The neurosphere assay: an effective *in vitro* technique to study neural stem cells

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Neural stem cells (NSCs) are known to be present in the adult mammalian brain where they constitutively differentiate into the neuronal, astroglial, and oligodendroglial lineages, in defined processes termed neurogenesis, astrogliogenesis and oligodendrogenesis, respectively (reviewed in Braun and Jessberger, 2014). During brain development, NSCs are present throughout the brain, becoming progressively restricted to defined brain regions. In the adult brain, NSCs are mainly present in areas classically known as neurogenic niches, i.e. the subventricular zone (SVZ), along the lateral walls of the lateral ventricles, and the subgranular zone, located in the dentate gyrus (DG) of the hippocampus. These areas are particularly enriched with NSCs, which not only are multipotent cells but also proliferative cells with the ability to self-renew, thus maintaining their own pool of cells. In fact, neurogenesis, astrogliogenesis and oligodendrogenesis are highly intricate processes comprising several steps, including proliferation, differentiation, migration, and functional integration of the newly formed cells in the existing circuitry, which are regulated by a plethora of factors. These newly differentiated adult-born cells have the capacity to continuously modulate brain function and plasticity, by constantly reacting to external or internal stimuli (reviewed in Braun and Jessberger, 2014).

In the 1960s, Altman and Das (1965) described for the first time the presence of dividing cells in the brain which were later demonstrated to be adult neural stem cells-derived neurons. Ever since its first description, neurogenesis has been extensively studied in physiological and pathological contexts, being considered a promising therapeutic avenue in both neurological and neuropsychiatric disorders (Braun and Jessberger, 2014). Physiologically, SVZ neurogenesis contributes to the olfactory system plasticity, whereas in injury or pathological conditions (e.g. stroke), the newborn neurons may repopulate areas from the striatum to cortex (Sawada et al., 2014). Beyond SVZ neurogenesis, SVZ NSCs can also give rise to oligodendrocyte precursor cells. In pathological contexts (e.g. multiple sclerosis), these cells are able to migrate to damaged sites, helping to restore the myelin sheaths (Xing et al., 2014). In the hippocampus, neurogenesis plays a critical role in learning, memory and cognition, as well as in mood regulation and response to stress, being affected in neurodegenerative (e.g. Alzheimer's disease) and neuropsychiatric (e.g. depression) disorders. In particular, DG newborn neurons support hippocampus-dependent memory by being involved in the reorganization of brain circuits (Braun and Jessberger, 2014). Apart from DG neurogenesis, DG NSCs have also the capacity to originate astrocytes, which play fundamental roles in the regulation of neurogenesis but also of synaptic activity and plasticity (Steiner et al., 2004).

Undoubtedly, by assessing NSCs behavior in the adult brain, a vast number of repair strategies can be tested for a myriad of neurological and/or neuropsychiatric disorders, specifically by replenishing either neuronal, astroglial or oligodendroglial loss or disfunction. Importantly, several *in vivo* and *in vitro* techniques have been developed to further explore NSC biology. Recently, our group demonstrated the potential of an *in vitro* technique established more than two decades ago, the neurosphere assay (NSA) to obtain high yields of NSCs from the main neurogenic niches and to study the inherent properties of the NSCs. The NSA is a simple, yet effective, method used to obtain a renewable source of undifferentiated cells that can be easily expanded in culture (Soares et al., 2020).

Firstly described by Reynolds and Weiss (1992), the NSA constitutes a very useful technique to study NSC biology and dynamics, bypassing some of the deterring issues involved in the use of animal models, namely the large number of necessary animals, the exposure of animals to toxic substances, and the limited amount of obtained tissue. The NSA is a powerful *in vitro* technique that allows studying NSC biology in controlled conditions. Cells are cultured in a chemically defined serum-free medium and in the presence of specific growth factors, the epidermal growth factor and the basic fibroblast growth factor (Soares et al., 2020). NSCs are epidermal growth factor- and basic fibroblast growth factor-responsive and, when exposed to these mitogens, enter a period of active proliferation, while differentiated cells do not survive (Soares et al., 2020). In these conditions, NSCs grow in spherical aggregates called neurospheres which can be passaged to further expand the pool of these cells. Indeed, we have demonstrated that both primary neurospheres and neurospheres that were passaged are composed by cells positive for NSC markers, namely SOX2 and Nestin. The expansion and precise selection of NSCs using this assay allows obtaining a higher yield of NSC clones from a limited amount of tissue, thus reducing the number of used animals (Soares et al., 2020). These cells, grown as characteristic sphere-shaped structures in suspension, when seeded and withdrawn from the mitogens in the medium, form a pseudo-monolayer of heterogeneous cells composed by cells that migrate out of the neurosphere, including NSCs and differentiated cells from the three neural lineages (Soares et al., 2020; **Figure 1**).

The NSA has been tested and used in several phylogenetically distinct animal groups in which neurogenesis has been proved to exist, such as mammals (e.g. mouse, rat and human), fish, fruit fly and birds (reviewed in Chapouton et al., 2007; Fernández-Hernández et al., 2013) to study NSC proliferation, self-renewal and viability along with both neuronal and glial differentiation, in physiological and pathological contexts. Importantly, beyond the NSA capability to evaluate the properties of postnatal NSCs, this assay can also be used to investigate neurodevelopment at the cellular and molecular levels. Moreover, genetically modified animals or animal models of disease can be studied using the NSA to further understand the mechanisms that regulate neurogenesis in those specific conditions. Importantly, cells can be processed for molecular analysis for RNA and/or protein expression, for immunolabelling to evaluate the expression of several neural markers, as well as electrophysiological studies and single-cell calcium-imaging to study the function of the newborn cells. The heterogeneity of the cells in culture makes this assay a potent tool to mimic the microenvironment of the niche. Importantly, there are several methods associated with the NSA that can be performed to access different properties of NSC, in particular stemness (self-renewal and cell-pair assay), proliferation (proliferation assay), differentiation (differentiation assay), and cell survival (cell survival assay) (Soares et al., 2020; **Figure 1**). In fact, using these assays allows investigating the actions of intrinsic or extrinsic factors on NSC properties (**Figure 1**). All these well-established advantages and the simplicity of the technique, when

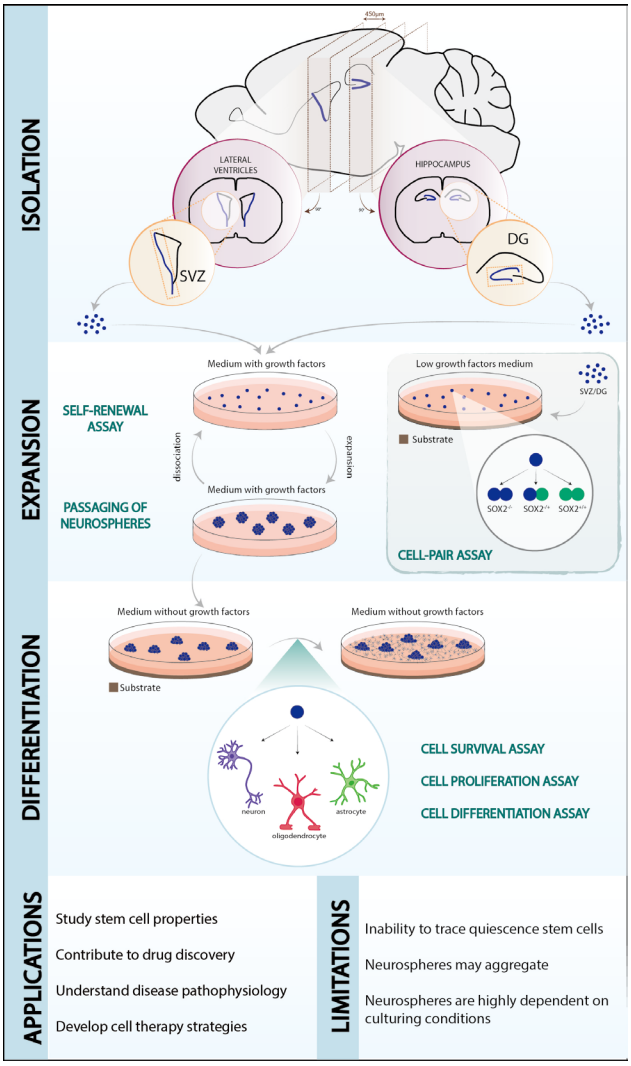

Figure 1 | **Schematic representation of the neurosphere assay, including its applications and limitations.**
Isolation of SVZ- and DG-derived neural stem cells (NSCs) from 450 µm coronal sections containing the lateral ventricles and the hippocampus, respectively. SVZ and DG NSCs are expanded in serum-free medium with growth factors to form neurospheres in suspension. Cell passage can be performed several times, in which the main step is the dissociation of the neurospheres. The self-renewal and the cell-pair assays can be used to access the self-renewal capacity of the NSCs. For the cell-pair assay, beyond the SOX2 marker, it can also be used another NSC marker, namely Nestin as well as a marker for differentiated cells (e.g. doublecortin for immature neurons). Primary or passaged neurospheres can be plated in coated plates in medium without growth factors, allowing the differentiation of SVZ and DG NSCs into neurons, oligodendrocytes and astrocytes. In differentiative conditions, cell survival, cell proliferation and cell differentiation assays can be performed. Some applications and limitations of the NSA are displayed on the bottom of the figure. DG: Dentate gyrus; SVZ: subventricular zone.

compared to other protocols (e.g. monolayer culture system), are the reason why this methodology has been widely used by many researchers in very different areas.

Recent studies have suggested that adult neurogenesis not only exists in the SVZ and the DG but also in other brain regions such as the hypothalamus, amygdala, striatum and septum, cortex, spinal cord or substantia nigra (reviewed in Jurkowski et al., 2020) as well as in the dorsal vagus complex, optic nerve or auditory system (Charrier et al., 2006; Völker et al., 2019; Bernstein et al., 2020). The source of newborn neurons is still under debate, with some evidence supporting the idea of endogenous pools of NSCs located at these non-canonical neurogenic niches while others support the migration of NSCs from the classic niches to these novel sites. Notably, these areas can be screened for NSCs using the NSA together with the evaluation of markers for NSCs (SOX2 and Nestin), proliferation (BrdU, a thymidine analogue) and multipotency (TUJ1, O4 and GFAP, for neuronal, oligodendroglial and astroglial lineages, respectively) (Völker et al., 2019; Jurkowski et al., 2020). Indeed, the NSA has been extensively used to characterize the sub-regions of the rodent hypothalamus in terms of neurogenic potential. Additionally, progenitor cells isolated from the adult human cortex when cultured in the presence of growth factors were able to form multipotent neurospheres. A recent study in mice demonstrated that precursors cells from the basolateral amygdala have the ability to proliferate in a neurosphere-like shape and to form mature neurons (reviewed in Jurkowski et al., 2020). Interestingly, other studies in rodents identified NSCs in the dorsal vagal complex, auditory pathway as well as in the optic nerve lamina region revealing stemness properties through the NSA (Charrier et al., 2006; Völker et al., 2019; Bernstein et al., 2020). Although neurospheres generated from the non-canonical neurogenic niches corroborate the hypothesis of the existence of a neurogenic potential in these areas, this potential needs to be further explored *in vivo*.

This method is highly versatile and can be adapted according to the experimental protocol. Indeed, variations of the NSA have been applied to other fields of study namely cancer biology, cardiology and enteric nervous system to generate tumorspheres, cardiospheres and enterospheres, respectively. In fact, since it was firstly described, several other sphere-formation assays have been published using other types of cells (reviewed in Deleyrolle et al., 2008).

Although this assay is highly relevant to study neurogenesis, oligodendrogenesis and astrogliogenesis derived from NSC and to identify region-specific factors involved in these processes, it has some limitations (**Figure 1**). Firstly, it does not take into account quiescent NSCs and the fact that neurospheres are composed not only of NSCs but also of progenitor cells, which have a more limited capacity of self-renewal. Therefore, counting the number of neurospheres is not an accurate measure to determine stem cell number and self-renewal properties. However, this limitation can be overcome by simultaneously measuring neurosphere diameter, given that progenitors, due to their reduced capacity to generate stem-like cells, originate smaller neurospheres. On the other hand, a direct correlation between the number of neurospheres with the number of NSCs is difficult since neurospheres may aggregate. The possibility of aggregation is proportional to cell density. Therefore, to solve this potential problem, cells can be expanded as single cells. Cell passaging, while advantageous to increase NSC yield, decreases cell viability with the increase in the number of passages (Soares et al., 2020). Moreover, the presence of high concentrations of growth factors have shown to deregulate the differentiation potential

and spatial identity of NSCs grown in the NSA (Hack et al., 2004). Additionally, the pseudo-monolayer used to study differentiation, although not completely bi-dimensional, might represent a limitation, which can be improved by using three-dimensional culture systems more similar to physiological conditions. Finally, despite its simplicity, this method requires an experienced researcher in this technique to fine tune all the different steps of the protocol, since the resulting neurosphere frequency is highly dependent on the dissection procedure, the dissociation process, as well as the components present in the medium (Soares et al., 2020). Additionally, the inherent properties of the regions where the NSCs are isolated from can also interfere with the neurosphere-forming capability. Indeed, SVZ-derived NSCs present a higher proliferation rate *in vitro* than the DG-derived NSCs (Seaberg and van der Kooy, 2002).

Importantly, similarly to what happens *in vivo*, there are differences in the neurogenic potential of cells derived from the SVZ and the DG. For example, the capacity of generating oligodendrocytes is much higher in SVZ- than DG-derived NSCs (Palmer et al., 1997). Consequently, this assay preserves *in vivo* cell lineage, showing limited capacity to increase cell potency. Furthermore, the substrate in which neurospheres are plated is essential to not only guarantee good cell migration out of the neurospheres, without compromising the differentiation process, but also to influence which cell type is predominantly generated during differentiation (e.g. promotion of oligodendrocyte *versus* neuronal differentiation) (Soares et al., 2020). In fact, this shows to be a good opportunity to study the influence of substrate and medium composition in the behavior of neurosphere-derived cells, in terms of proliferation, differentiation and migration.

Overall, the NSA contributed to the evolution of a new area of research, regenerative neurobiology and, ultimately, challenged and helped to overcome the "no adult neurogenesis" dogma suggested by Ramon y Cajal. Additionally, it has also broadened our understanding of the mechanisms underlying several brain disorders, opening the door to the possibility of designing innovative regenerative therapeutics. More than 25 years have passed since the NSA was first described and, since then, it has been subjected to intense use and scrutiny to be validated as a viable technique. It has now been proven to be a promising *in vitro* model to demonstrate the intrinsic properties of NSCs and to explore the mechanisms behind NSC dynamics (**Figure 1**).

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