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REVIEW

Neurospheres: a potential in vitro model for the study of central nervous system disorders

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Abstract

Neurogenesis was believed to end after the period of embryonic development. However, the possibility of obtaining an expressive number of cells with functional neuronal characteristics implied a great advance in experimental research. New techniques have emerged to demonstrate that the birth of new neurons continues to occur in the adult brain. Two main rich sources of these cells are the subventricular zone (SVZ) and the subgranular zone of the hippocampal dentate gyrus (SGZ) where adult neural stem cells (aNSCs) have the ability to proliferate and differentiate into mature cell lines. The cultivation of neurospheres is a method to isolate, maintain and expand neural stem cells (NSCs) and has been used extensively by several research groups to analyze the biological properties of NSCs and their potential use in injured brains from animal models. Throughout this review, we highlight the areas where this type of cell culture has been applied and the advantages and limitations of using this model in experimental studies for the neurological clinical scenario.

Keywords Neurospheres · Neural stem cell · Cell model · Differentiation · Neurogenesis · Neurological disorders

Abbreviations

AD	Alzheimer's disease
aNSCs	Adult neural stem cells
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblastic growth factor
βT4	β4 Tubulin
CA	Cellular automata
CC	Corpus callusum
CD133	Prominin-1
CNS	Central nervous system
DG	Dentate gyrus
EAE	Experimental autoimmune encephalomyelitis
eGFP	Enhanced green fluorescent protein
EGF	Epidermal growth factor
EGL	External germ layer of the cerebellum
ENU	N-ethyl-N-nitroso-urea
$GABA_A$	Gamma-aminobutyric acid type A
$GABA_B$	Gamma-aminobutyric acid type B
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate/aspartate transporter
hCNS-SCns	Human central nervous system stem cells grown as neurospheres
hiPSC	Human induced pluripotent stem cells
HD	Huntington's disease
HES1	Hairy/enhancer of split-1
HNPC	Human neural progenitor cell
MGE	Medial ganglionic eminence
MRI	Magnetic resonance image
MS	Multiple sclerosis
NOTCH1	Notch homolog 1
NSA	Neurosphere assay
NPCs	Neural precursor cells
NSCs	Neural stem cells
OLIG2	Oligodendrocyte transcription factor
PBT	Pediatric brain tumor
PS1	Presenilin1
p53 PFT-α	P53 inhibitor pifithrin-α
PD	Parkinson's disease
SCZ	Subcallosal zone
SGZ	Subgranular zone

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SOX2	SRY-box transcription factor-2
SVZ	Subventricular zone
TH	Tyrosine hydroxylase
TLE	Temporal lobe epilepsy
Tuj 1	III beta-tubulin

Introduction

The stem cells have a high capacity for proliferation and self-renewal, differentiation in the main lineages of their original tissue and tissue and/or organ regeneration. Neural stem cells (NSCs) are proliferative cells that give rise to neurons and glial cells and are present in the embryonic, neonatal brain and in some regions of the adult central nervous system (CNS). The use of NSCs to promote the repair of brain injuries has proved to be an efficient and promising approach in neurology field [1, 2]. Since the first publication in 1992 [3], many research groups have investigated the mechanisms of different neurodegenerative diseases using in vitro models, especially neurospheres. In this review, we describe the principles of neurosphere assay and analyze the different aspects of research with neural stem cells, highlighting the main implications of an in vitro model that can help explain the outcomes of some neurodegenerative diseases.

Neurodevelopment

During the development of the CNS of mammals, neurons and neuroglial cells (astrocytes and oligodendrocytes) are generated from multipotent cells, with neurons being the first cells generated in the process, mainly during the embryonic period. Historically, neurogenesis was believed to cease at the end of embryonic development. However, today we know that this process is continuous throughout life and occurs in different regions of the adult brain [4].

The understanding of adult neurogenesis has progressed significantly and we already have great knowledge about the biology of this phenomenon, from the location, proliferation and specification of the fate of neural stem cells migration, neuronal maturation and synaptic integration of newborn neurons [5, 6]. Neural stem cells per se generate distinct cell types, the progenitors. These are proliferative cells, but they originate cells more restricted to the lineage and these neuronal and glial progenitors cannot self-renew and express some lineage marker gene transcripts. With this, we can efficiently analyze these new cells through the isolation and in vitro analysis of the progenitors derived from the adult CNS, opening possibilities for studies in the repair of the CNS after injuries, using cell replacement therapy in the repair of degenerative diseases [7, 8].

In recent decades, the development of refined techniques has resulted in a lot of new research showing that neurogenesis, the birth of new neurons, occurs in limited and specific regions of the adult brain, with significant numbers of neurogenic cells. In mammals, the process of neurogenesis occurs in brain regions called “niches”, where NSCs are present. These regions include the subgranular zone of the hippocampal dentate gyrus (SGZ), subventricular zone of the lateral ventricles (SVZ), external germ layer of the cerebellum (EGL), subcallosal zone (SCZ), corpus callusum (CC), among others. However, the regions most frequently used by researchers to study the neurogenesis and isolation of NSCs are mainly the SVZ and SGZ of the hippocampal dentate gyrus, which we will report throughout this review [9, 10].

Both regions are responsible for maintaining neural precursor cells and generating new neurons. However, on the side walls of the lateral ventricles is the largest germinative zone of the brain of adult mammals. The SVZ is the region responsible for highest production of neuronal cells in the embryonic and adult phases, generating highly proliferative progenitors that can be used for neuroregenerative therapy [9, 11].

Neural precursor cells can actively participate in the repair process and intrinsically can synthesize many molecules useful for tissue regeneration. They constitute an extremely diverse population of cells, with specific morphology and markers of their germinal regions, exhibiting different characteristics and functions depending on their proliferative state and region. They were primarily isolated from the CNS of rodents by means of cell culture and these cells can be expanded and modified, maintaining their multipotentiality in many passages. Because it is easy to obtain, the manipulation of NSCs allows us to accurately analyze the intrinsic and extrinsic mechanisms that are involved in neurogenesis [3, 12, 13].

The existence of a type of multipotential stem cell in the CNS supports the findings that precursor cells derived from different regions of the developing and adult brain have similar properties. Adult neurogenesis in the hippocampus and olfactory bulb is an extremely dynamic process. Its regulation occurs through different points of stimulation and the understanding by which mechanisms this phenomenon happens can significantly enrich our knowledge about the etiology and pathophysiology of some neurological diseases [14, 15].

The neurogenic process needs precise control, since any physiological change can interrupt the process, causing apoptosis of the neuroprogenitors and deregulation related to hippocampal hypoplasia and neurodegeneration. In Parkinson’s disease (PD), for example, the depletion of dopaminergic neuronal circuits is associated with a neurogenic deficiency observed both in the SVZ and in the SGZ of the

dentate gyrus. However, the increase in neurogenesis is not necessarily beneficial and may be associated with other pathologies [16, 17]. Some studies suggest that exacerbated neurogenesis, in response to brain damage, is related to the development of epilepsy and its reduction may be involved in the pathophysiology of psychiatric disorders and neurodegeneration disease [18–20].

Studies show that NSCs respond to different stimuli during embryonic and adult development, especially those related to proliferation, such as growth factors. In vitro, these cells renew themselves by signaling, dividing and migrating from the matrix cell cluster—called spheres—generating new cells, such as neurons and glial cells, in addition to new NSCs that will form new matrix spheres after mechanical dissociation of in vitro-grown spheres. The spheres generated contain the same undifferentiated phenotype as the original sphere, thus demonstrating that NSCs have a high potential for self-renewal. Due to these characteristics, these cells are a promising tool for the study of neurodevelopment, as well as for identifying the etiology of different neurodegenerative diseases [15, 21].

The mechanisms that control neurogenesis are not yet fully elucidated, especially in the postnatal period, but it is known that changes in its process are involved in different pathologies. For this reason, therapeutic strategies focused on the neurogenic process need to be tested in preclinical and clinical trials.

Neurospheres

The SVZ and SGZ of the hippocampus are neurogenic regions in the adult brain that contain multipotent cells that renew and differentiate into all types of neural cells. The NSCs generated in these regions of adult mammalian brain play an important role in replacing post-mitotic cells and, consequently, in regenerative repair after injury [22, 23].

To isolate and expand NSCs, a culture system known as the neurosphere assay (NSA) is performed in order to generate primary cells capable of differentiating into the three main cell types of the CNS [24]. In vitro neurospheres were demonstrated to derive each from one neural stem cell and to consist in a clone of progenies including neural progenitors and new stem cells [25]. Neurosphere-constituting cells are non-adherent. Mature spheres display variable sizes (between 100 and 200 µm) and, once deprived of the mitogenic growth factors (EGF, bFGF), adhere to the substrate and display cell differentiation into neurons, astrocytes and oligodendrocytes [3, 25, 26].

The NSCs and patterns within the spheres may have specific regional and temporal characteristics in relation to growth, differentiation and specific gene expression in the region. But, regardless of regional origin, all neurospheres contain cells of different subtypes and are able to maintain the molecular patterns of expression of specific region genes across in vitro passages [27, 28].

Neurospheres generated from the different regions of the CNS express unique markers for each region, indicating that the original cells were in fact specified regionally. Since its discovery, new sources of neurosphere-forming cells have been investigated and different culture protocols have been developed to maintain, expand and differentiate them, thus allowing the characterization of these neural progenitors soon after isolation [29, 30].

Neurosphere culture is an excellent tool for obtaining and expanding neural and progenitor stem cells, in addition to allowing to analyze their properties under controlled conditions. Due to their high proliferation capacity, neurospheres can be dissociated and cultivated in many passages [31]. A large number of cells derived from SVZ and SGZ can be obtained after isolation and expansion, when in media enriched with growth factors. After being differentiated into a neuronal lineage, the cells have great potential for studies in the field of neurosciences such as to test new drugs, assist in clarifying and preventing the progression of neurodegenerative diseases and even elucidating the processes involved in embryogenesis [11, 32].

The proliferation of NSCs continues on the side walls of the lateral ventricle in the brain in the postnatal and adult periods. All neurosphere cultivation methods previously reported by researchers have been optimized for working with NSCs isolated from rodents of different ages. However, neonatal SVZ is more dense and matures from birth to mature organization, around 15 postnatal days [30, 33].

The most important characteristics that the cell model using neurospheres can offer are (1) the identification of neural stem cells; (2) the simplicity of the technique; (3) serve as a starting point for studies of molecular and biochemical mechanisms of neurodevelopment and (4) a means of tracking factors that may trigger changes in neurogenesis and trigger CNS diseases.

Neurosphere assay

In general, in order to obtain neural stem cells and neural progenitors in culture, and generate the neurospheres, it is necessary to follow 5 fundamental steps: obtaining the tissue, dissociation, cell isolation, cultivation and maintenance (Fig. 1). Firstly, it is necessary to extract the nervous tissue, in this case the brain, and secondly the region of interest, which may be SVZ or SGZ. Then, the tissue needs to be dissociated enzymatically using enzymes such as trypsin/EDTA, papain, accutase and collagenase, followed by mechanical dissociation [11, 34, 35]. The cell pellet generated is cultivated on non-adherent substrate using a serum-free growth medium with mitogenic growth factors in a humidified incubator at 37 °C and 5% CO_2 [11]. The cells

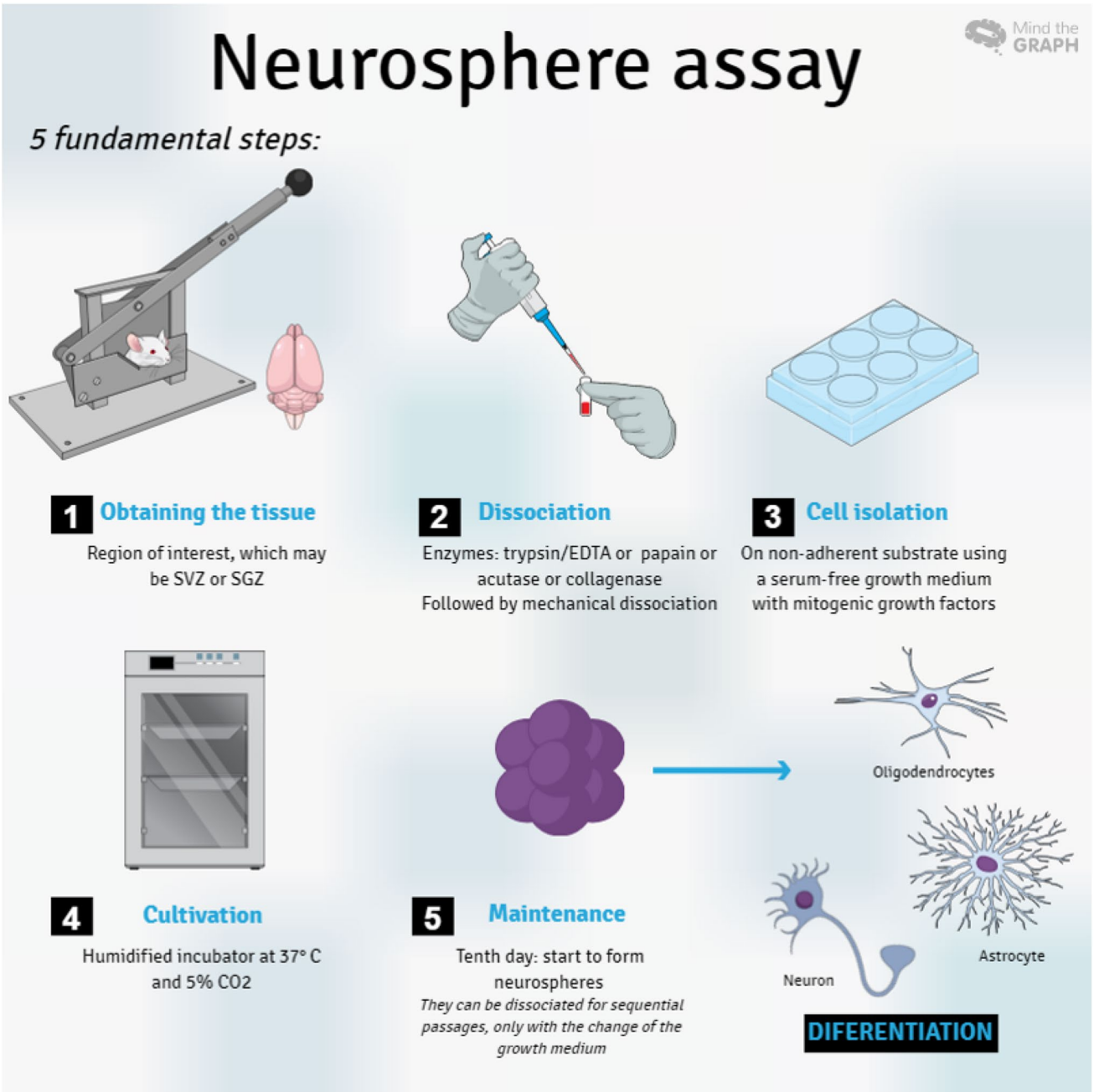

Fig. 1 Five fundamental steps for neurosphere assay from mouse cerebral tissue. The generated neurospheres, being dissociated in multiple passages or not, remain in suspension of single cells until they are cultured in adherent plates coated with poly-L-lysine and finally, when removing growth factors from the medium, adhesion to the substrate and differentiation into neurons, astrocytes and oligodendroglia occurs

of the seeded tissue start to form neurospheres from the tenth day and they can be dissociated for sequential passages, only with the change of the growth medium [36]. The generated neurospheres, being dissociated in multiple passages or not, remain in suspension of single cells until they are cultured in adherent plates coated with poly-L-lysine and finally, when removing growth factors from the medium, adhesion to the substrate and differentiation into neurons, astrocytes and oligodendroglia occurs [27, 35–39].

The cultures generated from the SVZ and SGZ regions have similar morphological characteristics. However, SVZ-derived cells proliferate more quickly, forming larger neurospheres, with a much larger number of neural precursor cells residing in SVZ compared to SGZ [38]. From these and

other evidences, the SVZ became the most isolated region for the realization of the NSA.

Culture medium and maintenance

The growth medium is basically composed by DMEM/F12—Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 supplemented with 100 U/ml penicillin/streptomycin, 2 mM GlutaMAX, 20 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF), 2% B-27 and 1% N2 supplement, the latter is not mandatory [11, 36, 39, 40].

The neurospheres culture is non-adherent, so to maintain this pattern, these cells are grown in the absence of serum and in untreated plates, because components of serum and the adhesion treatment of conventional plates favor the differentiation of NSCs and neural progenitors from irreversible way [11]. It is known that supplementation with serum promotes growth and stimulates the proliferation of cells in culture. In this cell culture model, these processes are promoted through supplementation with B-27/N2, compounds that act on cell growth, viability and survival of embryonic, post-natal and adults nerve cells of the hippocampus [39, 41].

To keep the neurospheres undifferentiated, it is necessary to supplement the medium with growth factors, especially EGF and FGF. In neurospheres, EGF increases the proliferation and survival of NSCs and neural progenitors, in addition to keeping them undifferentiated, while FGF activates neurogenesis and self-renewal [42]. Therefore, it is recommended that these growth factors be combined in the neurospheres growth medium and added to cells every 3 days to maintain undifferentiated status [11, 43].

In addition to these supplements, the culture of neurospheres requires the addition of micronutrients such as L-glutamine. This amino acid is an energy source for cells, being present in different culture media. Cells that divide rapidly, such as neurospheres, prefer media rich in L-glutamine, as these cells have a high rate of proliferation, need to rapidly synthesize proteins and nucleic acids and L-glutamine assists in these processes. However, after metabolization this compound is converted into ammonia, being toxic to cells, so the culture medium needs to be constantly renewed. While neurospheres are being formed, the basal environment is not renewed, it is only supplemented with mitogenic factors. So that L-glutamine does not hinder the development of neurospheres, this culture is supplemented with GlutaMAX, a stable component in an aqueous solution that is not spontaneously degraded and provides energy to the cells as well [11, 38].

All of these components must be added to the DMEM/F-12 basal medium, a versatile medium with high concentrations of glucose, amino acids and vitamins, ideal for the growth of neurospheres [11].

Morphology and phenotypic characterization

When we look at neurospheres in a phase-contrast microscope, it is possible to observe shiny spheres, with varying sizes (between 100–200 µm), composed of hundreds to thousands of cells. Generally, they have a clear and rounded nucleus with dispersed chromatin and two nucleolus. Neurospheres have morphological diversity, where larger spheres (that exceed 200 µm) generally have a darker core, with denser cells inside [44–46]. This indicates that in this region there is a high rate of apoptosis, because the larger the spheres the less nutrients reach the cells located in its center.

Regarding their phenotypic characterization, neurospheres are subjected to analysis of molecular markers and gene expression of each cell population. The NSCs do not have a specific molecular marker described so far, which makes it difficult to characterize [11]. However, these cells can transiently express the intermediate filament protein, nestin. This protein is found in nerve cells and can be used as a marker of neural precursors. In addition to NSCs and neural precursors, neurospheres have other cell types inside, which have molecular markers, which are: (1) type B cells or astrocytes expressing glial fibrillary acidic protein (GFAP), high-affinity glutamate/aspartate transporter (GLAST) and CD133; (2) type C cells expressing SRY-box transcription factor-2 (Sox2) and oligodendrocyte transcription factor (Olig2); (3) type A cells what expressing III beta-tubulin (Tuj1). Neurospheres are also positive for the cell cycle marker Ki-67, indicating that these cells have a high proliferation rate [11, 47, 48]. Thus, the presence of these different markers in the neurospheres indicates that this cell population is naturally heterogeneous and composed of cells in different stages of differentiation such as stem cells, proliferating neural progenitors and post-mitotic neurons and glia [41, 47, 48].

Advantages and disadvantages

The NSA assay was the first in vitro model able to demonstrate that the adult brain has effective neurogenic regions composed of a large number of neural stem cells, making it an important tool to evaluate proliferation, self-renewal and multipotency of stem cells and neural progenitors. The neurosphere culture is an excellent model to study neurogenesis, neuronal development and neuroregeneration, because they are easy to manipulate and respond well to extrinsic stimuli present in your microenvironment [41]. Unlike most primary cultures of CNS, neurospheres are relatively easy to grow, subject to multiple passages and able to differentiate into neurons and glial cells, making it an essential tool for

studying aspects of the developing brain and CNS pathologies that need effective medical treatment [11, 49].

However, due to its heterogeneous character and variations in methodology, the NSA should be used with great caution. A disadvantage of most protocols for this type of culture has been the need to use a relatively large number of animals, because the yield of cell isolation is generally low [38] and this variation in cell density can generate changes in the microenvironment, affecting the proliferation. In addition, the concentrations of mitogenic factors in the culture medium, dissociation technique and number of passages are factors that can alter the composition and properties of neurospheres, interfering mainly in their neurogenic potential [41, 50, 51]. Another disadvantage in this culture system is that the neurospheres, when transplanted into the brain for therapeutic purposes, mostly differentiate into glial cells, generating few neurons [41]. Therefore, this technique becomes more useful in the regenerative medicine of glial and non-neuronal diseases.

The combination of the progressive loss of neurogenic potential after several passages with the low neuron yield reveals the need for refinement of this culture system, which supports greater expansion of stem cells and neural progenitors, increasing their capacity for neuronal differentiation. Thus, with a very robust system, we can expand our knowledge about NSCs and their therapeutic applications.

Current research scenario

The isolation of CNS neural precursors, from embryonic and adult tissue, through the neurosphere formation assay was first described in 1992 by Reynolds and collaborators and since then the application of culture protocols for the isolation of NSCs has already been seen as a promising strategy for a better understanding of the behavior of different types of NSCs [3, 52].

Since these cells were first described in the brain of mice, the use of fetal brain tissue in both, cell therapy and transplants fields has received a lot of attention. Expectations and great interest were generated in the possibility of NSCs becoming an opportunity for treatment of neurodegenerative disorders [53, 54]. The biggest problem yet to be solved is the fact of how to direct and control the differentiation of specific cell phenotypes necessary for the replacement and repair in each disease.

Neurospheres offer an excellent source of neuroprogenitors for the study of neuronal development and differentiation. These multicellular spheres are capable of reproducing functions characteristic of the developing brain, such as proliferation, migration and differentiation. In neurosciences, the application of stem cells and neuroprogenitors has been studied with a focus on the treatment of neurodegenerative diseases such as sclerosis, stroke, cancer and trauma in order to recover damaged tissues [55].

In Table 1 we compile the main studies related to neurological disorders, including Alzheimer, Parkinson, demyelinating diseases, epilepsy and glioma associated with degeneration, which used the neurosphere assay as an investigation tool. Several of these studies have used human fetal brain tissue, human forebrain and rodent brain to isolate and cultured neural progenitors, as neurospheres, and apply them as therapy. However, most researchers use rats and mice as experimental models for obtaining, studying and therapeutically applying neurospheres [56–60]. Therefore, throughout this discussion we will focus on experimental modeling.

Among these studies, we can highlight the promising results found by Kuvacheva and collaborators (2015) who carried out a prominent study for Alzheimer's disease (AD). The study was carried out in wistar rats with the aim of evaluating the β-amyloid neurotoxicity and maintaining the development of neural progenitors. To this end, the authors evaluated the in vitro development of brain progenitor cells isolated from healthy Wistar rats and Wistar rats with Alzheimer's disease. The experimental model for AD was modeled through steroid-guided injection of β-amyloid in the CA1 field of the hippocampus. In animals with experimental AD, a slow growth of the neurosphere was observed compared to healthy animals. Subsequently, the neurosphere stabilization and expansion phase was practically absent in cells isolated from the brain of animals with AD, with the cell index gradually decreasing. These findings reveal a more intense proliferation and greater potential for neurospheres repair in healthy animals compared to the experimental model of AD [58].

In 2004, Wennersten and co-authors showed that neurospheres were strong candidates for therapeutic transplantation in neurological diseases that result in cell damage. The study evaluated the rate of proliferation, migration, and differentiation of human neural stem/progenitor cells after transplantation into a rat model of traumatic brain injury. The NSCs were obtained from 10-week-old human forebrain and, soon after a parietal cortical contusion injury was performed, the animals received the progenitor cell transplant. After a few weeks, the researchers realized that human cells were present in regions such as the perilesional zone, hippocampus and callosum body, showing that NSCs proliferate and survive after transplantation, with good migration rate and differentiation in the injured brain [60].

In neurological diseases where there is a disorder in CNS cell activity that generates recurrent symptoms, as in the case of epilepsy, the use of NSCs has been widely studied. In a model of epilepsy, Romariz et al. performed an assessment between the anticonvulsant potential and neurodifferentiation of neurospheres derived from medial ganglionic eminence (MGE) with newly isolated cells being

Table 1 Update of neural disorders using neurospheres as an in vitro model

Target	Major findings	References
Alzheimer's disease		
Proliferation of neural progenitors	The rate of formation of neurospheres from precursor cells is reduced in Alzheimer's disease. The experimental AD model showed that the proliferative rate of precursor cells and the formation of neurospheres is slower in animals with AD than in the healthy group and this is due to the neurotoxicity of the β-amyloid protein. In addition, the animals showed a decrease in the cell index, as well as in the stabilization and expansion of the neurospheres	[58]
Brain injury		
Transplanting neural progenitors	Stem cells and expandable neural progenitors from 10-week-old human forebrain survive in the brain of Sprague–Dawley rats, being able to migrate and proliferate in regions such as the hippocampus, corpus callosum, ipsilateral subependymal zone and contralateral cortex. Moreover, in these zones astrocytic and neuronal differentiation occurred. These cells demonstrate great potential for transplant therapy in cases of traumatic brain injury	[60]
Degenerative diseases		
Tissue regeneration	The rodent olfactory bulb is a source of stem cells and neural progenitors with the potential to become specific cell types for tissue regeneration of damaged tissues such as: brain, spinal cord and muscle. These cells can engraft different organs, migrate, proliferate and differentiate, as well as participate in the cell–cell interaction of the host	[79]
Demyelinating diseases		
Demyelinated spinal cord	Neural progenitor cells, derived from adult human brain, can generate functional cells, with characteristics similar to schwann cells, in demyelinated spinal cord of adult rats and promote extensive remyelination of axons	[63]
Monitoring of glial progenitor cells	Glial progenitor cells derived from newborn Lewis rat, magnetically labeled, can be screened by MRI after transplantation in rodents with myelin disorders. This technology proved to be adequate to evaluate clinical transplantation protocols, being possible to monitor the migration and distribution of transplanted cells into the brain parenchyma noninvasively	[80]
Cell proliferation and survival	Transplanted neurospheres are able to survive in the ventricles of mice (C57BL/6), migrate to the nervous parenchyma and respond to inflammation caused by EAE, the main experimental model of human MS. Due to their ability to survive, neurospheres have a clinical potential for transplants and therapies for demyelinating diseases	[64]
β4 Tubulin	βT4 are neural precursors that represent a cellular source for the last stages of myelination and for neural repair in the adult CNS. In vitro, these cells are present in neurospheres generated from SVZ and after cell transplantation in rats, there is extensive myelination and increased density of cells in SVZ, promoting activation, proliferation and differentiation of βT4 in the scenario of deficiency and dysfunction of oligodendrocytes	[56]
Differentiation of adipose-derived stem cells	Stem cells from rat adipose-derived can differentiate into schwann cells in vitro, with similar morphology, phenotype and functional capabilities, making them useful for the treatment of neurological diseases, such as MS	[59]
Diabetes		
Cognition and memory	The neurogenic process is impaired in type 2 diabetic adult Goto-Kakizaki rats. A hyperglycemic environment increases the proliferation of adult neural progenitors in SVZ and DG, but impairs and decreases their survival. The interruption of neurogenesis in type 2 diabetic animals may be a factor responsible for the impaired cognitive and memory functions observed in human diabetics	[81]

Table 1 (continued)

Target	Major findings	References
Epilepsy		
TLE model and inflammation	Transplantation of human fetal NSPCs into the brain of adult epileptic Sprague–Dawley rats has a therapeutic effect in suppressing inflammation-induced seizures, as well as for recurrent spontaneous seizures in the TLE model, reducing the duration of the seizure for weeks after transplantation, after-discharge duration on electroencephalograms, seizure stage in the kindling model and the frequency/duration of spontaneous recurrent motor seizures in pilocarpine model	[82]
Medial ganglionic eminence progenitors	Cells derived from MGE, derived from Sprague Dawley embryos, cultured as neurospheres can become a source of inhibitory neurons. In vitro, these cells differentiate into inhibitory interneurons and glial cells and, in vivo, reduce the frequency and total number of epileptic seizures	[61]
Oxidative damage	Neurosphere, derived from Wistar rats, manifest a neuroprotective potential against seizures in animal models of epilepsy. They have antioxidant properties that reduce the oxidative damage generated by crises and play an important role in cell viability during the repair of degenerative lesions	[62]
Glioma		
Inhibition of glioma cell proliferation	NSPCs, isolated from the striata of E14 C57BL/6 mouse embryos, transplanted into the intrathecal space of the magna cistern of adult mice secrete factors that significantly control glioma cell proliferation, due to its strong antitumor effect. These cells migrate to the murine CNS and secrete factors that inhibit the proliferation of glioma cells. Therefore, NSPCs can be useful for strategies to treat gliomas	[83]
Gliomagenesis and NSPCs from SVZ	SVZ neurospheres isolated from rats (Sprague–Dawley) born to mothers exposed to ENU exhibit altered phenotypic characteristics after 10 to 15 doublings. Neurospheres ceased their development, adhered to the substrate and assumed morphological characteristics of monolayer cells. They also showed increased cell viability and proliferation. These phenotypic changes indicate that the cells have become immortalized and can be considered precursors of glioma, being useful as a model for genetic and epigenetic investigations that result in total malignancy	[84]
Huntington's disease		
Human neural stem cell transplantation	Engrafts from fetal human neurospheres significantly improve motor function in a HD model for rodents (adult Lewis rats). Extensive migration to regions such as the globus pallidus, entopeduncular nucleus, and substantia nigra pars reticulata and differentiation in neurons and astrocytes has been observed in vivo after transplantation, structurally repopulating the striatum due to robust cell survival	[57]
Ischemia		
Neurogenesis	Neurospheres were cultured from NSC of amniotic fluid from anencephalic fetuses. These cells showed common characteristics with other sources of NSC, expressing specific stem cell markers, proliferation and differentiation capacity in astrocytes, oligodendrocytes and dopaminergic neurons in vitro and with therapeutic effects in ischemic rats, becoming a new source to isolate human NSCs and apply them in translational studies of neurological disorders	[65]
Proliferation of post-ischemic NSCs	Adult Wistar rats with induced ischemia exhibit a higher neurogenic rate than non-ischemic rats. The number of neurospheres generated in SVZ from ischemic animals significantly increases compared to healthy animals, suggesting that stroke increases NPCs proliferation	[85]
Neurodegenerative disorders		
Transplanting neural progenitors	HNPCs can be expanded, maintained in culture as neurospheres and differentiated into neuronal and glial cells. These cells express a high rate of survival, migration and proliferation after transplantation in rats with striated lesion	[86]

Table 1 (continued)

Target	Major findings	References
Parkinson's disease		
Human neural progenitor cell transplantation	HNPCs transplanted to the substantia nigra from adult Lewis rats, with partial lesion of Parkinson's disease, can survive long-term in the brain. Twelve weeks after transplantation, the cells showed a high rate of survival, proliferation and migration. In addition, there was a significant neuronal differentiation in the injured animals, with expression of MAP-2ab, a marker of mature neurons. This indicates that HNPCs are promising for the treatment of Parkinson's disease	[87]
GABA receptors	$GABA_B$ receptor activation decreases, and $GABA_A$ receptor activation increases the number of dopaminergic neurons generated from neurospheres from mesencephalic precursors (Sprague–Dawley rat embryos). $GABA_A$ receptor antagonists in the differentiation medium help to obtain a greater number of neurons for potential use in cell therapy for PD	[88]
Dopaminergic neurons	The use of dopaminergic neurons derived from human embryonic stem cells has great potential for clinical application for Parkinson's disease. These cells, when transplanted, have good survival and significant functional benefit, resulting in symptomatic improvement of the behavioral dysfunction of the disease in adult parkinsonian Wistar rats	[89]
Dopaminergic neurons	Multipotent NSCs transplantation has a high rate of survival and differentiation into functional dopaminergic neurons. NSCs isolated from rat embryo midbrain when cultured, generate neurospheres able to differentiate into neurons and astrocytes in vitro. After transplantation into the striatum of PD mice (C56BL/6), NSCs can survive, integrate into host brain and differentiate, in TH-positive neural cells, appropriately without any immunosuppressant	[90]
Pediatric brain tumors		
Multipotent tumor cells	Progenitor cells derived from PBTs are capable of producing proliferative neurospheres, with clonal density and differentiated cells with neuronal and glial properties. These neurospheres express genes characteristic of spheres derived from the CNS, with migration and proliferation activities when transplanted into the brain of neonatal rats, demonstrating that PBTs contain multipotent and self-renewable cells (neural stem-like cells), with altered characteristics that may contribute to tumorigenesis	[91]
Spinal cord injury		
Transplanting neural progenitors	Spinal cord NSPCs from Wistar/eGFP rats, when cultured and transplanted to adult Sprague–Dawley rats with acute spinal cord injury model, show a significant increase in cell survival between 7–9 days after transplantation. NSPCs also differentiate into astrocytes, oligodendrocytes and a small number in neurons	[92]
Axonal regeneration	Biodegradable polymers aligned with NSC and Schwann cells are an excellent approach to restore nerve function in adult Sprague–Dawley rats after a transection spinal cord injury. In transplanted animals, was detected a significant number of differentiated cells expressing nestin, neurofilament, S-100, βIII tubulin and GFAP. In addition, there was axonal regeneration in the spinal cord of animals treated with polymers containing NSCs and schwann cells. This indicates that the biodegradable polymers seeded together with NSCs and schwann cells facilitate regeneration across the transected spinal cord	[93]
Striatal injuries		
Intracerebral graft	NSPCs derived from human forebrain can be expanded as neurospheres and differentiated into dopaminergic neurons in vitro, surviving dissociation and replanting. Despite this, few cells expressing TH have been identified among neurons surviving intrastriatal grafts in adult Sprague–Dawley rats. Even so, NSPCs represent a long-term expandable source of cells for potential use in intracerebral grafts	[94]

Table 1 (continued)

Target	Major findings	References
Stroke		
Atorvastatin and Notch1 signaling	Therapy with atorvastatin in elderly Wistar rats post-stroke increases the proliferation of NPCs, up-regulates Hes1 and PS1 expression, and increases Notch1 signaling activity, promoting cell proliferation in the SVZ of rats after stroke	[95]
p53 PFT-α inibitor	Treatment with p53 PFT-α inhibitor modifies brain injury-induced neurogenesis, improving the proliferation and survival of NPCs in SVZ in animals after stroke. This study demonstrates significant behavioral recovery in the administration of PFT-α after the occurrence of ischemia in adult Sprague–Dawley rats	[96]
Post-stroke recovery, inflammation and vascular repair	The growth factor VEGF, secreted by transplanted fetal hCNS-SCns, is necessary for the recovery of the brain functionality of T cell-deficient adult Nude rats, after stroke. The functional recovery after stroke correlates with suppression of inflammation induced by hCNS-SCns, increasing vessel formation and improving the integrity of the blood–brain barrier, positively interfering with vascular repair after inflammation	[97]
Erythropoietin	Treatment with erythropoietin in Wistar rats after stroke significantly improves functional recovery, angiogenesis and neurogenesis in animals, promoting an increase in neurological function and decreasing neuronal apoptosis. Erythropoietin also increases brain levels of VEGF and BDNF growth factors that are involved in cerebral angiogenesis and neurogenesis	[98]

AD Alzheimer's disease, *BDNF* brain-derived neurotrophic factor, *βT4* β4 tubulin, *CNS* central nervous system, *DG* dentate gyrus, *EAE* experimental autoimmune encephalomyelitis, *eGFP* enhanced green fluorescent protein, *ENU N*-ethyl-*N*-nitroso-urea, $GABA_A$ gamma-aminobutyric acid type A, $GABA_B$ gamma-aminobutyric acid type B, *hCNS-SCns* human central nervous system stem cells grown as neurospheres, *HD* Huntington's disease, *HES1* hairy/enhancer of split-1, *HNPC* human neural progenitor cell, *MGE* medial ganglionic eminence, *MRI* magnetic resonance image, *MS* multiple sclerosis, *NOTCH1* notch homolog 1, *NPCs* neural precursor cells, *NSC* neural stem cell, *NSPCs* neural stem/progenitor cell, *PD* Parkinson's disease, *PBT* pediatric brain tumor, *PS1* Presenilin1, *p53 PFT-α* p53 inhibitor pifithrin-α, *SVZ* subventricular zone, *TH* tyrosine hydroxylase, *TLE* temporal lobe epilepsy, *VEGF* vascular endothelial growth factor

transplanted in the hippocampus of epileptic rats. According to the authors, because they differentiate into glial cells, neurospheres derived from MGE showed anticonvulsant effects, reducing seizures in the TLE model, providing results that demonstrate that the neurosphere test becomes a cell therapy of great benefit in the treatment of epilepsy [61].

Following the same line of research, Gois da Silva et al. [62] evaluated the role of neural stem cells and their development in the brain tissue of adult epileptic Wistar rats. In epileptic rats treated with neurospheres, elevated levels of thiobarbituric acid and nitrite and a reduction in glutathione, superoxide dismutase and catalase levels were observed when compared to untreated groups. It was also possible to observe a homogeneous distribution of neurospheres throughout the brain tissue, with viable cells and in neurodifferentiation in the pilocarpine group, acting on necrosis sites, demonstrating that neurosphere therapy becomes a protective tool due to its antioxidant effect, decreasing the damage caused by seizures, helping to repair the degenerative areas [62].

NSA is a technique that can also be applied in the study of demyelinating diseases. One example was the study conducted to evaluate the potential for myelin repair by transplanting neural precursor cells derived from tissue removed during adult human brain surgery. The cultured cells showed neuronal and astrocytic characteristics that when transplanted into the spinal cord of adult rats had extensive remyelination with a pattern similar to Schwann cells. It was observed that remyelinated axons began to conduct impulses close to normal conduction velocity, suggesting that the use of neurospheres that generate neural precursors for transplantation in demyelinated areas results in satisfactory functional myelinization [63].

One issue that generates discussion among researchers is proliferation, that is, the long-term survival of transplanted cells, especially in cases of chronic diseases. As Einstein et al. reported, exposing NSCs to certain factors causes cells to perform better in differentiating neuronal lines, surviving within the ventricles, and ensuring an extensive migration to areas of inflammation. From brain tissue from mice, they obtained floating spheres that were later transplanted into the lateral ventricles of rats. With this, they demonstrated that neurospheres were able to survive even in an environment with deficiency of growth factors, responding to stimuli for proliferation and differentiation in neuronal lineage [64].

Among the various areas from which neurospheres can be used, NSCs that come from them are particularly important in the study of neurogenesis. However, nowadays it is

difficult to obtain human tissue for studies with stem cells/neural precursors and with this it is essential to discover new techniques of isolation and cell culture. For this, Chang et el described a method from human amniotic liquid, liquid that contains several cells that make up the developing fetus, to isolate and proliferate human NSCs. The neurospheres, which underwent a long period of in vitro expansion, expressed specific markers for NSCs and neurons, astrocytes and oligodendrocytes. NSCs derived from amniotic liquid were grafted into the ischemic zone of the brain of rats for the purpose of functional recovery. The study reported a rapid improvement in motor function in the first week after transplantation with NSCs, stating that cells derived from amniotic fluid could easily integrate the injured ischemic area, serving as a good model in the treatment of neurodegenerative diseases [65].

Technical limitations

Although the use of neurospheres has many advantages, we need to consider their heterogeneous character and sensitivity, mainly when studying biological processes, making it difficult to compare results from different labs. There is a possibility of deregulation of the potential for differentiation and spatial identity of stem cells grown in the NSA in the presence of high concentrations of growth factors [66]. Also, there may have progressive loss of neurogenic potential with passage and low neuron yield after transplantation of expanded cells [41]. For example, spheres from neonatal mouse subependymal zone when transplanted into the lateral ventricle of mice cannot selfrenew in vivo. Also, these neurospheres do not contribute to the in vivo long-term neurogenesis [66, 67].To overcome some limitations of the NSA, Sipahi and Zupanc developed a cellular automata (CA) model. Effects of proliferative potential, contact inhibition, cell death, and clearance of dead cells on growth rate, final size, and composition of neurospheres were examined. According to the authors results a surprising prediction derived from this model is that cell death, while resulting in a decrease in growth rate and final size of neurospheres, increases the degree of differentiation of neurosphere cells. Interestingly, this approach makes it possible to simulate similarly the characteristics of spheres grown under culture conditions, being applied in a wide range of systems [68].

Another solution for the neurospheres limitations is the use of organoid technologies modeled from fibroblasts, for example, which can then be reprogrammed at an embryonic level, the so-called human induced pluripotent stem cell (hiPSC) and then differentiated into the desired cell type. The reprogramming of differentiated cells may occur by transfer of nuclear content to oocytes or by fusion with embryonic stem cells.This approach allows great potential for disease modelling, translational adoption in drug screening and regenerative medicine, and clinical research, revolutionizing the study of human brain and CNS in vitro [69, 70].

Brain organoids are widely studied because they mimic an embryonic and adult neuronal development system. This culture shows characteristics that help to mimic the study with cortical development in vivo and in vitro. For the technique of direct reprogramming of hiPSC can be used autologous cells to induce specific types of cells such as neurons, progenitors of blood cells, hepatocytes, and cartilage, also determining the destination of germ cells. Organoids technology has contributed to studies in ischemic stroke, traumatic brain injury, cell transplantation and other brain-related [71, 72].

One of the protocols developed for organoids generation was described by Lancaster and Knoblich, allowing brain organoids to be generated from reprogrammed cells of patients with different pathologies. The development of the organoids technique allowed a specific modeling for the understanding of the spectrum of some neurological disorders [73, 74]. For neuronal differentiation from this model, NSCs form a homogeneous population after some passages, resulting in more mature neural progenitors [75] The differentiation of hiPSCs in NSCs is an efficient method that generates greater differentiation in functional neurons, also allows a greater number of passages, cell freezing and thawing and manipulation in order to cell growth as neurospheres. NSCs derived from organoids are considered equally ideal as models for cellular and molecular tests in the understanding of various CNS diseases, being appropriate for studies with late characteristics of neurological diseases [76].

Currently, experimental models have been established for a better understanding of the differentiation and cell morphogenesis processes in vitro. Comparing the neurosphere assay and organoid technology, it is important to note that the neurosphere is mainly related to the expansion of NSCs, while organoids are prioritized in studies of histogenesis, differentiation and coordinated migration of NSCs. Authors report that the process of differentiating morphogenesis of the formation of neuronal clusters needs to recapitulate the specific aspects of cerebral histogenesis found in vivo and both techniques are in line with the expectations of the researchers [71, 77].

Future perspectives

For several decades research has indicated that neural tissues grafted on the CNS can repair or replace damaged neural structures and the development of characterized and functional neural precursors can be cultivated for a long period in vitro, being a beneficial alternative source for transplants.

The transplantation of neural stem cells has already proved significant in the development, function, and regenerative capacities in the CNS, effectively compensating the damaged tissue.

These findings have important implications for understanding the growth characteristics, differentiation and molecular specification of neurospheres derived from the CNS, as well as the therapeutic potential of these cells for neural repair [78]. Neurospheres can be seen not only as a single cluster of cells, but as an independent "ecosystem" full of neural progenitors that will give rise to the main cells of the CNS.

Therefore, we must continually explore the NSA, as a powerful in vitro model for a better understanding of the characteristics of neural cells, their specific markers for their selection at different stages of maturation and explore their potential use for transplants in neurodegenerative syndromes, in order to obtain a satisfactory response to the patient during the course of the disease.

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Declarations

Conflict of interest The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. LSS and APBS have received scholarships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Brazil), respectively. FM is a post-doctoral researcher funded by CAPES/Brazil. JCC and DRM has nothing to disclose.

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