervous tissue, bHLH transcription factors like Neurogenin 1 and 2, Mash-1 and NeuroD endow cells with the potential to adopt a neural fate, as thus are called "proneural". Such genes are potent regulators of neuronal differentiation. Mice lacking Mash-1 have defective progenitor specification in autonomic ganglia, olfactory epithelium and ventral forebrain (Cau et al., 1997; Lo et al., 1998; Casarosa et al., 1999). Ngn1 and Ngn2 mutant mice also present a neurogenic phenotype in ganglia and telencephalon (Ma et al., 1999; Fode et al., 2000).

Recent papers have shown that proneural bHLH transcription factors not only promote neurogenesis by activating the expression of a cascade of neuronal genes, but also reinforce neurogenesis by inhibiting the expression of glial genes (Tomita et al., 2000; Nieto et al., 2001 and Sun et al., 2001). For instance, deficiencies in various combinations of proneural genes resulted in a loss of certain types of neurons, and also in premature gliogenesis (Tomita et al., 2000; Nieto et al., 2001). In addition to reduced neurogenesis, *Math3; Mash1* double mutant mice show precocious glia differentiation in the retina, tectum and hindbrain. Likewise, *Ngn2; Mash1* double mutant embryos have decreased neuronal density in the developing cortex and premature generation of proliferative astrocyte progenitors. Ngn1 also suppresses astrocytic differentiation *in vitro* from cortical progenitors (Sun et al., 2001) by a different strategy from that utilized to induce neural differentiation. Sequestration of the transcription complex CBP-Smad1 away from astrocyte differentiation genes and inhibition of the activation of STAT transcription factors necessary for gliogenesis seem to be the mechanism by which Ngn1 suppresses gliogenesis. Taken together, the results presented suggest that neurogenins and related bHLH proteins play a role in the neuronal vs glial fate decisions.

Paradoxically, bHLH genes like *Ngn2* are also expressed in astrocyte progenitors suggesting that other factors might be present that counteract *Ngn2* effect and allow gliogenesis to proceed. Moreover, forced expression of bHLH genes in astrocyte progenitors is not sufficient to switch their fate into neurons (Cai et al., 2000). Given that Notch inhibits the expression of bHLH genes (Ma et al., 1998; Morrison et al., 2000), Notch signaling might be involved in inhibiting the neurogenic activity of *Ngn2* in astrocyte progenitors. This mechanism should then work as a way to relieve the anti-gliogenic activity of bHLH genes, allowing gliogenesis to proceed.

Recently, a series of studies have found that indeed Notch and Notch effectors promote gliogenesis and in some cases, at the expense of the neural fate. For instance, activation of the Notch pathway *in vivo* promotes glial fate in retina and telencephalon (Furukawa et al., 2000; Gaiano et al., 2000). *In vitro*, activation of the Notch pathway very potently and irreversibly instructs glial fate in neural crest stem cells (Morrison et al., 2000). Likewise, activated Notch1 and Notch3 promote the differentiation of astroglia in adult

hippocampal stem cells (Tanigaki et al., 2001). In retina, examples of instruction of Müller glia at the expense of neurons by *Hes5* and Notch are documented in the work of Hojo et al., 2000 and of Scheer et al., 2001, respectively. Also, misexpression of *hes2*, a member of a family of bHLH genes related to *hes*, promotes gliogenesis in the retina at the expense of rods (Satow et al., 2001).

As above mentioned, inhibition of the expression of proneural bHLH transcription factors might be one of the mechanisms by which Notch terminates neurogenesis and initiates gliogenesis, thus providing the neural vs glial switch. Intriguingly, Notch may also promote gliogenesis by a more direct mechanism. Tanigaki et al., 2001, describe that constitutively active Notch is capable of inducing GFAP transcription independently of STAT3, the key regulatory transcription factor for CNTF-mediated astroglia induction (Bonni et al., 1997). This result suggests that Notch promotes gliogenesis independently of its capacity to inhibit the sequestration by Neurogenin of CBP/Smad1 from the STAT binding site.

Recently, the first bHLH genes shown to be directly involved in glial development, *Olig1* and *Olig2*, have been described (Lu et al., 2000; Zhou et al., 2000). Both genes appear to be specifically expressed in oligodendrocyte precursors in the spinal cord and also in areas of the midbrain and hindbrain ventricular neuroepithelium from which oligodendrocytes arise. However, *in vivo* ectopic expression of *Olig1* or *Olig2* in chick embryos failed to demonstrate premature or expanded expression of early oligodendrocyte markers in the neural tube. Instead, ectopic expression of *Olig2* but not of *Olig1*, was able to induce *Sox10* [an early glial lineage specific transcription factor, Kuhlbrodt et al., 1998] in cells of the dorsal myotome, which have a mesodermal origin, although later markers of oligodendrocyte precursors were never ectopically expressed. The fact that ectopic expression of the *Olig* genes was never able to induce *Sox10* in cells of the neural lineage suggests these genes might not be able to override neurogenic programs where oligodendrocyte development inhibitors might be active.

In a recent study by Zhou et al., 2001, it was found that migrating oligodendrocyte progenitors (co-expressing *Olig2* and *Nkx2.2*) arise within the spinal cord ventricular zone when and where the previously mutually exclusive expression domains of *Olig2* and *Nkx2.2*

overlap. This observation suggested that the activity of both transcription factors might be involved in oligodendrocyte development. Indeed, co-misexpression of *Olig2* and *Nkx2.2* (but not of either gene alone) induced ectopic and premature oligodendrocyte differentiation accompanied by a robust expression of early as well as late oligodendrocyte markers like Sox10, PDGFR α , MBP and PLP/DM20. Interestingly, *ngn1* and *ngn2* expression needs to be extinguished from the Olig2⁺ domain so that oligodendrogenesis can occur. Forced expression of *ngn1* blocks normal as well as *Olig2/Nkx2.2* driven ectopic oligodendrocyte development. Hence, although it is true that *Olig2* plays a crucial role in oligodendrocyte specification (as further demonstrated by the lack of oligodendrocyte differentiation in Olig1/2 double mutant mice (Zhou and Anderson, 2002), cooperation with other transcription factors is equally essential.

Taken together, all the information discussed so far points towards the existence of molecules and mechanisms that participate in the neuron-glia switch in mammals. However, none of such mechanisms/molecules provide a binary master regulatory switch like *gcm* in *Drosophila*. In this regard, it should be pointed out that although many genes involved in neuronal specification (such as *atonal* (*ngn1*, *ngn2*) and *achaete-scute* (*Mash-1*)) are conserved between *Drosophila* and mammals, those involved in glia differentiation (like genes encoding myelin proteins and GFAP in mammals and REPO and TTK in *Drosophila*) are not, reflecting a strong divergence of mammalian glial cells from their *Drosophila* counterparts.

Unexpectedly, the approach we undertook to try to reveal possible functions of *mgcmb/Gcm2* in mammalian neural vs glial cell decision brought a whole new direction to our research. The attempt to enrich our test population in stem cells in order to simplify the study of their gene expression had a surprising bonus: the unique opportunity to study the biology of these rare cells that hold the promise for cell replacement therapies.

The following two chapters describe how the results presented in part II of this chapter provided the grounds for a more thorough characterization of cells that carry the LewisX epitope in their membranes.

Materials and Methods

RNA isolation and RT-PCR

Timed-pregnant Swiss Webster mouse (the morning after mating was considered E0.5) were euthanized by cervical dislocation. The embryos were removed by cesarean section and their cortices dissected out using RNase free instruments. Cortical tissues were collected in RNAlater solution (Ambion) and stored at -20°C . Total RNA was extracted using the RNEasy kit (Qiagen) following manufacturers instructions and treated with RNase-free DNase (Life Technologies) to eliminate contaminating DNA prior to reverse transcription. Reverse transcription (RevT) was performed as follows: $1\mu\text{g}$ RNA was incubated for 60 min at 42°C with 1x RevT buffer, 1mM d(NTPs), 5mM MgCl_2 , $0.5\mu\text{g}$ oligo d(T) primer, $1\text{U}/\mu\text{l}$ Rnase Inhibitor and 15U of AMV reverse transcriptase (all reagents from Promega). The reaction was stopped by a 10 min incubation at 99°C . Negative controls without reverse transcriptase were also performed. First strand cDNA served as template for PCR amplification. For the amplification of *mgcmb* cDNA, a pair of primers (5'-CTACGTGCGCTTCATCTACAGC-3' and 5'-CCAGAAGTTGGTGACAGGGTATC-3') was used for a single 35-cycle round of PCR (annealing temperature 60°C , 1.5 mM MgCl_2). GAPDH was amplified using the primer pair (5'-GAGCCAAACGGGTCATCATCT-3' and 5'-TCCACCACCCTGTTGCTGTAG-3'), as described above, except for annealing temperature, 58°C . PCR products were run in 1.5% agarose gels and visualized using ethidium bromide. For RT-PCR analysis of *mgcmb/Gcm2* expression in freshly isolated cells or cell cultures, both total RNA and cDNA were obtained as described above, except that random hexamers were used as primers for reverse transcription. For nested PCR, the following pairs of primers (outside and inside) were used: outside (5'-TGCGCACTCCTCGGCTTTATCT-3' and 5'-GCTTCTTGCCTCAGGCTCCT-3') and inside (the same as above). PCR reactions were conducted as previously described. In the second PCR round, one fourth of the first reaction volume was used as template.

Cloning of PCR products and generation of RNA probes

The 296bp PCR product resulting from *mgcmb* amplification was cloned into the pCR[®]II vector (containing Sp6 and T7 RNA polymerase promoters) using a TA-cloning kit

(Invitrogen), according to the manufacturer's instructions. The plasmid was used to transform competent DH5 α bacteria (Life Technologies). Single colonies were selected, further grown and used to extract DNA with the DNA MiniPrep kit (Qiagen). The plasmid was cut with appropriate restriction endonucleases and the orientation of the cloned fragment was determined. To generate digoxigenin-labeled RNA probes for *in situ* hybridization, the plasmid was linearized and transcribed using the DIG-RNA labeling kit (Roche Molecular Biochemicals), following the manufacturer's instructions. The probes were filtered through a G50 mini-column (Amersham Pharmacia) and stored at -20°C . DIG labeled Delta probe was generated as described (Delta plasmid was a gift from Dr Domingos Henrique).

***In situ* hybridization**

In situ hybridization in frozen sections was carried out as described by Henrique et al., 1997 with minor modifications. Briefly, mouse embryos (E10-E18) were collected, fixed for 4-12h in ice-cold 4% paraformaldehyde at 4°C and cryoprotected in 30% sucrose. 12-14 μm sections were cut in a freezing cryostat (Microm) and mounted onto Superfrost Plus slides (Fisher). The slides were incubated overnight at 65°C with 0.5 $\mu\text{g}/\text{ml}$ DIG-RNA probe in hybridization buffer (50% formamide, 5xSSC, 5x Denharts solution, 250 $\mu\text{g}/\text{ml}$ yeast RNA, 500 $\mu\text{g}/\text{ml}$ herring sperm DNA – all reagents from Sigma except DNA from Life Technologies). The slides were washed once for 15 min and twice for 30 min in 50% formamide, 1x SSC at 65°C . After 3 additional 30 min washes at room temperature (RT) in MABT (100mM maleic acid, 150mM NaCl, pH 7.5, 0.1% Tween-20), the slides were blocked for 2h at RT in MABT containing 2% blocking reagent (Roche Molecular Biochemicals) and 20% normal goat serum (NGS, Vector). Hybridization signal was revealed with anti-DIG antibody conjugated to alkaline phosphatase (1:2000, 4 hours at RT, Roche Molecular Biochemicals) using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) as substrates (Sigma). After satisfactory development of the purple reaction product, the slides were washed in phosphate buffered saline (PBS) containing 5mM EDTA and coverslipped with 50% glycerol in PBS.

Immunoselection of E13 LewisX positive cells

E13 mouse embryos were collected in hibernation medium. The brains were removed and the cortices dissected free of meninges (meninges removal was accomplished after at 30 min incubation in Earl's salts (Sigma) at 37°C). The tissue was treated with 10U/ml of papain (Worthington) and 60µg DNase (Sigma) for 30 min at RT. After three washes with DMEM containing B27 supplement (both from Life Technologies), the tissue was triturated 8-10x with a fire polished pasteur pipette. After a 10 min settling period, the top portion containing mainly single cells was removed into a glass siliconized tube (AquaSil, Pierce). Cells were incubated for 30 min at 4°C in a dual axis rotator with anti-CD15 antibody (IgM monoclonal, MMA clone, Becton Dickinson) and 5% HIFBS (Gemini) in DMEM. CD-15 labeled cells were washed twice in DMEM, pelleted by at 10 min centrifugation at 300xg and further incubated with magnetic beads coupled to goat anti mouse IgM antibodies (Dyna). The tube containing the cells was placed on a magnetic holder (Dyna) and CD15/LeX⁻ cells (not attached to magnetic beads) were collected. The tube was removed from the magnetic holder and the remaining cells were washed 3 times with cold DMEM. Non-magnetic cells resulting from these washes were discarded. CD15/LeX⁺ cells (magnetic) were collected. CD15/LeX⁻ cells were further incubated for 45 min at RT with a 1:8 dilution of guinea-pig complement (Cappel) in order to lyse contaminating CD15/LeX⁺. CD15/LeX⁺ and CD15/LeX⁻ and control (total, non sorted) cells were plated and the development of the cultures was followed over time. For determination of purity percentages, plates were stained with anti-CD15 antibodies 2 hours after plating.

Cell culture

Cells were plated (20-30 cells/well) in poly-L-lysine (PLL) Terasaki plates (Nunc) in DMEM containing glutamine, sodium pyruvate, B-27, N2, 1mM N-acetyl-cystein (NAC) (basal medium) supplemented with 10ng/ml FGF2 (all supplements and growth factors from Life Technologies except NAC from Sigma). Cultures were maintained at 35°C, 6% CO₂ in a humidified incubator. For proliferation studies, 100ng/ml 5-Bromo-2'-deoxyuridine (BrdU, Sigma) was added to the cultures 2 hours after plating.

Immunocytochemistry

LewisX: live cells were incubated with anti-CD15 antibody (1:20, 30 min at RT) and then fixed in ice-cold 4% paraformaldehyde for 30 min at RT. Staining was revealed using goat anti-mouse IgM biotinylated secondary antibodies, the ABC Elite Kit and VIP as substrate (all from Vector Laboratories).

O4: cells were incubated with ice-cold hybridoma supernatant (neat, 30 min at RT) and fixed. Staining was revealed as for LewisX.

β -Tubulin III and GFAP: cells were fixed as before, permeated for 5 min with -20°C 100% methanol, blocked for 15 min in 10% normal goat serum (NGS) and incubated with anti- β -tubulin III monoclonal antibody from Sigma (1:400, overnight at 4°C) or a polyclonal rabbit anti-GFAP antibody from DAKO (1:400, 1 hour at RT). The staining was revealed with secondary antibodies coupled to Alexa fluorochromes (Molecular probes).

BrdU: cells were fixed for 30 min in ice-cold 95% ethanol, 5% acetic acid, rinsed in PBS and incubated for 45 min with 2M hydrochloric acid (HCl) at RT. After 5 rinses with PBS, the cultures were blocked with 10% NGS and incubated with 1:20 anti-BrdU antibodies directly conjugated with fluorescein (Becton Dickinson). BrdU positive cells were counted and expressed as a percentage of the total cells as determined by DAPI staining (Molecular Probes).

Chapter Three



LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying
them as non-ependymal

Alexandra Capela and Sally Temple

Center for Neuropharmacology and Neuroscience
Albany Medical College
Albany NY 12208 USA

Phone: 518 262 5416

Fax: 518 262 6178

E-mail: Temples@mail.amc.edu

Capelaa@mail.amc.edu

Running title: Identification of stem cells

Capela and Temple (2002); *Neuron* 35(5): 865-75.

Abstract

Adult neural stem cells are rare and little is known about their unique characteristics, leaving their *in vivo* identity enigmatic. We show that Lewis X (LeX), a carbohydrate expressed by embryonic stem cells, is made by adult mouse subventricular zone (SVZ) stem cells and shed into their environment. Only 4% of acutely isolated SVZ cells are LeX⁺; this sub-population, purified by FACS, contains the SVZ stem cells. Ependymal cells are LeX⁻ and purified ependymal cells do not make neurospheres, resolving the controversial claim that these are stem cells. Thus LeX expression by adult CNS stem cells aids their *in vivo* identification, allows their enrichment, and raises new questions about the role of this unusual carbohydrate in stem cell biology.

Introduction

Rare adult neural stem cells have been isolated from the subventricular zone (SVZ) and the hippocampal dentate gyrus (Gage, 2000). These cells are an important source of adult neurons, and offer the promise of new central nervous system (CNS) repair therapies, so they are the subject of intense interest. Yet, the exploration of adult neural stem cells is hampered because we have had no means to positively identify these rare cells.

CNS stem cells are usually identified retrospectively by their ability to generate typical neurospheres or large adherent clones containing multiple neural cell types (Reynolds and Weiss, 1992; Davis and Temple, 1994; Palmer et al., 1997), which precludes the study or utilization of freshly isolated CNS stem cells, and their identification *in vivo*. Recently, a population of adult stem cells has been prospectively purified from the SVZ using a sequence of selection steps largely based on lack of differentiation markers (Rietze et al., 2001); this constitutes an important step forward in studying these cells. Still, we know little about the unique biology of CNS stem cells, for example which specific gene products they express, which would expand our understanding of these important cells, aid in their identification *in vivo* and allow their positive enrichment *in vitro* for study and use.

Given this impasse, we find ourselves confronted with controversies about the identity and characteristics of adult neural stem cells. For example, two different cell populations have recently been identified as including SVZ stem cells: GFAP-expressing astrocytes (Doetsch et al., 1999a,b) and Notch1-expressing, ciliated ependymal cells lining the ventricles (Johansson et al., 1999). These two distinct cell types are so intimately localized *in vivo* that it is difficult to separate them physically. Instead, we anticipate that defining specific features of stem cells will provide markers to help reveal their *in vivo* identity.

Genes expressed by adult CNS stem cells include Nestin, Musashi, Notch1 and GFAP (Sakakibara et al., 1996; Johansson et al., 1999; Doetsch et al., 1999a), but other CNS cell types also express these. Moreover, many of these markers are intracellular, limiting their usefulness for stem cell enrichment, although this problem can be overcome by creating transgenic mice with fluorescent reporter gene expression (Kawaguchi et al., 2001). A more generally useful marker would be a cell surface molecule allowing stem cell localization and purification from a wild-type mouse. This approach has been used to prospectively isolate embryonic human CNS stem cells using CD133⁺ mCD24^{lo} (Uchida et al., 2000) and mouse neural crest stem cells using the p75 neurotrophin receptor (Morrison et al., 1999).

Cell surface carbohydrate moieties are useful cell type markers (Jessell et al., 1990). In exploring candidates, we noted one with an intriguing distribution. The LeX antigen, which is trisaccharide 3-fucosyl-N-acetylglucosamine or FAL (Gooi et al., 1981), also known as SSEA-1 (stage specific embryonic antigen 1) or CD15 (leucocyte cluster of differentiation 15), is highly expressed on pluripotent stem cells: it is found on mouse and human embryonal carcinoma cells, mouse pre-implantation embryos, embryonic stem cells, teratocarcinoma cells and primordial germ cells (Solter and Knowles, 1978; Fox et al., 1981; Bird and Kimber, 1984; Muramatsu, 1994; Marani et al., 1986; Gomperts et al., 1994). Intriguingly, CNS cell sub-populations in various species also express this marker during development and in adulthood. LeX is expressed in germinal zones in the murine embryonic telencephalon (Yamamoto et al., 1985; Allendoerfer et al., 1995, 1999; Tole et al., 1995; Ashwell and Mai, 1997) and spinal cord (Dodd and Jessell, 1986), and in the cerebellar external granular layer (Marani and Tetteroo, 1983). In the adult mouse CNS, LeX is expressed by sub-populations of astrocytes, tanyocytes,

and a few neurons, but not ependymal cells (Bartsch and Mai, 1991; Gocht et al., 1996; Ashwell and Mai, 1997).

Given the expression of LeX on pluripotent stem cells in the embryo, in germinal zones of the developing CNS, and on astrocytes in the adult CNS, we decided to examine its expression within adult neurogenic zones, and to investigate the properties of purified LeX⁺ cells. In this study we describe a new aspect of the biology of adult CNS stem cells - their expression of the LeX carbohydrate moiety, which aids in their enrichment and identification.

Results

LeX is expressed in adult neurogenic zones

Frozen and paraffin sections of adult mouse brain were stained using the monoclonal anti-CD15 antibody produced by the hybridoma clone MMA, which recognizes LeX (Gocht et al., 1996). In the dorsal forebrain there is a distinct layer of LeX⁺ cells associated with the cortical pial surface, and strong labeling within the corpus callosum, while the intervening cortical tissue is only lightly stained, with occasional positive cells usually surrounding blood vessels (Figure 3.1 A-A'). The basal forebrain is more brightly labeled, with strong staining in the septal region and patchy staining in the striatum (Figure 3.1 B-E). Within the basal forebrain SVZ, sparse individual cells are prominently stained (Figure 3.1 C and E). Often LeX staining was diffuse and appeared extracellular, consistent with previous reports describing LeX in the extracellular matrix (Gocht et al., 1996). LeX⁺ cells were present in the rostral migratory stream, and rare positive cells were seen in the olfactory bulbs (not shown). Confirming previous reports (Bartsch and Mai, 1991), ependymal cells did not stain for LeX, neither in whole-mounts, paraffin sections (Figures 3.1 and 3.2) nor in cryostat sections (not shown).

Considering evidence that some SVZ astrocytes are stem cells (Doetsch et al., 1999a,b), we compared the localization of LeX and GFAP and found some noticeable similarities in overall distribution. In dorsal forebrain, like LeX staining, GFAP staining reveals an intense sub-pial layer, occasional cells surrounding blood vessels, and strong expression in the SVZ and corpus callosum (Figure 3.1). In the body of the cerebral

cortex both LeX and GFAP staining are weak. While both markers are expressed within the forebrain SVZ, GFAP staining is more abundant, and only a few SVZ cells appeared to express both LeX and GFAP.

To gain a clearer picture of SVZ LeX expression, we used a whole-mount labeling technique developed by Doetsch et al., 1999b, which allows staining of the entire ventricular striatal wall. Figure 3.2 A-A'' shows a striatal wall stained for LeX, and for comparison a striatal wall stained with a monoclonal anti-GFAP antibody (Figure 3.2 B-B''). LeX staining reveals a sub-population of large, bushy cells that are evenly spaced in a spotted pattern over the surface of the lateral wall. In contrast, GFAP staining is much more abundant, with processes forming a dense network.

To quantify LeX⁺ cells in the SVZ, we microdissected the area surrounding the ventricles from brain slices (Lois and Alvarez-Buylla, 1993), dissociated the cells and stained them acutely with the MMA antibody. As shown in Table 3.1 and Figure 3.2 C, C' only a minor proportion, $4.31 \pm 0.27\%$, of microdissected SVZ cells are LeX⁺. A similar percentage was obtained after cell dissociation using trypsin or papain. In order to characterize the LeX⁺ population, we double stained with other markers for SVZ cell types (Table 3.1). There was very little overlap between LeX and β -tubulin III, demonstrating that most neuroblasts were not included in this population. As predicted from the section staining, there was little overlap of GFAP and LeX in individual cells. Of the total GFAP⁺ population, only 6% also expressed LeX and of the total LeX⁺ population, only 18% expressed GFAP. None of the acutely isolated LeX⁺ cells co-stained with mCD24, an ependymal cell marker (Calaora et al., 1996) (Table 3.1, Figure 3.1 C, 3.2), confirming the lack of ependymal staining seen in sections.

The hippocampal dentate gyrus stained heavily for LeX (Figure 3.1 F, F''). Prominent staining was observed in the subgranular layer, which generates new granular neurons *in vivo* (Kempermann et al., 1997). As seen in the SVZ, there is strong expression of both LeX and GFAP in the hippocampus (Figure 3.1 F''); labeling of these markers within individual cells was not assessed.

Figure 3.1. Distribution of the LewisX antigen in the adult mouse brain.

Staining of coronal paraffin sections at anterior and posterior levels indicated in diagrams on the right. Left panels: LeX (red), middle panels: GFAP (green), right panels: merged images.

(A-A'') LeX and GFAP in the cerebral cortex, note LeX⁺ cells close to the pial surface (pia). Insert: High magnification of LeX and GFAP staining around a blood vessel.

(B-C'') In anterior sections, LeX labels cells in the striatum (str), septum (se) and corpus callosum (cc). **(C)** High magnification of the boxed area indicated in B'' showing LeX staining in the SVZ is stronger in the septal than the striatal side. Occasional very bright cells occur in the striatal SVZ (arrowhead in C).

(D-E'') In more posterior sections, the same pattern of staining is visible with strong staining in the septum and corpus callosum, patchy staining in the striatum, and occasional brightly-stained LeX⁺ cells in the SVZ region, as shown at higher magnification in E-E''.

In both anterior and posterior regions, there is a general overlap in the location of LeX and GFAP staining, but in the SVZ at high magnification, few cells appear to label with both markers; one example is indicated by the arrow in (E-E'').

(F-F'') The hippocampal dentate gyrus shows very strong LeX staining, which parallels that of GFAP, including its association with blood vessels (arrows). In the hippocampal dentate gyrus, more cells appeared to be labeled for both MMA and GFAP.

Scale bars: (A-A'', B-B'', D-D'') 200µm; (C-C'', E-E'') 30µm; (F-F'') 100µm.

Figure 3.1

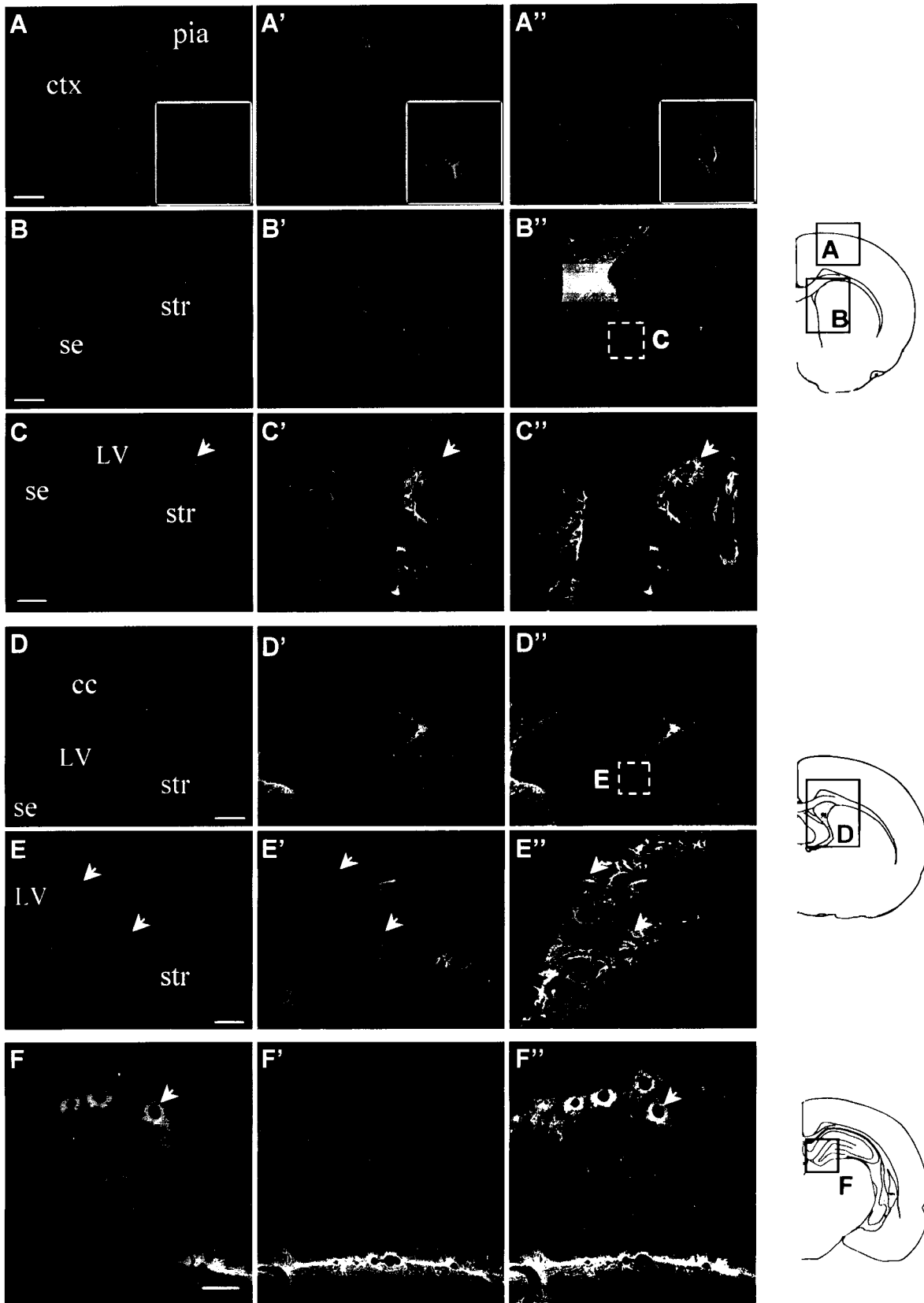


Figure 3.2. LeX versus GFAP staining in cells of the striatal ventricular wall.

(A) LeX staining in whole-mounts of the ventricle striatal wall reveals large, bushy cells that are evenly dispersed over the striatal side of the lateral ventricle. Stained cells are also visible in the corpus callosum (cc). (A', A'') high power images of typical LeX⁺ cells.

(B) GFAP staining in the striatal wall reveals a dense network of processes, prominent around blood vessels. (B', B'') high magnification showing typical GFAP⁺ fibrous staining. GFAP is more abundant than LeX staining.

(C, C') A small percentage of cells are LeX⁺ (arrowheads) in acute cell suspensions of striatal SVZ.

(D-E') LeX does not label ependymal cells. (D, D'). In acute cell suspensions of adult striatal SVZ, LeX⁺ cells shown in red (arrow) were never found to co-label with mCD24 (green), an ependymal cell marker. Insert: a ciliated ependymal cell in phase and stained with anti-mCD24, was negative for LeX (not shown). (E) LeX staining of the SVZ region, boxed area at higher power in (E'). The ependymal layer is LeX⁻ (arrows).

Scale bars: (A, B) 500 μ m; (A', A'', B', B'', D, D', E') 25 μ m; (C, C') 30 μ m; (E) 100 μ m.

Figure 3.2

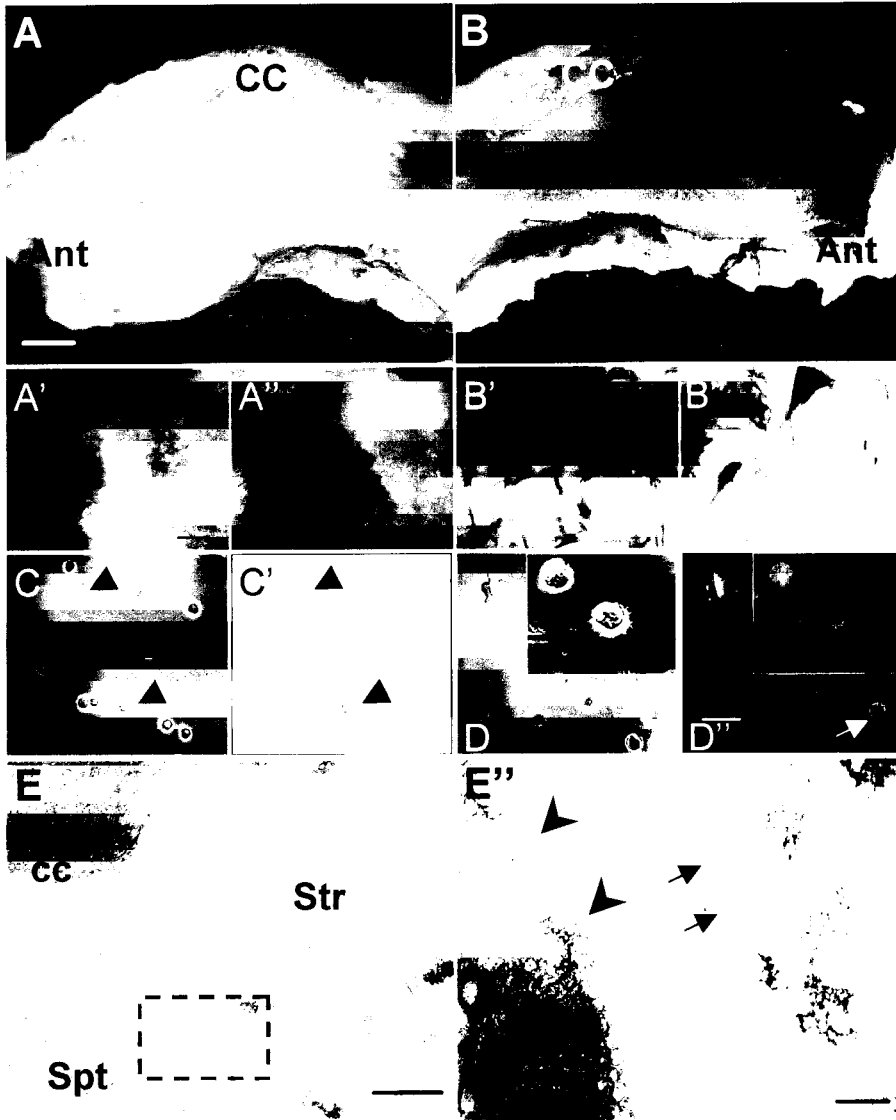


Table 1. Antigenic characterization of cells derived from microdissected adult mouse SVZ

	PSA-NCAM	β -Tub	GFAP	mCD24	LeX (MMA)
% of total cells	63.5 \pm 5.2	59.9 \pm 7.8	12.1 \pm 0.9	16.3 \pm 0.7	4.31 \pm 0.2
% of LeX ⁺ cells	nd	7.32 \pm 1.1	18.2 \pm 1.9	0	100

Table 3.1. Antigenic characterization of adult mouse striatal SVZ cells.

The striatal SVZ region was microdissected from slices and enzymatically dissociated to single cells that were then stained for SVZ markers. mCD24 has been reported on ependymal cells and at a lower level on Type A neuroblasts (Calaora et al, 1996); in this study we found labeling only on ependymal cells.

LeX is associated with a large protein carrier in neurogenic zones

Given that LeX is present on the surface of rare cells in the SVZ, we examined what carrier molecule it might be associated with. Previous studies have identified a large >200 KD carrier proteoglycan for LeX in the embryonic and early postnatal brain (Tole et al., 1995; Allendoerfer et al., 1995, 1999), whereas in adult brain this carrier has not been described, and LeX is associated with 70-80KD glycoproteins (Tole et al., 1995; Allendoerfer et al., 1999). We performed western blots on different CNS regions, probing with the MMA antibody to distinguish possible protein carriers for LeX. A >200KD carrier protein was seen in adult neurogenic regions (Figure 3.3): SVZ and hippocampus, and in the cerebral cortex, which may also acquire new neurons in adulthood (Gould et al., 1999). In contrast, in the cerebellum, which has been shown not to support neurogenesis (Suhonen et al., 1996), this large carrier molecule was not found, even though LeX staining was observed (not shown), possibly due to its expression in glycolipids. Thus the >200 KD carrier protein for LeX seen in the developing CNS may be retained in adult CNS regions containing neurogenic stem cells.

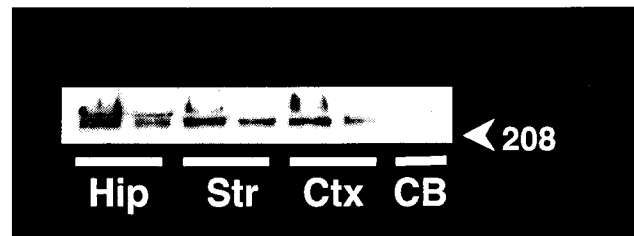


Figure 3.3. MMA recognizes a large protein carrier for LeX in neurogenic regions and not cerebellum.

Western blot shows all regions examined but cerebellum have a large >200 KD band revealed after LeX staining. First lane for all regions - 25 μ g protein, second lane -10 μ g (except cerebellum, only one lane - 25 μ g). Hippocampus (Hip), striatum (Str), cerebral cortex (Ctx) and cerebellum (Cb).

LeX⁺ cells divide *in vivo* and retain the proliferation marker BrdU

Some SVZ cells are slowly dividing *in vivo*, and these are thought to be stem cells. Prolonged exposure to 5-Bromo-2'-deoxyuridine (BrdU) allows its uptake into all dividing cell populations. Neuroblasts have a short cell cycle and young neurons rapidly migrate away from the SVZ, so this population loses the label quickly. SVZ cells that retain BrdU for at least a week after labeling appear to be slowly dividing stem cells (Morshead and Van der Kooy, 1992; Johansson et al., 1999; Doetsch et al., 1999a; Chiasson et al., 1999). We examined whether LeX⁺ SVZ cells have the BrdU uptake and retention characteristics expected of adult neural stem cells.

Adult mice were given BrdU in their drinking water for 2 weeks (Johansson et al., 1999; figure 3.4 A). After this treatment, dividing cells were found in the SVZ (Figure 3.4 C) and in the inner granular layer of the hippocampal dentate gyrus (Figure 3.4 D), as expected (Morshead et al., 1994; Kempermann et al., 1997). At 0, 4 and 7 days after ceasing BrdU administration, the SVZ region was microdissected and dissociated to single cells which were acutely stained with antibodies against LeX, GFAP, β -tubulin III and BrdU (Figure 3.4 E-G). Dissociated cells rather than tissue sections were examined to allow clear identification of multiple labels within individual cells.

Figure 3.4 B, top panel shows the percentage of SVZ cells positive for either LeX, β -tubulin III or GFAP that were BrdU labeled. At day 0 (following the 2 weeks of BrdU administration), $66.2 \pm 1.6\%$ of LeX⁺ cells were BrdU⁺ and at 7 days 30% of these retained the label, indicating that a third are slowly dividing. In contrast, $91.2 \pm 1.6\%$ of the β -tubulin III⁺ neuroblasts were BrdU⁺ at day 0, but as expected by 7 days only 2.8% retained the label. The GFAP⁺ population is 12% of the total dissociated SVZ cells. $45.5 \pm 2.2\%$ of the total GFAP⁺ cells incorporated BrdU label over this period, and the rate of decay was similar to that of LeX⁺ cells, with 24% retaining label at day 7.

Of the LeX⁺ cells that incorporated BrdU at day 0, only $5.1 \pm 1.4\%$ were GFAP⁺, but of the LeX⁺ cells that retained label at 7 days, $12.4 \pm 2.3\%$ were GFAP⁺ (Figure 3.3 B, lower panel). Apparently, only a small percentage of LeX⁺ cells that are dividing *in vivo* during a two-week period are astrocytes, but a greater proportion of these are slowly dividing like stem cells compared to LeX⁺GFAP⁻ cells.

FACS purified LeX⁺ cells are the neurosphere-generating cells of the SVZ

The number of LeX⁺ cells in the SVZ cell suspension (4%) is similar to the estimated number of stem cells present, assessed by neurosphere-generation (1%) (Reynolds and Weiss, 1992; Gritti et al., 1996; our data). To examine whether the sub-population of LeX⁺ cells included neurosphere-generating stem cells, we purified them by sorting and cultured them under neurosphere-promoting conditions. Figure 3.5B shows typical FACS histograms of SVZ cells unstained (control) and stained with MMA antibody directly conjugated to fluorescein. On average, 3.0-5.6% of the total cells were selected as positive, demonstrating a high recovery of LeX⁺ cells. To determine purity, FACS sorted cell suspensions were stained with MMA antibody; positive fractions ranged from 74-99% purity, and negative from 98-99.5% purity.

When cultured under neurosphere-promoting conditions in serum-free B27/N2 medium plus 20ng/ml of EGF and FGF2, the two FACS sorted populations behaved very differently. LeX⁺ cells made abundant neurospheres, while LeX⁻ cells rarely made neurospheres (Figure 3.5 C, E-G). This contrasting behavior was also seen after plating onto poly-L-lysine (PLL) coated tissue culture dishes in serum-free culture medium. LeX⁺ cells had various morphologies, were highly proliferative and frequently shed LeX⁻ containing material onto the substrate (not shown), consistent with the observation of extracellular LeX in neurogenic zones in sections. In contrast, during 5-7 days *in vitro* LeX⁻ SVZ cells rarely divided, and they generated largely neurons (Figure 3.5 H).

Interestingly, the number of neurospheres obtained from purified LeX⁺ SVZ cells depended on plating density. When 1000 LeX⁺ cells were plated per 35mm dish 1 in 50 generated neurospheres, when 100 LeX⁺ cells were plated per 35mm dish, 1 in 8 generated neurospheres and when 1-5 LeX⁺ cells were plated per Terasaki well, 1 in 4 generated neurospheres. One interpretation of this finding is that there is an inhibitory interaction between LeX⁺ cells that can reduce the efficiency of neurosphere generation. In contrast, when 100 LeX⁻ cells were plated per 35 mm dish no neurospheres were ever observed, but when negative cells were plated at a higher density, around 1,000 cells per dish, 1 in 550 cells made neurospheres; this was within the range of contamination of LeX⁺ cells in this fraction.

The neurospheres generated from the LeX⁺ fraction were typical neurospheres that contained multiple cell types and more stem cells, demonstrating that LeX⁺ stem cells were multipotent and self-renewing (Figure 3.5 I-K). For example 20/20 randomly picked neurospheres generated both neurons and glial cells after plating onto poly-L-lysine coated wells in medium lacking growth factors to encourage differentiation. Moreover, stem cells within the neurospheres expressed LeX. When primary neurospheres from adult SVZ were grown for 7-10 days in EGF, fixed, cryostat sectioned and stained for LeX, many positive cells were revealed, mainly at the periphery (Figure 3.6 A). After primary neurospheres were enzymatically dissociated to a single cell suspension, 29.22±1.14% of the cells were found to be LeX⁺, which is similar to the 20-30% estimated frequency of stem cells in neurospheres (Gritti et al., 1996). FACS purified LeX⁺ cells from primary neurospheres generated abundant neurospheres, while the LeX⁻ population rarely made neurospheres (Figure 3.6).

Figure 3.4. LeX⁺ cells in the SVZ are dividing in vivo and retain BrdU.

(A) Experimental scheme.

(B, upper panel) Acutely isolated SVZ cells were stained for BrdU label and SVZ markers. The percentage of each SVZ population that is BrdU labeled is shown.

(B, lower panel) Of the LeX⁺ cells that incorporate BrdU at day 0 and retain label at day 7, only a minor percentage is also GFAP⁺.

(C) At day 0 BrdU labeling was seen in the SVZ and (D) in the inner granular layer of the hippocampal dentate gyrus (arrows).

(E-G) Examples of labeling of acutely stained SVZ cells: LeX (red), BrdU (green) and GFAP (blue).

Scale bars: (C, D) 100µm; (E, F, G) 25µm.

Figure 3.4

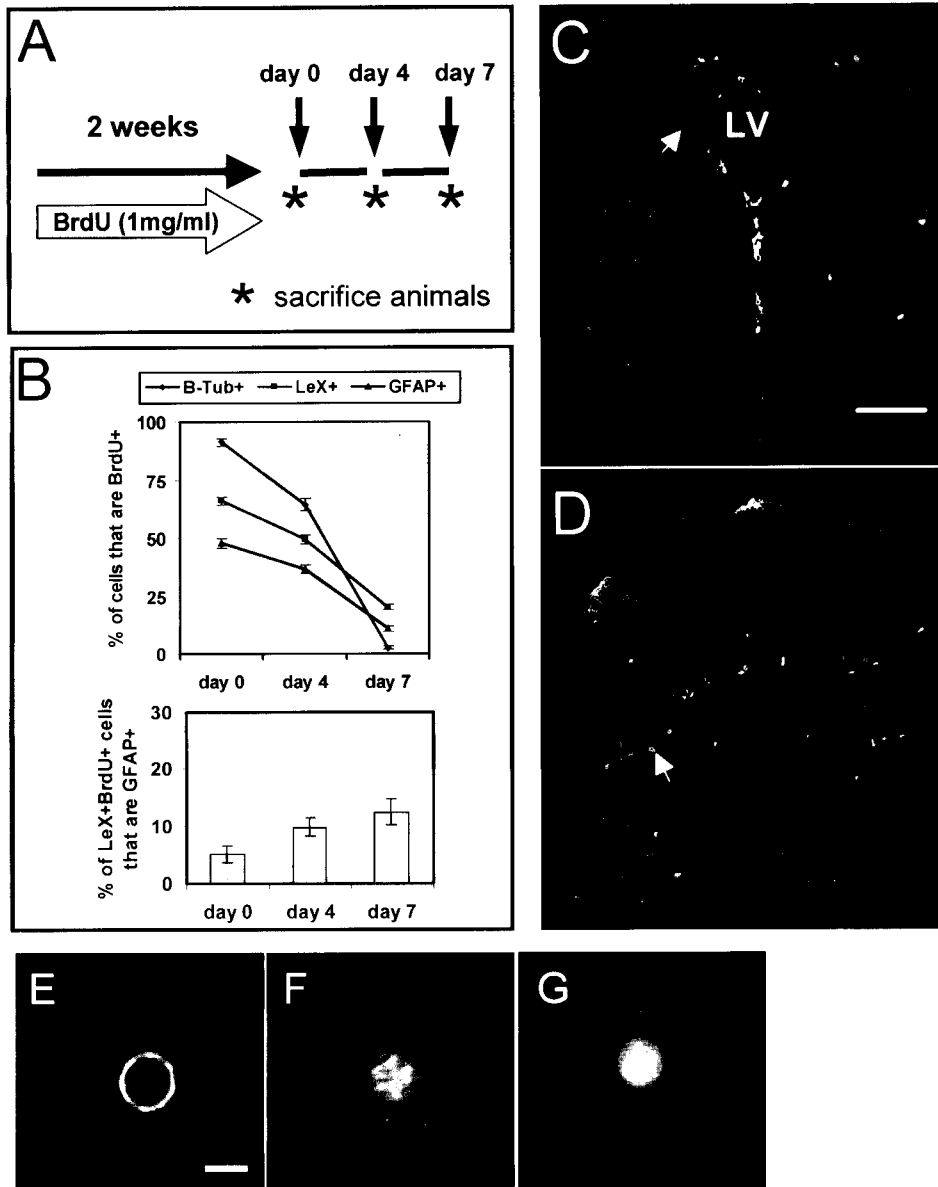


Figure 3.5. FACS sorting reveals that SVZ stem cells carry the LeX marker.

- (A)** The SVZ region was microdissected from slices. A single cell suspension was generated and labeled with MMA antibody. Typical FACS histograms are shown in **(B)** comparing the unlabeled (control) with the labeled population.
- (C)** A typical sample of the LeX⁺ sorted fraction stained with MMA shows high purity. After 7 days in culture, these cells generate neurospheres **(D)**.
- (E)** In adherent conditions, cultured LeX⁺ cells frequently shed LeX⁺ material (arrow) onto the substrate.
- (F)** A typical sample of the LeX⁻ sorted fraction stained with MMA shows high purity (insert, a phase micrograph). These cells rarely divided *in vitro* **(G)**.
- (H)** After culture for 5-7 days on PLL-coated culture wells, LeX⁻ cells produced many neurons that stained for β -tubulin III. Neurospheres generated from LeX⁺ cells gave rise to all three major CNS cell types: O4⁺ oligodendrocytes **(I)**, GFAP⁺ astrocytes **(J)** and β -tubulin III⁺ neurons **(K)**.
- Scale bars: (C, F, H) 30 μ m; (D, G) 50 μ m; (I, J, K) 25 μ m.

Figure 3.6. LeX⁺ cells in neurospheres are stem cells.

- (A)** In cryostat sections of neurospheres, LeX⁺ cells are numerous and located mainly at the periphery.
- (B)** Neurospheres were enzymatically dissociated to single cells and stained for LeX. FACS histograms show approximately 23% of cells in the labeled sample are positive. After FACS separation, the positive cells were highly pure **(C)** and when cultured, they generated abundant neurospheres **(D)**.
- (E)** A sample of the LeX⁻ fraction cells stained for MMA (insert, a phase micrograph). **(F)** After culturing under neurosphere generating conditions, few LeX⁻ cells divided. **(G)** The vast majority of neurospheres generated came from the LeX⁺ neurosphere cells.
- Scale bars: 30 μ m.

Figure 3.5

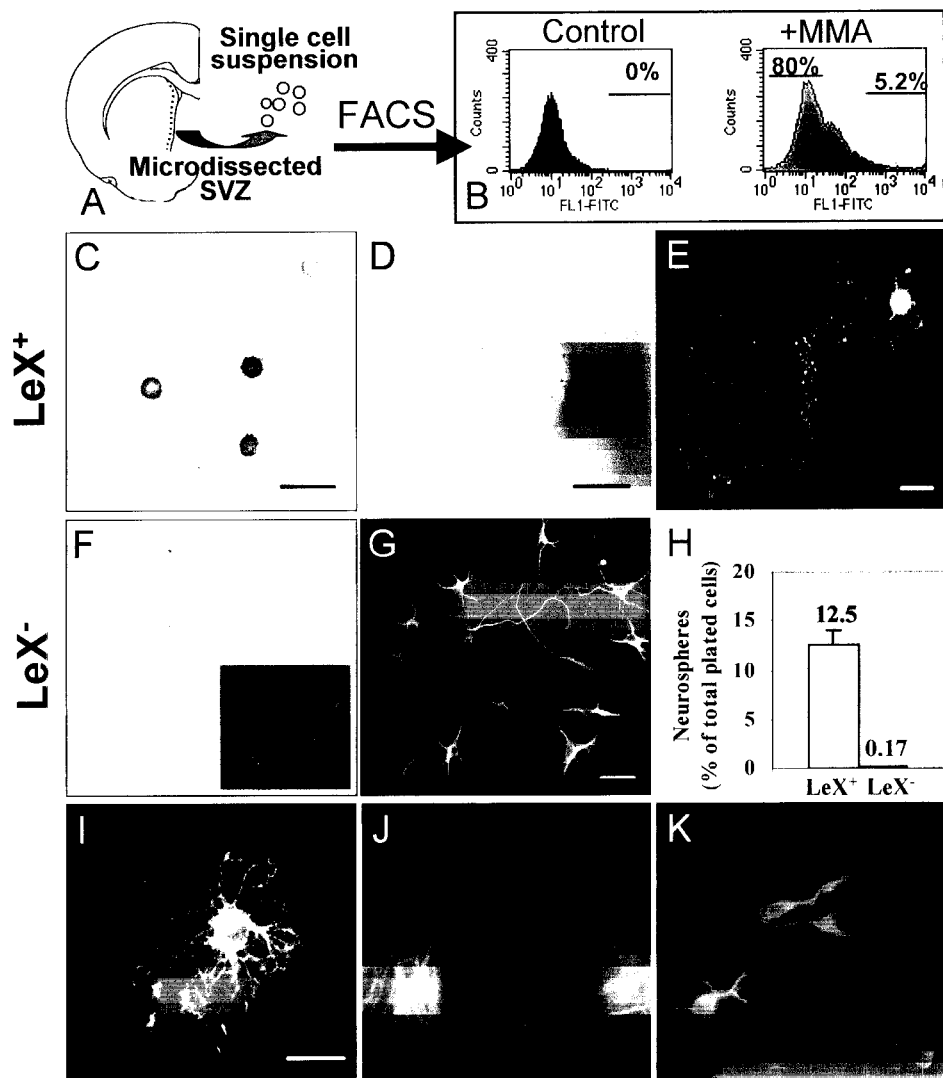
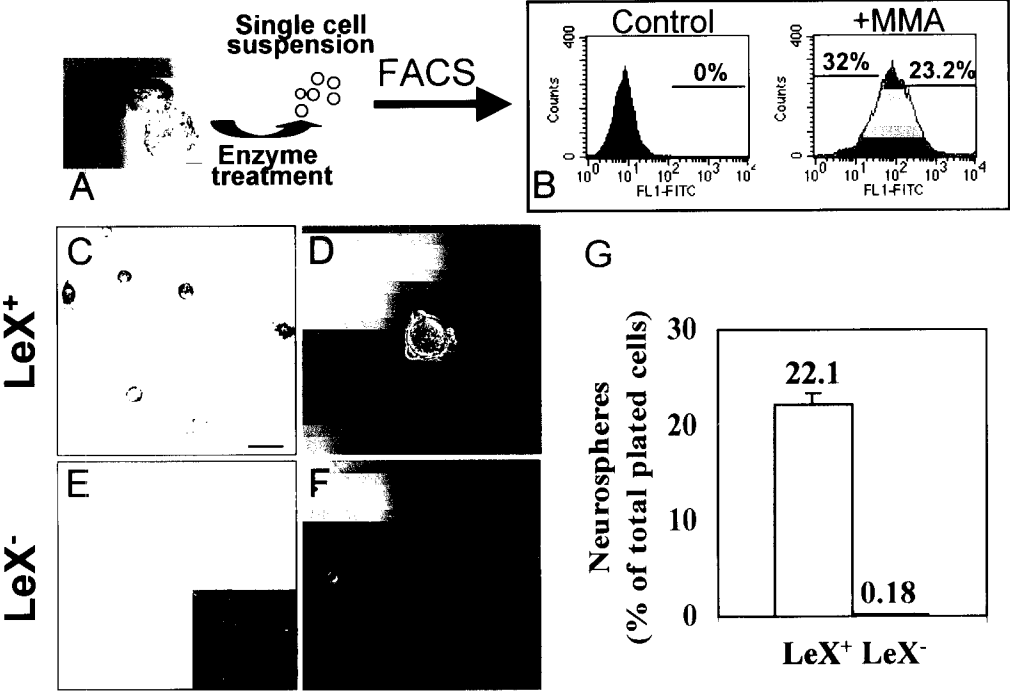


Figure 3.6.



Ependymal cells are not stem cells

In 1999, Johansson et al. presented evidence that ependymal cells include a stem cell population. They enriched for these cells by different methods including their expression of Notch1, by filling the ventricles with DiI and then selecting DiI⁺ cells or by picking individual ciliated ependymal cells with a glass micropipette. After culturing these isolated cells in neurosphere-conditioned medium, neurospheres were produced. This result has proven somewhat controversial because it has been difficult to reproduce. Chiasson et al., 1999 and Laywell et al., 2000, reported that ependymal cells isolated from adult or early postnatal mice are capable of generating neurosphere-like structures, but these were not typical neurospheres, lacking multipotency and self-renewal. A more recent paper has added to the controversy by presenting data that support both possibilities (Rietze et al., 2001). Adult SVZ stem cells were purified based on their lack of the ependymal marker mCD24 (HSA), and were found to lack cilia suggesting a non-ependymal identity. Yet, after filling the ventricles with DiI to preferentially label ependymal cells, some of the purified stem cells were found to be DiI⁺.

In our studies, we found that LeX labels most stem cells, yet ependymal cells do not express LeX in sections nor after acute isolation *in vitro*, suggesting that they may not in fact be stem cells. In order to investigate this further, we purified mCD24⁺ ependymal cells by FACS and cultured them in standard neurosphere-generating culture conditions (20ng/ml FGF2 and EGF). The mCD24⁺ fraction gave rise to negligible numbers of neurospheres (0.15%), which is within the range of mCD24⁻ cell contamination (2%). In contrast, the mCD24⁻ fraction produced 7.1% neurospheres (Figure 3.7 A). Clearly, neurosphere production is associated with the non-ependymal cell fraction. Given that neurosphere conditioned medium was used in Johansson's study to encourage neurosphere production by ependymal cells, we used a co-culture protocol as illustrated in Figure 3.7 B. Ciliated ependymal cells were individually picked using a glass pipette and transferred to a multiwell plate; a transmembrane insert was placed in the well and neurosphere-generating SVZ cells were added to the upper part of the insert. This allowed neurosphere-conditioned media to be shared by the two cell populations while keeping them physically separate. Even though the SVZ population above the transmembrane produced abundant neurospheres (Figure 3.7 D), the ependymal cells below produced none (Figure 3.7 C). Our experiments clearly

demonstrate that ependymal cells selected by two different methods - expression of mCD24 and possession of cilia - do not generate neurospheres, even in the presence of neurosphere-conditioned medium. This is consistent with our finding that adult neural stem cells are distinguished by their expression of LeX.

Figure 3.7. Ependymal cells isolated by two different methods do not generate neurospheres.

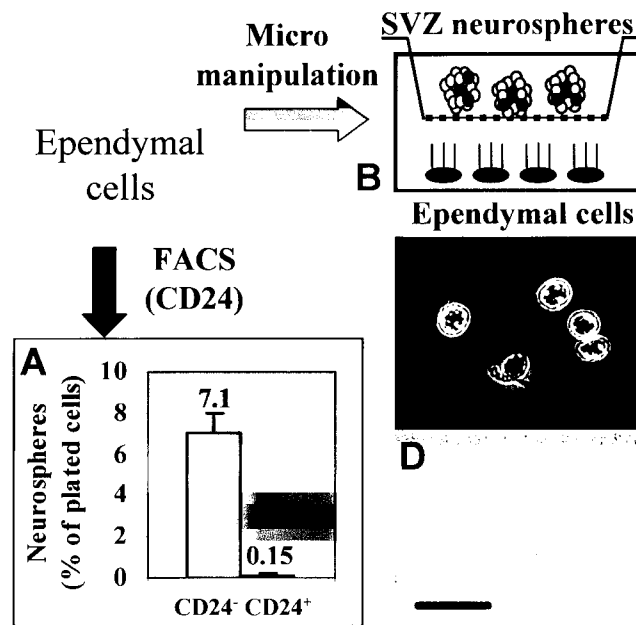
(A). Only sorted mCD24⁺, non-ependymal cells generate significant neurospheres.

(B) Scheme of co-culture of ependymal cells (below the transmembrane insert) with total SVZ cells (above the insert).

(C-D) The SVZ cells above the insert make neurospheres (D) while ependymal cells below do not (C).

Scale bar: (C) 25 μm , (D) 100 μm .

Figure 3.7



Discussion

LeX is a fucose-containing trisaccharide present on the surface of mouse embryonic stem cells and primordial germ cells. Given its expression on pluripotent stem cells in the early embryo, and on a subset of cells in the developing and adult CNS, we examined whether some of the LeX⁺ cells in the adult CNS might be stem cells.

Most neurosphere-generating adult SVZ cells are LeX⁺

Currently, the best biological assay for adult CNS stem cells is the ability to generate neurospheres (Reynolds and Weiss, 1992). FACS-purified LeX⁺ cells made abundant neurospheres, while LeX⁻ cells rarely produced neurospheres, and these could have been generated from the few contaminating LeX⁺ cells in the negative fraction. The neurospheres produced from LeX⁺ cells are typical in terms of growth characteristics and cell content: neurons, glia and more stem cells. Moreover, the primary neurosphere stem cells were also LeX⁺, as shown by FACS separation and secondary neurosphere generation. Hence, LeX expression is maintained as adult stem cells self-renew. Typical neurospheres capable of making neurons and glia *in vitro* preferentially generate glia after transplantation *in vitro* (Winkler et al., 1998; Cao et al., 2001). It will be important to address the ability of freshly isolated LeX⁺ cells to make neurons and glia after transplantation *in vivo*, which will be addressed in future studies.

The number of freshly isolated LeX⁺ cells collected by FACS was 3-6% of the total population, indicating a good recovery of LeX-expressing cells. Moreover, 1 in 4 of these generated neurospheres when plated at clonal density, indicating that most of the stem cells are within this LeX⁺ fraction. However, when the highly pure positive and negative cell populations are separated, there is an intervening population that contains some (approximately 7%) low LeX-expressing cells that are difficult to separate from LeX⁻ cells. This intervening population makes neurospheres at a frequency closely related to the percentage of LeX⁺ cells it contains (not shown). We conclude that there is a strong association between LeX-expression and neurosphere-generating stem cells, and that this can be used to substantially enrich these rare cells.

Not all the LeX⁺ cells behaved like stem cells. When 1-5 LeX⁺ cells were plated per Terasaki well, approximately 1 in 4 generated neurospheres, a 25-fold enrichment

over the total, unsorted SVZ cells. LeX⁺ cells that do not generate neurospheres may be stem cells that are too damaged to divide - certainly most appeared to have died by 24 hours after plating. Alternatively, they may be cells other than stem cells, or perhaps stem cells that require different environmental conditions for their growth.

The stem cell frequency obtained using LeX positive selection (25%) is higher than the 0.31% reported by Kawaguchi et al., 2001, purified from the SVZ of adult transgenic mice expressing EGFP under the control of a Nestin enhancer, and the 4.3% reported by Uchida et al., 2000 for human embryonic CNS stem cells using CD133⁺, mCD24^{-/lo}. The combination of two markers in Uchida's study increased enrichment of neurosphere-generating cells, just as a panel of antibodies isolates highly pure hematopoietic stem cells. A recent study used cell size, binding of the lectin PNA and mCD24^{lo} to separate a highly enriched population of adult neural stem cells (Rietze et al., 2001), although only 63% of the total SVZ stem cells were selected. Likewise, greater enrichment of adult CNS stem cells may be achieved by combining LeX expression with additional selection methods.

Using BrdU uptake *in vivo* to identify SVZ stem cells

There is an ongoing discussion regarding BrdU uptake as an indicator of cell division, and whether it may sometimes reflect DNA repair rather than DNA synthesis (Nowakowski and Hayes, 2000). A recent study indicates that the immunocytochemical method commonly used to detect BrdU uptake, and which we used in this study, is not sensitive enough to detect DNA repair (Palmer et al., 2000). With this caveat, we conclude from the BrdU uptake study we conducted that two-thirds of LeX⁺ cells in the SVZ divide over a two-week period, and of these, one-third are slowly dividing. LeX⁺ cells that did not divide during the two-week labeling period may be post-mitotic - or they may have an even longer cell cycle time.

While there is strong evidence that some SVZ stem cells are slowly dividing, this does not preclude a more rapidly dividing population co-existing in the SVZ. Stem cells from different sources have various cell cycle times (Morrison et al., 1997). Hence some of the LeX⁺ cells that lose BrdU label during the chase period may also be stem cells.

A non-ependymal identity for SVZ stem cells

The original claim that ependymal cells include a stem cell population (Johansson et al., 1999) quickly proved controversial, with observations that ependymal cells inspected at the electron microscopic level did not take up BrdU and that physically isolated ependymal cells could not generate typical neurospheres (Doetsch et al., 1999a; Chiasson et al., 1999; Laywell et al., 2000). Given our finding that most of the neurosphere-generating cells in the SVZ are LeX⁺, the fact that ependymal cells are LeX⁻ argues against their being stem cells. Supporting this conjecture, purified mCD24⁺ cells did not generate neurospheres, nor did individually picked ciliated ependymal cells, even in the presence of neurosphere-conditioned medium. This is consistent with the recent observation that purified SVZ stem cells lack ependymal features (Rietze et al., 2001). The apparently contradictory observation that after ventricular DiI labeling, 32% of purified SVZ stem cells were DiI⁺ (Rietze et al., 2001) could be explained by the fact that this is not a specific labeling technique: DiI can reach other cells in the SVZ, for example astrocytes whose end-feet sometimes penetrate the ependymal layer to contact the ventricular space (Doetsch et al., 1999b).

If SVZ stem cells are not ependymal cells, are they astrocytes?

There is substantial evidence that at least some SVZ stem cells are astrocytes. After depletion of SVZ cells using cytosine arabinoside, the entire cell network can regenerate from a stem cell population that expresses GFAP (Doetsch et al., 1999b). Purified SVZ astrocytes can generate neurons (Laywell et al., 2000). However, not all SVZ astrocytes are stem cells: 12% of SVZ cells are GFAP⁺, but only 1% of SVZ cells generate neurospheres and only a fraction of astrocytes participate in the regeneration process *in vivo* (Doetsch et al., 1999b). Our studies indicate that SVZ stem cells are LeX⁺. LeX and GFAP are expressed in similar domains *in vivo*, suggesting a functional relationship. Yet only 6% of SVZ astrocytes are LeX⁺. Could these LeX⁺GFAP⁺ cells be SVZ stem cells? This would represent around 1% of the total SVZ cells, which could correspond to the 1% that make neurospheres. Furthermore, at clonal density around 1 in 4 LeX⁺ cells produce neurospheres, and around 18% of LeX⁺ cells are GFAP⁺, which is numerically consistent with this being the major stem cell population. It is true that only 5% of the LeX⁺ SVZ cells that incorporated BrdU were also GFAP⁺ - but some of these were slowly

dividing like some stem cells. Given that we know so little about the cell cycle length of endogenous CNS stem cells, we should perhaps give less weight to this finding and explore the reasonable hypothesis that LeX⁺GFAP⁺ defines the major SVZ stem cell population.

What LeX staining *in vivo* reveals about CNS stem cells

Can LeX be used to identify stem cells *in vivo*? Because LeX is shed from stem cells, and may be abundant in the extracellular matrix, this can obscure which cells are producing LeX. Furthermore, only approximately 1 in 4 LeX⁺ cells that are isolated from the SVZ generate neurospheres, and we do not know whether the remaining cells are stem cells or not. Nevertheless, LeX staining can help us hone down the localization of stem cells.

In the SVZ, there are a few distinct cells that strongly express LeX. It seems likely that these correspond to the LeX⁺ population isolated *in vitro*. In whole-mount staining of the striatal wall, LeX⁺ cells are large bushy cells evenly distributed over the entire wall, suggesting that their spacing and number are regulated. Interestingly, purified LeX⁺ cells generated more neurospheres as they were diluted. Perhaps neural stem cells secrete factors that control the division and position of neighboring stem cells. It has been suggested that this type of negative feedback controls skin and intestinal crypt stem cells (Jones et al., 1995b; Bach et al., 2000) and the number and spacing of neuroblast stem cells in insect neuroectoderm (Campos-Ortega, 1995).

In addition to its expression in the striatal SVZ, LeX is abundantly expressed in the septal SVZ, which can generate neurospheres (Chiasson, 1999) and in the hippocampus. Besides these areas of known stem cell location, there is strong LeX labeling in other areas, such as the body of the septal and striatal regions, and it would be worthwhile to examine the fate of these LeX⁺ cells. In this context, it is interesting to note the strong LeX staining in the corpus callosum, which is known to contain primitive glial progenitor cells (Reynolds and Hardy, 1997). Similar glial progenitors obtained from the adult optic nerve can revert to a multipotent, stem-like state (Kondo and Raff, 2000). It will be important to examine whether LeX is expressed on these plastic glial progenitor cells.

In the cerebral cortex, LeX⁺ cells were present at the pial surface and associated with blood vessels in the body of the cortex. Previous studies indicate that stem cells

can be obtained from postnatal cortical parenchyma (Marmur et al., 1998); perhaps LeX⁺ cells from the pial surface or around blood vessels could be responsible. Interestingly, stem cells in the adult mouse hippocampus are also frequently located close to blood vessels, which have been suggested to be a stem cell niche (Palmer et al., 2000). Our findings that LeX⁺ cells are often associated with blood vessels both in cerebral cortex and in hippocampus reinforce this idea and further suggest that LeX is part of that niche.

Given the strong correlation between LeX and the stem cell state in forebrain, it will be valuable to examine its expression on neurosphere-generating cells from other CNS regions, as stem cells have been obtained even from non-neurogenic areas such as the spinal cord (Weiss et al., 1996). Use of this marker may thus help locate stem cell sources in other CNS areas.

Biological significance of LeX

The fact that LeX is expressed on embryonic pluripotent stem cells and also on adult CNS stem cells suggests it has an important function for stem cells, and this will be pursued in future studies. Because LeX influences blastocyst cell adhesion, (Bird and Kimber, 1984; Hakomori, 1992), we speculate that it influences CNS stem cell adhesion. Carbohydrate ectodomains on proteoglycans can be shed into the extracellular matrix where they interact with growth factors (Kato et al., 1998). LeX has been described in the extracellular matrix (Gocht et al, 1996) and we observed shedding of LeX⁺ material by adult SVZ cells *in vitro* and diffuse staining of LeX in neurogenic zones. Low concentrations of free LeX can promote FGF2 oligomerization and stimulate its mitogenicity for embryonic stem cells (Milev et al., 1998; Jirmanova et al., 1999). However, excess LeX inhibits FGF2 mitogenicity (Dvorak et al., 1998). Thus, different concentrations of LeX in the extracellular environment might regulate growth factor access to and influence on CNS stem cells. In the embryo, LeX-containing carbohydrates can bind Wnts (Capela and Temple, unpublished observations).

In conclusion, we speculate that this unusual fucose-containing carbohydrate helps to regulate and maintain the stem cell population: LeX may prove to be an important player in the stem cell niche. Moreover, this unique feature of adult neural

stem cells offers clues to their *in vivo* identity and allows their enrichment for further study, providing access to more information about these essential cells.

Acknowledgments

We are most grateful to Bob Dilwith at the NYS Health Department for FACS analysis and to Fiona Doetsch, Qin Shen and Karen Kirchofer for invaluable help with manuscript preparation. This work was supported by Fundação para a Ciência e Tecnologia and Gulbenkian Science Institute, Portugal and NIH grant R01 NS33529.

Materials and Methods

SVZ dissection and dissociation

Coronal slices of adult female Swiss Webster mice brains were cut with a razor blade. The SVZ was microdissected from each slice and the tissue enzymatically treated for 1 hr at 37°C in 14U/ml of papain (Worthington) or with 1.3mg/ml trypsin and 0.67mg/ml hyaluronidase (Sigma), with gentle rocking. The tissue was then dissociated to a single cell suspension using a fire-polished Pasteur pipette. The cells were centrifuged for 10 min at 300xg and resuspended in DMEM (Life Technologies).

Cell culture

Neurosphere generation: SVZ single cell suspensions were plated in bacteriological grade 35mm dishes (Falcon) in basic serum-free medium: DMEM containing B-27, N2, N-acetyl-cystein (NAC), 20ng/ml EGF or 20ng/ml EGF plus 20ng/ml FGF2 (supplements and growth factors from Life Technologies) and maintained at 35°C, 6% CO₂ in a humidified incubator. Ciliated ependymal cells were individually picked using pulled capillary pipettes and plated in 6 well plates (Costar); the basic medium with 20ng/ml FGF2 plus 20ng/ml EGF was conditioned by co-culture with SVZ cells plated in the upper part of a transmembrane insert (Costar). For secondary neurosphere generation, primary neurospheres were dissociated as described for SVZ tissue, but with a 30-minute papain incubation, and plated as for primary neurospheres.

Cell sorting

SVZ single cell suspensions or primary neurosphere suspensions labeled with MMA (Becton Dickinson or American Type Culture Collection) or mCD24 (Pharmingen) were sorted using a Becton Dickinson FACS Vantage. Gating parameters for sorting were set by side and forward scatter to eliminate debris, dead and aggregated cells. A second gating parameter was set by green (530nm, MMA) or red (575nm, mCD24) fluorescence to separate positive from negative cells. The flow rate was ~1000 events/sec for high purity and recovery.

Immunostaining

Dissociated SVZ cells: cells were plated for 2 hrs on PLL-coated Terasaki plates (Nunc), incubated with MMA antibody (1:20 dilution) for 30 min at room temperature (RT), fixed with ice-cold 4% paraformaldehyde (PFA) for 30 min and then incubated with biotinylated antibodies, ABC Elite kit and VIP (Vector). For double labeling with MMA and cell specific markers, Streptavidin-Alexa 546 (Molecular Probes) were used to reveal LeX staining. mCD24: cells were blocked and incubated overnight at 4°C with primary antibody (1:500, gift from G. Rougon).

For intracellular antigens, cells were permeabilized for 5 min with 100% methanol at -20°C, blocked, then incubated with primary antibody. β -tubulin III: (1:400, Sigma) overnight at 4°C; GFAP: (1:400, DAKO) 1hr at RT. Staining was visualized with Alexa 488 conjugated secondary antibodies (1:200, Molecular Probes) for 45 min at RT. Before incubation with anti-BrdU antibodies (1:100, DAKO), cells were treated for 45 min at 37°C in 2M HCl.

Neurosphere sections: primary neurospheres were fixed in 4% PFA for 30 min, cryoprotected in 30% sucrose and 12 μ m cryostat sections were cut. Sections were blocked in Ca- and Mg- phosphate buffered saline (DPBS) containing 0.1% Triton X-100 and 10% normal goat serum (NGS) and incubated with 1:200 MMA antibody overnight at RT. The staining was revealed using the same method as for dissociated SVZ cells.

Brain sections: mice were anesthetized and transcardially perfused with 4% PFA. Cryostat sectioning and staining was performed as for neurosphere sections. Paraffin sections (4 μ m) were de-waxed in xylene and hydrated. Antigens were unmasked by incubating with 0.25% pepsin in 0.1M HCl. MMA staining was performed as described for neurospheres. GFAP polyclonal antibody (1:800, DAKO) was incubated overnight at 4°C and revealed with Alexa-488 anti-rabbit secondary antibodies. BrdU staining was performed as described above.

Whole-mount staining: mice were anesthetized and transcardially perfused with saline followed by cold 4% PFA or Zamboni's fixative. The entire wall of the lateral ventricles was exposed by dissection under the microscope. The tissue was permeabilized with 100% methanol and 100% acetone for 30 min at -20°C, blocked in DPBS containing 0.5% Triton X-100, 10% NGS and incubated with MMA antibody (1:200) or monoclonal anti-GFAP (1:100, Chemicon) for 48 hrs at 4°C. Staining was

revealed with peroxidase-conjugated goat anti-mouse IgM or anti-mouse IgG antibodies (Jackson Immunoresearch Laboratories), using DAB as substrate.

Long term BrdU labeling

Adult mice were given drinking water containing 1mg/ml BrdU for 2 weeks, then BrdU-free water for 0, 4 or 7 days. At each time point, tissue was prepared for immunohistochemistry of cryostat sections and of dissociated cells.

Western blots

Adult tissues were dissected in CMF-PBS and homogenized in ice-cold lysis buffer (150mM NaCl, 50mM Tris, mM EDTA, pH7.4) containing protease inhibitors (Complete™ mini, Roche Molecular Biochemicals). Homogenates were centrifuged 10 min at 200g at 4°C to remove whole cells and nuclei and then at 100,000g for 1hr at 4°C to pellet membranes, which were washed in lysis buffer and re-pelleted. Protein concentration was determined using the BCA kit (Pierce). Protein samples were boiled for 5 min in reducing loading buffer, separated on an SDS-polyacrylamide gel and then transferred to PVDF membranes (Immobilon P, Millipore). After blocking overnight at 4°C with 5% non-fat dry milk in Tris buffered saline (TBS), the membranes were incubated with MMA antibody (1:200, 5 hrs at RT). After six 5-minute washes, membranes were incubated with peroxidase conjugated goat anti mouse IgM (1:4,000) and the bands detected by luminescence using the ECL reagent (Amersham Pharmacia).

SUPPLEMENTAL DATA PRELIMINARY RESULTS FOR *IN UTERO* TRANSPLANTATION OF FRESH ADULT SVZ CELLS AND OF CULTURED LEX⁺ PRIMARY NEUROSPHERE CELLS.

Introduction

Due to the lack of specific neural stem cell markers, previous work addressing the *in vivo* behavior of neural stem cells has relied on their *ex vivo* expansion in the presence of chemically defined culture medium. Nonetheless, the results are very exciting and suggest that stem cells have a broad capacity to generate different cell types in many different regions of the CNS (reviewed by Temple, 2001).

Our study shows that neural stem cells can be purified from adult tissue, circumventing embryo manipulation, which, in case of human stem cell research, poses many ethical questions. Also, it allows the purification of neural stem cells from freshly dissociated tissue, avoiding possible reprogramming due to their *ex vivo* expansion. The hematopoietic system is notorious in providing the best example of *in vivo* engraftment of repopulation by freshly isolated stem cells. Unfortunately, there are only a few examples of *in vivo* analysis of neural stem cell outcome without *in vitro* expansion (Durbec and Rougon, 2001; White et al., 2001; Rietze et al., 2001).

The findings presented and discussed above demonstrate that neural stem cells in the adult SVZ are LeX⁺. However, the *in vivo* potential of freshly isolated LeX⁺ stem cells was not studied.

In order to test the ability of freshly isolated SVZ cells to generate multiple differentiated progeny *in vivo*, we used an *in utero* transplantation technique. Adult mouse SVZ cells were injected into the ventricles of E13-E15 mouse embryos, a developmental window in which neurogenesis is peaking, ensuring that the intrinsic and extrinsic cues for neurogenesis are present. Our prediction was that cells derived from the SVZ would recapitulate their *in vivo* neurogenic program in the host environment and would set the behavioral pattern of LeX⁺ cells. Transgenic adult mice carrying human placental alkaline phosphatase (hPAP) as a reporter gene (DePrimo et al., 1996) were used as donors.

Results

Injection of freshly isolated SVZ cells

E13-E15 mouse embryos received *in utero* intra-ventricular injections of a mixture of freshly isolated adult AP⁺ SVZ cells (derived from the transgenic hPAP mouse) and embryonic cortical cells and were allowed to survive until postnatal 3 (P3). Because transgenic mice were in very limited numbers, embryonic cells derived from wild-type mice (WT) were co-injected with hPAP SVZ cells to optimally increase the number of cells per injection. Pups were sacrificed at P3 and P5, their brains were removed, fixed and 20 μ m cryosections were prepared. The sections were incubated with AP substrates after high temperature block of endogenous AP activity. Injected animals showed consistent although modest engraftment of hPAP positive cells in different locations of the brain. Figure 3.8 A, B and C shows hPAP⁺ cells in the olfactory bulbs (this is the target for neuroblasts generated by endogenous SVZ stem cells), cortex, and hippocampus respectively. In some cases, the morphology of the hPAP⁺ cells is consistent with labeling of neurons (figure 3.8 A), although the use of neuron specific antibodies was not possible after NBT/BCIP staining. Nevertheless these results confirm that freshly isolated SVZ cells engraft in an embryonic environment and migrate to diverse locations.

Strikingly, we also found that cell aggregates resembling neurospheres were formed in the ventricular system, eventually attached to the ventricles and appeared to start infiltrating in the brain parenchyma (figure 3.9 A, B and C), specially in the colliculus. In some neurospheres, all cells were hPAP⁺ (figure 3.9 A), suggesting that they were clonal but some contained hPAP⁺ and WT cells (figure 3.9 B, C), suggesting cell aggregation. Given that the injected cell suspension is highly pure in single cells (> 98%), the possibility that these neurosphere-like structures resulted from proliferation of clusters in the initial cell suspension is very unlikely. Interestingly, neurospheres contained GFAP⁺ astrocytes, NeuN⁺ neurons and more LeX⁺ cells (figure 3.9 D, E, F). In certain cases, neurosphere-derived cells infiltrated into the brain parenchyma as large bushy cells that we were unable to identify (figure 3.9 G, H). Interestingly, when these *in vivo*-formed neurospheres were incubated with PECAM, an endothelial cell marker, positive staining was observed (figure 3.9 I) and suggested that perhaps new blood

vessels are being generated/recruited to the neurosphere. These endothelial cells were hPAP negative, suggesting that they came either from the host or from the co-injected WT embryonic cells. The formation of such neurospheres *in vivo* has never been reported previously. We may have observed it here because the freshly isolated cells may grow more aggressively after injection *in vivo* than those that were cultured for long periods prior to injection. It is important to consider that a stem cell that slips outside of the ventricular zone into the ventricular space might behave in this manner. Could this be one of the origins of neuroectoderm tumors? The presence of endothelial cells within the neurospheres makes this possibility even more plausible, and it should be pursued in future studies.

Injection of primary neurosphere LeX⁺hPAP⁺ cells

Generation of neurospheres has been extensively used as a means to expand neural stem cells *in vitro*. In the best case scenario, 20-30% of the cells in a typical neurosphere are stem cells that can generate more neurospheres; our study indicates that such *in vitro* expanded stem cells are also LeX⁺.

Primary neurospheres were generated from adult SVZ of hPAP mice, collected at 7 days and dissociated in a single cell suspension. LeX⁺ cells were purified using magnetic nanobeads (only visible using electron microscopy and biodegradable) and injected into E13-E15 mouse embryos as before. Pups were allowed to survive until P0, P8 and P12 and their brains were processed for immunohistochemistry.

In clear contrast with freshly isolated unsorted SVZ cells, the engraftment of LeX⁺hPAP⁺ cells was not as successful. Very few hPAP⁺ cells were found throughout the brain parenchyma, mainly in the colliculus (figure 3.10 A, B). We did not find donor cells in the olfactory bulbs, cortex or hippocampus. Likewise, the typical neurosphere-like structures observed when fresh SVZ were transplanted were not obtained in this case.

Figure 3.8. Freshly isolated adult SVZ cells engraft in embryonic mouse brain.

At P3, hPAP⁺ SVZ cells can be found in: **A.** olfactory bulb (insert shows high magnification of boxed area showing labeling in cells with neuronal morphology), **B.** Cortex and **C.** hippocampus.
Size bar 200 μm .

Figure 3.9. Freshly isolated adult SVZ cells generate neurosphere-like structures *in vivo*.

(A) At P3, pure hPAP⁺ (dark purple) neurospheres are found.
(B,C). Neurospheres containing hPAP⁺ and wild-type cells (mixed neurospheres) are also generated.
(D). At P5, mixed neurospheres (hPAP in red for all panels) contain astrocytes (blue), **E.** NeuN⁺ neurons (green), **F.** and more LeX⁺ cells (green).
(G, H). Neurosphere cells infiltrate in the parenchyma of the culliculus and generate unidentified large bushy cells.
(I). Mixed neurospheres also contain PECAM⁺ endothelial cells (green). DAPI (blue) stains nuclei.
Size bar: all 100 μm except I, 50 μm .

Figure 3.10. Cultured LeX⁺ cells engraft poorly into embryonic brain.

(A,B). At P5, hPAP⁺ large bushy cells (red) are found in the parenchyma of the colliculus. hPAP⁺ do not express NeuN but surrounding endogenous neurons do so (green, arrows).
Size bar: 100 μm .

Figure 3.8

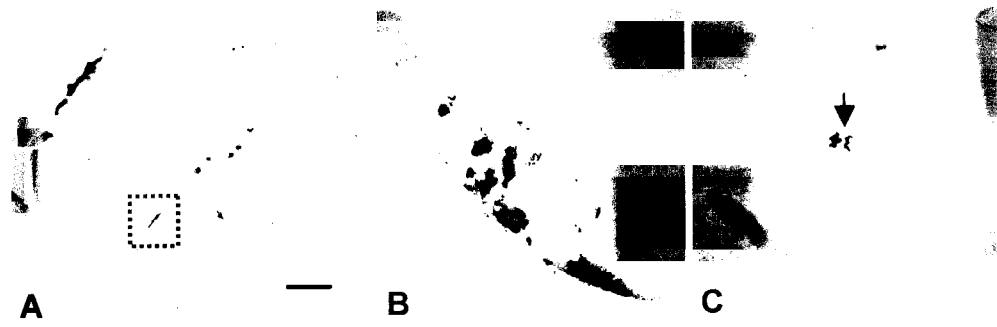


Figure 3.9

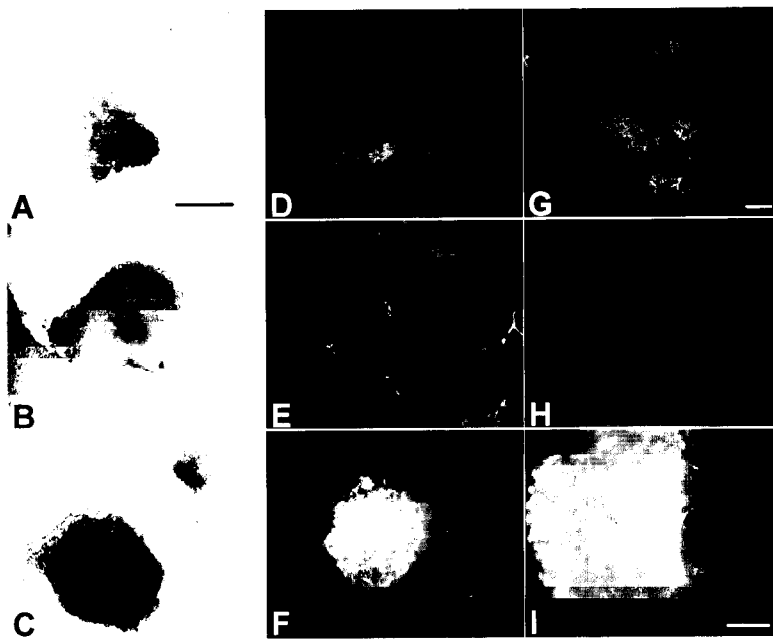
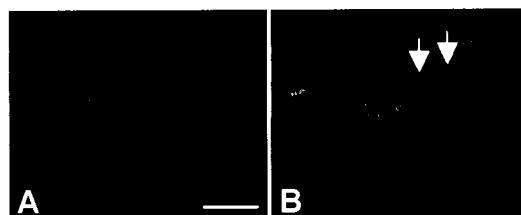


Figure 3.10



Discussion

The transplantation experiments described did not assess the neurogenic/stem cell potential of freshly isolated LeX⁺ SVZ cells *in vivo*, because of technical difficulties encountered at the time these experiments were attempted. These included limited access to transgenic adult hPAP mice and poor cell sort yields with only 2,000-5,000 LeX⁺ cells obtained from the SVZ of 8 adult animals, which meant sub-optimal cell numbers for injection into each embryo. Breeding of hPAP transgenic mice is now possible at the Albany Medical College and will allow the transplantation of freshly isolated LeX⁺ SVZ cells in the future.

Interestingly, from the preliminary experiments we performed, we can conclude that freshly isolated SVZ cells engraft, migrate and in some cases adopt neuronal morphology. Because total cells were injected, it is not possible to determine which cells were responsible for the generation of grafted hPAP⁺ cells, i.e. whether they were LeX⁺ or not. Moreover, LeX⁺ cells expanded *in vitro* do not engraft as well as freshly isolated cells. Contrary to the transplantation of fresh SVZ cells, cultured LeX⁺ cells were not co-injected with freshly isolated embryonic cortical cells. We can speculate that embryonic cortical cells helped the engraftment of fresh SVZ cells by providing correct cues, although control experiments should be conducted to test this interesting hypothesis. Alternatively, the cultured cells did not grow as well after injection.

Another study directly addressed the differential engraftment of cultured or fresh adult SVZ cells (Durbec and Rougon, 2001) by testing their capacity for generation of PNS derivatives upon transplantation into chick neural crest migration routes. They found that cultured cells were more plastic and generated PNS cells, while fresh cells did not survive, which is different from our results. However this could be due to the fact that they attempted inter-species injections, and the cells were injected into neural crest pathways rather than the CNS ventricles. Interestingly, with SVZ cells derived from neonatal mice, fresh cells generated progenitors and neurons while cultured cells generate crest derivatives. Taken together, these results show that cultured cells of neonatal or adult SVZ are plastic and able to generate PNS derivatives *in vivo*. Fresh SVZ adult cells do not survive in a crest environment while neonatal cells do and generate

CNS cells; however fresh cells never generate crest derivatives, demonstrating a more restricted potential which agrees with other studies (reviewed by Temple, 2001).

The novel result in our studies was the formation of "neurosphere"-like structures *in vivo*. These neurospheres contained astrocytes, neurons and LeX⁺ cells suggesting a similar composition of their *in vitro* counterparts. hPAP⁺PECAM⁺ cells were found within such neurospheres suggesting that these endothelial cells are either derived from co-injected WT embryonic cortical cells or are recruited from endogenous tissue. The cortex does contain developing blood vessels, and the co-injected population might therefore have an endothelial population. Another possibility is that neural cortical progenitor cells were somehow able to produce endothelial cells. Although this might sound highly unlikely, there are reasons to think that a common progenitor for endothelial/ neural cells may be present in the embryonic brain (Palmer et al., 2000). Moreover, the recruitment of blood vessels can be related to tumor formation and hence should be taken in consideration when considering the therapeutic application of freshly isolated cells.

Acknowledgements

We thank Dr Stringer for allowing us to use the hPAP mice and Fiona Doestch and Gord Fishell for donating the adult hPAP mice used in this study. We acknowledge Fiona Doetsch's help in setting up the transplantation technique in the lab. We thank Wenlei He for teaching us the technique, Susana Nery for invaluable advice with the transplantation technique and AP immunohistochemistry and Yu Sun for her skilful and generous help with the transplantations.

Materials and Methods

Cell preparation

SVZ cells from adult transgenic hPAP and wild-type mice were isolated as described earlier in this chapter. Primary neurospheres derived from hPAP mice were prepared and used to generate a single cell suspension as described earlier.

Single cell suspensions were obtained from the cortices of wild-type mouse embryos (E13-E15) as previously described (Qian et al., 2000). Briefly, dissected cortical tissue was incubated for 30 min at room temperature (RT) in pre-warmed DMEM containing 7-10U/ml papain (Worthington), 1mM glutamine, 1mM sodium pyruvate and 60µg of DNase (Sigma), on a rocking platform. Papain inhibitor (Worthington) was added at the end of the incubation, and the tissue was triturated using a 10ml sterile plastic pipette to generate a single cell suspension. After three washes with DMEM, the cells were resuspended in hibernation medium. An aliquot of cells used for transplantation was cultured to ensure that the cells were viable and generated neurospheres/stem cell clones.

Cell sorting

Primary neurosphere LeX⁺hPAP⁺ cells were obtained using the immunomagnetic method described in chapter 2, with some modifications. Milteny magnetic nanobeads were used (Milteny Biotech) instead of Dynal beads.

Surgery

Timed-pregnant Swiss Webster mice (Taconic Farms) were anesthetized with metofane and administered a muscle relaxant (2.5% MgCl₂). Anesthesia was carefully monitored throughout the surgery. A small midline incision was made in the skin, followed by another similar incision to expose the abdominal cavity. The uterine horns were identified and gently pulled out through the incision to expose 2-3 embryos at a time. By very gentle handling of the uterus, the embryos were positioned in a way to facilitate access of the injection needle to the ventricles. Each embryo was injected with a 0.5-1µl of cell suspension containing wild-type E13-E15 cortical cells plus adult hPAP mouse SVZ cells (20,000-40,000 cells; 1% of cells are derived from the hPAP mouse) or dissociated

cells from hPAP mouse SVZ primary neurospheres (10,000) containing fast green (Sigma) as a contrast dye. Attempts were made to inject all embryos. After returning the uterus to the abdominal cavity, the incisions were sutured with silk thread and codein was applied to the stitched wound. Animals were placed on a heated mat set at 37°C and allowed to recover from anesthesia under a heating lamp. The cages were returned to the animal facility and the animals were monitored daily. Births were recorded. All procedures were approved by the Institutional Animal Care and Use Committee at the Albany Medical College.

Immunohistochemistry

P0 to P14 injected mouse pups were sacrificed by decapitation and their brains were removed and fixed in 4% paraformaldehyde (Tousimis Research) 12-24h at 4°C. The brains were subsequently cryoprotected in 30% sucrose solution and mounted with OCT (Sakura). Twenty micron sections were cut in a cryostat and the sections were mounted onto Superfrost Plus slides (Fisher) at stored at -20°C.

For detection of human placental alkaline phosphatase (hPAP), two methods were used. For detection using the hPAP specific activity, the sections were rinsed 2 times with PBS and the endogenous AP was blocked by a 30 min incubation in PBS at 65°C. After 2 rinses with PBS at RT, sections were incubated in detection buffer (100mM Tris pH 9.5, 100 mM NaCl and 50mM MgCl₂) for 10 min and then in detection buffer plus NBT and BCIP (Sigma). After a 12-24h incubation with AP substrates, the sections were rinsed with PBS containing 5mM EDTA. For detection of hPAP and other antigens simultaneously, tissue sections were rinsed with PBS containing 0.1% Triton (PBST), blocked for 1h at RT with 10% horse serum in PBST and further incubated with a sheep polyclonal antibody against hPAP (1:100, overnight at 4°C, American Research Products). The staining was revealed using biotinylated anti-sheep antibodies (1:200, 2 hours at RT, Vector) and streptavidin Alexa 546 (1:200, 45 min at RT, Molecular Probes). For NeuN staining, sections were incubated with anti-NeuN antibody (1:500, overnight at 4°C, Chemicon) and anti-mouse IgG-Alexa 488 (1:200, 45 min at RT, Molecular probes). For PECAM staining, sections were incubated with anti-CD31 antibody (1:20, overnight at 4°C, BD-Pharmingen) and anti-rat IgG Alexa 488 as for Neu-N.

Sections were coverslipped with vectorshield (Vector), observed and photographed using a Zeiss axiophot equipped with a Optronics digital camera.

Chapter Four

Stem cells in high growth regions of the CNS express abundant LeX,
a carbohydrate moiety that binds Wnts and FGFs

Alexandra Capela and Sally Temple

Center for Neuropharmacology and Neuroscience
Albany Medical College
Albany NY 12208 USA

Phone 518 262 5416

Fax: 518 262 6178

E-mail: temples@mail.amc.edu

capelaa@mail.amc.edu

Running title: LeX expression and neural stem cells

Keywords: Stem cells, progenitor cells, mouse, cerebral cortex, LeX, extracellular
matrix, carbohydrates, CD15, MMA

[Adapted from manuscript in preparation. To be submitted to Neuron]

Abstract

LeX/SSEA1, is an extracellular matrix-associated carbohydrate expressed by ES cells and recently found on adult neural and bone marrow stem cells. We now show that LeX is expressed by radial stem cells and highly proliferative progenitor cells in the developing nervous system. Moreover, LeX is highly expressed in regions with prolonged growth and neurogenesis, including basal forebrain, hippocampus, mid/hindbrain isthmus and olfactory epithelium, regions which express the growth inducers FGF8 and Wnts. LeX is known to bind FGFs, and we show it binds Wnt-1. LeX-containing vesicles reminiscent of argosomes, a means of *Drosophila* Wg dispersal, are found within neuroepithelia. Our findings suggest that LeX is critical for stem cell function, binding and distributing factors that regulate their proliferation and self-renewal.

Introduction

Patterning of the CNS involves expansive growth of certain regions of the neuroepithelium - notably the forebrain, and the cerebellum, which ends up with the highest concentration of cells in the central nervous system (CNS). Some CNS regions even grow throughout life - neurogenesis continues in the subventricular zones surrounding the forebrain lateral ventricles and the hippocampus, and in the olfactory epithelium of the peripheral nervous system (PNS). Understanding why some neural regions and progenitor cells are capable of exceptional growth and prolonged neurogenesis is important for elucidating developmental mechanisms and for generating novel neural cell replacement therapies.

What distinguishes these high growth areas of the nervous system, and maintains long-term neurogenesis and cell production? One possibility is that the progenitor cells in these areas have higher growth potential than those in other areas.

Stem cells have been described in the nervous system as early as neural plate stages (Tropepe et al., 1999), and have been isolated from most later embryonic CNS regions (Temple, 1989; Davis and Temple, 1994; Reynolds et al., 1992; Reynolds and Weiss, 1996; Mayer-Proschel et al., 1997) and from neural crest, from which PNS tissue derives (Stemple and Anderson, 1992; Morrison et al., 1999). Moreover, neurogenic

stem cells have been isolated from adult SVZ, hippocampus and olfactory neuroepithelium, indicating that these regions are supported by stem cell populations throughout life (Reynolds and Weiss, 1992; Morshead et al., 1994; Gritti et al., 1996; Palmer et al., 1997). Neural stem cells in high growth areas of the nervous system may have greater growth potential, either due to intrinsic differences or to localized environmental factors.

Recently, we showed that the carbohydrate moiety LeX (fucose N-acetyl lactosamine) that is expressed by pluripotent mouse blastocyst, embryonic stem cells and primordial germ cells (Solter and Knowles, 1978; Fox et al., 1981; Gomperts et al., 1994), is also expressed on the surface of adult SVZ neural stem cells (Capela and Temple, 2002). LeX has now been described on rare adult pluripotent stem cells from bone marrow (Jiang et al., 2002). This carbohydrate is shed into the extracellular matrix around stem cells where it can influence their environmental niche. LeX can regulate FGF mitogenicity for ES cells in a concentration-dependent manner (Dvorak et al., 1998; Jirmanova et al., 1999). Neural stem cells also divide in response to FGFs, raising the possibility that LeX may influence neural stem cell proliferation. Hence we examined its expression on progenitor cells in the developing nervous system (NS).

LeX, also known as FAL, SSEA-1 or CD15, has already been described in some CNS germinal areas, including the ventricular zone (VZ) of the embryonic telencephalon, hippocampus and spinal cord and the cerebellar external granular layer (reviewed by Gotch et al., 1996), although the types of progenitor cell expressing LeX were not explored. Interestingly, LeX is expressed by a subset of radial glial cells in the mouse embryonic forebrain (Mai et al., 1998), which are now recognized as neuronal progenitor cells (Gaiano et al., 2000a; Malatesta et al., 2000; Alvarez-Buylla et al., 2001; Miyata et al., 2001; Noctor et al., 2001) and have been suggested to have stem cell characteristics (Gaiano et al., 2000a; Alvarez-Buylla et al., 2001).

In this study we show that regions of the nervous system with high growth potential are characterized by high LeX expression. Interestingly, many of the LeX-expressing cells have radial features, both in the developing CNS and the nasal epithelium of the PNS. Using FACS selection, we show that LeX⁺ cells have higher growth potential than LeX⁻ cells: LeX is expressed on the surface of neural stem cells and their immediate progeny, highly proliferative restricted neuroblasts and glioblasts but is

largely absent from more restricted progenitors and differentiated neurons and glia. Hence LeX expression is associated with high proliferative output of neural progenitor cells. This association occurs throughout life, and because LeX is a surface marker, neural stem cells can now be isolated and enriched at all stages.

It is known that LeX is shed into the extracellular matrix (ECM) and can bind FGFs. We show a close association between LeX and FGF8 and Wnt-1 expression, and further that LeX-containing molecules from the embryonic nervous system bind Wnts. We suggest that LeX is made by stem cells and expressed abundantly in stem cell niches in NS regions with high growth where it binds factors critical for enhancing stem cell proliferation and self-renewal.

Results

LeX is abundant in high growth regions of the developing nervous system

Embryos were stained with the anti-CD15 antibody produced by the hybridoma clone MMA, which recognizes the LeX epitope. The staining pattern was confirmed using a different antibody (anti-SSEA-1) to LeX. At E9.5-10 there is strong staining throughout the CNS (Figure 4.1 A), with some prominent areas: the floor plate, isthmus (mes/metencephalic junction) and hindbrain (Figure 4.1 A), where LeX delineates rhombomeres. The pituitary primordium also has strong LeX expression in its anterior, oral ectoderm component (Rathke's pouch) but not the in its posterior, diencephalic component (the infundibulum) (Figure 4.1 B). LeX was highly expressed in the nasal epithelium (figure 4.1 K and 4.2 D) and in other placodal tissue - the otic pits but not lens (not shown).

Staining of horizontally sliced embryos revealed additional features: the forebrain has high LeX expression in radial cells (Figure 4.1 C). Strong expression of LeX was observed at the lumen (apical) border of the neuroepithelium in forebrain, (Figure 4.1 C), spinal cord (Figure 4.1 D) and olfactory epithelia. High power reveals apical LeX staining was often in villi structures and omega profiles (Figure 4.2 E). Interestingly LeX⁺ staining was observed in vesicles inside these neuroepithelial cells, with a gradient declining from apical-basal (Figure 4.2 F).

At this early age, LeX was also found outside the nervous system. As previously reported (Gomperts et al., 1994), primordial germ cells (PGC's) en route to the genital ridges prominently express LeX (Figure 4.1 D). In addition, LeX is present in the skin (Figure 4.1 C).

At E11.5, the areas of prominent LeX expression are more restricted: the forebrain midline (commissural plate), including the hippocampal primordium, hypothalamus and isthmus (Figure 4.1 E-H) and basal forebrain, including the striatal primordium. PGC's are also evident (Figure 4.1 I). A gradient of LeX expression, stronger caudally, is now seen in the cortex (Figure 4.1 J). As at the earlier age, the pituitary and the olfactory epithelium express high levels of LeX (Figure 4.1 G and K).

Hence LeX expression is abundant and widespread in the very early nervous system, and quickly becomes concentrated in regions with high growth potential.

LeX⁺ neural progenitor cells have a radial morphology *in vivo*.

LeX has been reported in a subset of forebrain radial glia (Mai et al., 1998). Inspection of whole embryos and horizontally sliced embryos suggests a radial phenotype for LeX⁺ cells in many locations. To explore this further, we stained sister cryostat sections for LeX and the radial glial marker RC2 (both are mouse IgM antibodies).

In the cerebral cortex and basal forebrain MMA labels cells with radial phenotypes that were similar to RC2-positive radial glia (Figure 4.2 A, B). By E18, some LeX⁺ cells had a morphology intermediate between radial glia and astrocytes; such transitional forms, also seen with anti-GFAP, provide evidence that radial glia transform into astrocytes (Schmechel and Rakic, 1979; Voigt, 1989).

Radial LeX-expressing cells were also seen in the diencephalon, olfactory epithelium and isthmus (Figure 4.2 C, D; not shown). In neonates and adults, LeX staining is observed in Bergman glia (our observations, Bartsch and Mai, 1991) Hence, many LeX⁺ cells are radial in the embryo.

Figure 4.1 Distribution of the LewisX antigen in the mouse embryo.

(A-D) LeX expression pattern in E9.5-E10 and **(E-K)** E11.5 mouse embryos.

(A) LeX is highly expressed in the CNS. Note particularly strong expression in the forebrain (arrow), floor plate (black arrowhead), isthmus (white arrowhead) and hindbrain (bracket).

(B) High magnification of LeX staining in Rathke's pouch (anterior pituitary primordium) (arrowhead), and CNS floor plate (arrow).

(C, D) LeX is strongly expressed at the apical edge (ventricular lumen) of forebrain (C) and spinal cord (D) neuroepithelium, and in radial fibers, resembling the staining of radial glia (arrowheads). A sub-population of cells in skin are LeX⁺(C). Primordial germ cells (PGC) are also LeX⁺(D) (arrows).

(E-I) In E11.5 embryos LeX expression is more restricted to certain locales, including the commissural plate (F), pituitary (black arrowhead), hypothalamus (G), and isthmus (H) Migrating PGC's are LeX⁺ (I).

(J) In forebrain, LeX expression is graded being strong at caudal levels (arrowhead) and weaker rostrally (arrow).

(K) The olfactory epithelium expresses LeX (arrow). Note sharp LeX staining in the isthmus (arrowhead).

(C, J and K) Top is anterior; (C) slice is ventral side down; (J, K) slices are ventral side up.

Size bars: (A, J, K) 500 μ m; (B, C, D) 200 μ m; (E) 1mm; (F-I) 400 μ m.

Figure 4.2 LeX positive cells have radial morphology *in vivo*.

(A, B) E11.5 coronal sister forebrain sections showing RC2⁺ radial cells (A) and LeX⁺ cells (B). LeX strongly labels the end-feet of RC2⁺ cells, but is weaker in the radial process although more prominent in wholemount (below).

(C, D) In wholemounts, radial LeX⁺ cells are clearly stained in the forebrain (C) and the olfactory epithelium (D).

(E, F) Neuroepithelial LeX staining at the apical edge of the neuroepithelium (the ventricular lumen) is often seen in villi-like processes (E) and in vesicles inside neuroepithelial cells (F).

Size bars: (A-D) 100 μ m; (E, F) 25 μ m.

Figure 4.1

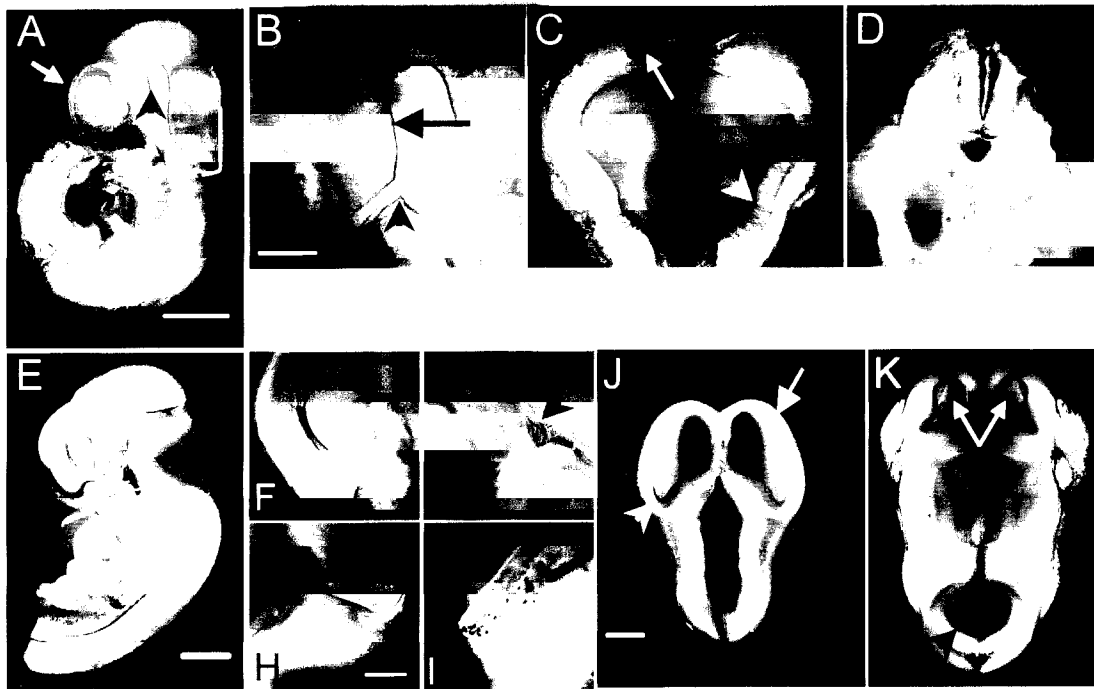
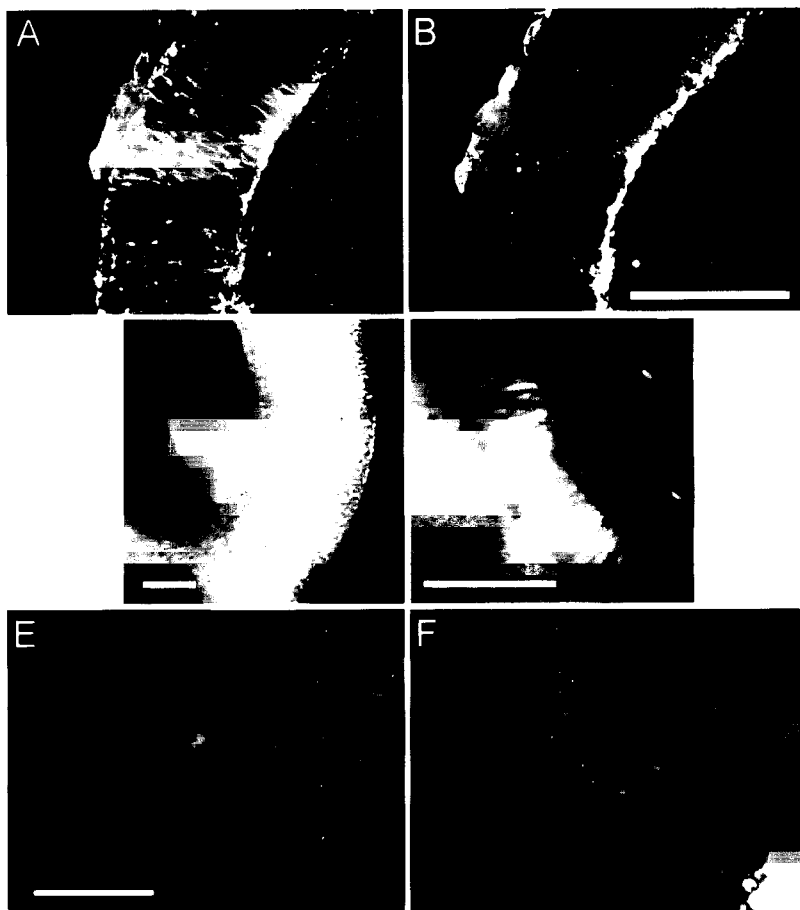


Figure 4.2



LeX expression identifies stem cells and highly prolific progenitor cells

To test whether LeX expression is related to the growth potential of neural progenitor cells, we analyzed LeX⁺ and LeX⁻ sorted cells derived from embryonic mouse cerebral cortex, a progenitor population that we have extensively characterized. At E10 the cerebral cortex is composed mostly of proliferating progenitor cells, which, when cultured individually under standardized conditions, generate different types of clones (Davis and Temple, 1994; Kilpatrick and Bartlett, 1993), suggesting that they are intrinsically different: 10-20% are multipotent stem cells, 15-20% are restricted neuroblasts that produce solely neurons, and the remaining cells generate single neurons (restricted glioblasts arise later). However it has never been established whether this behavioral heterogeneity reflects true heterogeneity, or is an artefactual response to cell culture.

Progenitor markers label most cells in the early cortex, hence we were interested to find that only approximately half (53.5±5.7%) acutely isolated E10.5 cortical cells were LeX⁺. Most LeX⁺ cells also express other progenitor markers (93% are also RC2⁺ and 99% are Nestin⁺). The LeX⁻ population contains most of the extant neurons, but includes a high percentage of progenitor cells (82% are Nestin⁺ and 62% are RC2⁺). Hence at E10.5, two progenitor subpopulations are defined by LeX expression (Table 4.1). These populations were FACS separated and their fate assessed by clonal analysis. Single cells were plated at clonal density, mapped two hours later to record their number and location, then followed daily as clones developed. At 7-10 days, clones were fixed and stained with cell-type specific markers.

We found a significant association between LeX expression and the proliferative state of E10.5 cortical progenitor cells. Most cells that did not divide at all *in vitro* were LeX⁻. Almost all LeX⁻ cells produced neurons, indicating that LeX⁻ RC2⁺ and LeX⁻ Nestin⁺ progenitors are committed to the neuronal lineage. LeX⁺ neuroblasts were significantly more proliferative than LeX⁻ neuroblasts (Table 4.2).

The vast majority (over 90%) of stem cell clones, (defined by growth characteristics and production of both neuronal and glial lineages), were found in the LeX⁺ fraction. At E10.5, only 1.6% of cells in the LeX⁻ subpopulation generate stem cell clones, 10-fold less than the LeX⁺ population (Figure 4.3 H and Table 4.2). Similarly,

only 4% of E10.5 LeX⁻ cells generate neurospheres compared to 25% of the LeX⁺ cells (Figure 4.3 and Table 4.2).

Table 4.1 Antigenic characterization of LeX⁺ and LeX⁻ cortical cells.

Single cortical cells were sorted based on LeX expression then stained for other markers. Both MMA and RC2 are IgM monoclonals, but are distinguishable in isolated cells because MMA localizes to the cell surface and RC2 is intracellular. Results: mean±SEM.

Table 4.2 Developmental potential of sorted LeX⁺ and LeX⁻ cortical subpopulations.

Sorted cells were cultured as adherent clones or in suspension (for neurosphere generation). Adherent clone development was followed for 7-10 days and final clone composition determined by immunostaining. Neurosphere number was scored at 7 days. Results: mean±SEM.

Figure 4.3 E10.5 cortical stem cells are LeX positive.

(A) E10.5 cortical cells were acutely stained with MMA-FITC and sorted into LeX⁺ and LeX⁻ populations.

(B, C) Sorted samples were highly pure (>95%), determined by acute staining with MMA.

(D, F) LeX⁺ cells generate adherent stem cell clones (D) and neurospheres (F).

(E, G) LeX⁻ cells have limited proliferative potential and generate negligible numbers of stem cells clones (E) or neurospheres (G) but mainly neurons in adherent conditions (insert in E).

(H) Graph of the percentage of LeX⁺ and LeX⁻ E10.5 cortical cells that generate stem cell clones assayed by plating either onto an adherent substrate or in suspension to give neurospheres. The LeX⁺ subpopulation contains the majority of the stem cells. Results are expressed as mean±SEM.

Size bar: (B, C) 25µm; (D-G) 100µm.

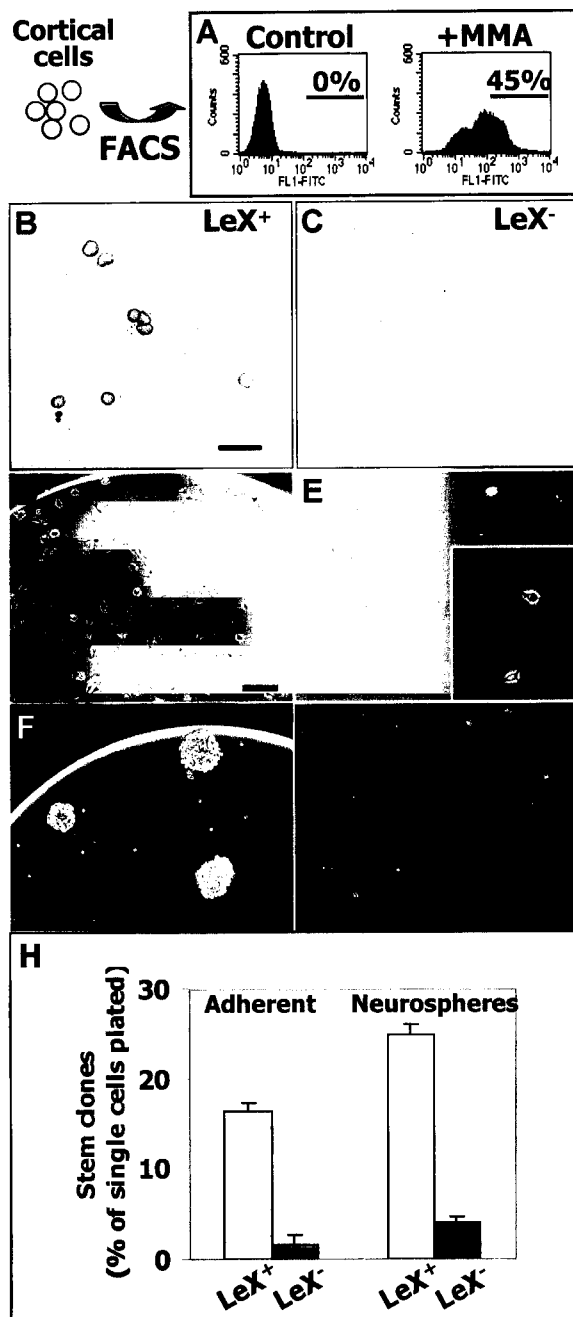
Table 4.1. Antigenic characterization of unsorted and sorted cortical cell populations

	Unsorted			LeX ⁺			LeX ⁻		
	E10.5	E14	E18	E10.5	E14	E18	E10.5	E14	E18
MMA	53.5±5.7	37.3±2.7	13.1±0.9	99.2±0.3	92.5±3.3	97.4±0.5	0.1±0.1	0	0.4±0.1
β-Tub	17.3±1.9	49.7±5.1	79.5±0.3	1.2±0.4	9.1±1.7	6.3±0.7	34.9±2.2	96.5±1.3	98.1±0.1
RC2	73.1±3.3	27.0±6.4	8.7±0.4	93.2±0.6	67.2±7.2	57.8±1.7	62.5±3.9	7.9±1.3	0.1±0.1
Nestin	93.5±1.3	54.3±0.4	18.8±3.4	99.2±0.5	98.2±0.4	92.9±3.9	82.2±3.3	20.5±1.3	3.5±0.7

Table 4.2. Frequency of clone types and neurospheres generated from sorted cortical cells.

	LeX ⁺			LeX ⁻		
	E10.5	E14	E18	E10.5	E14	E18
Stem clones	16.3±1.0	11.6±3.6	0.88±0.4	1.6±1.1	0	0
Single neurons	13.9±2.9	30.2±1.4	63.5±1.8	71.6±3.2	99.6±0.4	100
Neuroblasts	68.6±3.1	19.2±2.4	6.5±1.1	26.6±2.7	0.3±0.3	0
Glioblasts	1.2±0.5	39.9±2.5	27.0±1.6	0	0	0
Neurospheres	24.8±1.0	11.4±0.7	35.6±1.5	4.11±0.5	0.2±0.1	2.6±0.3

Figure 4.3



Stem cells continued to express LeX as they self-renewed. At early stages of clone development, LeX was expressed on progenitors and less on differentiated neurons (Figure 4.4 A). After 7-10 days in vitro, clones contained many flattened progenitor cells, some expressing the glioblast marker NG2, while others were LeX⁺, with only a few cells expressing both markers. To examine whether the late-born LeX⁺ cells were indeed stem cells, 10-day-old clones were removed from the culture plate using trypsin to give a single cell suspension, stained with CD15 and FACS sorted. 22% of the LeX⁺ cells derived from 10-day clones generated neurospheres, while only 2% of the LeX⁻ cells did so (6% if only non-neuronal LeX⁻ cells are considered), figure 4.5.

The vast majority of stem cells, assayed by adherent clones or neurosphere formation, are included in the LeX⁺ population throughout embryonic development, as are the more prolific neuroblasts (Table 4.2). The frequency of LeX-expressing cells declines dramatically during cortical development, mirroring the decline in stem cell frequency (Table 4.3). Early glioblasts produced during the late embryonic period are also LeX⁺. However, in the first month after birth during peak gliogenesis, while the vast majority of stem cells are LeX⁺, only a fraction, <8% of the glioblasts and few differentiated glia are LeX⁺. This suggests that, similar to neuroblasts, as glioblasts mature they lose LeX.

Table 4.3 The percentage of LeX⁺ cells in the cortex decreases with development.

Figure 4.4 LeX expression in developing clones of early (E10.5) cortical cells.

(A) A clone of 10 cells at 2 DIV contains actively proliferating LeX⁺ cells (red) and immature LeX⁻/β-tub⁺ neurons (green).

(B) At later stages in clone development (3-5 DIV), stem cell clones contain LeX⁻/β-tub⁺ neurons (blue), NG2⁺ glial precursors (green) and LeX⁺ cells (red).

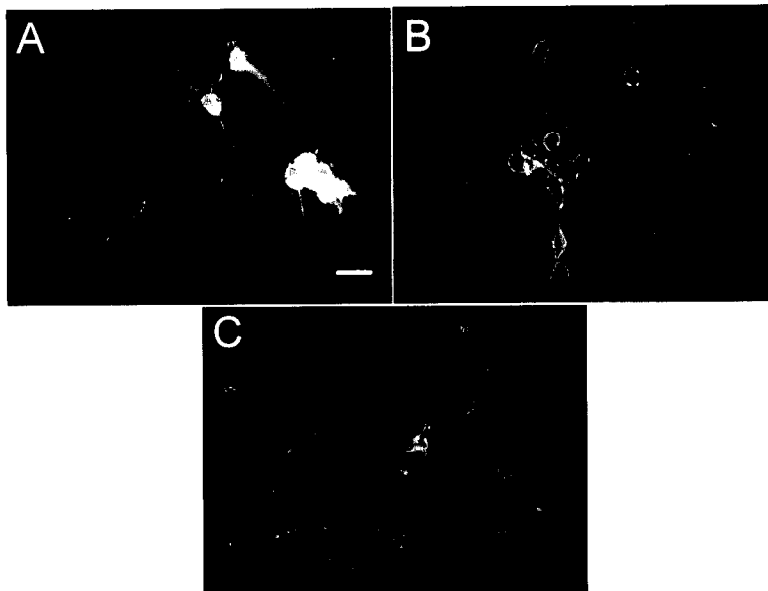
(C) At 10 DIV, LeX⁺ cells (red) are morphologically similar to NG2⁺ glial progenitors (green) but the two markers rarely overlap.

Size bar: (A, B and D) 50μm; (C) 25μm.

Table 4.3 Percentage of LeX⁺ cells in acutely dissociated mouse cortical cells at different developmental ages.

	E10.5	E14	E18	P0	P4	Adult
LeX	53.5±5.7	37.3±2.7	13.1±0.9	1.5±0.2	2.1±0.1	4.3±0.3

Figure 4.4



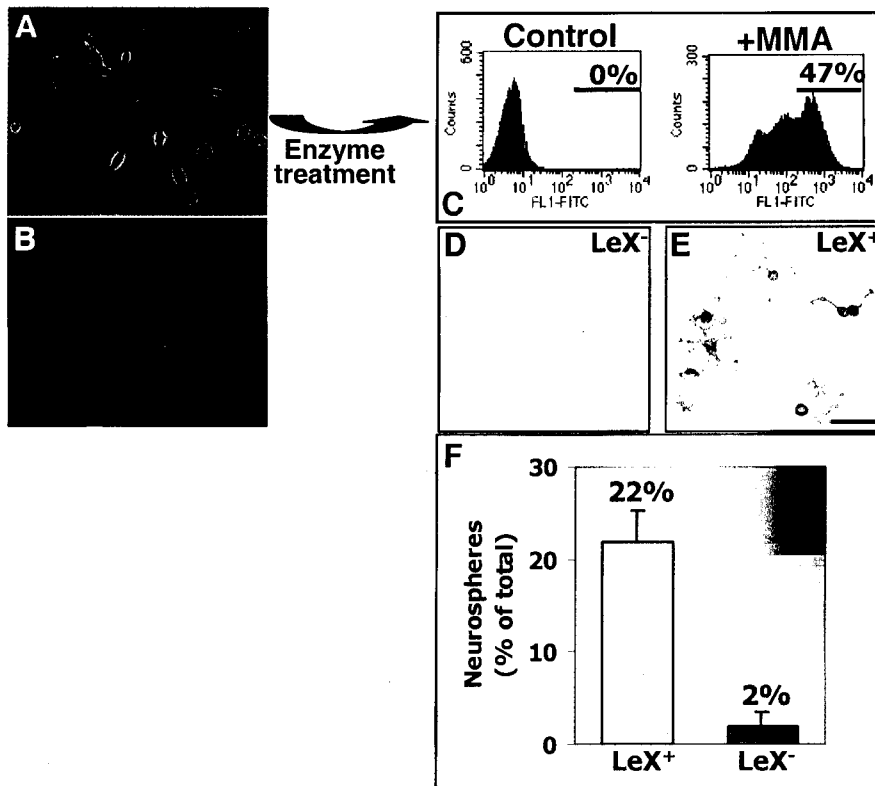


Figure 4.5 Stem cells in late cortical cultures are LeX⁺.

(A, B) E11 cortical cells growing as a monolayer for 10-11 DIV, phase (A) MMA staining (B).

(C) E11 clones illustrated in A,B were removed with trypsin, stained with MMA-FITC and sorted into LeX⁺ and LeX⁻ subpopulations.

(D) LeX⁻ and **(E)** LeX⁺ subpopulations were highly pure.

(F) The potential to form neurospheres segregates with the LeX marker. Results are expressed as mean±SEM.

Size bar: (A, B, C and D) 50µm.

Regions of high LeX expression express *Fgf8* and *Wnt-1*

We noted that the pattern of LeX staining in whole mounts was similar to that reported for *Fgf8* and *Wnt* genes, which are expressed (together or separately) in the forebrain midline, isthmus, pituitary, nasal epithelium and oral ectoderm and are involved in growth and patterning of these tissues (McMahon et al., 1992; Crossley and Martin, 1995; Neubuser et al., 1997). Hence we examined the relationship of LeX staining to the expression of these two secreted growth factors in more detail.

In the forebrain, LeX is prominent in the midline, as is *Fgf8* (Figure 4.6C) and *Wnt-1* (McMahon et al., 1992). In the mid-hindbrain isthmus, FGF-8 plays a crucial inductive role, along with *Wnt-1* (Crossley et al., 1996; Lee et al., 1997). *Pax2* is the earliest gene to be expressed in the isthmus and is critical for its development, along with the related *Pax5* (Rowitch and McMahon, 1995; Schwarz et al., 1997). Later, *Wnt-1* is expressed in the mesencephalic domain, anterior to the domain of *FGF8* expression. Interestingly, LeX spans both the *Wnt-1* and *FGF8* domains (Figure 4.6 K, L), similar to *Pax2* expression (Ye et al., 2001 and figure 4.6 M).

Besides these CNS observations, we noted an intriguing association of LeX expression in other embryonic areas where FGF8 induction occurs. In the embryo, the posterior (infundibular) pituitary releases FGF8 that with BMPs from the ventral juxtavituitary mesenchyme, coordinates the patterning of the anterior pituitary (Ericson et al., 1998). At E11.5, *Fgf8* mRNA is seen in posterior pituitary (Figure 4.6 H, Crossley and Martin, 1995), while, interestingly, FGF8 protein localizes exclusively to the anterior pituitary (Figure 4.6 C), as does LeX (Figure 4.6 H). Given that LeX is known to bind FGFs, this suggests that localized expression of LeX binds and concentrates FGF8 protein at the anterior pituitary target.

Fgf8 is expressed in mandibular and maxilar prominences (prospective dental ectoderm), providing an inductive signal for odontogenesis (Crossley and Martin, 1995) that is antagonized by BMP2 and BMP4 from the ectoderm and/or mesenchyme (Neubuser et al., 1997). We found that LeX is expressed in the same region of the dental ectoderm as *Fgf8* (Figure 4.6 E, F).

FGF8/BMP signaling is also involved in inducing the olfactory placode. At E10.5, *Fgf8* expression is localized in the surface ectoderm surrounding the nasal pits and later to the external ridge of the olfactory epithelium (Figure 4.6 D). LeX expression extends

throughout the olfactory epithelium, being exceptionally strong apically (Figure 4.6 D, insert).

Figure 4.6 NS regions strongly expressing LeX also express *Fgf8* and *Wnt-1* transcripts.

(A, B, C) Wholemound *in situ* hybridization for *Fgf8* mRNA (A, B) and

immunohistochemistry for FGF8 protein (C) reveal labeling in the same embryonic regions as LeX, except for the limb AER (compare with Figure 4.1).

Arrowheads indicate high *fgf8* expression (A) in the olfactory epithelium and mandible and (B) in the isthmus and commissural plate. (C) Expression of FGF8 protein in the isthmus (white arrowhead) and in the anterior pituitary (black arrowhead).

(D) *Fgf8* (red) is expressed in the olfactory epithelium but confined to the external ridge. LeX (green in all panels) is expressed throughout the epithelium and is especially abundant apically at the lumen border (insert).

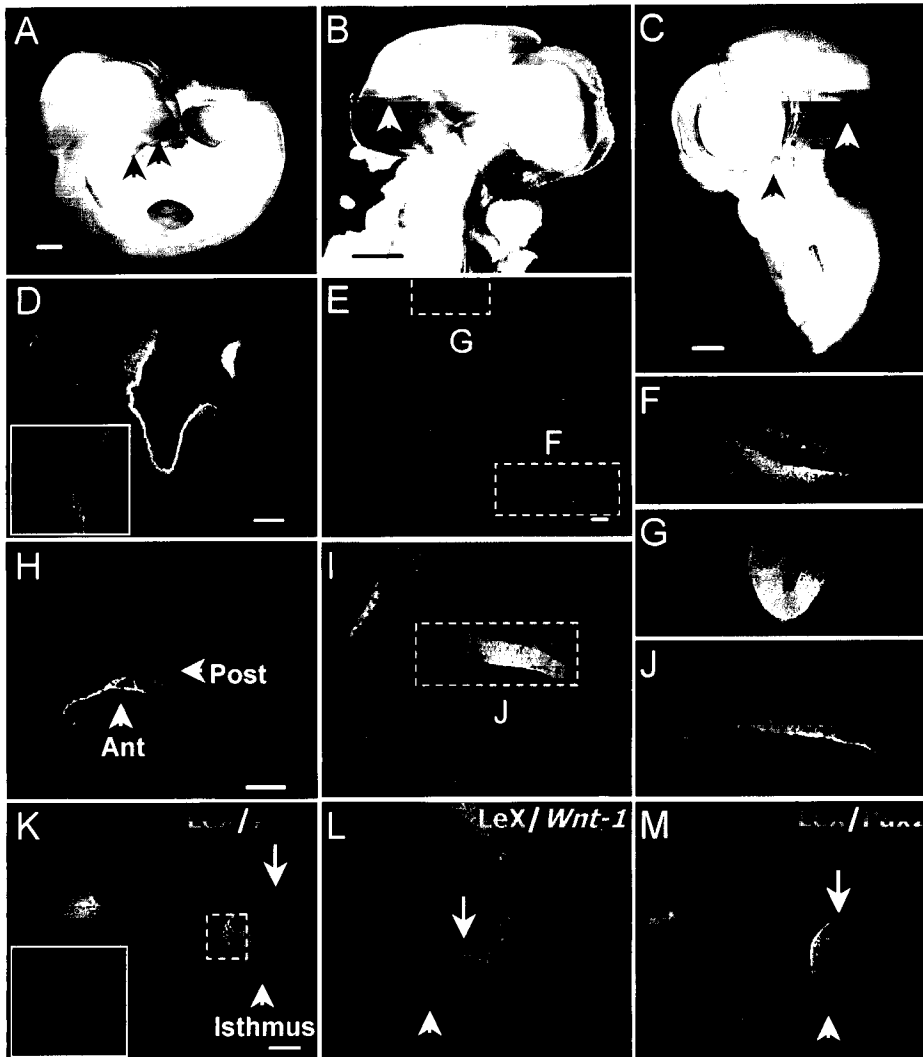
(E, F, G, I, J) *Fgf8* and LeX are expressed in the prospective dental ectoderm (E, high magnification in F) and in the forebrain midline (E and I, high magnification in G and J).

(H) In the pituitary primordium, LeX is expressed in the anterior pituitary (Ant) while *Fgf8* is expressed in the posterior pituitary (Post).

(K-M) *Fgf8* expression in the isthmus partially overlaps with that of LeX. LeX expression extends rostrally (arrow). Insert: high magnification of boxed area, highlighting the strong apical LeX expression. (L) The domain of LeX expression rostral to *Fgf8* (arrow) overlaps with *Wnt-1* (red). (M) Pax-2 (red) encompasses *Fgf8* and *Wnt-1* expression, and is similar to LeX.

Size bar: (A-C) 500 μ m; (D-M) 100 μ m.

Figure 4.6



LeX-containing carbohydrates in the embryonic CNS bind Wnt.

LeX is known to bind FGFs (Dvorak et al., 1998; Jirmanova et al., 1999), which may explain its association with FGF8. However a relationship between LeX containing molecules and Wnts has not been previously described. The possibility that LeX may bind Wnts is particularly intriguing given the presence of LeX in proteoglycans (Tole et al., 1995; Allendoerfer et al., 1995, 1999, our data) and the fact that *Drosophila* Wg is dependent on proteoglycans for function (Binari et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001; Greco et al., 2001). We therefore examined whether an *in vivo* association between LeX containing molecules and Wnt-1 might exist.

Embryonic CNS extracts were generated and LeX-associated proteins were immunoprecipitated using the anti-CD15 MMA antibody. The proteins co-immunoprecipitated with anti-CD15 contained LeX⁺ molecules of the expected size (Figure 4.7 A, lanes 2-5). They also included Wnt-1, as shown by immunoblotting with anti-Wnt-1 antibodies (Figure 4.7 B, lanes 9-11).

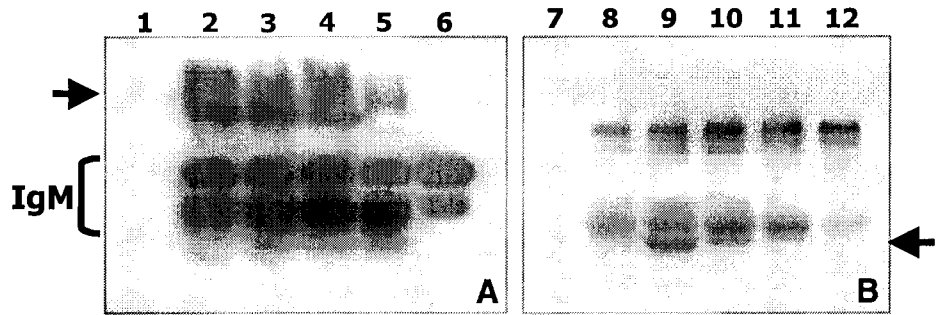
Figure 4.7 Co-Immunoprecipitation of Wnt-1 using the MMA antibody.

Total brain protein was isolated from E15-E16 mouse embryos and immunoprecipitated with anti-MMA antibody or with mCD24 (an IgM expressed in brain but not in LeX⁺ cells) as negative control. The two blots, shown in Panels A and B are duplicates. Panel A: probed with MMA to confirm precipitation of LeX⁺ material. Panel B: probed with Wnt-1 antibody to test for co-immunoprecipitated proteins.

(Lanes **1, 8**): Negative control - immunoprecipitation using CD24. No LeX⁺ (lane 1) nor Wnt-1⁺ (lane 8) proteins. Lane 8 shows nonspecific binding of the Wnt-1 antibody, seen also in panel B. Lanes **9, 10** and **11** show Wnt-1 immunoreactive material of the expected size (arrow), and decreasing in intensity with protein loading, becoming undetectable at 2mg loaded protein, (lane **12**).

Protein loading: **Lanes 2, 9**: 15mg; **Lanes 3, 10**: 10mg; **Lanes 4, 11**: 5mg; **Lanes 5, 12**: 2mg. Note LeX⁺ material (arrow) and heavy and light chains of the IgM in panel A (0.5µg of MMA antibody were run in lane **6**, confirming the identity of the IgM-derived bands).

Figure 4.7



Discussion

Extraordinary growth of certain regions of the embryonic nervous system is essential for generating its final form and function. The mechanisms that allow localized expansion of neural tissue are not yet clear. Here we show that LeX is abundant in high growth areas of the NS, and that neural progenitor cells expressing this molecule are more prolific than those that do not.

LeX reveals heterogeneity of early CNS progenitor populations

It is still not clear whether the earliest neural progenitor cells are a homogeneous population, as first suggested by Sauer, 1935, or are intrinsically diverse. Only a minor population of early neural cells generate neurospheres or stem cell clones in vitro (Tropepe et al., 1999; Davis and Temple, 1994). This suggests intrinsic heterogeneity, but may also be explained by environmental differences cells encounter in vitro. A more direct demonstration of heterogeneity would be to define molecular distinctions between progenitor cells that predict their subsequent behavior. Known progenitor markers label most early neuroepithelial cells uniformly: for example, Nestin labels 98% of E10 cortical cells and RC2 90%. Here we show that at this stage only half the cells have detectable LeX, and these generate the largest clones. Hence LeX expression defines two sub-populations of early CNS progenitor cells that exhibit intrinsic differences in growth potential.

Selection of highly prolific cells using LeX

LeX expression allows enrichment of neural stem cells from diverse embryonic and adult stages. Embryonic neural stem cells have also been selected from transgenic mice expressing green fluorescent protein (GFP) under a nestin enhancer, which selects all detectable neurosphere-generating cells. However only 3.4% of the selected cells are multipotent stem cells, probably reflecting the ubiquity of Nestin expression amongst progenitor populations (Kawaguchi et al., 2001). LeX selection is a better means of stem cell enrichment, but it is still not an exclusive marker of stem cells: only 16% of FACS sorted LeX⁺ E10 cortical cells produce stem cell clones. LeX also labels the highly prolific progeny of stem cells - the transit amplifying neuro- and glioblasts, while its expression

declines as these progenitors progress towards differentiation. Because more prolific progenitors appear to have stronger LeX staining, neural stem cells might be further purified by fluorescence intensity. Use of two different antigens, as with human embryonic CNS and mouse neural crest stem cells (Uchida et al., 2000, Morrison et al., 1999), might also aid their purification.

LeX⁺ neural stem cells self-renew and maintain LeX expression.

We show that LeX is expressed on embryonic neural stem cells throughout development. The fact that LeX is also seen on the rare, adult neural stem cells indicates that its expression is maintained throughout life. Further evidence that stem cells maintain LeX expression as they self-renew comes from clonal studies in which the development of single progenitors is followed over time. We have shown previously that single stem cells isolated from the embryonic brain produce first neurons, then glia, mirroring the timing of production of these cells *in vivo*. Later stage stem cell clones, in their gliogenic phase, contain cells that can generate neurospheres after sub-cloning (Qian et al., 2000). One interpretation of this observation is that CNS stem cells self-renew as the lineage develops, and are present in small numbers at late stages. Another interpretation is that late stage clones contain a population of glioblasts that can revert to a stem cell state, as shown for the O-2A precursor (Kondo and Raff, 2000). In the present study, we show that late stage stem cell clones contain LeX⁻NG2⁺ glioblasts and a separate population of LeX⁺NG2⁻ stem cells. These data support the model that stem cells self-renew throughout the lineage, retaining LeX expression, and that neurosphere generating cells found late in clones are stem cells rather than reverted glioblasts.

Radial glia as stem cells

Previous studies revealed LeX on radial glial cells in the embryonic forebrain (Mai et al., 1998). In this study we confirm that finding, and extend it by showing that LeX labels radial cells in many different areas of the CNS, and in the nasal epithelium.

Radial glia are now known to give rise to neurons (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001). There have been suggestions that some radial glia are stem cells. FACS studies indicate that some may be common progenitors for neurons

and glia: 4-5% of radial glia at E14-E16 can generate mixed clones of neurons and non-neuronal cells (Malatesta et al., 2000). Constitutive Notch activation promotes radial glial development, and enhances neurosphere production, (Gaiano et al., 2000a,b). Radial glia generate astrocytes, and in the adult sub-ventricular zone astrocytes are stem cells, suggesting a lineal relationship. In songbirds, adult radial glia are believed to be neurogenic stem cells (Alvarez-Buylla et al., 1990).

However, there have been no previous direct demonstrations that normal, embryonic radial glia are self-renewing stem cells. Here we show that LeX⁺ cells in the forebrain have the morphological characteristics of radial glia. Selection of LeX⁺ cells by FACS allowed prospective isolation of normal radial glial cells, and the LeX⁺ cells encompassed virtually all the self-renewing stem cells detectable in the embryonic cortex. Hence we conclude that some radial glia are stem cells, although we acknowledge that non-radial LeX⁺ stem cells may also be present in the neuroepithelium. Astrocytes arise almost exclusively from the LeX⁺ population, reinforcing the link between the astrocytic lineage and stem cells.

Previous studies have shown that radial glial in the cortex express Pax6 (Gotz et al., 1998) which is important for their normal morphology, proliferation and neurogenesis (Heins et al., 2002). Interestingly, LeX expression is reduced in the pax6 mutant due to downregulation of the LeX-synthesizing enzyme FucT-IX (Shimoda et al., 2002). Hence, one aspect of Pax-6 function may be regulation of LeX expression.

The radial morphology of LeX⁺ cells is prominent in other CNS areas such as the diencephalon and the isthmus, which contain stem cells (data not shown) and in the nasal epithelium, which contains stem cells that generate new neurons throughout life. In the embryo, the proliferative layer of the nasal epithelium is apical and it moves basally during late embryogenesis, but the nasal stem cell identity is unknown. Our finding that LeX labels a radial cell population suggests that it would be worthwhile to examine whether these may be the embryonic olfactory stem cell. In the adult, the basal layer is believed to contain the stem cells, with candidates being horizontal cells and globose cells (MacKay-Sim and Kittel, 1991; Caggiano et al., 1994) and LeX expression may help elucidate their identity.

LeX and FGF signaling

LeX is present on cell surfaces and in the ECM. Some proteoglycan components of ECM, notably HSPGs, can bind growth factors, modulating their concentration and availability to surrounding cells (Schlessinger et al., 1995). Free LeX promotes stimulates FGF2 mitogenicity for ES cells at low concentrations while inhibiting it at high concentrations (Jirmanova et al., 1999). LeX was particularly prominent on the apical surface of the neuroectoderm, where FGF2, a known neural stem and progenitor mitogen, has been localized (Raballo et al., 2000). LeX may bind FGF2 on this surface of the neuroepithelium, where M-phase occurs, thus regulating exposure to mitogen at a critical point in the cell cycle.

It is highly likely that similar regulation can occur with other members of the FGF family, including FGF8, which is structurally related to FGF2. Unlike FGF2, FGF8 does not appear to have a strong mitogenic function. Instead, it has a potent inductive role, in which it is able to activate particular development programs in responsive tissue e.g. application of FGF8 to mesencephalon can duplicate this region (Crossley et al., 1996). The discrete location of FGF8 in the developing nervous system reflects this role and the co-incidence of abundant LeX expression in these regions strongly suggests a close functional relationship. For example, LeX may bind and target FGF8. This possibility is illustrated in the developing pituitary, where FGF8 mRNA is expressed in the anterior lobe, while FGF8 protein is localized to the posterior lobe where LeX is expressed.

LeX and Wnt signaling

Immunoprecipitation of LeX-containing molecules from the embryonic NC brings down Wnt-1, indicating an association *in vivo*. In *Drosophila*, binding to ECM and cell surfaces via association with HSPGs is critical for Wg function (Reichsman et al., 1996; Schryver et al., 1996). Flies deficient in HSPG synthesis have phenotypes similar to that of *wg*. Injection of heparinases into *Drosophila* wild-type embryos can mimic the *wg* phenotype while injection of heparan sulfate leads to a phenotype similar to Wg over-expression (Binari et al., 1997). By analogy, it is possible that LeX-containing ECM components play a regulatory role in Wnt function in vertebrate neuroepithelia. We noted that LeX is concentrated at the apical border of the dorsal forebrain neuroepithelium, where Wnt 1 is expressed. Overexpression of beta-catenin, a down-stream factor in the Wnt signaling

pathway, causes dramatic expansion of early cortical progenitors (Chenn and Walsh, 2002).

Interestingly, in *Drosophila*, graded Wg distribution in the wing imaginal disc epithelium may arise by its distribution in argosomes, vesicles that include a portion of ECM and associated Wg (Greco et al., 2001). LeX was often found in villi-like structures at the apical surface of neuroepithelia and was present in vesicles inside neuroepithelial cells, with the highest concentration near the apical surface, similar to the graded distribution of Wg in argosomes in the fly. It is therefore possible that vesicles containing LeX-associated proteoglycans with associated growth factors such as Wnt-1 may aid in distribution of these factors within vertebrate neuroepithelia.

LeX in the isthmus

Given the extensive understanding of MHB development, we might best elucidate LeX function there. The isthmus organizer arises at the MHB, the interface between mesencephalic *Otx2* and metencephalic *Gbx2* expression domains, (Millet et al., 1996, 1999; Wassarman et al., 1997; Broccoli et al., 1999). FGF8, Wnt1, Pax2, Pax5, En1 and En2 are important components of the organizer activity (reviewed in Rhinn and Brand, 2001). *Pax2* is the first gene to be expressed in the presumptive MHB (Rowitch and McMahon, 1995). While *Pax2* null mutants have no obvious MHB alteration, double *Pax2/Pax5* mutants lack the isthmus organizer (Schwarz et al., 1997).

Formation of the isthmus involves genetic feedback programs that refine the localization of expression of crucial genes. *FGF8* and *Wnt-1* are initially expressed in a broad region in the MHB but their expression becomes restricted to sharp concentric rings immediately next to each other, *Wnt-1* being rostral to *FGF8*. Repression of *FGF8* by *Otx2* and repression of *Wnt-1* by *Gbx2* refine the *FGF8* and *Wnt-1* border (Li and Joyner, 2001), which in turn might restrict expression of *Pax2* and *En1* (Danelian and MacMahon, 1996).

Interestingly, LeX expression is initially broad and later becomes a sharp ring covering the *Wnt-1* and *FGF8* expressing domains, mirroring refinement of other isthmus genes. Its expression domain is similar to that of *Pax2* and En-1. Perhaps LeX expression is promoted by Pax2 (as it is by Pax6 in cortex) and helps stabilize *Wnt-1* and

FGF8 at the border, aiding feedback programs. It would be interesting to examine LeX expression in *Pax2* and/or *Pax2/Pax5* double mutant mice.

Concluding remarks

In this study, we provide evidence that the trisaccharide LeX is present on and around embryonic neural stem cells, providing continuity of expression from pluripotent stem cells in the early embryo through to adult neural stem cells (Capela and Temple, 2002) and adult bone marrow stem cells (Jiang et al., 2002). These observations indicate a critical role for this unique carbohydrate in stem cell biology. Our finding that in the mouse embryo, LeX expression parallels that of *FGF8* and *wnt-1* suggest that LeX might participate in the inductive functions of these molecules, which operate even before gastrulation (Wilson and Edlund, 2001). Analysis of mice deficient in LeX synthesis will be important to help clarify its role in stem cell function.

Acknowledgements

We are most grateful to Ivor Mason for *FGF8b* and Andy MacMahon for *Wnt-1* plasmids and to Joel Levine for NG2 antibody. Our sincere thanks to Robert Dilwith for his invaluable help with cell sorting and to Natasha Abramova for help with *in situs*. AC acknowledges the financial support of Fundação para a Ciência e Tecnologia and Instituto Gulbenkian de Ciência, Portugal. This work was supported by NIH grant R01 NS33529.

Materials and Methods

Cell dissociation

Single cell suspensions were obtained from the cortices of Swiss Webster mouse embryos as previously described (Qian et al., 2000). Briefly, dissected cortical tissue was incubated for 30 min at room temperature (RT) in 5ml 37°C DMEM containing 7-10U/ml papain (Worthington), 1mM glutamine, 1mM sodium pyruvate and 60µg of DNase (Sigma), on a rocking platform. Papain inhibitor (Worthington) was added at the end of the incubation, and the tissue was triturated using a 10ml sterile plastic pipette to generate a single cell suspension. After three washes with DMEM, the cells were resuspended in hibernation medium and either immediately plated or sorted.

Cell sorting

Single cell suspensions derived from fresh or cultured embryonic cortex were labeled with the anti-CD15 MMA antibody, which recognizes LeX, (Becton Dickinson or American Type Culture Collection), washed, resuspended in calcium and magnesium-free PBS (CMF-PBS) and sorted using a Becton Dickinson FACS Vantage. Gating parameters for sorting were set by side and forward scatter to eliminate debris, dead and aggregated cells. A second gating parameter was set by green fluorescence (530nm) to separate positive from negative cells. The flow rate was ~1000 events/sec for high purity and recovery. Sorted cell sub-populations were washed three times in DMEM and centrifuged at 300xg for 10 minutes. The final cell pellet was resuspended in hibernation medium.

Cell culture

Clonal analysis: unsorted (total) and sorted populations were plated at clonal density (<10 cells/well) in Terasaki plates (Nunc) in basal culture medium: DMEM containing glutamine, sodium pyruvate, B-27, N2, (all from Life Technologies) 1mM N-acetyl-cystein (NAC, Sigma), supplemented with 10ng/ml FGF2 (Life Technologies) and maintained at 35°C, 6% CO₂ in a humidified incubator. Maps depicting the position of single cells were made two hours after plating on day zero and updated daily for 7 to 10 days. The clones were then fixed and stained with cell type specific antibodies.

Neurosphere generation: cells were plated at clonal density in uncoated wells in basal medium supplemented with 20ng/ml EGF plus 20ng/ml FGF2. The number of neurospheres generated was scored at day 7.

FACS separation of late-generated LeX positive and negative cells: E11 cortical cells were plated in 15 ml of basal medium plus 10ng/ml FGF2 in PLL-coated T75 culture flasks (Costar) at 2000 cells/ml. The cultures were re-fed every three days. After 10-11 days *in vitro* (DIV), the medium was removed, the cells were rinsed three times with CMF-PBS, and incubated for 5 minutes at 37°C with 0.25% trypsin-EDTA (Life Technologies). Trypsinization was stopped with DMEM plus 10% heat-inactivated fetal bovine serum. After three washes in DMEM, the cell suspension was stained with the anti-CD15 antibody and sorted. LeX⁺ and LeX⁻ cells were plated at clonal density for neurosphere generation.

Sub-cloning of neurosphere cells: neurospheres derived from single LeX⁺ cells were collected under the microscope and incubated for 30 minutes at 37°C in papain. After several washes, a single cell suspension was generated by trituration with a fire polished Pasteur pipette. Cells were re-plated at clonal density in the same conditions as the parent cultures. Neurosphere generation was assessed 7 days later.

Immunostaining

Acute staining: cells were plated for 2 hours on PLL-coated Terasaki plates, incubated with anti-CD15 (1:20 dilution) for 30 minutes at RT, fixed with ice-cold 4% paraformaldehyde (PFA, Tousimis Research) for 30 minutes and then incubated with biotinylated antibodies and the ABC Elite kit and VIP (Vector). For intracellular antigens, cells were permeabilized for 5 minutes with 100% methanol at -20°C, blocked, then incubated with the primary antibodies: β -tubulin III: (1:400, Sigma) overnight at 4°C; RC2 (1:4, hybridoma supernatant, Developmental Hybridoma Bank) overnight at 4°C; NG2 (1:400, rabbit polyclonal, gift of Dr Joel Levine), 1hour at RT; GFAP (1:400, Dako), 1 hour at RT. Staining was visualized with Alexa 488 (1:200, Molecular Probes) and Cy3 (1:200, Jackson Research Laboratories) conjugated secondary antibodies for 45 minutes at RT.

Staining of monolayer cells: the same basic procedure was followed as for acute staining. For triple staining of CD15, β -tubulin III and NG2, streptavidin-AMCA (Jackson Research Laboratories) was used to reveal the β -tubulin III staining.

Whole-mount staining: the protocol was adapted from that described by Doetsch et al., 1999b for wholemount staining of the lateral ventricular walls of adult mice. Briefly, mouse embryos were collected, cleaned of membranes and incubated for 4-6 hours at 4°C in ice-cold 4% PFA. The embryos were then rinsed 3 times in saline, permeabilized with 100% methanol and 100% acetone for 30 minutes at -20°C and washed in Dulbecco's phosphate buffered saline containing 0.3% Triton X-100 (DPBST). The embryos were cut in half lengthwise or in coronal sections with microscissors and microknives. The tissues were blocked for 2 hours in DPBST plus 10% normal goat serum (NGS) or horse serum and incubated with anti-CD15 (1:200), anti-SSEA-1 (1:400, ascites fluid, Developmental Hybridoma Bank) or anti-FGF8b antibodies (1:100, R&D Systems) for 48h at 4°C with gentle rocking. After extensive washing, staining was revealed with peroxidase-conjugated goat anti-mouse IgM or donkey anti-goat IgG (1:100, Jackson Immunoresearch Laboratories), using DAB substrate. Stained tissues were photographed using an Optronics digital camera mounted on a Leica stereoscope.

Staining of cryostat sections: mouse embryos were fixed for 4-12h at 4°C in ice cold 4% PFA, cryoprotected in 30% sucrose and embedded in OCT (Sakura). Fourteen μ m sections were cut and mounted. Sections were rinsed in DPBST (containing 0.1% Triton X-100), blocked for 2 hours in DPBST plus 10% NGS, and incubated with anti-CD15 (1:200, overnight at RT), RC2 supernatant (1:4, overnight at 4°C) and Pax2 (1:200, overnight at 4°C, Zymed Laboratories). Staining was revealed as for acute staining with streptavidin-Alexa 546 or Alexa 488 to detect the LeX epitope and appropriate Alexa conjugated anti-mouse and anti-rabbit secondaries for RC2 and Pax2, respectively.

In situ hybridization/ immunohistochemistry

In situ hybridization in frozen sections was carried out as described by Henrique et al., 1997. Briefly, 14 μ m E11.5 mouse embryo sections were generated as previously described using RNase-free reagents and instruments. FGF8b and Wnt-1 plasmids (gifts from Dr Ivor Mason and Dr Andy McMahon, respectively) were linearized and transcribed using a DIG labeling kit (Roche Molecular Biochemicals). The slides were

incubated overnight at 65°C with 0.5µg/ml DIG probe in hybridization buffer. The hybridization signal was revealed using anti-DIG antibodies conjugated to alkaline phosphatase (1:2000, 4 hours at RT) and fast red substrate (Roche Molecular Biochemicals). After several washes in DPBST, the slides were blocked and stained with anti-CD15 antibody as described above, using streptavidin-Alexa 488 as fluorochrome.

Co-immunoprecipitation

Total brain extracts were obtained from E15-E16 mouse embryos: brains were dissected and placed on co-immunoprecipitation buffer (co-IPB, 10mM Tris-HCl, pH 7.4, 50mM NaCl, 1% IGEPAL, 10% glycerol), containing Complete Mini protease inhibitor cocktail (Roche Molecular Biochemicals). The tissue was homogenized using a dounce homogenizer, incubated on ice for 30 minutes and further homogenized to ensure optimal extraction. The lysate was centrifuged at 14,000xg for 10 min at 4°C and the supernatant was cleared by sequential incubations with mouse IgG and agarose beads coupled to goat anti-mouse IgM antibodies (Sigma). The cleared extracts were incubated 6 hours at 4°C with 1µg of anti-CD15, or negative control IgM, anti-CD24 (Pharmingen), followed by a 2 hour incubation with agarose anti-IgM beads at 4°C. The beads were washed 3 times with cold co-IPB and resuspended in SDS loading buffer, boiled for 5 min, centrifuged and the supernatant run on a 15% polyacrylamide gel. The proteins on the gel were transferred into PVDF membranes (Immobilon P, Millipore) that were then blocked overnight at 4°C with 5% non-fat dry milk in Tris buffered saline. The membranes were incubated with anti-CD15 antibody (1:200, 5h at RT) to confirm immunoprecipitation of LeX-containing molecules and with 4µg/ml anti-Wnt-1 monoclonal antibody (Upstate Biotechnologies) and 0.5µg/ml goat anti-FGF8b to probe for co-immunoprecipitated material. After 6 washes of 5 minutes each, the membranes were incubated with peroxidase-conjugated secondary antibodies (1:4,000, Jackson Immunoresearch Laboratories) and the bands detected by luminescence using the ECL reagent (Amersham Pharmacia).

Chapter Five

DISCUSSION

The realization that stem cells can reside in the adult brain has stimulated a whole wealth of research mostly fueled by the need to develop alternative therapies for neurodegenerative diseases like Parkinson's and Huntington's and for brain injury caused by stroke (Bjorklund and Lindvall, 2000). The added confirmation that neurogenesis can occur in adult animals from fish to humans contributed to the neural stem cell research "boom". Suddenly, scientists realized that the cure for brain disease may be within ourselves. In addition to the transplantation of new stem-cell derived cells, mobilization of endogenous neural stem cells is another possible strategy for treatment of CNS cell loss. Stem cells may be particularly useful for patients in which multiple cell types are lost, such as after trauma or some neurodegenerative diseases. But before this possibility can be explored with rigor, one needs to learn much more about the biology of neural stem cells.

In contrast to the skin, intestinal epithelia and the blood system, which continuously generate new cells to replace those lost in the "line of normal duty", the brain demonstrates limited capacity for self-repair. Despite the continued generation of neurons in the hippocampal dentate gyrus and olfactory bulb, adult neurogenesis does not appear to make a significant contribution in case of injury. But why not? It is as if we were given the most amazing gift but we do not know how to use it - the instructions did not come with it. Are stem cells in the germinal regions incapable of responding to injury? Is it because neural stem cells are unusually sensitive to injury themselves? Or is it that injury irremediably changes the environment in a way that is absolutely non-permissive for the neurogenic program to occur? Do non-neurogenic regions express appropriate/sufficient signals to attract stem cells? Or are neural stem cells somehow deaf to such signals?

It is out of the scope of this thesis to answer these questions and much progress is being made to address them as I write these words. Instead, my contribution to the field was to find a surface marker that allows the purification and *in vivo* localization of neural stem cells. The ability to purify or enrich a population in stem cells from fresh

tissue will bring enormous benefits to the study of the biology of these rare cells, providing invaluable clues to their use in transplantation and/or endogenous mobilization.

I found that embryonic and adult neural stem cells express the LewisX epitope in their surface. Antibodies against this sugar antigen are commercially available making it possible to other scientists to use this methodology to purify neural stem cells.

From neural-glia production to neural stem cell markers

This discovery came about as a by-product of my principal interest which was to understand how the cortical neural stem cell makes the two major cell types in the CNS: neurons and glia. This is described in chapter 2. The progress of my research was impeded by the fact that even in the early embryo, the cortex is composed of different types of progenitor cells and only a small percentage of those are stem cells. Given that stem cells were my target population, I had to devise a way to discard other progenitors from my analysis and keep only or mainly neural stem cells. The enrichment of a subpopulation in stem cells could have been approached in two ways: either by collecting cells that do not express any differentiation markers (hematopoietic stem cells are usually purified using this method) or by selecting stem cells based on their expression of a specific marker. Both alternatives were equally difficult because the majority of the early cortical cells express the same markers even though individual cells performed differently in culture.

The LewisX epitope was one of the few surface markers that consistently labeled a specific cell subpopulation in the mouse cortex from embryo to neonate. Importantly, the percentage of LewisX positive cells decreased with age, and was maintained at very low levels in adult tissues bearing stem cells like the SVZ, as expected of a stem cell marker.

I then used fluorescence-activated cell sorting or FACS in order to purify LeX⁺ cells from the embryonic and neonate cortex (chapter 4) and also from the adult SVZ (chapter 3). When subjected to the *in vitro* tests used to diagnose the presence of stem cells, I showed that only the LeX⁺ population contained stem cells demonstrating multilineage differentiation and self-renewal *in vitro*.

Neural stem cells are LeX⁺

But not all LeX⁺ cells are stem cells. Clonal studies and immunocytochemistry demonstrate that the LeX antigen is also present on direct lineage descendants of stem cells. Interestingly, the percentage of adult SVZ LeX⁺ cells exhibiting stem cell characteristics *in vitro* is dependent on the initial plating density and increase from 2% to around 25% when 1-5 LeX⁺ cells are plated per well instead of 1000. This phenomenon is observed in cultures of skin stem cells and is likely to reflect control mechanisms that constrain the proliferation of stem cells *in vivo*. My work has advantages if compared with another study performed by Rietze et al., 2001 in which a different combination of markers and cell size are used to purify SVZ stem cells. Using LeX, we are able not only to demonstrate *in vivo* localization of stem cells (which Rietze and colleagues can not) but we recover all stem cells in the LeX⁺ subpopulation. Although the method used by Rietze and colleagues produces a highly pure sample of stem cells, they found they had not collected all the stem cells. Around one third of stem cells were in their discarded fraction. Given this, this method may only select a subpopulation of stem cells. Finally, my study helps end the controversy over the identity of the stem cell in the adult SVZ by demonstrating that 1) ependymal cells are LeX⁺, 2) do not generate neurospheres when purified by micromanipulation or by sorting using antibodies against CD24, an ependymal cell marker.

In embryonic and neonatal cortex, the use of LeX to purify stem cells presents also advantages when compared with other studies. For instance, Kawaguchi and colleagues, 2000, use a transgenic mouse expressing GFP under the control of a nestin enhancer to purify highly fluorescent cells with stem-like properties *in vitro*. The frequency of neurosphere generation obtained using this method is much lower when compared with that obtained with LeX. Moreover, LeX⁺ cells can be isolated from wild type mice, bypassing the need for the production of transgenic mice.

In recent years, the concept of radial glia as neurogenic progenitor cells in the embryonic CNS has been well established (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001). Interestingly, accumulating evidence also points to an association between radial glia and stem cells. First, Notch activation in the embryonic cortex, which can maintain progenitor cells in an undifferentiated state, promotes radial glial development and enhances neurosphere production, consistent with radial glia being

stem-like cells (Gaiano et al., 2000a,b). Second, radial glia generate astrocytes, and astrocytes are stem cells in the adult SVZ, suggesting a lineal relationship. Third and perhaps the most compelling evidence is that in songbirds, adult radial glia are neurogenic stem cells (Alvarez-Buylla et al., 1990). However, there have been no direct demonstrations as yet that normal, embryonic radial glia have the self-renewing properties of stem cells. In chapter 4 I show that some LeX⁺ cells in the forebrain have the morphological characteristics of radial glia, as previously reported (Mai et al., 1998), express radial glia markers and behave as self-renewing, multilineage stem cells. Hence it is likely that some embryonic radial glia are stem cells, although non-radial LeX⁺ stem cells are also present.

Time constraints did not allow me to address the *in vivo* potential of purified LeX⁺ cells from fresh tissue but these experiments were initiated and are underway. My prediction is that upon transplantation, LeX⁺ cells will engraft aggressively and contribute to the generation of appropriate repertoires of cells at the transplantation sites. Studies by Tamaki, Uchida and colleagues support my optimism. Purified freshly isolated human embryonic neural stem cells injected into neonatal mice engraft potently and generate neurons with high morphological complexity in many different locations (Tamaki et al., 2002).

The biological significance of LeX

The fact the LeX is expressed in neural stem cells from embryo to adult suggests important roles for this small carbohydrate in the biology of these cells. LeX immunoreactive material can be found in the extracellular matrix and has been found to shed off our cells. Previous studies indicate that LeX is involved in compaction of the early mouse embryo and we speculate that it also influences CNS stem cell adhesion.

Carbohydrate ectodomains like LeX bind the growth factor FGF2, and can regulate its mitogenicity. The co-localization of LeX and FGF8 in areas of high proliferative (and organizational capacity such as the isthmus) in the embryonic CNS suggests a similar mechanism. Likewise, Wnt-1, another possible mitogen for CNS stem cells, interact with LeX containing molecules (chapter 4). Differential density of LeX in the extracellular environment and at the cell surface might thus regulate growth factor

access to and influence on CNS cells. The high density of LeX coating on CNS stem cells and in the environment might provide a mechanism for maintaining them in an undifferentiated and proliferative state. In this context, it is possible that the progressively weaker LeX expression in the stem cells' immediate progenitors might reflect a growth factor "weaning" process that stimulates differentiation.

The importance of markers...in plasticity and endogenous function

Recent studies indicate that cultured mouse and human neural stem cells (mostly if not all derived from adult tissue) have an unexpectedly plasticity: they can generate many non-neuronal tissues. For instance, *ex-vivo* expanded neural stem cells (neurospheres) derived from adult mouse SVZ can generate blood cells when injected into an irradiated mouse (Bjornson et al., 1999) and skeletal muscle when transplanted into regenerating muscle sites (Galli et al., 2000). Moreover, "chopped up" neurospheres can generate derivatives of the three germ layers upon injection into mouse and chick blastocysts (Clarke et al., 2000), although the chimeras generated are not true mosaics. Instead, donor cells are found in discrete patches and are curiously absent from the blood lineage. Such experiments might now be viewed with some skepticism given the recent papers describing a novel type of cell fusion can occur between implanted cells and blastocyst cells (Terada et al., 2002; Ying et al., 2002). If this fusion can occur, then it is difficult to be sure that the results of such embryonic transplantation experiments are truly a reflection of the capacity of the implanted cells, rather than some strange cell hybrid generated through cell fusion.

A further criticism of most of the studies on stem cell plasticity to date is the fact that expanded rather than freshly isolated stem cells were used. Again, it is impossible to determine if such plasticity is only the result of long-term culture of stem cells in the presence of massive amounts of growth factors or if it does occur *in vivo*. Moreover, although neurospheres allow expansion of the rare neural stem cell, they do contain restricted progenitors and ultimately, one has to question if lineage jumping is a stem cell feature at all, although conceptually, it is more easily acceptable that a stem cell does so than an a neuron suddenly making a muscle cell or a myeloid cell.

Very recently, the neuron-to-blood phenomenon was re-addressed (Morshead et al., 2002) and the results were contrary to previous ones (Bjornson et al., 1999); the authors suggest that such re-programming events are extremely rare and perhaps the consequence of genetic or epigenetic alterations due to extended culture and thus not useful for therapeutic purposes.

For this amazing plasticity to be considered more than a culture phenomenon, and to be definitely attributed to neural stem cells, one has to repeat these studies using freshly isolated neural stem cells, and to use other assays of plasticity that avoid the cell fusion phenomenon mentioned earlier. If it emerges that neural stem cells are indeed plastic, and that this is shared with other somatic stem cell types, then this implies that organ specific stem cells are more close to their ancestors, the embryonic stem (ES) cells than previously thought. Perhaps somatic stem cells are specified for a particular tissue, but may be reprogrammed when placed in a novel environment. Alternatively, perhaps somatic stem cells are really ES cells that are dispersed by the morphogenetic movements of gastrulation into different tissues, or primitive germ cells that have migrated widely in the early embryo, as suggested by Weissman (2000). Following this line of thought, it may be more than a co-incidence that LeX is present on ES cells and primitive germ cells in the early embryo as well as on neural stem cells. Perhaps LeX is retained on these cells because they are phenotypically related. Moreover, I found that LeX⁺ cells were present in low numbers in adult skin and gut (unpublished observations) and although I did not have time to pursue this observation, the possibility remains that these rare cells are stem cells in this tissue. In that case, either LeX is functionally important for a variety of specialized stem cells, or perhaps as suggested above stem cells in a variety of tissues are closely related to primitive stem cells.

Not only it is crucial that stem cells are purified from fresh tissue based on specific markers for the *in vivo* demonstration/refutation of "lineage boundary crossing", it is also important for the assessment of their potential upon transplantation in the brain. For instance, although cultured adult neural stem cells seem to have a relatively broad potential when engrafted into different areas of the brain, freshly isolated cells show a much more restricted potential (Herrera et al., 1999) even if transplanted into embryonic environment when endogenous stem cells are actively engaged in the

generation of various types of neurons (Lim et al., 1999). Transplantation of freshly dissociated embryonic cells back into the embryo reveals high plasticity (Fishell, 1995; Brustle et al., 1995; Olsson et al., 1997) although many variants of dorsal-to-ventral/ventral-to-dorsal and rostral-to-caudal/caudal-to-rostral transplantations have not been tested (reviewed by Temple, 2001). But with development, even embryonic cells lose their plasticity. Is it because stem cells change their properties over time, or due to feedback signaling of maturing precursors that are co-transplanted in vast majority? This is another question that transplantation of freshly purified neural stem cells can help address.

Use of freshly isolated neural stem cells to study lineage relationships and developmental differences

In addition to the obvious benefits for transplantation studies, prospective identification and purification of neural stem cells provides yet another possibility: that of studying the relationship between neural stem cells at different developmental ages and even between stem cells of different lineages. This has not been properly addressed if addressed at all due to the lack of specific stem cell markers. Is there a universal stem cell gene expression profile? Is there a gene expression blueprint that distinguishes neural stem cells from hematopoietic stem cells or other stem cells? Is this blueprint developmentally regulated? These are incredibly complex and fascinating questions that we may now explore.

For instance, in chapters 4, I provide evidence that stem cells of different embryonic ages are LeX⁺, although the immediate precursors of the stem cell express LeX, albeit at a lower level. Using powerful genetic subtraction techniques, it will be possible to discover stem cell specific genes if one compares amplified cDNA from LeX⁺ cells with that of LeX⁻ cells at a given age. But the potential for this technique goes beyond stem cell vs restricted progenitors differences in gene expression. Subtractive cloning of purified LeX⁺ cells will also allow that gene expression be compared between let's say, E10 cortical cells and SVZ cells and all the variations that one may think of. This will generate an enormous amount of essential information on neural stem cell biology that we can only suspect of at this time.

In a breakthrough paper, Geschwind et al., 2001 set the stage for the unraveling the gene expression pattern of CNS progenitors using a combination of RDA (representational difference analysis) and cDNA microarray analysis to identify novel and known genes involved in CNS stem/progenitor cell proliferation and function. To achieve this goal, cDNA isolated from neurospheres derived from neonatal mouse cortex was "subtracted" from sister neurospheres subjected to 24h of differentiation conditions and subjected to additional techniques. This approach led to the discovery of novel genes specifically expressed in germinal areas of the embryonic and adult mouse brain like the VZ, SVZ and DG. Building on this work, Terskikh et al., 2001, showed that some of these genes are common to hematopoietic stem cells, suggesting that CNS and hematopoietic stem cells share genetic programs and signaling strategies. Again, these studies use cultured neurospheres as the source of neural stem cells and thus are subjected to the criticisms amply discussed in this thesis. The use of LeX⁺ cells as a source of neural stem cells will hopefully improve the important task initiated by Geschwind and colleagues.

FUTURE STUDIES

Many of the ideas for future work (some of them already underway) are implicit in previous sections of this discussion. In summary, these are:

1. Transplantation of freshly isolated LeX⁺ neural stem cells for *in vivo* assessment of developmental potential. This should be done using two host systems:
 - a) Mouse blastocysts: to address how broad the suggested potential of neural stem cells truly is.
 - b) Embryonic and adult CNS: to determine the extent of engraftment and potential for the generation of full repertoires of neuronal subtypes as well as macroglia.
2. Application of subtractive molecular biology techniques to discover neural stem cell specific markers that can be used to further refine neural stem cell purification.
3. Expand on point 2. and use the same technique to compare neural stem cells isolated from different regions of the brain and/or different developmental stages within one region.
4. Further explore the significance of LeX in the biology of stem cells by:
 - a) Identifying the molecular carriers of LeX at different stages of development.
 - b) Identifying additional growth factors that associate with LeX-containing molecules.
 - c) Manipulating/disrupting LeX synthesis in the brain

FINAL REMARKS

Although not presented in this thesis, we have data that indicates that other stem cells are also LeX⁺. For instance, LeX⁺ cells are found in appropriate stem cell locations in the adult mouse intestinal crypts, in singled out cells in embryonic skin, in cultures of basal keratinocytes and in the adult mouse pituitary, known for its modest regeneration. It is also well documented that primordial germinal cells strongly express LeX. Taken together, these results seem to indicate that stem cells in a variety of systems might maintain a common denominator: LeX.

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