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Neural Stem Cells from Mammalian Brain: Isolation Protocols and Maintenance Conditions

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1. Introduction

Traditionally, the adult brain has been considered a quiescent organ, lacking the production of new cells, or more exactly, new mature and functional neurons. This dogma has been widely refused in the last decades with the discovery of proliferative cells with stem cell properties in the adult brain.

First evidences come from the demonstration of neurogenesis in non-mammal vertebrates such as birds or lizards (as reviewed in Garcia-Verdugo et al., 2002). Neurogenesis was also confirmed to occur in adult mammals, like mice and rats, and, finally, in primates and humans (for a complete revision see Gil-Perotín et al., 2009). Though the process of neurogenesis in the adult is primarily confined to the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus, glial progenitors exist in other brain regions. These widespread glial progenitors remain quiescent and do not generate mature glial cells, but, in certain situations such as traumatic injury, they may act as true stem cells (Belachew et al., 2003; Rivers et al., 2008).

The terminology of stem cell, progenitor cell and precursor cell has been adapted from others tissues. Basically, a *bona fide* neural stem cell (NSC) must meet all these three features: capacity of self-renewal, capacity to differentiate into the three neural lineages (neuron, astrocyte and oligodendrocyte) and, finally, the ability to regenerate neural tissue. When cells show a limited self-renewal and are already committed toward a specific fate, they are classified as progenitor cells, while the term "precursor" represents intermediate stages.

Neural stem/progenitor cells (NSPc) primary cultures provide the best *in vitro* model to study proliferation and differentiation signaling pathways, a difficult issue to address *in vivo*. Additionally, these cells might be used in future replacement cell therapies, thus motivating the development of protocols aimed to isolate and expand these cells *in vitro*. These protocols display significant variations among them, and the introduction of new technologies has increased drastically their number. The differences in the protocols have rendered different results in terms of stem cell subpopulations, differentiation potential and the amount of cells. The last is especially relevant in the case of human samples because of their low availability.

Therefore, the aim of this chapter is to recapitulate some of these technical differences that could induce variances in the final results. We have analyzed the main isolation protocols from the two canonical neurogenic zones in the adult (subventricular zone and hippocampus), described for both animal models (mouse and rat) and human.

2. Neural stem/progenitor cell isolation

The NSPc isolation procedures follow common steps including tissue dissection, digestion and cell enrichment. However, comparing the different protocols found in the bibliography, it is notable the presence of significant differences between them even when they are consecutive works from the same group. The introduction of new technologies has also increased drastically the number and variety of protocols. Additionally, some tissues like normal human brain are particularly difficult to manage due to their low availability, which requires improvements in the protocol to include modifications that increase the rate of isolated cells. Interestingly, the diversity of isolation procedures results in the obtaining of different stem/progenitor cell subpopulations with distinct differentiation potential, and might be also responsible for the, sometimes contradictory, results observed in the literature.

Although the development of standard protocols would be the best option to assure that results can be easily compared, in the practice, this is almost impossible. Different groups have generated independently alternative procedures for the isolation, dissociation and enrichment of NSPc. Furthermore, the animal model, the specific location of the brain sample, or even the characteristics of the experiment have requirements that would make unmanageable the use of universal procedures. Usually, the same group employs very similar strategies to isolate cells from different samples, independently of their developmental stage or animal/human origin. Nevertheless, it will be interesting to establish flexible guidelines to indicate what can be modified from the standard procedures and how to do so.

The basic scheme followed by NSPc isolation protocols is reflected in figure 1, and we will discuss the specific methodology associated with every step in the following headings

2.1 Tissue dissection methods

The origin of the tissue influences the type of isolated cells as well as their proliferation and differentiation capacity. A number of profound differences have been reported between brain samples from different species (mouse, rat of human) or from different stages of development within a given specie (Gritti et al., 2009; Svendsen et al., 1997). However, the accurate dissection of specific regions of the brain has become more relevant as the knowledge on the NSPc biology and location increases. In fact, regardless of the animal model, one of the main factors that might determine the final results is the specific location of the brain tissue from where NSPc are isolated.

Different regions of the brain have been used as a primary source of NSPc and, consequently, discrepancies in the isolated cells have been reported. In this sense, analyzing the distinct approaches for the tissue dissection might be useful to contextualize such a controversy.
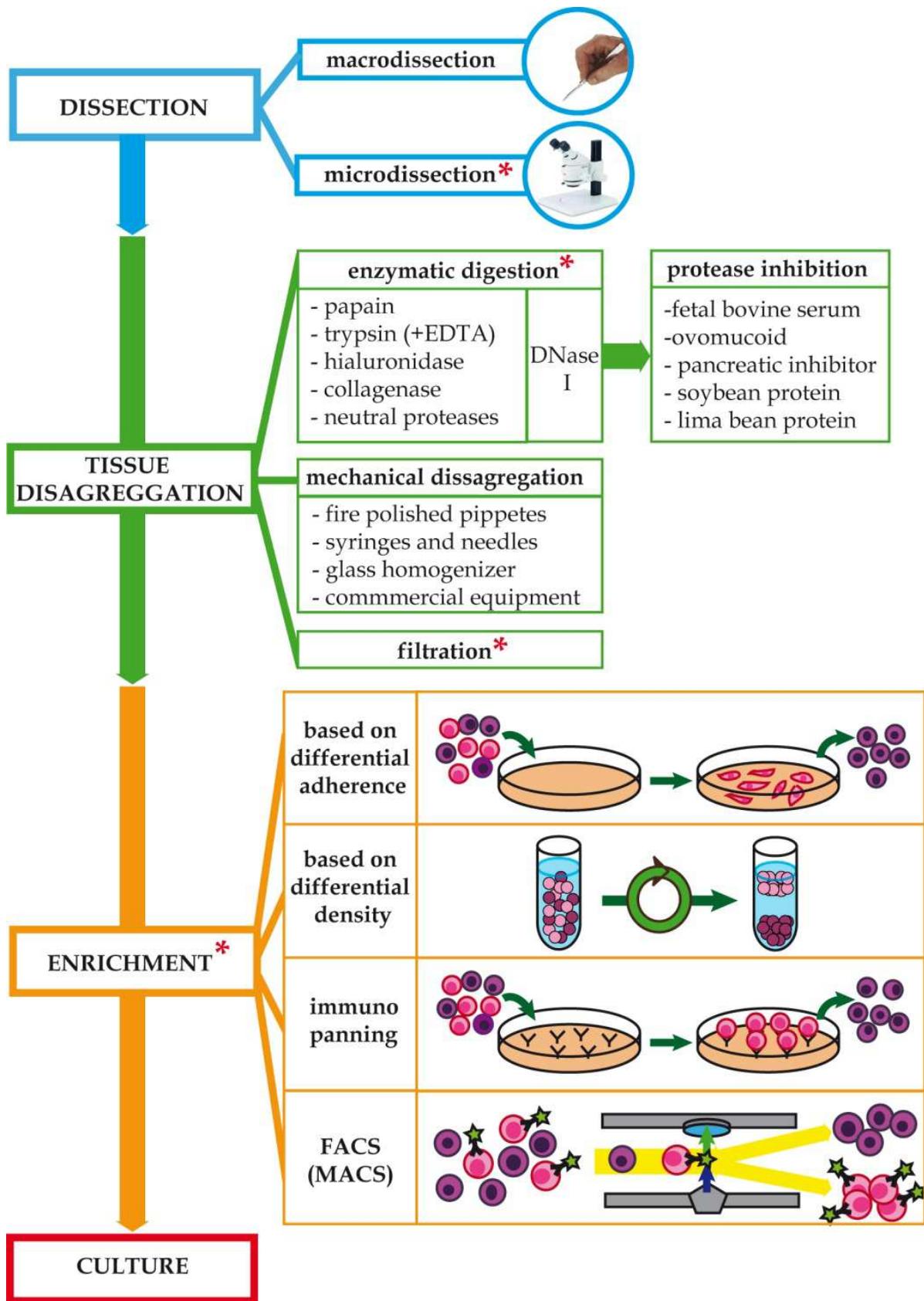
Fig. 1. Diagram depicting the main steps of standard neural stem/progenitor cell isolation protocol. Headings marked with an asterisk are not always included.

We might consider three different levels of dissection according to the amount and location of tissue, ranging from large unselected brain tissue to microdissection. In a first level, a number of works start from whole brain (e.g. Von Visger et al., 1994) or large areas that include heterogeneous regions (e.g. whole human temporal lobe, Kirschenbaum et al., 1994). In these cases, the results can be highly variable, because of the different types of progenitors coming from distinct locations and giving rise to an artefactual impression of cell heterogeneity. An intermediate step of complexity is found in those works that use tissue from specific areas, but without the exclusion of contiguous tissues, i.e. macrodissection. In this regard, some authors reported the presence of multipotent stem cells from different regions of the adult parenchyma that differ from canonical neurogenic zones (SVZ and SGZ), e.g. from striatum (Reynolds & Weiss, 1992). However, these cells might arise from the cross-contamination of adjacent neurogenic regions (Lois & Alvarez-Buylla, 1993). Likewise, as will be discussed later, the existence of real neural stem cells in adult dentate gyrus of hippocampus has become a controversial subject. Some authors claim that there are true stem cells from this zone. However, others state that these isolated cells should be considered progenitors because of their low proliferation *in vitro* and their doubtful multipotentiality. The main argument of these authors is the lack of fine dissection, and the inclusion of neural stem cells from other adjacent tissues, like SVZ. Therefore, considering the current knowledge on NPSc niches location, an exhaustive microdissection is essential to take out the region of interest in a reliable way before starting the isolation procedures. Then, it is highly recommendable the use of thin slices of tissue for the accurate microdissection of different compartments under a dissecting microscope (e.g. Seaberg & van der Kooy, 2002).

Tissue dissection is particularly challenging in the case of human surgical samples, where orientation and anatomical organization is usually altered after surgery, making difficult the recognition of particular zones and, consequently, a good dissection. Alternatively, some authors have demonstrated the isolation of viable cells from postmortem tissue, especially in the case of human samples (e.g. Schwartz et al., 2001). While these procedures might be the only way to access some type of tissues, there might be some logistical inconveniences, the main one being that collection of tissue and cell isolation protocols need to be performed within few hours, because the number of NSPc decreases with time (Leonard et al., 2009; Xu et al., 2003), especially when samples are exposed to environmental temperature instead of 4°C (Laywell et al., 1999).

2.2 Tissue digestion methods

2.2.1 Enzymatic dissociation

NSPc are surrounded by a highly structured extracellular matrix mainly composed by lecticans, hyaluronic acid, tenascin-C and tenascin-R (Rutka et al., 1988). These molecules interact among them and with membrane molecules on cell surfaces, and can regulate part of their behavior.

Therefore, one of the most successful strategies for removing NSPc from the rest of the tissue implies the use of proteases to degrade this matrix.

The first step, to prepare the tissue for enzymatic digestion, involves the mincing into small pieces (less than 1 mm³) in order to provide more degradable surface for the action of

proteases. In this sense, the use of two different enzymes stands over the rest in the literature: trypsin (examples of its applications in different samples and developmental stages can be read at Kirschenbaum et al., 1994; Kukekov et al., 1997, Reynolds et al., 1992; Reynolds&Weiss, 1992; Svendsen et al., 1998) and papain (Babu et al., 2007; Roy et al., 2000a; Wang et al., 2000; Windrem et al., 2004). Trypsin is the most employed one, and is often combined with ethylenediaminetetraacetic acid (EDTA), a Ca2+ chelating agent that weakens intercellular unions. Regarding the concentration and the incubation time, it is not always possible to compare between different protocols as the enzyme units are not always specified and the incubation time ranges from 10 to 90 minutes. Additionally, other enzymes can be found in the bibliography such as hialuronidase (e.g. Gritti et al., 1995; Weiss et al., 1996), collagenase (e.g. Uchida et al., 2000), and neutral protease (dispase) (e.g. Babu et al., 2007), alone or in combination with others.

Generally, the use of proteases is linked to the utilization of Desoxiribonucleiase I (DNase I), usually from bovine origin, in order to eliminate the DNA mucus originated by cell lysis, which could hinder cell survival and further experiments.

In any case, the employment of enzyme specific buffers (with adjusted pH and containing activators) is necessary to allow the action of these enzymes. In some cases, antibiotic/ antimitotic is added to the digestion solution to prevent contamination. At this stage, some authors also include kynurenic acid in order to reduce glutamate excitotoxicity through NMDA receptor channels (e.g. Reynolds&Weiss, 1992). Afterward, the use of protease inhibitors is necessary to stop enzymatic reaction. Papain is usually neutralized with fetal bovine serum, whereas in the case of trypsin, the most employed method includes ovomucoid, although there are commercially available soy, lima bean, and basic pancreatic protein -based inhibitors.

The criterion for the choice of one or another enzyme is not clear, and frequently it has more to do with the previous experience and skills of the group. Nevertheless, as a general rule, embryo and early fetal samples require less amount of enzyme due to its laxity. For this reason, some protocols reduce protease concentrations and/or exposure time (e.g. Svendsen et al., 1998) or even recommend the use of mechanical disaggregation techniques alone (e.g. Ciccolini&Svendsen, 1998; Reynolds&Weiss, 1996).

The enzymatic digestion is a critical step because it affects directly to the NSPc survival rate. In this sense, some studies have been done to compare cell survival after dissociation with different protease. Maric et al., 1998, used murine embryonic tissue to evaluate the efficacy of papain, trypsin, and collagenase treatment, or mechanical disaggregation alone. The results indicate that papain dissociation is optimal, achieving the maximum reproducible cell recovery and viability. On the contrary, trypsin, collagenase, and mechanical dissociations resulted on suboptimal and highly variable yields. Another study, carried out by Panchision et al., 2007 also compared the results obtained for mouse embryonic stem cell isolation when using papain, Tryple™ (a commercial analog of trypsin), or collagenase/neutral protease commercial cocktails (Accutase™ and Liberase-1™). Data also confirmed that mechanic dissociation induced more variability, cell death and more number of aggregates. However, Tryple™ and papain produced more quantity of DNA mucus (but not an increased cell death) and a lower adherence to culture plate after planting. They conclude that the best results were obtained with papain, independently of the exposure time to the enzyme.

Moreover, this work also revealed another important factor to take into account when optimizing protease dissociation: cell surface markers can be altered by these enzymes, inducing false negatives when immunocytochemistry or Fluorescent Activated cell sorting is performed just after isolation. Table 1 includes a list of sensitive markers described in this paper and similar reports. In addition, another work detected that trypsin cleavage can lead to an increased positivity of some tumor –related surface markers, depending on the state of glycosylation (Corver et al., 1995).

	enzyme -sensitive	very weakly enzyme - sensitive
Trypsin	hCD133, CD31, O4, CD81, c14, Ca125, BMa180	A2B5, CD15
Papain	PSA-NCAM, CD24, BMP IA, BMP IB	CD15, O4, CD81
Liberase-1™	BMP IA, BMP IB	
Accutase™	none of the studied	
Tryple™	none of the studied	

Table 1. List of enzyme – sensitive markers that are reported in Corver et al., 1995; Panchision et al., 2007; and commercial report by Reiβ et al (Miltenyi). CD133 has been reported to be sensitive to trypsin treatment in human cells, but not in rodent cells.

2.2.2 Mechanical disaggregation

Usually, the enzymatic digestion is not enough to remove the NSPc from the remaining tissue. After or during enzymatic digestion, the tissue must be triturated to break up the digested pieces into a single cell suspension. It is a dramatic process that ends up with an important number of dead cells. However, different strategies have been described in the literature in order to reduce, to some extent, this number. The most common method consists in passing the suspension through fire polished glass pipettes, due to their high availability and lower price (e.g. Ciccolini & Svendsen, 1998; Gage et al., 1995; Reynolds&Weiss, 1992). Moreover, they can be narrowed into different diameters, adapting their thickness to samples of different size. Many protocols include the sequential trituration through pipettes with decreasing diameters in order to disaggregate the tissue in successive steps and reduce cell death (e.g. Wang et al., 2000). Nevertheless, this system also presents some technical problems. First, cells display a relative adherence to glass and might be lost. Furthermore, as glass pipettes are usually prepared specifically for each experiment, their diameter can vary, and therefore, different cell survival rates can be obtained. The cell adherence issues might be partially resolved by coating pipettes with silicone. Alternatively, some commercially available plastic pipettes (Kukekov et al., 1997) are treated to reduce the adherence, but they cannot be fire polished.

Another strategy is based on the utilization of sterile syringes and needles (e.g. Shi et al., 1998). In this sense, a large range of needle gauges is available commercially, ensuring the reproducibility of the technique; however, their edges are too sharp and that results in an increase in cell death. Although less frequent, it is worth mentioning the use of different devices like the glass homogenizer, used for embryonic neural stem cell isolation (Carpenter

et al., 1999), and some commercial equipment that appeared in the last years, promising a higher efficiency via the automation of the isolation procedure (Reiβ et al (Miltenyi)).

2.2.3 Filters utility

Some groups, after enzymatic digestion and mechanical disaggregation, include a filtering step to remove the debris from the cell suspension. This additional step might eliminate undissociated tissue pieces as well as avoid the presence of necrotic particles in the final pellet that would potentially induce cell death. However, it also reduces the final number of viable cells trapped into the filter. In any case, the use of filters usually requires a DNase I treatment, to remove the mucus that can difficult the filtering, and it is strongly recommended the dilution of cell suspension in a considerable volume of medium. Regarding the type and size of the filters, some authors describe the use of cell strainers, whereas others prefer sterile gauze (e.g. Kukekov et al., 1997). The mesh size also differs among protocols (40 um (Wang et al., 2000), 70 um (Rietze et al., 2001), 100 um, etc), and should be chosen in accordance with the efficiency of preceding methodology.

2.3 Neural stem/progenitor cells enrichment procedures

The initial protocols for NSPc isolation were designed with the only purpose of isolating and culturing these cells to study their biology *in vitro*. However, as the knowledge on the biology and differentiation potential of NSPc increased, it was evident that cell cultures comprised a number of different subpopulations with different degree of stemness. Consistently with this reality, many authors have recently included separation steps into their NSPc isolation protocols. This separation is usually based on the NSPc phenotypic characteristics closely related to their stem cell features.

In this sense, the first works on NSPc isolation and culture described a selection based on their capacity to proliferate in the chosen medium and growth factors. Obviously, it was not enough to discriminate heterogeneity. Consequently, many technical approaches have been developed since then, for the enrichment of a specific subpopulation. This way, the biological significance behind the molecule chosen to enrich for a specific type of cell and the technology used for the procedure become an important step determining the differentiation potential of the final cell culture. The current techniques for the separation and enrichment of NSPc are described below.

2.3.1 Methods based on differential adherent properties of cells

One of the first methodologies for the enrichment of particular subpopulations was based on the differential attachment of cells to the culture plate due to their particular adhesion molecule patterns. By optimizing some parameters like substrates and time in culture it is possible to distinguish between different types of cells. Astroglial cells show the biggest adherence, even in untreated culture plate, whereas oligodendrocytes can be easily detached through the agitation on a rotary shaker at slow revolutions (200-300 rpm) for 12-20 h. This procedure has demonstrated to be useful, easy and affordable. As a consequence, it has been common in the purification of specific cell types like oligodendrocytes (McCarthy & de Vellis, 1980; Chen et al., 2007b).

Taking advantage of these properties, Lim&Alvarez-Buylla, 1999, reported the isolation of 4 cell fractions using serial streaming of medium or PBS over the surface of poly-D-lysine treated plates, and a final step with trypsin. The first fraction (or fraction 1), which contains the less adherent cells, was enriched in PSA-NCAM and Tuj1 (identified as migrating neuroblasts). On the contrary, cells from the most adherent fraction (fraction 4) were GFAP+ and show characteristics of neural stem cells (type B/C according to the model of SVZ organization (Fig.2). However, it is important to mention that this procedure does not allow the obtaining of high purity cultures.

2.3.2 Differential gradient centrifugation

Another group of technical approaches for NSPc enrichment is based on fractionating cell populations according to their buoyant density. Previously, the cells are dissolved in specific solvents that, after centrifugation, generate a density gradient. The cells distribute in this gradient and can be collected separately. The gradient might be formed by using different types of reagents, being Percoll the most widely used (e.g. Palmer et al., 1999; K. Chen et al., 2007a). It consists of colloidal silica particles coated with a layer of polyvinylpyrrolidone (PVP) that can be used to form solution densities between 1.00 and 1.20 g/ml. A combination of Percoll gradients can be generated in order to separate more subpopulations. Using a discontinuous density gradient, Maric et al., 1998 reported the isolation of 20 different bands and the delimitation of density bands can be facilitated by commercial color-coded density marker beads. While its application has become very common because of its low interaction with cells and low toxicity, it is restricted to research as it may contain variable quantities of endotoxin (PVP). Alternatively, density gradients can be also generated using sucrose solutions (Johansson et al., 1999) and Bovine Serum Albumin (Ericsson, 1977).

2.3.3 Immunopanning

Initial immunopanning applications were essentially directed to eliminate specific cell subpopulations by antibody union and complement-mediated lysis (e.g. Gard&Pfeiffer, 1993). Nevertheless, the present acceptation of the immunopanning technic comprises the purification of a cell population by exploiting their differential binding to the culture dishes previously coated with a cell-surface antibody. Cells expressing this surface antigen are retained on the dish and are thereby separated from the remaining cell population. It has been especially applied to the isolation of oligodendrocyte progenitor cells, using A2B5 or O4 (Barres et al., 1992; Wu et al., 2009; Mayer-Proschel, 2001) as molecular surface markers, but it can also be adapted to segregate immature neurons (PSA-NCAM) (Ben-Hur et al., 1998; Schmandt et al., 2005). Although the use of immunopanning has become less popular with the introduction of Fluorescence-activated cell sorting (FACS) technology, some authors had reported that immunopanning provides a higher survival (Mayer-Proschel, 2001).

2.3.4 Fluorescence activated cell sorting (FACS)

The main improvement in terms of separation and enrichment of specific NSPc comes with the introduction of the FACS technology. As a specialized form of flow citometry, it provides a method for sorting heterogeneous cells based upon the specific union of a fluorophore-labeled antibody to a cell surface maker. In addition to antibodies, other

molecules like lectins can be used to recognize the glycosylation state of some membrane epitopes (as reviewed in Kitada et al., 2011).

The main advantage of this procedure is its high sensitivity, reaching values of purity above 95%. Moreover, the possibility of labeling cells with simultaneous antibodies allows the isolation of a particular subset of cells with a combination of membrane markers (e.g. Uchida *et al*, 2000). Moreover, the use of this technology makes possible the sorting of cells according to the expression of either cytoplasmic or nuclear markers. This advantage allowed the design of transgenic animal models that express a given fluorophore under the control of specific promoters. Additionally, the introduction of small DNA molecules can also induce the expression of a fluorescent molecule in both animal and human cells.

Alternatively, magnetic labeled antibodies might be used through a variation known as Magnetic-activated cell sorting (MACS). This technology uses a more reduced and affordable equipment, although it does not allow the labeling of more than one surface marker. Table 2 lists the stem cell markers used in the isolation of NSPc subpopulations by FACS or MACS.

NEURAL STEM CELLS	ANTIBODY	Integrin α1β5	Yoshida et al., 2003
		CD15	Capela et al., 2002; Corti et al., 2005; Panchision et al., 2007
		CD24LOW	Murayama et al., 2002; Rietze et al., 2001
		CD133	Cortie tal., 2005; Panchision et al., 2007; Uchida et al., 2000
		CXCR4	Corti et al., 2005
		EGFR1 (EGF)	Ciccolini et al., 2005; Pastrana et al., 2009
		NOTCH1	Johansson et al., 1999
	LECTINS	PHA-E4	Hamanoue et al., 2008
		WGA	Hamanoue et al., 2008
	FLUOROPHORE UNDER PROMOTOR CONTROL	p/GFAP	Doetsch et al., 1999; Pastrana et al., 2009
		p/MELK	Nakano et al., 2005
		p/MSI1	Keyoung et al., 2001
		p/Nestin	Kawaguchi et al., 2001; Keyoung et al., 2001; Roy et al., 2000a, 200b; Sawamoto et al., 2001; Yoshida et al., 2003
		p/SOX1	Barraud et al.,2005

			p/SOX2	Brazelet al., 2005; Ellis et al., 2004; ; Keyoung et al., 2001; Suh et al., 2007; Wang et al., 2010
LINEAGE RESTRICTED PRECURSOR	glial	ANTIBODY	A2B5	Maric et al., 2003; Nunes et al., 2003; Windrem et al., 2002; Windremet al., 2004; Wright et al., 1997
			CD44	Liu et al., 2004
			GD3	Maric et al., 2003
			NG2	Aguirre et al., 2004
			O1	Duncan et al., 1992
		FLUOROPHORE UNDER PROMOTOR CONTROL	p/CNP	Aguirre et al., 2004; Nunes et al., 2004; Roy et al., 1999; Yuan et al., 2002
	neuronal	ANTIBODY	PSA-NCAM	Panchision et al., 2007:Windrem et al, 2004
		FLUOROPHORE UNDER PROMOTOR CONTROL	P/Tα1	Piper et al., 2001; Roy et al, 2000ª, 2000b; Sawamoto et al., 2001; Wang et al., 2000
			P/Neurogenin2	Thompson et al., 2006
		OTHERS	Cholera toxin	Maric et al., 2003
			Tetanus toxin	Maric et al., 2003

Table 2. Main markers used for NSPc isolation by FACS or MACS.

3. Primary neural stem/progenitor cell culture

3.1 Culture conditions

3.1.1 Culture media

The culture media commonly used to grow NSPc includes two components, the basal media and supplements and the use of growth factors. The basal culture media formulation does not change significantly between different groups. It is based on the use of Dulbecco's modified Eagle's Medium (DMEM), which is composed by a defined mixture of inorganic salts, amino acids and vitamins among other nutrients. DMEM is usually combined (1:1) with Ham's F-12 (F-12), which basically increases the level of some nutrients and provides different inorganic salts. Although less frequent, other alternatives with similar characteristics have been reported as basal media, such as Neurobasal™, or Ex Vivo™ 15 (e.g. Babu et al., 2007).

The basal media is frequently supplemented with N2 or B27 supplements which contain nutrients like insulin, transferrin or putrescine, among others. These supplements cannot be

added to basal formulation until they are used because of their short life at 4ºC. Although both of them might be used, even in combination, they have different properties that may influence cell culture behavior. B27 has a more complex composition than N2 supplement and only enhances cell survival during the period immediately following isolation (Svendsen et al., 1995), while N2 offers the same results, at a lower price. Babu et al. (2007) concluded that monolayer cells maintained with N2 supplement generated more neurons after differentiation, whereas B27 supplement promoted proliferation.

3.1.2 Serum and growth factors

Although basal media and supplements are quite similar in most cases, the most important issue in terms of culture media is the use of either specific growth factors or serum. The first works on NSPc isolation and maintenance described the use of serum in their culture media. However, as the knowledge on NSPc biology increased, researchers found that the use of serum, generally fetal bovine serum (FBS), had several disadvantages. As a complex solution of undefined composition that can vary drastically among batches, the use of serum does not contribute to improve our knowledge about trophic signals requirements. Additionally, it is not a physiological condition, since neural stem cells are not exposed directly to serum *in vivo*. Finally, serum includes a combination of different growth factors that are able to maintain stem cell phenotype and also induce differentiation. All these reasons made the authors substitute serum for a specific combination of purified growth factors. The utilization of two main growth factors stands out from the rest: fibroblast growth factor 2 (FGF-2, also called basic FGF or bFGF) and epidermal growth factor (EGF), alone or in combination. Moreover, FGF-2 must be used in combination with heparin, which mediates the binding of the growth factor to its receptor (Yayon et al., 1991).

Initial works (Reynolds, 1992; Reynolds & Weiss, 1992) described the isolation of an EGF-responsive neural stem cell population from striata/lateral ventricle, although some authors reported that similar cell cultures could be also maintained with FGF (Gritti et al., 1995; Vescovi et al., 1993). Similarly, some works also found a synergic effect of both EGF and FGF in proliferation, but only at low cell densities (Svendsen, 1997; Tropepe, 1999). Finally, a series of studies (Martens et al., 2000; Tropepe et al., 1999; Ciccolini, 2001; Maric et al., 2003) demonstrated that FGF- responsive cells arise earlier at development, and then give rise to both EGF/FGF- responsive cells. Moreover, it was revealed that the acquisition of EGF responsiveness is promoted by FGF *in vitro* (Ciccolini & Svendsen, 1998). First isolations could be explained with the discovery of a small autocrine/paracrine FGF production by neural stem cells, allowing the survival of FGF-2 dependent cells without FGF until the acquisition of EGF responsiveness (Maric et al., 2003).

Other growth factors that have been reported to support cell culture are Transforming growth factor alpha (TGF-α) (Reynolds et al., 1992), Leukemia inhibitory factor (LIF) and its equivalent Ciliary neurotrophic factor (CNTF) (Carpenter et al., 1999), or Brain-derived neurotrophic factor (BDNF), although its capacity to enhance later neuronal production and survival has been questioned (Kirschenbaum & Goldman, 1995; Ahmed et al., 1995; Reynolds & Weiss, 1996).

Platelet-derived growth factor alpha (PDGFα) is frequently used in the maintenance media for oligodendrocyte progenitor cells. The signaling pathway through the PDGFα/PDGFRα

has different effects depending on the stage of differentiation of these progenitors: it provides signals favoring proliferation and migration in murine and human oligodendrocyte progenitors (Wilson et al., 2003; Calver et al., 1998), whereas later in development is related with cell survival (Gogate et al, 1994). Similarly, FGF promotes proliferation and blocks differentiation of oligodendrocyte progenitors, in part through the modulation of PDGFRα receptors expression (McKinnon et al., 1990).

Finally, some works have attempted to co-culture NSPc in the presence of other supportive cells like astrocytes (Richards et al., 1992; Lim et al., 1999), that seem to favor the NSPc growth by physical contact, or endothelial cells, that also enhance cell proliferation via VEGF production (Sun et al., 2010).

3.1.3 pH and oxygen levels

The metabolic processes undergone by the cells in culture give rise to acidic components that eventually are released to the media, thus decreasing the pH. This alteration, easily followed by the inclusion of a pH indicator like phenol red, has a direct influence in the behavior of the cells. Therefore, buffering agents are commonly added to medium formulation in order to control variations in the pH. In this sense, two main systems are routinely used in the elaboration of the media: sodium bicarbonate buffer, which is dependent on the CO_2 concentration present in the incubator, and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), independent of atmospheric CO_2. Although HEPES is better at maintaining physiological pH controls, the exposure of HEPES-containing media to light must be reduced as HEPES-containing media generates hydrogen peroxide when exposed to ambient light ({Zigler et al., 1985).

In contrast, the level of O_2 tension remains to be optimized for NSPc cultures. The standard conditions to culture NSPc had included atmospheric levels of O_2 (21%), although physiological levels are much lower (around 3%). Several studies have confirmed that NSPc expansion under low level of O_2 correlates with the expression of stemness markers and higher survival rate both *in vitro* and *in vivo* after engraftment (reviewed in De Filippis & Delia, 2011).

3.2 Monolayer versus neurosphere cultures

To maintain and propagate stem cell cultures different authors have published two alternative methods of NSPc culture and expansion: as free-floating cell clusters (neurospheres) or as adherent cultures forming a monolayer on the plate surface.

The neurosphere assay has been the most extended method to demonstrate the presence of NSPc in culture (Reynolds et al., 1992) and it is still used with different modifications (Rietze& Reynolds, 2006). Some authors claim that each neurosphere represents a microenvironment that recapitulates neurogenic niche and allows survival of stem cells *in vitro* (Bez et al., 2003) through direct cell-to-cell interaction. Nevertheless, a single neurosphere contains only a small percentage of true stem cells, whereas the remaining cells are in different stages of differentiation. Necrotic and apoptotic cells are also present (Lobo et al., 2003). Interestingly, it has been reported that committed progenitors, like oligodendrocyte precursors, can generate cell clusters similar to neurospheres (Chen Y. et al., 2007).

This culture method has also several technical disadvantages. First, when neurospheres become larger, the diffusion of nutrients and growth factors through the neurosphere is compromised (Svendsen et al., 1997b), which makes difficult the interpretation of some experimental results. Second, packed neurospheres do not allow the tracking of individual cells, which also hinders studies relating to differentiation processes. Finally, recent publications demonstrate that neurospheres are not static particles originated from a single cell and isolated from the rest of the neurospheres and cells (Rietze, 2006). On the contrary, they are dynamic structures within the culture, were cells are exchangeable from one to another sphere. This effect, may be circumvented by either using a limiting dilution analysis to obtain a single cell in each well or using semisolid cultures by adding methylcellulose (Gritti et al., 1999; Kukekov et al., 1997) or collagen (Neural Colony-Forming Cell Assay (Louis et al., 2008).

By contrast, monolayer cultures obviate some of these restrictions. They can be used to study the properties of stem cells at individual cell level, although it does not allow cell interaction during differentiation. Moreover, cells are exposed homogeneously to growth factors and serum, with the consequent reduction in cell heterogeneity.

In all, there is not a prevalent method over the other. It has not been demonstrated a total equivalence between both type of cultures and the two methods have advantages and limitations that researchers should take into consideration in the experimental design. The formation of neurospheres may be promoted by following several strategies, being the most common one the use of nonadherent surfaces like poly-2-hydroxyethyl methacrylate (Kukekov et al, 1999). Furthermore, it has been also reported the addition of mercaptoethanol to avoid cell attachment (Kukekov et al., 1997). However, not all attempts to transform an adherent culture into neurospheres have been successful (Walton et al., 2006). Alternatively, cell attachment may be induced by coating the plate surface with charged molecules such as poly-l-ornithine, poly-d-lysine or laminin.

3.3 Cell passaging

Before cells become totally confluent, it is necessary to subculture them after disaggregation of cell clusters into single cell suspensions. Regardless of the type of culture, monolayer or suspension, passaging should be performed before cells achieve their maximum confluence (monolayer) or cell cluster become necrotic (neurospheres) in order to avoid senescence associated with prolonged high cell density. The methodology employed for the disaggregation step depends on the cell type.

Adherent cells are usually detached from the surface of the culture vessel by enzymatic means. Trypsin, alone or in combination with EDTA, has been the most used protease (e.g. Palmer et al., 1997); but in the last years it has been substituted in current protocols by Tryple™, since this commercial product is free of animal- and human- derived components, less damaging to cells, and does not require the use of inhibitors.

In the case of neurospheres, cell disaggregation is performed by using mechanical procedures which involve triturating spheres with fire polished pipettes. However, this is an aggressive method that renders high levels of cell death. Enzymatic digestion can be also

used before triturating, however, this may alter the experimental results if FACS assays are conducted right after disaggregation.

An alternative method was reported by Svendsen & ter Borg, 1998 for passaging neurospheres isolated from human fetal tissue. Briefly, neurospheres were cut into 4 pieces instead of standard trituration into single cell suspension. According to their data, this sectioning method reduces cellular trauma and preserves cell interaction, allowing NSPc to proliferate more replication rounds *in vitro.*

3.4 Cryopreservation

Cryopreservation allows the maintenance of NSPc in a suspension mode awaiting for future experiments and saving expensive culture reagents. Considering the low number of cells obtained from each sample, especially in human tissue, increasing the survival ratio after long-term preservation of NSPc becomes a major concern. The main cryopreservation protocols employ dimethylsulfoxyde (DMSO) diluted at 10-20% in culture media to avoid ice crystallization, accompanied by a slow cooling step in isopropanol recipients. Although less popular, glycerol can be used instead of DMSO. Cellular viability can be improved adding animal serum to freezing medium, but it can potentially introduce contaminants, and induce differentiation. In any case, cryopreservation must follow some general rules to ensure the successful preservation of cells. It must be performed during the logarithmic growth phase and high cell density in each ampoule seems to facilitate cell recuperation. Smaller neurospheres survive better than larger, so triturating cells until getting a suspension of small neurospheres improves cell survival.

Recently, a new alternative preservation method, named vitrification, has been adapted for NSPc (Tan et al., 2007). In brief, cells are sequentially submerged in a series of freezing solutions with increasing concentrations of cryoprotectant (ethylene glycol and sucrose), and finally transferred into borosilicate glass capillaries, snap-frozen and stored in liquid nitrogen. The results showed that vitrification offered the best combination of cell viability, multipotency, and preservation of structural integrity of neurospheres.

3.5 Differentiation

After isolation of proliferating cells, it is necessary to confirm the stemness characteristics of the cells, that is, the multipotent and self-renewal capacities. In this sense, cells with lower self-renewal or with potential to generate just one type of cell should be considered as progenitor cells. To evaluate the differentiation capacity, cells are exposed to differentiation signals coming from animal serum or chemically defined compounds.

The use of serum has the same problems highlighted above. Nevertheless, this is still the standard methodology, because the specific signals inducing NSPc differentiation into a specific lineage remains largely unknown. Cells maintained in defined medium tend to differentiate when exposed to serum in a variable concentration (from 1% up to 10%)(e.g. Ciccolini & Svendsen, 1998; Palmer et al., 1999; Roy et al., 2000; Wanget al., 2000), although a preference towards astroglial differentiation has been reported (Palmer et al., 1995). The use

of serum is usually accompanied by the addition of molecules such as Poly-L-ornithine, laminin or matrigel to promote adhesion to substrate, which seem to enhance differentiation of neurospheres cultures (Ciccolini & Svendsen, 1998; Reynolds & Weiss, 1996; Tropepe et al., 1999). In some cases, the removal of growth factors in conjunction with an adherent substrate has been also used to differentiate NSPc (Gritti et al., 1996).

Alternatively, media previously exposed to other cell cultures (conditioned medium) may be used to induce differentiation. Probably the most employed one is B104 conditioned medium, which is exposed to a neuroblastoma cell line and induces oligodendroglial differentiation (Young & Levinson, 1997).

Few authors have conducted NSPc differentiation assays by using growth factor cocktails in the absence of serum. Uchida et al., 2000 reported that a combination of BDNF and glial-derived growth factor (GDNF) was enough to differentiate CD133+ cells from human fetal tissue. Ling et al., 1998 reported a more specific differentiation protocol, proving that the combination of Interlekine-1b, Intelkeulin-11 and GDNF promoted the appearance of dopaminergic neurons (tyrosine hydroxylase -positive cells).

Furthermore, a number of chemical signals have been also reported to stimulate the differentiation toward a particular neural lineage.

In the case of neuronal maturation, BDNF, retinoic acid, Neurotrophin (NT3), and Sonic Hedgehog (SHH) have been associated to an enhanced neural obtaining (Babu et al., 2007; Bull & Bartlett, 2005; Dutton et al., 1999; Roy et al., 2000a, 2000b).

Oligodendroglial differentiation can be also enhanced using PDGFa, which promotes their survival (Gogate et al., 1994) in collaboration with NT3 and Triiodothyronine (T3), factors necessary for the correct development of oligodendrocytes and the expression of myelin proteins (Billon et al., 2002; Park et al., 2001).

4. Isolation from neurogenic zones

Neural stem cells seem to reside within specific niches of the adult brain. These regions are located in the subventricular zone of the lateral ventricles and the subgranular zone in the hippocampus. The origin of NSPc in these two areas has been the focus of intense debates in the literature and the isolation procedures of such cells from these specific locations need special attention.

Since the discovery of adult neural stem cells, the isolation procedures have been modified along with the increased knowledge of NSPc biology. Initially, these cells were supposed to be scattered within the brain parenchyma. However, soon after it was restricted to the SVZ, although the individual cell identity is still a source of division among researchers due to the lack of a specific marker to label neural stem cells. The nature and origin of the neural stem cell in the SGZ of the hippocampus has been also a subject of an intense debate, questioning whether they could be considered true neural stem cells or committed progenitors. Additionally, other types of neural progenitors like oligodendrocyte progenitor cells (OPCs) seem to be dispersed through the white matter, and their isolation procedures and characterization have become recently relevant in the context of demyelinating diseases.
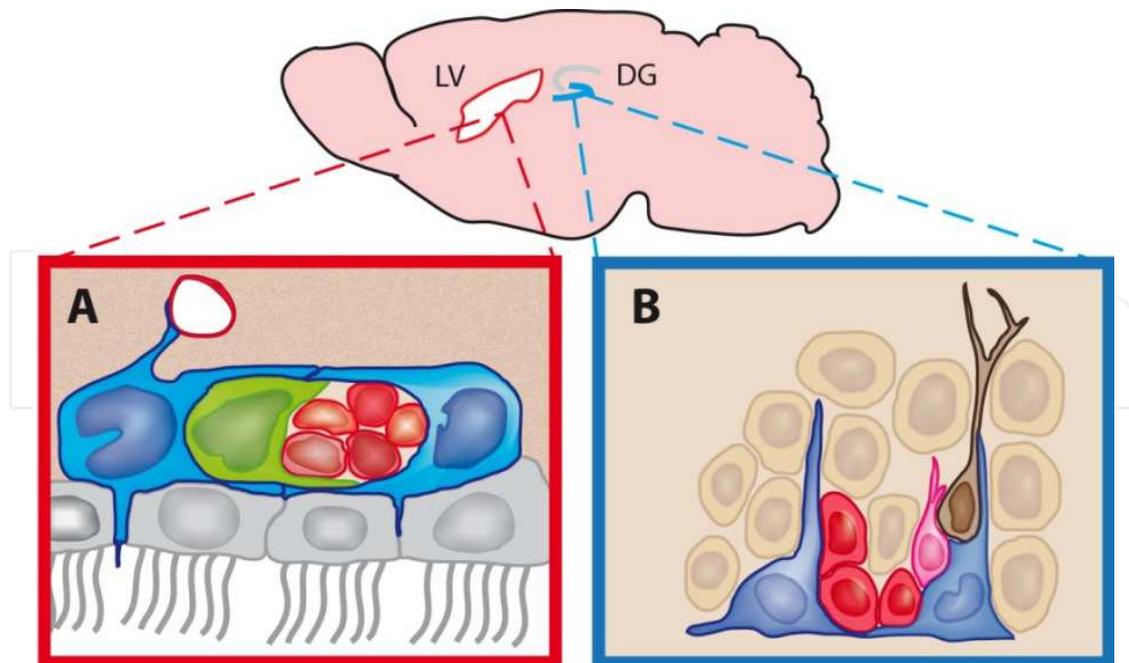
Fig. 2. Schematic representation of both adult neurogenic niches and their location in a sagittal section of rodent brain. A) The subventricular zone (SVZ) of the lateral ventricles (LV) contains an astrocyte-like stem cell population termed as type B1 cells (blue) that, unlike ependymal cells (grey), contact with lumen occasionally, showing a single cilium. Through asymmetrical divisions, Type B1 cells generate rapidly dividing, transit-amplifying cells, termed as Type C (green) which, in turn, give rise to immature neuroblasts or Type A cells (red). Those neuroblasts migrate through an astroglial scaffold toward the olfactory bulb, where they fully differentiate into granular neurons. B) The subgranular zone (SGZ) of the dentate gyrus of hippocampus also harbors an astroglial stem cell subpopulation (green). These cells generate directly immature neuroblasts , which can divide once (Type D1, red), migrate while undergoing differentiation (Type D2, type D3,pink) and integrate as granular neurons (brown).

4.1 Subventricular zone

The first population of adult neural stem cells in mammals is located in a specific niche along the SVZ of the lateral ventricles. Several types of cells can be distinguished within these niches including type B cells (slowly dividing astroglial cells and the *bona fide* adult neural stem cells), type C cells (transit-amplifying cells derived from asymmetric division of Type B cells), type A cells (immature neuroblasts derived from Type C cells) and ependymal cells lining the lumen of the ventricle. Although this is a general model found in rodents, some differences have been described in primates and humans (for a complete revision see Quiñones-Hinojosa et al., 2006). However, the accumulating knowledge on the neural stem cells biology, and their interaction with other elements of the niches, has transformed the description of the neurogenic regions into a dynamic process were the acquisition of new findings changes the model over the time. The evolution of this model has also derived in the inclusion of changes in the NSC isolation and maintenance protocols.

Once the presence of neural stem cells was demonstrated within the adult brain of mammals, the first question to address was the location of such cells and the neurogenic

region. Early studies reported the isolation of NSC from the striatal tissue of both rat (Gritti et al., 1995; Reynolds & Weiss, 1992;) and mouse (Richards et al., 1992). Nevertheless, the striatum is a relatively large region that is not consistent with data about NSC biology. Afterwards the investigations in this field confirmed that only SVZ tissue was able to generate cell cultures with stem cell properties, whether via explants (Kirschenbaum et al., 1994; Kirschenbaum & Goldman, 1995; Lois & Alvarez-Buylla, 1993) or neurospheres cultures (Morshead et al., 1994) were used. Subsequent articles which described the isolation of neural stem cells from striatum specified the inclusion of the SVZ region within the dissected tissue (Weiss et al., 1996) and confirmed that the results of neural stem cell isolation exclusively from SVZ were identical to those obtained from extensive anatomical regions containing the SVZ (Kukekov et al., 1997). These initial works emphasized the relevance of a fine orientated dissection for a successful NSC isolation protocol.

Nonetheless, the SVZ comprises a heterogeneous population, and those early reports did not reveal the cellular identity of the NSC. Probably, the first work addressing the NSC identity *in vitro,* was performed by Johansson et al., 1999. Marking ventricular cells with DiI (a lipophilic membrane stain that diffuses laterally to stain the entire cell), they concluded that NSC were actually ciliated ependymal cells. By contrast, Doetsch et al. (1999) reported that only GFAP expressing cells (marked via adenovirus which allowed the expression of the green fluorescent protein (GFP) under the control of glial fibrillary acidic protein (GFAP) promoter) give rise to neurospheres culture. Moreover, it was previously described that those astrocyte-like cells occasionally contacted the ventricle and displayed a single cilium, suggesting that DiI isolated cultures could be originated from them instead of ependymal cells (a more complete model can be consulted at Mirzadeh et al. (2008)). Following previous findings, two new studies described that both types of cells were able to proliferate *in vitro*, but only SVZ astrocytes generated neurospheres with self-renewal and mutipotential capacity (Chiasson et al., 1999; Laywell et al., 2000). These findings also marked the need for an identification and selection step in the NSPc isolation protocol. With the introduction of FACS technology, two phenotypical features of NSC supported the astrocyte-like theory, the isolation of a CD15+ population with stem cell characteristics (Capela & Temple, 2002), a carbohydrate only expressed in astrocytes, and the identification of NSC as CD24^{low} (Rietze et al, 2001), whereas ependymal cells are CD24+. Using an opposite strategy to deplete adult GFAP+ cells, two independent studies demonstrated that the ablation of adult astrocytes resulted in the loss of multipotent neurosphere formation (Morshead et al, 2003; Imura et al., 2003). However, with the controversy surrounding the identity of NSCs, two later studies reported the isolation of CD133+ cells from adult brain, as previously reported by Uchida et al. (2000) for fetal tissue. Nevertheless, opposite results were obtained from each one. The first one (Corti et al., 2007) concluded that CD133 stained a small number of cells underlying the ependymal layer, and the sorting of those CD133+ cells leads to the isolation of a NSC population. Interestingly, Mirzadeh et al. (2008) found that 29% of the apical processes of B1 cells were positive for CD133. By contrast, Coskun et al. (2008) found that both CD133+ ependymal cells and NSC originate from ependymal cells. In any case, it will be necessary to find new markers in order to improve the identification and selection of either the real adult neural stem cells and the different range of progenitor cells. This will allow the study of their specific biological features and maybe modulate their behavior *in vivo.*

4.2 Subgranular zone

In the adult hippocampus, NSCs are located in the SGZ, a cellular layer found between the granule cell layer and the hilus, in the dentate gyrus. Similarly to SVZ, NSCs have been identified as astrocyte-like cells, with cell bodies located in the SGZ and vertical processes extended through the molecular layer. However, unlike what happens in the SVZ, these astrocytic cells generate an earlier immature neuroblast that divides only once and expresses neuronal markers (PSA-NCAM and doublecourtin) (Type D1 cells). Those cells migrate short distances within the granule cell layer while undergoing morphological changes (type D2 and Type D3 cells) until they reach a final position and differentiate into mature granular neurons.

The first data from adult hippocampal cell cultures were published by Palmer et al., 1995. They isolated a monolayer culture from adult female rat hippocampus. These cells were capable of proliferating in serum free media supplemented with FGF. In Gage et al., 1995, they were also able to derivate a FGF-2 -dependent adherent culture that differentiates into mature neurons when engrafted into adult rat brain. Short after, *in vitro* differentiation was attempted by Palmer et al., 1997. Using several combinations of growth factors, they demonstrated the multipotency of those progenitor cells, and the enhancement of neuronal maturation when BDNF was added (similarly to NT3 and retinoic acid results), whereas serum addition promoted astroglial differentiation.

As described previously for initial reports on SVZ, those studies also isolated cells with similar features from non-neurogenic zones, including the septum of striatum. However a few years later was published the first work that isolated specific hippocampal neural stem cells from adult human samples (Kukekov et al., 1999). They observed the presence of neurospheres when cultured in non-adherent conditions by using mercaptoethanol, similarly to previous studies carried out with SVZ samples (Kukekov et al., 1997). Following the enrichment step based on the expression of GFP under the control of an specific promoter (P/Tα1:hGFP and E/nestin:EGFP), described previously to identify NSC from the SVZ, Roy et al., 2000b, isolated, for the first time, neural stem cells from human hippocampal samples. However, these results did not reproduce in mice and later works criticized the gross microdissection done in these publications. Afterwards, Seaberg & van der Kooy, 2002 tried to generate neurospheres from microdissected mouse dentate gyrus. They were unable to generate neurospheres capable of self-renewal, and multipotency was also compromised. Nevertheless, hippocampus obtained with gross dissection was able to generate neurospheres, suggesting that previous results could be explained if the dissection procedure included contaminating cells from the ventricular layer next to the hippocampus, or even white matter progenitors. They also assayed different culture conditions and dissection techniques, including dentate gyrus microdissection, and were unable to obtain proliferating neurospheres. After a number of publications showing controversial results in this regard Babu et al., 2007, using dentate gyrus microdissection from p/Nestin:EGFP transgenic mouse, were able to obtain monolayer cultures with self-renewal (up to 66 passages) and multipotency characteristics. Moreover, they observed spheres-like colonies when performing a modified neurosphere assay using semisolid medium. Additionally, after trying different media and supplements they concluded that although B27 and EGF promoted a slightly higher proliferation, N2 supplement and bFGF maintained cells differentiate better into mature neurons. Moreover, they reconfirmed that BDNF, NT3, SHH promoted neuronal differentiation, while LIF and Bone morphogenetic protein 2 (BMP-2) promoted glial differentiation

More recent works found that isolated neural progenitor/stem cells display paracrinal production of BMP, and the addition of noggin to culture media favors the formation of multipotential and self-renewal neurospheres (Bonaguidi et al., 2005; Bonaguidi et al., 2008).

The differences, in terms of culture media requirements, between NSC isolated from the SVZ and those from the SGZ might be due to their behavior *in vivo*. NSPc from lateral ventricle are prepared to migrate a larger distance to the olfactory lobes, while new neurons produced from dentate gyrus integrate nearby the stem cell niche and, therefore, are not prepared to maintain their stemness capacity in the absence of the niche signals.

5. Conclusions

Cell isolation and culture provides a powerful tool for the study of neural stem and progenitor cells. Although *in vitro* analysis has several limitations, and results cannot be directly extrapolated to the *in vivo* behavior of the isolated cells, it allows the analysis of their features and potential capacities in a controlled environment that can be modified and monitored more accurately.

Every step of the isolation procedure is likely to be optimized. Any protocol amendment should be tested and not considered trivial, as it can have a high impact on the cell population obtained. Consequently, isolation methods should be planned according to further experimental applications and not based on the routine practices of each research group, especially in the case of adaptation of protocols used previously for tissues collected from different species.

Moreover, it should be considered that the final purpose of most experiments is to improve our knowledge about stem cells and their clinical applications. For this reason, steps in the protocol which include reagents with undefined composition or with the possibility of introducing contaminants, such as serum, must be redesigned, because it is the only way to understand the chemical signals underlying the biological behavior of neural stem or progenitor cells.

6. Acknowledgements

7. References

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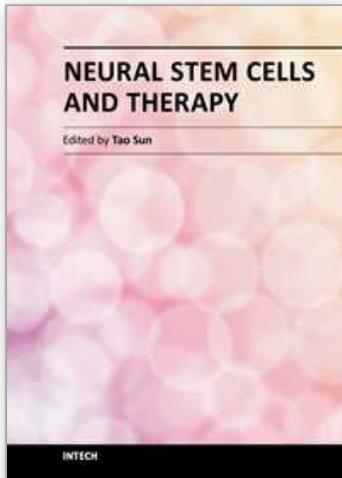
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This book is a collective work of international experts in the neural stem cell field. The book incorporates the characterization of embryonic and adult neural stem cells in both invertebrates and vertebrates. It highlights the history and the most advanced discoveries in neural stem cells, and summarizes the mechanisms of neural stem cell development. In particular, this book provides strategies and discusses the challenges of utilizing neural stem cells for therapy of neurological disorders and brain and spinal cord injuries. It is suitable for general readers, students, doctors and researchers who are interested in understanding the principles of and new discoveries in neural stem cells and therapy.

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