mgr Klaudia Anna Radoszkiewicz

Optimization of the isolation and long-term culture method of neural stem/progenitor cells for therapeutic application

Optymalizacja metody izolacji i długotrwałej hodowli neuralnych komórek macierzystych/progenitorowych do celów terapeutycznych

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Supervisor: Prof. dr hab. n. med. Anna Sarnowska

Subsidiary supervisor: Dr hab. n. med. Marta Kot

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"And no one showed us to the land

And no one knows the where's or why's

But something stirs and something tries

And starts to climb toward the light."

- Pink Floyd, Echoes (1971)

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List of abbreviations used in the dissertation:

2D	two-dimensional
3D	three-dimensional
ALS	amyotrophic lateral sclerosis
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BIH	benign intracranial hypertension
CNS	central nervous system
CMFDA	5-chloromethylfluorescein diacetate
CNTF	ciliary neurotrophic factor
CSF	cerebrospinal fluid
CXCR4	C-X-C motif chemokine receptor 4
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
ESCs	embryonic stem cells
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
HBSS	Hank’s balanced salt solution
HGF	hepatocyte growth factor
hNSCs	human neural stem/progenitor cells
IGF-I	insulin-like growth factor 1
IL-10	interleukin 10
iPSCs	induced pluripotent stem cells
Ki67	marker of proliferation ki67
LDH	lactate dehydrogenase
LIF	leukemia inhibitory factor
MAP2	microtubule-associated protein 2
mNSCs	mouse neural stem/progenitor cells

MSCs	mesenchymal stem/stromal cells
NeuN	neuronal nuclei
NG2	neural/glial antigen 2
NGF	nerve growth factor
NPCs	neural precursor cells
NSCs	neural stem/progenitor cells
OGD	oxygen-glucose deprivation
OHC	organotypic hippocampal slices culture
RT	room temperature
rNSCs	rat neural stem/progenitor cells
ROCK	Rho-associated protein kinase
SAH	subarachnoid hemorrhage
SOX2	SRY-Box transcription factor 2
TNF-alpha	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
WGE	whole ganglionic eminence

Abstract in Polish (streszczenie)

Jednym z kluczowych wyzwań terapii komórkowej jest uzyskanie komórek o wysokim potencjale terapeutycznym. Takie komórki powinny charakteryzować się zdolnością do:

- **wysokiej przeżywalności po transplantacji;**
- **skutecznej repopulacji tkanki;**
- **długotrwałej aktywności wydzielniczej wspierającej endogenne procesy naprawcze.**

Badania wskazują, że optymalizacja rodzaju komórek oraz warunków hodowli *in vitro* może poprawić ich skuteczność terapeutyczną. Szczególne trudności pojawiają się w kontekście regeneracji ośrodkowego układu nerwowego (OUN). Dotychczasowe terapie z wykorzystaniem somatycznych komórek macierzystych nie przyniosły satysfakcjonujących wyników, głównie ze względu na ograniczony potencjał do różnicowania neuralnego i funkcjonalnego. Wiele doniesień wskazuje, że w tej kwestii potencjalnie najlepsze narzędzie terapeutyczne mogą stanowić **neuralne komórki macierzyste/progenitorowe (NSCs- neural stem/progenitor cells)**. NSCs wyróżniają się wysokim potencjałem do różnicowania w dojrzałe komórki nerwowe oraz zdolnościami wydzielniczymi dopasowanymi do aktywacji endogennej neurogenezy. Jednakże, ich przeżycie oraz proliferacja po transplantacji stanowi duże wyzwanie. Dodatkowo, wyniki badań nad NSCs są niespójne, a większość eksperymentów przeprowadzono na komórkach zwierzęcych (mysich i szczurzych), co wynika z ograniczonej dostępności ludzkiego materiału komórkowego oraz aspektów etycznych.

Znaczenie mikrośrodowiska dla terapii NSCs

Kluczowym elementem skutecznej terapii jest lepsze zrozumienie mikrośrodowiska mózgu (w tym niszy komórek macierzystych) i jego wpływu na NSCs. Nisza neuralna reguluje ich losy poprzez:

- **bodźce biochemiczne** (czynniki wzrostu, hormony, peptydy);
- **czynniki biofizyczne** (ciśnienie, naprężenia mechaniczne);
- **interakcje komórkowe.**

Istotnym elementem niszy neuralnej jest również **płyn mózgowo-rdzeniowy (CSF-cerebrospinal fluid)**, regulujący przeżycie, proliferację i różnicowanie NSC, choć jego rola w neurogenezie osób dorosłych nie jest do końca poznana.

W związku z powyższym, głównymi kierunkami moich badań były:

1. **Izolacja i długoterminowa hodowla ludzkich NSCs**,
2. **Zrozumienie roli NSCs w regeneracji tkanki nerwowej**, w tym określenie niszy sprzyjającej ich przeżyciu i proliferacji.
3. **Porównanie NSCs ludzkich, mysich i szczurzych**, by wyjaśnić różnice międzygatunkowe.
4. **Ocena wpływu warunków przestrzennych, suplementacyjnych i dysocjacyjnych** na właściwości NSCs.

Metodyka:

- Do badań wykorzystano **ludzkie płodowe NSCs (fhNSCs- fetal human neural stem/progenitor cells)** oraz NSCs izolowane z **nowonarodzonych osesków myszy (mNSCs- mouse neural stem/progenitor cells)** i **szczurów (rNSCs- rat neural stem/progenitor cells)**.
- Oceniono wpływ czynników obecnych w środowisku takich jak **bazowy czynnik wzrostu fibroblastów (bFGF- basic fibroblast growth factor) i epidermalny czynnik wzrostu (EGF- epidermal growth factor), glutamina** oraz **CSF** na rozwój NSCs.
- Analizowano przeżycie, proliferację, zdolności wydzielnicze i różnicowanie **NSCs preinkubowanych z CSF *in vitro*** w różnych warunkach hodowlanych, ***ex vivo*** w obecności organotypowej hodowli skrawków hipokampa szczura **(OHC- organotypic rat hippocampal slices culture)** poddanej okresowej deprywacji tlenu oraz glukozy **(OGD-**

oxygen-glucose deprivation) oraz ***in vivo*** (w modelu ogniskowego uszkodzenia mózgu po podaniu ouabainy)

Kluczowe wyniki:

1. **Literaturowe rozbieżności w badaniach NSCs wynikały m.in. z różnic międzygatunkowych** i warunków hodowlanych.
2. NSCs pozyskane od wszystkich badanych gatunków wymagały **bezpośredniej hodowli po izolacji**, co znacząco podtrzymywało ich przeżycie.
3. Kluczowe dla hodowli NSCs okazały się:
    - odpowiednie stężenie **bFGF i EGF-20 ng/mL dla obu czynników;**
    - obecność **glutaminy w pożywce;**
    - odpowiednią metodę **dysocjacji**.
4. **Potencjał migracyjny NSCs pozyskanych od wszystkich badanych gatunków zmienia się w czasie**, co ma istotne znaczenie dla interpretacji badań *in vivo.*
5. **NSCs hodowane w pożywce z CSF wykazują wysoki potencjał neuroprotekcyjny oraz zmieniony potencjał wydzielniczy w stosunku do NSCs kontrolnych,** po współhodowli z uszkodzoną tkanką nerwową.

Wnioski i perspektywy:

- **NSCs wykazują znaczący potencjał terapeutyczny** w leczeniu schorzeń OUN, takich jak udar mózgu oraz choroby neurodegeneracyjne.
- Skuteczność terapii jest ściśle uzależniona od **odpowiedniego mikrośrodowiska hodowli komórkowej**, naśladującego warunki panujące w mózgu.
- **Sam przeszczep NSCs do uszkodzonej tkanki nerwowej nie gwarantuje sukcesu**- kluczowe jest zrozumienie interakcji komórek z naturalnym mikrośrodowiskiem oraz optymalizacja warunków hodowli w warunkach laboratoryjnych, tak aby zachować ich właściwości terapeutyczne.
- **CSF jako integralny element mikrośrodowiska NSCs może odgrywać szczególną rolę** w poprawie ich przeżywalności i funkcjonalności, co stanowi obiecujący kierunek dalszych badań.
- Wykorzystanie powyższych ustaleń może przyczynić się do rozwoju skuteczniejszych strategii terapeutycznych w leczeniu chorób neurodegeneracyjnych i uszkodzeń OUN.

Abstract

One of the key challenges in cell therapy is obtaining cells with high therapeutic potential. Such cells should be characterized by the ability to:

- **efficient survival after transplantation;**
- **effective tissue repopulation;**
- **long-term maintenance of the secretory activity supporting endogenous repair processes.**

Research indicates that optimizing both the type of cells and the *in vitro* culture conditions can enhance their therapeutic efficacy. This challenge becomes particularly complex in the context of central nervous system (CNS) regeneration. To date, therapies utilizing somatic stem cells have not yielded satisfactory results, primarily due to their limited potential for neural and functional differentiation. Numerous reports suggest that in this matter, **neural stem/progenitor cells (NSCs)** may contribute as the most promising therapeutic tool. NSCs are characterized by a high potential to differentiate into mature neural lineage cells and a secretory profile that supports the activation of endogenous neurogenesis. However, their survival and proliferation maintenance after transplantation remain significant challenges. Furthermore, results of NSC-related studies are often inconsistent, with the majority conducted using animal-derived cells (mouse and rat) due to limited access to human cell material and ethical considerations.

The role of the microenvironment in NSC therapy

A critical factor for effective therapy is a better understanding of the brain's microenvironment (including the stem cell niche) and its influence on NSCs. The neural niche regulates the NSC fate through:

- **biochemical signals** (growth factors, hormones, peptides);
- **biophysical factors** (pressure, mechanical stress);
- **cell-cell interactions.**

Another key component of the neural niche is the **cerebrospinal fluid (CSF),** which regulates NSC survival, proliferation, and differentiation, although its role in adult neurogenesis is not yet fully understood.

Considering the above, the main objectives of my research were:

1. **Isolation and long-term culture of human NSCs**;
2. **Understanding the role of NSCs in neural tissue regeneration,** including defining the niche favorable for their survival and proliferation;
3. **Comparing human, mouse, and rat NSCs** to clarify interspecies differences;
4. **Evaluating the impact of spatial, nutritional, and dissociation conditions** on NSC properties.

Materials and methods:

- The study utilized **fetal human neural stem/progenitor cells (fhNSCs),** as well as NSCs isolated from **neonatal mouse (mNSCs) and rat (rNSCs) pups.**
- The effects of environmental factors such as basic **fibroblast growth factor (bFGF), epidermal growth factor (EGF), glutamine,** and **CSF** on NSCs development were investigated.
- **CSF-treated NSC** survival, proliferation, secretory activity, and differentiation were analyzed in various ***in vitro*** culture conditions, ***ex vivo*** in the presence of **organotypic hippocampal slice cultures (OHC)** subjected to **oxygen-glucose deprivation (OGD),** and ***in vivo*** in a model of focal brain injury induced with ouabain.

Key findings:

1. Discrepancies in NSC-related literature data were partly due to **interspecies differences** and **variable culture conditions.**
2. **Immediately after NSC isolation, direct culture** was essential for maintaining cell survival across all species.
3. Critical factors for a successful NSC culture include:
    - optimal concentrations of **bFGF and EGF- 20 ng/mL for both factors;**
    - the presence of **glutamine** in the medium;
    - the appropriate method of **dissociation**.
4. **NSC migratory potential changes over time**, which is crucial when interpreting *in vivo* results.
5. After co-culture with damaged neural tissue**, NSCs cultured in medium with CSF exhibit a high neuroprotective potential and an altered secretory profile compared to control NSCs**.

Conclusions and Perspectives:

- **NSCs demonstrate significant therapeutic potential** in treating CNS disorders, such as stroke and neurodegenerative diseases.
- The efficacy of NSC-based therapies strongly depends on the surrounding cell culture **microenvironment** that mimics the conditions of the brain.
- **Transplanting NSCs into damaged neural tissue alone does not ensure therapeutic success**. Understanding their interactions with the natural microenvironment and optimizing culture conditions is essential to preserving their therapeutic properties.
- **CSF as an integral component of the NSC niche may play a vital role in improving NSC survival and function**, representing a promising direction for future research.
- Implementing these findings could contribute to developing more effective therapeutic strategies for neurodegenerative diseases and CNS injuries.

Novelty of the dissertation

This dissertation introduces novel findings across the following articles:

Article I: ***"Critical Points for Optimizing Long-Term Culture and Neural Differentiation Capacity of Rodent and Human Neural Stem Cells to Facilitate Translation into Clinical Settings"***

- A first comprehensive analysis of **selected interspecies variability and its impact on NSC culture** standardization.
- Identification of unreported **challenges and inconsistencies in long-term NSC culture maintenance**, emphasizing the need for optimized protocols that ensure reproducibility across species.
- A new framework **for bridging experimental NSC research with clinical translation**, providing a valuable resource for regenerative medicine and therapeutic applications.

Article II: ***"Understanding Intra- and Inter-Species Variability in Neural Stem Cells' Biology Is Key to Their Successful Cryopreservation, Culture, and Propagation"***

- The first comparative analysis of NSC biology across **human, mouse, and rat species has been conducted in a single study**, revealing that **inter-species variability** significantly influences NSC behavior alongside selected environmental factors.
- Discovery that NSCs from humans, mice, and rats share **one critical common feature**- the need for **immediate post-isolation culture** to enhance their survival significantly.
- There is new evidence that rat NSCs exhibit biological responses that more closely mimic human NSCs than mouse NSCs (such as neurosphere formation potential and neural differentiation stages).

Article III: ***"Deciphering the Impact of Cerebrospinal Fluid on Stem Cell Fate as a New Mechanism to Enhance Clinical Therapy Development"***

- CSF was identified as a previously **underappreciated regulator of NSC fate**.
- Demonstration **of CSF's role in shaping the NSC microenvironment** by influencing their **survival, differentiation, and integration into host tissue,** which are the critical factors in regenerative medicine.
- Proposal of **CSF modulation as a novel mechanism to enhance NSC-based therapies,** which could open new possibilities for improving clinical applications in neurological disorders.

Article IV: ***"Unraveling the Impact of Human Cerebrospinal Fluid on Human Neural Stem Cell Fate"***

- Introduction of CSF modulation as an **innovative strategy to improve the therapeutic potential** of hNSC-based treatments for neurological disorders.
- The discovery that human CSF directly influences NSC fate by inhibiting hNSC proliferation and regulating differentiation and **altering their secretory profile, especially in response to damaged neural tissue.**
- The first evidence that **hCSF actively supports neural tissue regeneration has been presented in this study**.
- The identification of **hNSCs' dynamic response post-ischemic injury**: the initial secretion of proliferation-inducing factors, followed by immunomodulatory, proangiogenic, and neuroprotective factors' release has been found.

1. Introduction

Present challenges of regenerative therapy for neurological disorders

Basic mechanisms of Neural Stem/Progenitor Cells' (NSCs') action

In regenerative medicine, a significant hurdle persists in developing effective treatments for neurological diseases. The lack of proper treatment is primarily due to the complexity of the central nervous system (CNS). Damage to the CNS poses significant risks, often resulting in cognitive, motor, sensory, and organ dysfunction [1,2]. The following changes in its cellular structure, cellular and biochemical composition, can be irreversible, posing challenges in understanding and investigating pathophysiology [3,4].

A major goal of neuroregeneration is to boost endogenous neurogenesis, however, current therapeutic options face issues with their efficacy. To some degree, such spontaneous recovery can occur naturally. In the adult subventricular zone (SVZ) and subgranular zone (SGZ), endogenous neural stem cells typically remain in a quiescent state, and their number is limited [5]. Their activation and proliferation occur predominantly in response to brain injuries such as trauma or ischemic stroke, where they endeavor to repair the damaged brain tissue. The neural niche, through the secretion of paracrine and autocrine signals, plays a pivotal role in modulating neural stem/progenitor cells (NSCs). While the exact origin of niche signals remains elusive, numerous studies have highlighted the influence of factors released by NSCs on the niche microenvironment [6,7]. NSCs have also demonstrated a remarkable capacity for migration, even over considerable distances, to integrate into injured brain regions in various age groups [8]. Still, the regenerative capacity of these activated cells is inherently restricted [9]. Although some tissues' niches, such as the intestine and skin, show active renewal, and others, including the liver and pancreas, regenerate more slowly, the CNS exhibits little to no self-renewal, leaving the reasons for its limited regenerative capacity largely unaddressed [10,11].

Thus, current research is focused on finding efficient indirect methods to both stimulate the proliferation of endogenous NSCs and enhance their contribution to neural repair. As endogenous NSCs are in impenetrable areas, autologous administration so far seems to be almost impossible.

Variability in NSC sources, their promise, and implications

Although protocols for direct replacement therapy remain inadequate, stem cell administration has emerged as a glimmer of hope. The limited access to endogenous neural cell sources contributed to a comprehensive exploration of different therapeutically active cell types. For the treatment of neurological disorders, the choice of stem cells depends on the desired therapeutic effect, which includes the direct replacement of affected neural cells in damaged or degenerated tissue or the production of neurotrophic and immunomodulatory factors. Such cells should be characterized by great cell survival, proliferation, inflammation reduction, and stable secretory effect to support the host cells over an extended period, all while minimizing the adverse side effects of administration [12].

To date, a dominant focus has been placed on mesenchymal stem/stromal cells (MSCs) due to their rich secretory capabilities and great availability [13–15]. These cells can be easily accessed with almost non-invasive methods from several adult tissues, such as adipose tissue, bone marrow, or Wharton's jelly [5]. Nonetheless, clinical trials have underscored the limitations in MSC application, including limited survival post-transplantation and poorly understood neurorestorative properties [16–18]. While most research indicates the safety of MSC-based therapies, individual adverse effects have also been reported, such as differentiation into undesirable cell types and initiation of an uncontrolled immune response [19–22]. Human embryonic stem cells (hESCs), characterized by the capacity for extensive cell culture expansion and differentiation into diverse neuronal subtypes, face hurdles in clinical application. Ethical concerns regarding their derivation from embryos, scientific challenges such as immune-compatibility issues, and the risk of teratoma formation hamper their clinical use [23]. Raised

concerns about the long-term safety of ESC-derived oligodendrocyte progenitor cells used in the spinal cord injury clinical trial led to early discontinuation in 2011 [24]. Tumor risk was part of broader safety considerations. Similarly, human induced pluripotent stem cells (hiPSCs) and their derivatives have also emerged as promising therapeutic candidates for neurological diseases. Despite the promising therapeutic potential, safety concerns have also been raised. Specifically, despite the preclinical findings suggesting their safety, issues such as tumor formation and inappropriate localization of transplanted cells have also been reported, underlining the importance of rigorous safety assessments and further research [25–30].

Moreover, encouraging findings suggest they can modulate the local environment through secreted factors and act as chaperone cells for injured tissue [31]. Additionally, they offer clonal expansion and genetic stability, making them suitable for precise genetic manipulations[32]. Multiple sources of NSCs have been explored for regenerative therapies, however, an ideal source has yet to be definitively identified [33]. Each source has its own set of advantages and limitations:

1. Fetal and adult CNS-derived NSCs- these cells are directly isolated from the CNS and exhibit a high degree of specialization. However, they are limited in availability and raise ethical concerns.
2. Pluripotent stem cell-derived neural progenitors- iPSCs and ESCs can be differentiated into neural progenitors, offering a potentially unlimited source of NSCs. Nevertheless, issues such as genetic instability and the risk of teratoma formation need to be further investigated.
3. Non-neural stem cells- MSCs and other non-neural stem cells have been investigated for their ability to transdifferentiate into neural lineages. While they are more accessible and less controversial, their neural differentiation efficiency is comparatively low.

Taken together, due to the highest degree of differentiation and genetic stability, the most promising source of NSCs remains in the CNS. However, when it comes to developing proper research strategies, significant challenges persist. One of the major challenges is the poor survival rate of transplanted cells. The exact reason for their rapid loss with time remains difficult to address, whether due to a lack of essential support factors in the host environment, the presence of factors inhibiting their viability, inadequate pre-transplantation preparation, or specific vulnerabilities of the transplanted cells. Furthermore, even if the critical cell survival factors are identified, the data on whether their modification could effectively improve the outcomes is still inadequate or unknown[34–36].

Addressing these obstacles possibly requires a two-step action: while we need more research that could allow us to observe the direct NSCs-niche interactions, first, we should take a step back to standardize protocols in NSC characterization, isolation, preparation, and transplantation dosage determination. These methodological variations, together with species-specific differences in NSC biology and inconsistencies in their characterization, hamper data comparability and the translation of preclinical findings into clinical applications. It is essential to acknowledge that conditions optimized for one species may yield different results in another. For example, translating findings from animal models to humans is complex, as protocols optimized for rodent-derived NSCs might not yield similar results in human cells. Overcoming these challenges is crucial for enhancing the therapeutic potential of NSCs in treating neurological disorders. [31,37–42].

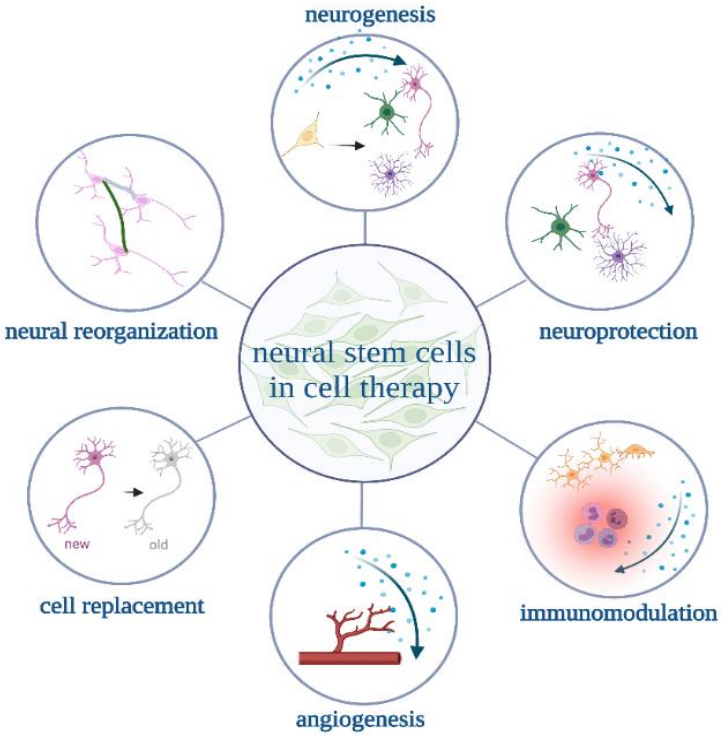


Figure 1. Possible benefits of NSCs' use in clinical trials suggest the need to further their studies on the preclinical level.

The still underexplored role of the niche and its components in NSC Function

In exploring the factors involved in regulating the behavior and fate of NSCs, the neural niche, which reflects the microenvironment surrounding NSCs, plays a crucial role. Key components such as extracellular matrix, signaling molecules, CSF, and interactions with other cell types significantly influence NSC proliferation, differentiation, and integration [43–46]. Thus, understanding the dynamics of NSC-niche interactions is essential for optimizing NSC-based therapies.

These interactions are also relevant for exogenous NSCs, which must adapt to the host environment and establish appropriate interactions with endogenous cells. Successful transplantation depends on their ability to form a proper communication network with local cells, integrate into the existing neural circuitry, and determine the niche factors that influence these NSCs. Current research is focused on mimicking the natural brain environment *in vitro* to study these interactions and develop more physiologically relevant models [47,48].

Investigating each component of the neural niche provides insights into how these factors influence NSC behavior. CSF, in particular, plays a significant yet underappreciated role in maintaining the homeostasis of the neural environment and delivering essential nutrients and signaling molecules to NSCs [49–51]. Moreover, the high dynamic range of CSF turnover supports neuropeptide and hormone signaling over considerable distances and periods, aligning with diurnal fluctuations [52]. However, the direct mechanisms by which CSF influences NSC function remain poorly understood. By having a deeper understanding of these interactions, possible new strategies can be developed to enhance the integration and functionality of exogenous NSCs. For example, it might involve pre-conditioning NSCs before transplantation, engineering biomimetic scaffolds to support their survival and integration, or modulating the host environment to be more conducive to NSC function [53–56].

In conclusion, while NSCs hold significant promise for treating neurological disorders, addressing several issues is essential (Figure 2). Developing treatment for neurological diseases is challenging due to the complex nature of CNS damage and the limited regenerative capacity of NSCs. Research focuses on stimulating endogenous NSCs and utilizing exogenous stem cells, while the clinical application still faces hurdles such as poor cell survival and safety concerns. Understanding the neural

niche's role in regulating NSC behavior is crucial for developing effective therapies and improving patient outcomes.

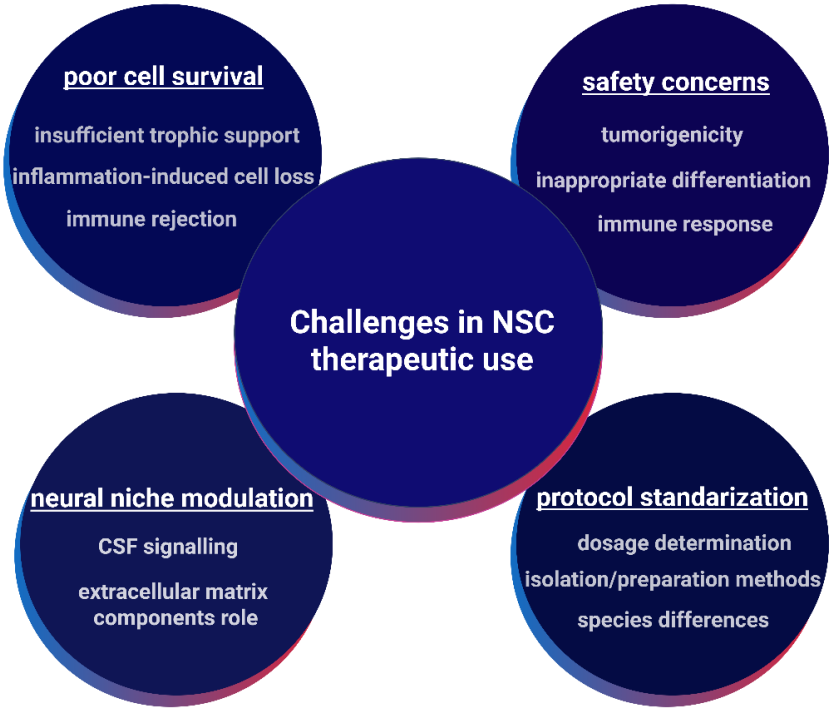


Figure 2. Possible challenges in NSC use in therapy.

2. The objective of the dissertation

The general objective of the dissertation

The main objective of this dissertation is to **optimize the isolation and long-term culture method of NSCs to enhance their therapeutic application**. The specific aims are described in Articles I, II, III, and IV.

Specific objectives of the dissertation

Article I **Systematic identification of the critical elements of NSC protocols, including cell isolation techniques, culture conditions, and growth factors, essential for maintaining cell viability, proliferation, and multipotency.**

This objective aims to address potential challenges and limitations during protocol optimization.

Article II **Investigation of the inter-species variability in NSC functional properties**, **focusing on the effects of spatial, nutritional, and dissociation conditions on cell survival, proliferation, and differentiation.**

This objective seeks to determine the applicability of findings from rodent NSC research to human cells and identify species-specific responses crucial for developing clinically relevant NSC-based therapies.

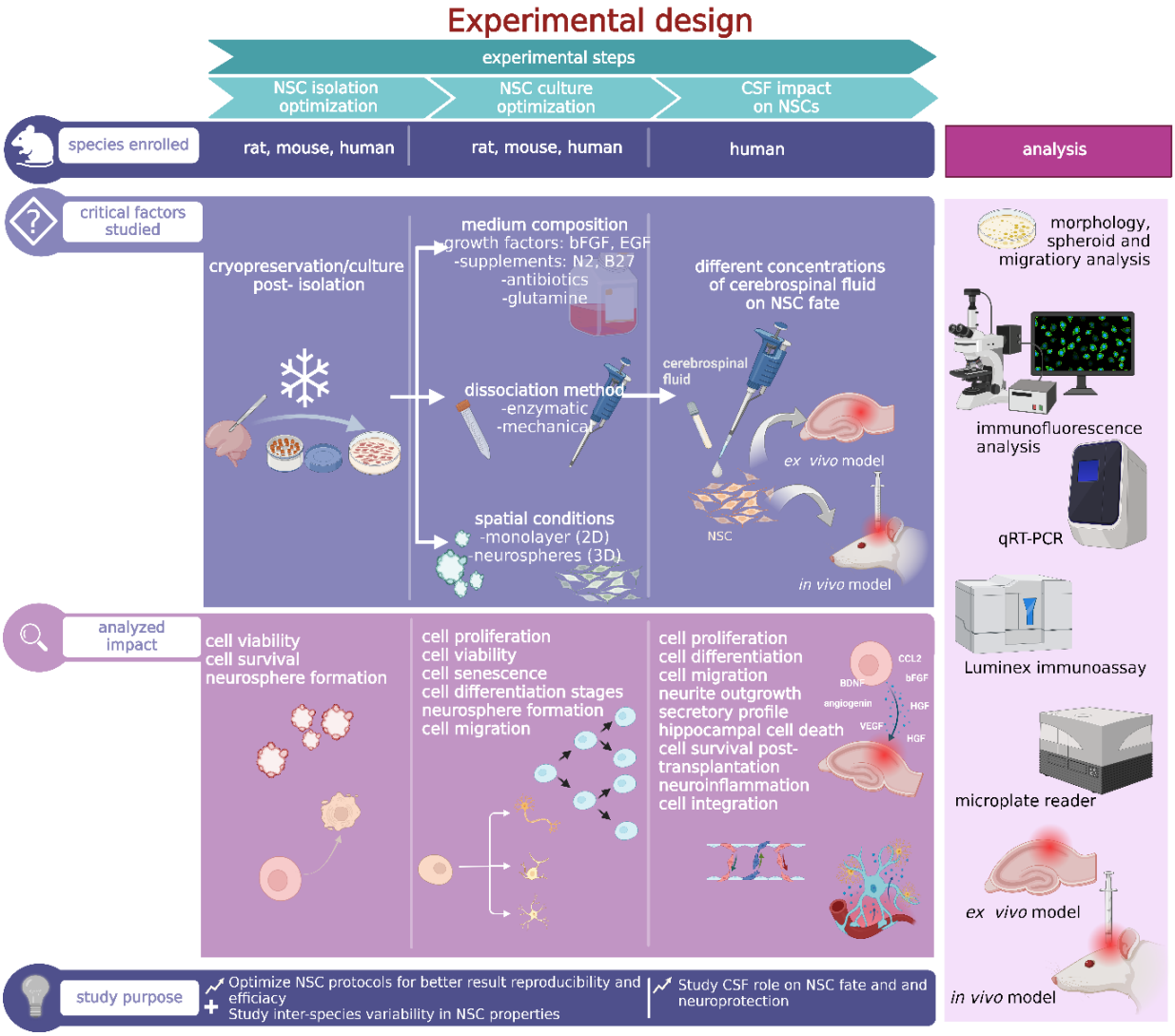
Article III **Investigation of the CSF role in the NSC niche.**

This objective aims to compare the effects of CSF from different species, sources, and conditions on the survival, integration, and functionality of NSCs and describe the factors within CSF that could enhance NSC-based therapies by improving these properties, underlying potential mechanisms involved in CNS regeneration and repair.

Article IV **Assessment of the effects of preincubating human NSCs with human CSF from healthy donors that aims to recreate culture conditions which closely mimic the physiological brain environment.**

This objective evaluates the impact of CSF on the secretory, neurogenic, neuroprotective, and anti-inflammatory potentials of NSCs in the context of ischemic brain injury to enhance NSC survival, proliferation, and therapeutic efficacy using *in vitro*, *ex vivo*, or *in vivo* models.

3. Materials and Methods



The combination of *in vitro*, *ex vivo*, and *in vivo* methods allows for a comprehensive assessment of NSC behavior and their therapeutic abilities, but requires careful interpretation of results due to the limitations of each model. Moreover, the comparison between the three species presented in this Dissertation can be hampered due to each of their unique biology. Additionally, the broad spectrum of both inter- and intra-species variability issues is described in **Article I**.

In vitro model

Isolation of NSCs

NSC sources and isolation procedures

Human Neural Stem/Progenitor Cells (hNSCs)

In the presented studies, hNSCs were isolated from the fetal human brain following previously established protocols [57,58]. In **Article II**, hNSCs were obtained from the Stem Cell Research Laboratory at the Department of Neurosurgery, University of Warmia and Mazury in Olsztyn, Poland. The isolation procedure involved careful dissection and enzymatic digestion (with Accutase) of the fetal brain tissue. Next, the cells were divided into two groups. The first group of cells was immediately cultured after isolation. The second one was directly cryopreserved to assess the influence of cryopreservation on their survival and proliferation potential. In **Article IV**, hNSCs were obtained from IRCCS Casa Sollievo della Sofferenza in San Giovanni Rotondo, Foggia, Italy. In both studies, the material was acquired legally and ethically, in accordance with local informed consent procedures.

Using NSCs from human brain tissue is particularly valuable as these cells closely mimic the cellular environment of the human brain. However, their use is greatly limited by their restricted availability. The isolation and culture of hNSCs are technically demanding, and these cells are highly delicate and require precise handling to maintain cell viability, expansion, and functionality. An additional challenge lies in ensuring their survival after transplantation. The variability in human samples, including gestational age and genetic background, presents additional limitations, potentially affecting the consistency of results. Working with human neural tissue is expensive due to the costs associated with ethical approvals, tissue acquisition, and specialized handling.

Advantages:

- The method provides a human-specific model ideal for detailed studies of human-specific cellular behaviors and responses.
- It is useful in modeling human neurological diseases and testing therapeutics.

Limitations:

- The method is limited due to tissue availability and ethical concerns.
- The variability in human samples (in gestational age, genetic background, and health conditions) can affect the consistency of results.
- Technical complexity and high costs are associated with isolation and maintenance.
- The lifespan of hNSCs in culture can be shorter compared to rodent models, which can limit the duration and scope of experiments.

Mouse Neural Stem/Progenitor Cells (mNSCs) and Rat Neural Stem/Progenitor Cells (rNSCs)

In **Article II**, newborn Wistar rats and C57BL/6J-type mice from the Mossakowski Medical Research Institute Animal Breeding House were used. The isolation protocol involved all the aforementioned procedures described for hNSCs to make a reliable comparison between the species.

Due to the aforementioned limited utilization of human NSCs, numerous efforts have been made to identify animal models that would closely mimic human neural cell biology. Rodent NSCs are widely used in research due to their accessibility and the availability of established protocols. They are ideal for genetic manipulation and large-scale experiments. However, their relevance to human biology is limited, as there are still significant interspecies differences between rodent and human neural development and disease mechanisms.

Advantages:

- The method provides a well-established model with extensive characterization.
- It is useful for high-throughput studies and genetic manipulations.

Limitations:

- Species differences may limit the direct application to human biology. The genetic homogeneity of laboratory rodent strains may not fully represent the genetic diversity found in human populations.
- Variations in NSC characteristics between different rodent strains or species, as well as the age of the donors, can affect the consistency of experimental outcomes.
- There might be ethical considerations regarding animal use.

Culture/cryopreservation after cell isolation

Given these challenges, we aimed to identify and compare the critical differences and similarities in the culture requirements, viability, and behavior of human *versus* rodent NSCs. Thus, in **Article II**, cell culture conditions were optimized to promote the survival and proliferation of NSCs. After the isolation, NSCs derived from humans, rats, and mice were divided into two groups: one group was directly cultured, while the second one was cryopreserved to assess and compare their effects on these parameters.

Cryopreservation facilitates long-term storage of NSCs, allowing experiments to be conducted over extended periods and helping preserve valuable cell lines. However, this process can compromise cell viability and functionality, particularly in primary cells like NSCs, which typically tolerate only a limited number of freeze-thaw cycles before losing their regenerative potential. Additionally, even subtle variations in culture media, such as changes in pH, nutrient composition, or growth factor

concentrations, can significantly influence NSC behavior and experimental outcomes. The degradation of media components over time due to storage conditions or shelf life may also contribute to variability.

Advantages:

- Well-defined culture conditions support cell growth and maintenance, ensuring reproducibility.
- Cryopreservation allows for long-term storage and future experimentation.

Limitations:

- Cryopreservation can impact cell viability and functionality.
- Variations in culture media preparation can affect cell growth and experimental outcomes.
- The stability of culture media components can affect the final results.
- There is a limited number of freeze-thaw cycles of NSCs.

Thawing

In Article II, the cryopreserved cells were then undergoing the thawing procedure, which involves immediate warming of the cryotubes in a 37°C water bath for 2 minutes. This quick thawing is crucial to prevent the formation of ice crystals, which can damage cells.

Advantages:

- Rapid warming in a 37°C water bath minimizes ice crystal formation and cellular damage, which helps preserve cell viability.
- Centrifugation effectively removes toxic cryoprotectants, enhancing cell viability.

Limitations:

- It can still cause stress to the cells, potentially affecting their viability and functionality.

2D and 3D Culture

Both 2D and 3D cultures were employed in **Articles II and IV**. **In Article II**, based on the literature, the optimal medium composition and seeding density for NSCs in these conditions were determined. The cell culture expansion in both articles was performed using 3D culture, while 2D culture was used to perform selected functional assays and phenotypic analysis.

2D cultures are ideal for screening and studying the differentiation of cells, while 3D cultures better mimic the *in vivo* environment, providing a more physiologically relevant model. It is worth noting that 2D culture may not fully replicate the complex three-dimensional environment of tissues, potentially limiting the physiological relevance of the findings. However, the complexity of setting up 3D cultures and analyzing results, such as immunofluorescence, makes them challenging to use. This method gives additional variables such as neurosphere size and nutrient diffusion to the core of the neurosphere, making experimental reproducibility difficult.

Advantages:

- **2D Culture**: Simple, ideal method for high-throughput screening and studying cell differentiation.
- **3D Culture**: It better mimics the *in vivo* environment, providing a more physiologically relevant model.

Limitations:

- **2D Culture**: May not fully replicate the complex 3D environment of tissues.
- **3D Culture**: Complex setup and maintenance; analysis can be challenging due to their structure.

Enzymatic and mechanical dissociation

For 3D cultures, neurospheres were mechanically **(Article II and IV)** and enzymatically **(Article II)** dissociated when they reached the desired diameter. For 2D cultures, when the cells reached the desired subconfluency, they were detached enzymatically with Accutase® solution and seeded into 24-well or 96-well plates coated with Poly-d-lysine and laminin.

Enzymatic dissociation is efficient and can be gentle on cells, helping to maintain their viability and surface markers. However, it can also induce stress or damage if not carefully optimized, potentially affecting cell function and survival. On the other hand, mechanical dissociation helps preserve cell surface markers and minimizes enzymatic damage, but it can physically damage cells and lead to uneven dissociation, complicating cell counting and experimental reproducibility. Mechanical dissociation is also more time-consuming and labor-intensive, especially with large volumes or multiple samples.

Advantages:

- **Enzymatic Dissociation**: This method allows for efficient and consistent detachment of cells from the culture surface and provides gentle yet effective cell dissociation, minimizing cell damage and maintaining cell viability.
- **Mechanical Dissociation:** It preserves cell surface markers and can reduce enzymatic damage.

Limitations:

- **Enzymatic Dissociation:** It can cause cell stress or damage, potentially affecting cell viability and functionality. Overexposure or improper usage can lead to significant cell death. Optimization of enzymatic dissociation protocols may be required for different cell types.
- **Mechanical Dissociation**: It can lead to physical damage to cells and impact cell fate and experimental outcomes. The inconsistency in cell counting and experimental reproducibility may result from uneven dissociation, with some cells remaining clumped together while others remain fully dissociated. It can be time-consuming and labor-intensive, particularly when handling large volumes or numerous samples.

Characterization of NSCs

As presented in this subsection, methods are mostly well-established and commonly used by the laboratories, the description is shorter and focused on relevant advantages and limitations.

Phenotypic and analytical analysis

Immunofluorescence analysis

Immunofluorescence analysis was used to analyze proliferation, inflammation, and neural markers. **In Articles II and IV**, it was performed on cells cultured under 2D and 3D conditions. For 3D cultures, in Article II, a new staining protocol was developed to visualize neurospheres without cryosectioning. The same protocol was used for *ex vivo* slices.

In **Article IV**, *in vivo* brain sections were treated according to the protocol.

Advantages*:*

- The method provides detailed visualization of cellular markers.
- **2D culture:** Well-established, commonly used protocol.
- **3D culture/*ex vivo*:** Allows visualization of NSCs in a more physiologically relevant 3D environment.
- ***in vivo:*** Enables the study of NSCs in their native tissue environment, providing insights into their *in vivo* behavior.

Limitations:

- **2D culture:** 2D cultures do not replicate the complex 3D microenvironment of neural tissue, potentially affecting cell behavior and function. Lack of cell-cell interactions can alter cellular phenotype.
- **3D culture/*ex vivo*:** More complex and time-consuming than 2D staining; requires careful optimization to ensure antibody penetration.
- ***in vivo****:* Invasiveness of the method, raising ethical concerns.

Quantitative Real-Time PCR (qRT-PCR) Analysis

In **Article IV**, qRT-PCR was used to quantify the expression of genes related to NSC proliferation and differentiation. Total RNA was isolated, converted to cDNA, and analyzed using the 2-ΔΔCt method [13,59].

Advantages:

- Quantitative and sensitive, allowing for the assessment of specific genes.

Limitations:

- High-quality RNA is required.

Cytokine and Chemokine Quantification

In **Article IV**, a Luminex assay was used to measure cytokine concentrations in the culture medium obtained from different conditions and time points, allowing simultaneous quantification of selected cytokines and chemokines.

Advantages:

- The method allows for the simultaneous measurement of multiple cytokines' concentration.

Limitations:

- Sample handling, culture conditions, and storage can influence the results.

Functional Assays

Live/Dead Assay

In **Article II**, cell viability was assessed using the Live/Dead™ assay.

Proliferation Assay

In **Articles II and IV**, cell proliferation was measured using the PrestoBlue™ assay.

Lactate Dehydrogenase (LDH) Assay

In **Article II**, cell viability was assessed by measuring LDH release with a colorimetric assay kit.

Senescence Assay

In **Article II**, cell senescence was evaluated using the CellEvent™ Senescence Green Detection Kit, measuring the β-galactosidase activity.

Advantages:

- It is a simple and quick method that provides a direct measure of cell viability, proliferation, membrane integrity, and senescence in different conditions.

Limitations:

- It does not distinguish between cell types and their differentiation stage.
- The differences in metabolism between species can affect the interpretation of statistical comparisons
- The detection of β-galactosidase activity is not entirely specific to senescent cells, as some non-senescent cells can also express this enzyme under certain conditions.

NSC Scratch Injury Assay

In Article IV, a scratch assay was used to study the influence of CSF on NSC migration.

Advantages:

- It is a simple and cost-effective way to study cell migration and wound healing.

Limitations:

- ❖ The method lacks the complexity of the *in vivo* environment.

Investigating neural niche effects on NSCs with CSF

In **Article IV**, CSF in different concentrations was used to explore its effects on NSC differentiation, proliferation, secretory potential, and neuroprotection. The leftover CSF was collected from adult volunteers whose samples were not needed for further medical diagnosis.

The neural niche's influence on transplanted NSCs remains poorly understood, along with the reasons behind their low post-transplant survival, which are still unclear. Using CSF offers a more physiologically relevant environment, but its application comes with challenges. CSF composition varies significantly between individuals due to factors like age, health status, and collection timing, presumably leading to inconsistent results. The limited volume of CSF, especially from human donors, restricts experimental use and often requires pooling, which may dilute specific biological signals. Moreover, CSF is highly sensitive to handling—exposure to light, temperature changes, or improper storage can degrade key components, compromising data reliability.

Advantages:

- CSF provides a medium that closely mimics the *in vivo* environment.
- The method allows for identifying specific effects on NSC behavior, including differentiation, proliferation, and neuroprotection.

Limitations:

- The variability in CSF samples (individual differences, such as age, gender, underlying medical conditions, and the time of day when the sample was collected).
- Sample volume of the obtained CSF may require pooling samples from multiple donors, which can reduce individual variability/dilute specific biomarkers or factors present in the CSF.
- Exposure to light, temperature fluctuations, and improper storage conditions can lead to the degradation of proteins and other critical components in the CSF.
- Contamination during collection, handling, or storage can cause artifacts.
- The preparation and analysis of CSF samples require careful handling and precise protocols to avoid degradation and contamination.

The differences in the available results and their possible explanation are described in **Article III**.

Ex vivo model

Organotypic Hippocampal Slices Culture (OHCs)

The OHCs were utilized to study NSC interactions in a three-dimensional, tissue-like environment. In **Article IV**, OHCs from 7-day-old Wistar rats were cultured using a modified Stoppini method [60,61]. The slices, prepared from rat hippocampi and cultured for 7 days, were then preselected and exposed to OGD to induce ischemic conditions.

Ex vivo model, compared to *in vitro*, provides the next level of niche complexity by incorporating interactions between different neural cell types as well as including local immune response, ensuring conditions that are more relevant to the *in vivo* environment. It enables a high number of experiments with a relatively low number of animals, which reduces the number of variants investigated *in vivo*. Organotypic cultures preserve the tissue architecture and cellular interactions of the hippocampus, allowing for a detailed examination of hippocampal structure and function, including responses to injury and treatments. However, the viability of organotypic slices is limited, which can restrict the duration and scope of experiments.

Advantages:

- Preserves tissue architecture and different cellular interactions, as well as local immune response, offering a more physiologically relevant model than an *in vitro* model.
- Enables a high number of experiments with relatively few animals, reducing the need for more extensive *in vivo* studies.
- Allows the examination of hippocampal responses to injury and cell transplantation.

Limitations:

- Relatively low viability of OHC limits the duration of experiments.

Oxygen-Glucose Deprivation (OGD) procedure

The procedure was conducted as previously described [61]. Post-OGD, slices were co-cultured with neural stem cells (NSCs) either directly or indirectly. Neuroprotection was assessed 24 hours later using propidium iodide staining and confocal microscopy.

Advantages:

- Allows quick assessment of NSC neuroprotective potential.

Limitations:

- Assesses only short-term effects.

The transplantation of 5-chloromethylfluorescein diacetate (CMFDA)-stained NSCs

In **Article IV**, NSC migration on hippocampal slices *ex vivo* was visualized by staining NSCs with CMFDA.

Advantages:

- ❖ Provides dynamic information about NSC behavior in a closer to physiological environment.

Limitations:

- ❖ When used *ex vivo*, it cannot fully mimic the physiological tissue complexity, signaling, and cell interactions.

In vivo model

Ouabain-Induced Brain Injury and hNSC Transplantation

In **Article IV,** a model of ischemic brain injury was performed by administering ouabain to induce focal brain damage, followed by the transplantation of hNSCs into the affected brain region.

Being the next level of complexity after the *ex vivo* model, this model provides the most detailed examination of the therapeutic potential of hNSCs in a controlled setting, including systemic immune response. It allows precise control over the injury and transplantation process, which is valuable for analyzing the effects of cell therapy on brain damage. However, the invasiveness of the procedures raises ethical concerns, and the variability in injury severity among animals, which arises from individual differences, may introduce inconsistencies in the results. Factors such as the exact location and dosage of ouabain, as well as individual differences in rat physiology, can affect the extent of brain injury and complicate data analysis. Furthermore, the rat (host's) immune system might recognize hNSCs as foreign, affecting the outcomes. Long-term monitoring is necessary to evaluate transplanted cells' survival, integration, and functional impact, but this process can be stressful for the animals.

Advantages:

- Allows for precise control over injury and transplantation, enabling detailed study of hNSC effects and including the systemic immune response of the host.
- Closely mimics human ischemic brain injury, allowing the finding of new potential therapeutic strategies.

Limitations:

- Invasive procedures raise ethical issues regarding animal welfare.
- Differences in injury severity and animal physiology can lead to inconsistent results.
- hNSCs may be recognized as foreign by the host immune system, potentially affecting therapeutic outcomes.
- Long-term monitoring required for assessing cell integration and functionality is demanding and can be stressful for animals.

Statistical Analysis

All experiments included appropriate controls and were repeated at least three times. Data were statistically analyzed to evaluate significant differences between experimental groups. Detailed information on the specific statistical analyses is provided in the figure captions and within the Materials and Methods sections of **Article II and Article IV**.

4. Summary of the most important results concerning the current state of knowledge

Article I: *Systematic identification of the critical elements of NSC protocols.*

Developing effective treatments for neurodegenerative diseases is a significant challenge, particularly in translating stem cell therapies into clinical practice. NSCs are greatly valued for their ability to support damaged tissue and their capacity for extensive clonal expansion and genetic stability, making them ideal candidates for precise genetic modifications. However, several critical issues remain unresolved, including the proper characterization of original cell lines, specific protocols for NSC isolation and preparation, as well as the standardization of cell numbers for transplantation and its method. The challenges observed nowadays in clinical trials highlight the need for optimization, beginning on the *in vitro* level of studies. Before moving on to the core topic of the article, I would like to describe the fundamental controversy that should be addressed — **the inconsistency in the terminology used to describe CNS undifferentiated cells**. Terms such as neural stem cells (often described as NSCs), neural progenitor cells (often mistakenly described as NPCs), and neural precursor cells (mostly described as NPCs or NSCs) are used interchangeably, though they determine distinct stages of cell differentiation and their capabilities. Neural stem cells have indefinite self-renewal potential, while neural progenitor cells have limited replication capacity. Neural precursor cells include all undifferentiated progeny of NSCs, including both NSCs and neural progenitor cells [42,62–64]. Thus, clear, consistent definitions seem to be crucial for accurate comparison across studies. Another significant challenge is the **variability of the laboratory conditions** used, which greatly impacts the reproducibility and interpretation of experimental results. Establishing a long-term culture of NSCs requires careful consideration of factors that could maintain lineage specificity and preserve both phenotypic and functional multipotency.

2D vs. 3D culture conditions

One of the critical factors is **spatial conditions**. NSCs can be cultured as either a 2D adhesive monolayer or as 3D neurospheres **(Article I, page 3)**. Although 2D cultures promote a more homogenous NSC population and faster proliferation, they lead to a loss of the cells' undifferentiated state [65–67]. By the fifth passage, NSCs in 2D culture are shown to present decreased growth and self-renewal capabilities, while the same features remain consistent for over ten dissociations in 3D cultures [68]. Additionally, 2D cultures do not accurately replicate the natural 3D brain environment. Despite challenges such as restricted nutrient and oxygen diffusion and limited access to the neurosphere core for analysis, 3D cultures remain the preferred method for long-term NSC cultivation and expansion, and are still considered the gold standard [69–71].

Dissociation methods

Regarding **NSCs**, the enzymatic method is widely interpreted as the standard for both 2D and 3D cultures due to high cell viability maintenance [72,73]. However, it can permanently affect the culture, potentially impacting karyotype stability, cell survival, and surface antigen integrity, or even induce apoptosis **(Article I, page 3)**. Rho-associated protein kinase (ROCK) inhibitors are commonly used to address these issues. Among the proteolytic enzymes, Accutase® is preferred over trypsin as it better preserves cell viability and has a lower impact on surface markers like EGF and bFGF receptors, though the optimal dosage remains under debate [72,74]. In contrast, trypsin can cause cell death and damage cell membranes. Mechanical dissociation, involving methods like filtration, chopping, and pipetting, offers an alternative but more aggressive approach. While some studies show it can be effective, it generally results in lower cell viability than enzymatic methods. In contrast, higher proliferation rates have been noted in several cases following the mechanical dissociation of human NSCs [75]. Remarkably, a combination of both enzymatic and mechanical dissociation techniques has shown improved results, achieving high cell viability and effective tissue dissociation with minimal cell death, and is now considered one of the most efficient methods for NSC culture.

Medium composition

Growth factors

Standardization of **medium composition**, particularly the concentration, and combination of growth factors like bFGF and epidermal growth factor (EGF), is essential for maintaining consistent cell proliferation, differentiation, and overall stem cell function [76]. These factors are essential for preserving the undifferentiated state of NSCs and promoting their proliferation, although their effects can vary depending on concentration and specific culture conditions [77]. bFGF primarily activates pathways that stimulate multipotential stem cells, while EGF supports both proliferation and differentiation processes **(Article I, page 4)**. Combining bFGF and EGF with LIF can further enhance the expansion, multipotency, and longevity of human NSCs while preventing cellular senescence [78]. Heparin plays a critical role in stabilizing bFGF through its interaction with heparan sulfate proteoglycans, thereby improving the efficacy of bFGF in culture systems [79–81].

Glutamine

Although glutamine is a nonessential amino acid, it plays a vital role in stem cell metabolism and early embryonic development [82]. Glutamine is crucial for cellular energy production, maintaining redox balance, and supporting proliferation, making it a highly recommended component for maintaining NSC cultures **(Article I, page 5)**.

Supplements

Commonly used supplements include N-2 and B-27 **(Article I, page 5)**. N-2 promotes neural cell proliferation and differentiation, though its effectiveness can be influenced by the type of transferrin used [83–85]. B-27, which provides additional vitamins and proteins, enhances cell survival and expansion but can introduce complexities in culture maintenance [86–88]. The newer N21 supplement effectively supports neuron isolation and expansion [89]. Combinations of bioactive compounds such as insulin, transferrin, progesterone, putrescine, and selenite offer similar benefits to N-2 but may differ in effectiveness [90]. Additionally, antibiotics and antimycotics are generally avoided in preclinical research due to their potential impact on cell functionality [91].

Neural induction medium

The composition of the **differentiation medium** is also critical **(Article I, page 10)**. Factors such as BDNF, NGF, and IGF-I can enhance neuron production, particularly when used in a combination [92 93]. While fetal bovine serum (FBS) is commonly utilized in laboratories worldwide, its animal origin limits clinical use, prompting the development of alternative options [94 95]. The origin of NSCs also affects their differentiation capabilities, making it important to consider these factors for clinical applications.

Coating

In addition, even **coating materials** are crucial for optimizing NSC culture conditions as they significantly influence cell fate **(Article I, page 19).** Laminin, Matrigel, and polylysine influence NSC culture, with each material affecting cell proliferation and differentiation differently. The choice of coating should be tailored to specific experimental requirements.

Cell cryopreservation method

Cryopreservation represents a crucial step on the path toward clinical application, thus requiring careful consideration **(Article I, page 19).** The rate of cooling during cryopreservation affects NSC survival, with rapid cooling minimizing ice crystal formation but requiring precise control to avoid cell damage. Conversely, slow cooling can lead to toxic intracellular solute concentrations. Optimizing cooling protocols and medium composition, including cryoprotectants like dimethyl sulfoxide (DMSO) and serum, is vital for ensuring consistent post-thaw cell viability and function.

Interspecies variability

What is more, culture conditions optimized for one species may not apply to another, leading to inconsistencies in research findings. This variability complicates the standardization of protocols and pooling of data, and the ability **to transfer findings from animal models to humans is often limited**. Results obtained in rodent models may not be directly applicable to human NSCs due to fundamental biological divergences. Validating findings across multiple species can bridge this gap and enhance the relevance of preclinical studies for human applications.

The isolation source and developmental age

In addition, **the origin and developmental stage of isolated NSCs** significantly affect their characteristics **(Article I, page 18)**. For example, NSCs from the forebrain produce more neurons than those from the midbrain or hindbrain [96]. NSCs from cortical regions primarily differentiate into one type of neuron, while those from the whole ganglionic eminence (WGE) display a different neuronal phenotype [75]. As brain development progresses, the number of proliferative cells decreases, while neuronal marker expression increases [75]. These variations highlight the importance of *in vitro* testing to confirm NSC potential before clinical application.

In summary, standardization of culture conditions is crucial for obtaining reliable results, considering factors like proliferation rate, differentiation, and cryopreservation. Variations in medium components, spatial conditions, coating type, cryopreservation, and dissociation can significantly impact the outcomes. Further research is necessary to validate the effects of cryopreservation and thawing on NSC viability and function.

Article II: *Investigation of the intra- and inter-species variability in NSC functional properties.*

Following the conclusions outlined in Article I, our experiment showed that NSCs from different species can respond differently to the same culture conditions. These differences make it difficult to apply results from one species directly to another. This finding is important, especially when using animal-derived NSCs instead of human ones. To make sure the results are relevant, it's important to choose animal NSCs that react similarly to human NSCs under the same conditions. The following factors, which were identified as critical for NSC culture, were examined in this study.

Cryopreservation

Starting with cell isolation, the current study reported that **direct cryopreservation of human and rodent NSCs significantly affects their viability and growth potential (Article II, page 8, Figure 4).** Cryopreservation immediately after isolation leads to significantly reduced viability and slower growth of human NSCs compared to those that were cultured before freezing. This pattern was consistent across both 2D and 3D cultures. Despite optimizing thawing protocols, such as avoiding centrifugation and adjusting medium composition, the cryopreserved cells struggled to establish long-term cultures. A similar reduction in viability was observed in rodent NSCs (rNSCs and mNSCs) subjected to direct cryopreservation, with delayed sphere formation and lower growth potential compared to cells that were cultured before freezing.

Medium composition

Growth factors and glutamine

When analyzing the effect of medium composition on NSCs, several clear patterns were observed. In 2D culture, human NSCs showed the highest proliferation in complete medium containing 20 ng/ml of both bFGF and EGF **(Article II, page 10, Figure 5)**. In contrast, incomplete media- especially those lacking growth factors or glutamine-led to a marked reduction in proliferation. Interestingly, despite these differences in proliferation, cell viability was mostly unaffected by medium composition. However, the absence of glutamine increased signs of cell senescence **(Article II, page 11, Figure 6)**. mNSCs followed a similar pattern, with significantly reduced proliferation only when both growth factors and glutamine were missing. rNSCs also showed decreased proliferation in media lacking either glutamine or growth factors, with the strongest effect seen when glutamine was absent.

Interspecies differences

Inter-species comparisons revealed that human NSCs generally had the highest viability across all medium variants, while rodent NSCs, particularly rNSCs, exhibited the lowest viability **(Article II, page 12, Figure 7)**. Proliferation potential was notably greater in mNSCs compared to hNSCs and rNSCs in several media variants. Additionally, cell senescence was more pronounced in rodent NSCs, highlighting differences in how these cells respond to the same culture conditions.

In 3D cultures, human NSCs formed neurospheres in all medium conditions, but neurosphere growth was inhibited in media lacking either growth factors or glutamine **(Article II, page 13, Figure 8)**. This effect was even more visible for rodent NSCs, with severe reductions in neurosphere formation observed when both growth factors and glutamine were absent.

Dissociation/expansion method

The choice of dissociation method had a species-specific impact on cell viability. Although there were no significant differences in the hNSC viability, for rNSCs, enzymatic dissociation resulted in higher initial cell death. Despite these early effects, long-term viability differences between the methods were less pronounced. The mechanical dissociation of mNSCs was more detrimental and resulted in a significant viability decrease after a week of culture **(Article II, pages 14 and 15, Figures 9 and 10)**.

Differentiation stage

Characterization of NSCs in 2D culture revealed species-specific differences in marker expression **(Article II, pages 16, 17, and 19, Figures 11, 12, 13, and 14)**. The highest Nestin expression was observed in hNSCs cultured with FGF and FGF10/EGF20 media, while significantly lower levels were detected in cells grown with EGF alone or without any growth factors. These results positively correlated with the expression of SOX2+ cells. Similarly, mNSCs displayed medium-dependent changes in marker expression, with the highest Nestin levels observed in EGF-containing medium and the lowest SOX2 levels in glutamine-free conditions. In contrast, rNSCs consistently expressed lower levels of both markers compared to human and mouse NSCs, highlighting interspecies differences in neural differentiation potential and sensitivity to culture conditions.

Migratory potential

NSC migration was assessed by measuring the diameter of the area occupied by the migrating cells **(Article II, page 20, Figure 15)**. The results showed that migration potential peaks at different times depending on the species, with human and mouse NSCs' maximum migration on Day 5 and rat NSCs on Day 3. Overall, the cells presented a strong ability to adapt to the new spatial conditions, demonstrating high migration potential early in the culture.

The article confirmed that optimizing NSC culture conditions depending on the species is crucial for obtaining accurate results. Key factors for human, rat, and mouse NSC culture include using a medium with 20 ng/mL of bFGF and EGF, ensuring the presence of glutamine, and applying a proper dissociation method for neutrospheres. These practices can standardize comparisons across studies and improve experimental outcomes. However, variations in proliferation, senescence, and differentiation among species could be linked to the NSC isolation method and source. In this study, human NSCs exhibit better viability and lower senescence than rodent NSCs but also require a complete medium with growth factors and glutamine to sustain proliferation, unlike rodent cells, which are less sensitive to medium composition. Moreover, hNSCs shared more similarities with rat NSCs in neurosphere formation and differentiation, but were closer to mouse NSCs in terms of senescence, viability, and migration.

Article III: *Investigation of the CSF role in the NSC niche.*

The article highlights the importance of CSF as a critical yet underexplored component of the NSC microenvironment. Since CSF is a key part of the natural brain stem cell niche, it can strongly affect how NSCs behave and develop. Including CSF in research could help make preclinical models more relevant to clinical outcomes. Traditionally viewed as a fluid with basic mechanical and chemical properties, CSF is now recognized for its complex roles throughout early development and adulthood. Regarding neurological disorders, CSF undergoes various changes, affecting its composition and function. By transporting essential nutrients, hormones, and other factors throughout the CNS, it serves as a conduit for intricate cell signaling pathways that maintain brain homeostasis. As such, CSF presents an excellent model for studying NSC fate. The analysis of 26 studies revealed significant but diverse effects of CSF on stem cell proliferation, differentiation, and survival depending on developmental stage, health status, and species.

Composition of different CSF types

The reviewed studies demonstrate that CSF significantly impacts the fate of NSCs due to specific signaling molecules within the CSF that regulate cell growth and differentiation **(Article III, pages 8-10, Table 2)**. This effect depends on its origin. For instance, artificial CSF brings different results compared to human-derived CSF, making direct substitutions discouraged. Additionally, a significant difference exists between embryonic(eCSF) and adult CSF (aCSF). Studies have shown that eCSF contains diffusible factors that regulate neuroepithelial stem cell fate, influencing brain development *in vivo* [97]. Although the precise mechanisms remain unclear, components such as proteins, particles, amino acids, and fibroblast growth factor have been implicated [97]. eCSF has a more complex composition than aCSF, but it still strongly affects the behavior of adult NSCs [98,99]. In experiments, eCSF usually promotes neuronal differentiation, while aCSF tends to push NSCs toward glial cell fates. This shows that the age of the CSF plays a key role in how it influences stem cell behavior. Most studies so far have used adult CSF from healthy donors. These studies show that aCSF often reduces NSC proliferation, probably because it contains signals that support glial differentiation.

This pattern reflects how the brain develops. During the embryonic stage, the brain mainly produces neurons, and after birth, it shifts toward making glial cells. CSF changes along with these developmental stages. eCSF contains more signals that support neurogenesis, while postnatal CSF promotes gliogenesis, helping the brain mature and maintain its functions.

Interestingly, CSF from patients with neurological conditions, like benign intracranial hypertension (BIH) or subarachnoid hemorrhage (SAH), has the opposite effect. It increases NSC proliferation. This may happen because disease-related CSF contains higher levels of growth factors and cytokines, such as FGF, NGF, TGF-β, GDNF, BDNF, and VEGF [51]. These molecules are known to support cell survival, stimulate proliferation, and protect neural tissue. For example, scientists found higher levels of VEGF and BDNF in the CSF of SAH patients. These factors help form new blood vessels, repair tissue, and support neurogenesis. They seem to play a key role in boosting NSC proliferation after injury. This suggests that pathological CSF could serve as a valuable source of regenerative signals and might have potential in future therapies. Still, we need more research to understand how these changes in CSF affect NSCs over time [100–102].

Impact of CSF on NSC fate

The presence of CSF in most studies enhanced differentiation into glial cells, though the exact mechanisms remain poorly understood **(Article III, page 11, Figure 2)**. Several studies have identified key molecules in CSF that influence NSCs fate by regulating their differentiation, proliferation, survival, and migration. Buddensiek and colleagues suggested that bone morphogenetic proteins (BMPs), possibly secreted by the choroid plexus, may contribute to these effects [98]. Although the expression of specific BMPs such as BMP2–BMP6 in the choroid plexus remains unclear, BMP4 is known to promote NSC differentiation through ERK pathway activation and GSK3β inhibition [50,98,103,104]. BMP7 has also been shown to stimulate dendritic growth in rat neurons, and BMP antagonists can block this effect,

highlighting its role in CSF-driven neuronal differentiation [50]. The study by Zhu et al. found that insulin-like growth factor-1 (IGF-1) in human CSF positively influenced the migration capacity and C-X-C chemokine receptor type 4 (CXCR4) expression in human amniotic MSCs and fetal neural progenitor cells, further demonstrating CSF's role in stem cell proliferation, migration, and viability [105]. Likewise, IGF-2 in embryonic CSF plays a crucial role in supporting growth and survival in the developing rodent cortex [106]. CSF also contributes to NSC fate by maintaining specific regional identities and proliferative signals. In the developing chick brain, CSF helps preserve midbrain markers such as Otx2 and Fgf8, while FGF2 within CSF enhances precursor proliferation [97,107]. Cytokines present in CSF further shape NSC outcomes. LIF and ciliary neurotrophic factor (CNTF), both found at high levels in CSF, promote astrocytic differentiation [108]. Another key factor, TGF-β, plays a dual role in regulating NSC fate. It promotes gliogenesis and supports brain homeostasis, but also inhibits neurogenesis and controls NSC proliferation by inducing cell cycle exit in hippocampal neurons [109–113].

Different studies have reported varying effects of CSF on NSCs, even when derived from the same origin. These discrepancies may stem from differences in NSC sources, CSF concentrations in culture (ranging from 0.5% to 100%), or the origin and biodiversity of the CSF itself. Nevertheless, the findings provide compelling evidence that healthy CSF, circulating through different parts of the CNS, offers a supportive environment for administered NSCs by providing stimulatory factors that favor neural lineage differentiation, albeit at the cost of reduced proliferation.

Together, these findings highlight that CSF is not a passive fluid but an active signaling environment that shapes NSC fate through a range of developmental stage- and context-specific factors. Further research is necessary to explore the effects of CSF on NSC fate under specific conditions. To fully understand CSF's impact on these cells, it is crucial to investigate the interactions between NSCs and the various growth factors, signaling molecules, and other components within CSF. Understanding the conditions under which CSF can support NSCs and promote regeneration and restoration may enhance targeted cellular therapy for CNS disorders.

Article IV: *Assessment of the effects of preincubating human NSCs with human CSF from healthy donors that aims to recreate culture conditions which closely mimic the physiological brain environment.*

This study evaluated the effects of CSF on the proliferation, migration, differentiation, and secretory profile, along with the neuroprotective potential of hNSCs.

The influence of CSF on NSCs *in vitro*

In vitro, the treatment with 100% CSF led to a noticeable decrease in the proliferation rate of hNSCs after 7 days compared to both the control medium and the medium with 30% CSF addition, which showed similar proliferation rates. Despite this, 100% CSF significantly enhanced the migratory capacity of hNSCs, as well as neurite outgrowth, with the most pronounced effects observed after 48 hours. Due to impaired cell adhesion and altered morphology observed under 100% CSF, this condition was not included in subsequent analyses.

Thus, the study was continued with only 30% CSF and a control group (using the standard medium), presenting significant changes in neural marker expression. After 7 days, in 2D culture, immunofluorescence staining revealed that the presence of early neural marker NESTIN was significantly reduced in the CSF group (70.6 ± 5%) compared to the control (82.1 ± 6.6%). Conversely, the early neuronal marker β-TUBULIN III exhibited a remarkable increase in the CSF group (23.4 ± 6.1%) relative to the control (15.4 ± 3.1%). However, the neuronal marker MAP2 was significantly lower in the CSF-treated cells (9.3 ± 3.4%) compared to the control (17 ± 4.2%). Additionally, the expression of the astrocytic marker GFAP was significantly upregulated in the CSF group (6.9 ± 5.8%) compared to the control (11.2 ± 3.6%). These results suggest early neuronal and astrocytic differentiation of hNSCs in the presence of CSF. The changes in protein levels were correlated with mRNA expression analysis.

However, in the 3D culture, NESTIN expression was upregulated 3.3-fold in the CSF group, in contrast to its downregulation in the 2D culture. Conversely, β-TUBULIN III expression was significantly reduced (0.065-fold) in the CSF group compared to the control. GFAP showed a great increase (38.03-fold) in the CSF-treated cells. Additionally, markers of oligodendrocytes (NG2) and proliferation (KI67) were both downregulated in the CSF group (0.54-fold and 0.68-fold, respectively). MAP2 expression, however, showed no significant differences between the two groups. These results indicate that in 3D culture, CSF enhances the expression of neural and astrocytic markers while reducing early neuronal, oligodendrocytic, and proliferation markers. Secretory analysis showed elevated levels of neurotrophic and inflammatory cytokines, including VEGF, BDNF, and HGF, in the CSF-treated group compared to the control.

The influence of CSF on NSCs *ex vivo*

Ex vivo experiments, in which NSCs were co-cultured with OGD-treated OHCs, showed significantly reduced neuronal death in the hippocampal CA1 region, suggesting their substantial neuroprotective effects. Both direct and indirect co-culture with CSF-treated cells led to lower cell death compared to the control group. Transplanted hNSCs were tracked using CMFDA-staining and migrated and integrated into damaged hippocampal areas. Staining with anti-Nestin and anti-Ki67 revealed high proliferation (Nestin+ Ki67+ cells) during the first 14 days. However, by day 21, Ki67 expression decreased, indicating a loss of proliferation abilities. CSF pretreatment promoted early proliferation and integration but did not sustain long-term proliferation, leading to the focus on the 14 days for direct co-culture experiments. In the indirect co-culture, CSF treatment significantly increased the concentrations of neurotrophic and inflammatory factors like bFGF, EGF, VEGF, BDNF, and HGF over time compared to the control, while LIF and beta-NGF remained undetectable. At the mRNA level, NESTIN was downregulated in the NSC CSF-treated OGD group, while β-TUBULIN III and KI67 were downregulated in NSC OGD cells. GFAP was significantly upregulated in the OGD group, with no

change from CSF treatment. Thus, CSF seems to modulate certain gene expressions related to neural differentiation and proliferation in hNSCs, changing the outcome specific to overall experimental conditions.

The influence of CSF on NSCs *in vivo*

Despite the positive *in vitro* and *ex vivo* effects, CSF pretreatment did not improve the survival or proliferation of transplanted hNSCs in a rat model of ischemic brain injury *in vivo*. Most transplanted cells differentiated into astrocytes (GFAP+), and the presence of activated microglia (Iba-1+) indicated an ongoing immune response. The results suggest that CSF-pretreated hNSCs may even strengthen the immune response after transplantation *in vivo*, potentially due to the human origin of the CSF.

Overall, CSF demonstrated a capacity to enhance the migratory and secretory properties of hNSCs *in vitro*, with mixed effects on differentiation. However, translating these benefits to *in vivo* applications remains challenging due to the presence of inflammatory responses and environmental conditions in the injured brain. The use of immunosuppressive drugs such as cyclosporine or tacrolimus could provide a solution to help reduce the immune response to transplanted cells. However, these must be carefully considered to avoid side effects.

5. Conclusions

General conclusion

Optimizing NSC isolation and long-term culture is crucial for enhancing their therapeutic potential. This requires species-specific protocols and accurate modeling of CSF influence. These conclusions are based on the following specific Article I-IV conclusions:

Conclusions from Article I

1. The critical elements of NSC protocols were systematically identified, including:
   - ❖ Growth factors such as bFGF, glutamine, culture type, and coating methods are required to maintain high proliferation rates of NSCs.
   - ❖ Supplements, growth factors, serum, and coatings are essential for effective cell differentiation.
   - ❖ Optimal cryopreservation conditions, including freezing methods, medium components, and cooling rates, are essential for maintaining cell viability post-thaw.
2. The potential challenge in protocol optimization lies in variations in NSC proliferation and differentiation that may be attributed to differences in isolation methods, brain regions, and developmental stages.
3. Differences between species necessitate that protocols optimized for rodent NSCs not be directly applied to human NSCs. Each species requires tailored conditions for obtaining reliable results.

Conclusions from Article II

1. The interspecies NSC variability investigation revealed a crucial effect on NSC functional properties of the following conditions/factors:
   - ❖ Cryopreservation: cultivating NSCs derived from all investigated species before cryopreservation is essential for maintaining their viability and functionality, while freshly isolated NSCs do not survive cryopreservation.
   - ❖ Supplementation: an optimal concentration of growth factors, specifically 20 ng/mL for both bFGF and EGF, is crucial for effective NSC proliferation, neurosphere growth, and cell viability; glutamine in the culture medium is important for sustaining cell proliferation and function.
   - ❖ Dissociation: an enzymatic method for neurosphere dissociation helps preserve cell viability.
2. The migratory potential of NSCs changes over time depending on the species, which is important for interpreting *in vivo* experiments.
3. Among rodent models, rat NSCs exhibit behavior more closely aligned with human NSCs than mouse NSCs in response to environmental conditions (neurosphere formation potential and differentiation stage).

Conclusions from Article III

1. CSF appears to serve a supportive role for NSCs, influencing their survival, proliferation, and differentiation.
2. The effects of CSF on NSCs vary depending on the source (embryonic, adult, artificial, or disease state), concentration, and specific components present:
   - ❖ Embryonic CSF tends to encourage neuronal differentiation, while adult CSF promotes glial differentiation and may reduce proliferation, potentially due to factors driving the development of glial cells.
   - ❖ CSF from patients with brain injury or other neurological diseases enhances NSC proliferation, likely due to elevated levels of growth factors and cytokines that support neuroprotection and tissue repair.

- ❖ Artificial CSF does not fully replicate human-origin CSF, leading to different results.
- ❖ Components in CSF, such as BMPs and IGF-1, play significant roles in NSC behavior. For example, BMP-7 in CSF contributes to dendritic growth, while IGF-1 in human CSF positively influences NSC migration and CXCR4 expression. Conversely, TGF-β has a dual role: promoting gliogenesis and inhibiting neurogenesis.

Conclusions from Article IV

1. Human CSF plays a crucial role in inhibiting the proliferation and stimulating the differentiation, secretory, and migratory potential of human NSCs, potentially influencing their therapeutic effectiveness.
2. CSF alters the secretory potential of NSCs, especially after interaction with damaged neural tissue. After post-ischemic injury *ex vivo*, NSCs initially release factors that promote cell proliferation, later shifting to the secretion of immunomodulatory, proangiogenic, and neuroprotective factors over time.
3. *In vivo*, human NSCs exposed to human CSF show decreased survival and proliferation while increasing their immunogenicity upon transplantation into the rat brain, suggesting a potential additive immunogenic effect of CSF.

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7. Reprints of articles included in the collection

7.1. Certified by the MMRI PAS Library, the five-year impact factor (IF) of publications included in the collection.

Collection of articles presented in the dissertation:

I. Radoszkiewicz K, Hribljan V, Isakovic J, Mitrecic D, and Sarnowska A. Critical Points for Optimizing Long-Term Culture and Neural Differentiation Capacity of Rodent and Human Neural Stem Cells to Facilitate Translation into Clinical Settings. Experimental Neurology 2023, 114353. doi: 10.1016/J.EXPNEUROL.2023.114353. 5-year IF: 4.8

II. Radoszkiewicz K, Jezierska-Woźniak K, Waśniewski T, Sarnowska A. Understanding Intra- and Inter-Species Variability in Neural Stem Cells' Biology Is Key to Their Successful Cryopreservation, Culture, and Propagation. Cells 2023; 12(3):488. doi: 10.3390/cells12030488. 5-year IF: 6.0

III. Radoszkiewicz K, Bzinkowska A, Chodkowska M, Rybkowska P, Sypecka M, Zembrzuska-Kaska I, and Sarnowska A, Deciphering the impact of cerebrospinal fluid on stem cell fate as a new mechanism to enhance clinical therapy development Front. Neurosci. 2024, 17:1332751. doi: 10.3389/fnins.2023.1332751. 5-year IF: 4.3

IV. Radoszkiewicz K, Bzinkowska A, Sypecka M, Sulejczak D, Ferrari D, Gelati M, Vescovi AL, Sarnowska A. Unraveling the impact of human cerebrospinal fluid on human neural stem cell fate. Front Cell Dev Biol. 2025 13:1527557. doi: 10.3389/fcell.2025.1527557. 5-year IF: 5.2

BIBLIOTEKA
Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk
02-106 Warszawa, ul. A. Pawińskiego 5
tel. 22 608-66-11 NIP 525-000-81-69
e-mail: library@imdik.pan.pl

KIEROWNIK BIBLIOTEKI
Katarzyna Nieszporska
mgr Katarzyna Nieszporska

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7.2. Reprints of Articles

7.2.1. Article I

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Review Article

Critical points for optimizing long-term culture and neural differentiation capacity of rodent and human neural stem cells to facilitate translation into clinical settings

Klaudia Radoszkiewicz [a,1], Valentina Hribljan [b,1], Jasmina Isakovic [c], Dinko Mitrecic [b], Anna Sarnowska [a,*]

[a] Translational Platform for Regenerative Medicine, Mossakowski Medical Research Institute, Polish Academy of Sciences, Pawinskiego 5 Street, 02-106 Warsaw, Poland
[b] Laboratory for Stem Cells, Croatian Institute for Brain Research, University of Zagreb School of Medicine, Šalata 12, Zagreb, Croatia
[c] Omnion Research International Ltd, Heinzelova 4, 10000 Zagreb, Croatia

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ABSTRACT

Despite several decades of research on the nature and functional properties of neural stem cells, which brought great advances in regenerative medicine, there is still a plethora of ambiguous protocols and interpretations linked to their applications. Here, we present a whole spectrum of protocol elements that should be standardized in order to obtain viable cell cultures and facilitate their translation into clinical settings. Additionally, this review also presents outstanding limitations and possible problems to be encountered when dealing with protocol optimization. Most importantly, we also outline the critical points that should be considered before starting any experiments utilizing neural stem cells or interpreting their results.

1. Introduction

One of the major challenges of the healthcare landscape is to develop successful treatments for neurodegenerative diseases. Due to their plasticity, stem cells possess the ability to modulate multiple cellular functions and bring quantifiable advantages in the treatment of injured tissue. They accomplish this through two major mechanisms:

1. Phenotypic multipotency – direct replacement of the affected neural cells (e.g., neurons, oligodendrocytes, or astrocytes) in damaged or degenerated tissue;
2. Functional multipotency – interactive homeostatic effects via the production of neurotrophic and immunomodulatory factors, exosomes, extracellular vesicles, and formation of gap junctions (Ferrari et al., 2018), (Teng, 2019).

Even though both direct and indirect mechanisms bring major benefits, viable protocols for translation of this research into clinical settings are lacking. As such, the current solutions dealing with direct replacement therapy are still insufficient. Yet, a glimmer of hope was given by the commonly used mesenchymal stem/stromal cells (MSCs) in lieu of their therapeutic potential based on their rich adjuvant abilities (Figiel-Dabrowska et al., 2021; Wedzinska et al., 2021; Tomecka et al., 2021). However, many clinical trials have already shown that the application of MSCs is limited by their short-term survival after the transplantation and not well-understood neurorestorative properties (Guy and Offen, 2020; Lukomska et al., 2019; Gugliandolo et al., n.d.). Moreover, since the cell-based therapy protocol implemented across the experiments exhibits great variability, it is very difficult to compare the obtained outcomes. Similar limitations can also be found in clinical trials focused around neural stem cells (NSCs). These cells, unlike MSCs, demonstrate a plethora of neurorestorative properties that make them even more attractive for any application in neurological disorders (Zhou et al., 2021; D et al., 2012; Mitrečić, 2011; Hribljan et al., 2018). So far, satisfactory findings have been related to the therapeutic effect of exogenous NSCs, based on their ability to secrete several trophic factors

Abbreviations: NSCs, neural stem cells; NPCs, neural precursor cells; MSCs, mesenchymal stem cells; CNS, central nervous system; DMSO, dimethyl sulfoxide; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; NGF, nerve growth factor; LIF, leukemia inhibitory factor; BDNF, brain-derived neurotrophic factor; FBS, fetal bovine serum; WGE, whole ganglionic eminence.

* Corresponding author.
E-mail address: asarnowska@imdik.pan.pl (A. Sarnowska).
[1] These authors contributed equally.

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as well as to serve as chaperone cells for injured and dysfunctional tissue (Hess and Borlongan, 2007). Furthermore, mammalian NSCs are also characterized by their rich clonal expansion and genetic stability. Since they can be easily transfected, NSCs became attractive candidates for precise genetic manipulations (Bressan et al., 2017). Nevertheless, many unsolved problems persist. These include: i) insufficiencies in characterization of the original cell lines; ii)deficiencies in availabilities of specifically tailored protocols for optimizing the isolation and preparation of a particular line of NSCs; and iii) inadequate evidence for standardization of the numbers of cells to be used for transplantation into different target organs or systems (Casarosa et al., 2014). These should be confronted before any further therapeutic application. As such, all of the aforementioned issues pertaining to clinical trials with NSCs further highlight the need to optimize the conducted procedures, starting with the isolation method and *in vitro* culture.

1.1. Misleading cell nomenclature

The next important issue pertains to the various terminology used to describe the undifferentiated cells of the central nervous system (CNS). This commonly leads to misunderstanding, especially when interpreting the obtained results. To clarify, neural stem cells (NSCs), neural progenitor cells, and neural precursor cells (NPCs) possess different features and, as such, are not the same cell type. In general, neural progenitor cells are defined to be the progeny of stem cell division, undergoing a limited number of replication cycles, while NSCs possess unique and interminable self-renewal abilities. According to numerous reports, the term neural precursor cells refers to a mixed population consisting of all undifferentiated progeny of neural stem cells, including both NSCs and neural progenitor cells (Martínez-Cerdeño and Noctor, 2018; ffrench-Constant, 2008; Zhao and Moore, 2018; Dibajnia and Morshead, 2013). As such, and before any clinical application, several preclinical laboratory tests should be performed, including *in vitro, ex vivo,* and *in vivo*, all in order to adequately characterize the cell types in question.

1.2. The influence of pre-application characterization

It is important to stress that, when performing the aforementioned tests, the conditions which are adapted for one species could have a different impact on another, i.e. the standardization protocol performed on cells derived from animal models (for example, a mouse) could not simply be applied to human stem cells. Therefore, and in order to tackle the outstanding issues within the field, we analyzed a dozen differences between the conditions used in laboratories worldwide, for both rodent and human NSCs, as well as their influence on changing the cells' properties which often leads to inconsistent results and conclusions. Although our knowledge regarding numerous processes related to the physiological effects of neural stem cells has been drastically expanded throughout the past 30 years (Fig. 1), a complete pooled analysis was shown to be very difficult. The interpretation of results regarding physiological effects on NSCs is often hampered since several key environmental factors such as growth factors or heparin, are in varied ways by different research groups. Additionally, since changing their state is a pivotal part of their biology, one should also pay regard to the high plasticity of NSCs.

In a perfect situation, one would succeed to maintain a cell culture in which cells are multiplying in a linear way, with minimal or nonexistent cell death, and without any visible variations in cell growth, cell death, and phenotypical features. Therefore, the ideal differentiation protocols should lead to a successful establishment of all the required phenotypical features, including those collected by morphological, RNA expression and protein expression markers. However, it is still abundantly clear that a myriad of protocols are used within the field because perfect conditions do not exist. Thus, before starting a cell culture experiment, one needs to clearly outline the necessary steps and perform due diligence in order to define the crucial parameters which need to be controlled during the experiment's execution.

With the goal to bring to the readers a detailed, yet focused, overview, this review includes information on two major groups of parameters which influence cell cultures: a) factors linked to growing the cells *in vitro*, especially from the perspective of the long-term cultures; and b) factors which influence cell differentiation and the acquiring of desired

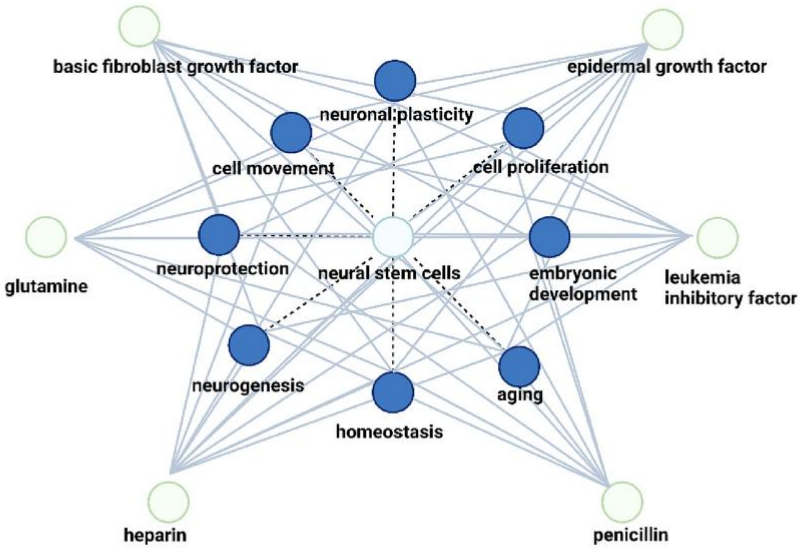

Fig. 1. The correlation between processes connected with neural stem cells (green circles) and their physiological effects (blue circles). based on a graph created in biovista vizit.

end-phenotypes.

1.3. The influence of various factors on long-term culture of NSCs

Since quite a significant number of factors must be considered when establishing a long-term culture of NSCs, especially as they pertain to maintaining a satisfactory lineage specificity as well as phenotypic and functional multipotencies, they represent an important target for any future protocol optimization. Of note here, factors which influence cell differentiation are described in a separate chapter.

1.4. 2D vs. 3D

NSCs can be cultured either as an adhesive monolayer (2D) or as a suspension, in the form of 3D neurospheres (3D). When compared to 3D cultures which comprise cells with little direct access to nutrients, 2D cell cultures are used to obtain a more homogeneous population of NSCs (Fig. 2). This also enables the cells to proliferate faster (Liu et al., 2014), (Yang et al., 2019). Yet, since NSCs loose their undifferentiated state and stem features during this process of 2D culture, the cell culture ends up containing more differentiated cells (Islami et al., 2017; Molina-Jimenez et al., 2012; Cruz-Acuña and García, 2017). An additional consideration comes in the form of NSCs growth rate and self-renewal capacity which, when compared to the 3D culture wherein these properties remain stable for over 10 passages, reduces after the 5th passage (Sun et al., 2011). Moreover, since the 2D culturing method does not adequately show the natural 3D niche environment, cell-to-cell interactions, and oxygen concentration, it can provide misleading results regarding *in vivo* responses (Edmondson et al., 2014). Considering all of the aforementioned confounding factors, it is easy to see why a 3D cell culture represents the preferred method for both long-term NSCs cultivation and cell expansion following derivation, having been used by many groups for over 30 years (Reynolds and Weiss, 1992a).

Even though it was proven to be more optimal, the 3D culturing method possesses several potential pitfalls. Firstly, the visualization of the inner cells of the neurosphere could be problematic, resulting in an inexact morphological or functional analysis. Moreover, the penetration of nutrients and oxygen to the interior of the neurosphere is limited. This can hamper any studies examining the impact of nutrition on cell proliferation, survival, or differentiation (Svendsen et al., 1997). Additionally, since the clonogenic potential of NSCs growing in a 3D cell culture is difficult to assess, as both stem cells and progenitors can form a neurosphere – with quiescent stem cells passing largely undetected. As such, the "stemness" of these cells is also being brought into question (Pastrana et al., 2011; Gil-Perotín et al., 2013; Ladiwala et al., 2012).

1.5. Dissociation/passaging method

A wealth of literature points to the enzymatic method of dissociation as the one which provides good cell viability (Gil-Perotín et al., 2013), (Wachs et al., 2003a). Therefore, this method has been established as the golden standard for dissociation and passaging of both 2D and 3D cultures. Enzymatic dissociation has remarkable long-term effects on cell culture, including affecting the stability of the karyotype, cell survival, and maturation, impacting the loss of surface antigens or even, in some cases, causing apoptosis-induced cell loss. This suggests the need of using a Rho-associated protein kinase (ROCK) inhibitor (Jager et al., 2016; Hasegawa et al., 2006; Chen et al., 2012; Panchision et al., 2007; Ohnuma et al., 2014; Svendsen et al., 1998a; Sen et al., 2004). Employing such a method requires the use of a proteolytic enzyme, such

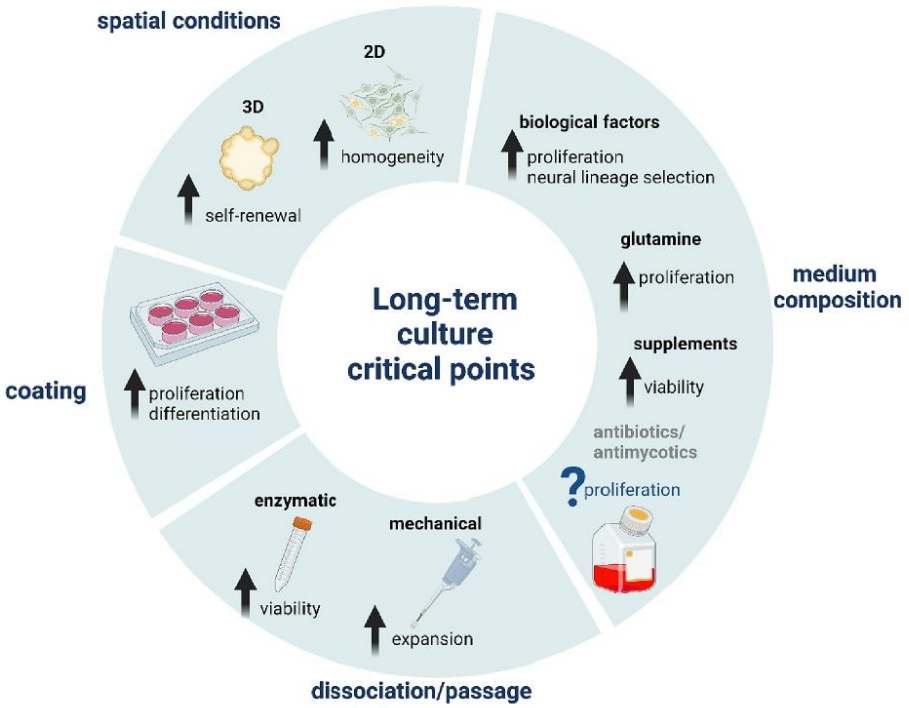

Fig. 2. Critical points for establishing viable NSC culture conditions.

as trypsin, dispase, TrypLE, or Accutase®. Even though Accutase® and trypsin represent the most commonly used enzymes (Table 4, Table 6) they both, to a different extent, cause some changes in cell viability following passaging. Since several studies have shown that trypsin can induce cell death and disrupt the cell membrane, the use of Accutase® became widespread as it facilitates increased cell survival and efficient growth in 2D or regrowth of dissociated neurospheres (Wachs et al., 2003a), (Zhou et al., 2020). Although Li et al. showed that the extent of apoptosis detected in human NSCs culture immediately after dissociation of trypsin was lower than that observed in cells treated with Accutase®, the viability of these cells was significantly decreased (Li et al., 2015). In line with its high efficiency in promoting cell dissociation, Accutase® also seems to be less harmful to NSCs than trypsin, seen in the higher cell viability following dissociation (Wachs et al., 2003b). Moreover, Accutase® does not affect the integrity of cell surface markers, including EGF and bFGF receptors, to the same extent as trypsin (Wachs et al., 2003b). However, there is little agreement on the most optimal Accutase® dosages, ranging everywhere from 200 µl up to 1 ml per pellet, with 2-5 min incubation time in 37°C (Jager et al., 2016), (Wachs et al., 2003c), (Deshpande et al., 2019). In case of trypsin, the most commonly used concentration is 0.05% and the suggested incubation time ranges from 3 to 5 min (Wachs et al., 2003d; Chen et al., 2007; Walker and Kempermann, 2014; Azari et al., 2011a; Teng et al., 2018).

An additional, alternative method of 3D cell culture dissociation comes in the form of mechanical dissociation. It includes the use of filters, chopping, and trituration strategies with different pipettes (Azari et al., 2011b). Even though this procedure is known to be relatively aggressive, it was shown to be quite efficient when well-optimized (Sen et al., 2004), (Azari et al., 2010), (Svendsen et al., 1998b). This is supported by the fact that our group has also observed a higher proliferation rate following the mechanical dissociation of human NSCs. Moreover, it has also been reported that the mechanical method could provide a higher expansion rate of human NSCs, as compared to the enzymatic method (Martín-Ibáñez et al., 2017). However, the majority of researchers still suggest using enzymatical dissociation due to it resulting in better viability of both rodent and human NSCs (Wachs et al., 2003a), (Zheng et al., 2006). Yet, this method is not recommended when a single-cell suspension is required (Wachs et al., 2003b).

As such, many researchers have resorted to combining these two methods of dissociation(Rietze and Reynolds, 2006; Aligholi et al., 2014; Stoll et al., 2015). For example, both mechanical and enzymatic dissociation of mouse brain hippocampal tissue facilitates obtaining a high viability (>90%) single-cell suspension (Trujillo et al., 2021). Thus, a combination of enzymatic dissociation using Accutase® with mechanical pipetting is now considered as one of the most efficient procedures for performing sufficient tissue dissociation with minimal cell death (Zhou et al., 2020).

1.6. Medium composition

1.6.1. Growth factors (bFGF, EGF and LIF)

The inevitable importance of two mitogens in the medium, basic fibroblast growth factor (bFGF, also named FGF-2) and epidermal growth factor (EGF), for long-term neural cultures, has been documented throughout the past 30 years (Reynolds and Weiss, 1992a). Both factors facilitate the maintenance of the cell's undifferentiated state and induce cell proliferation, while their removal induces differentiation (Reynolds and Weiss, 1992b). As such, an in-depth analysis of recent studies shows that the majority of researchers use both bFGF and EGF for their 3D culture medium. However, some differences in their application exist. While both factors are mainly known to be crucial for neurosphere formation, special attention should be paid to their functional variety as it pertains to culture conditions and the origin of the cells.

One of the most prominent issues pertaining to the first attempts at establishing a NSC culture is the induction of NSC proliferation *in vitro* since many researchers observed their high tendency for differentiation and division (Teng, 2019). We now know that the signaling pathways related to many growth factors which are present in the cellular niche play a crucial role in maintaining of the neural stem cell population (Campos et al., 2006), (Campos, 2005). Moreover, bFGF-signaling pathways seem to be more complicated than EGF's; wherein it has been established that bFGF stimulates multipotential stem cells from various regions of the CNS (Temple and Qian, 1995), (Gritti et al., 1996). On the other hand, EGF was suggested to be binding to CNS cells and, as such, stimulating their proliferation, division, and, in the presence of this mitogen, promoting further differentiation into neurons and astrocytes (Reynolds et al., 1992). There are also additional reports suggesting that EGF induces increased levels of Notch-1 intracellular domain in neural progenitors and establishes a bidirectional cross-talk with Notch and EGF in NSCs (Campos et al., 2006), (Lathia et al., 2008). Notch is a cell surface protein involved in the promotion of NSCs survival, self-renewal and cell fate choices during CNS development (Lathia et al., 2008).

When used together, bFGF and EGF have been shown to promote *in vitro* proliferation of NSCs *via* the MAPK pathway (Campos, 2005), (Yanagisawa et al., 2005; Learish et al., 2000; Xiao et al., 2007). Moreover, their use has also yielded improved cell survival and their differentiation into 3 neural lineages (Vescovi and Snyder, 1999). The responsiveness to the presence of both mitogens has been defined as a screening method for the selection of the most immature and multipotent neural stem/progenitor cells from heterogenous CNS cultures (Teng, 2019).

However, it should be noted that NSCs/NPCs' response to bFGF and EGF can vary throughout different culture conditions and application methods since NSCs' response is connected to their region of origin as well as their lineage (Kalyani et al., 1997). It has been revealed that the temporal differences in the activation of the Ras-MAPK pathway between these two factors could cause different cellular responses (Yamada et al., 2004). Even though both bFGF and EGF stimulate proliferation of neural progenitors of embryonic rodent, primate and human central nervous system (CNS), the specific effects on the culture-monolayer *vs.* neurospheres- also depend on the species in question (Ray and Gage, 2006; Chiasson et al., 1999; Carpenter et al., 1999a; Gritti et al., 1999). Although NSCs and their progeny, which form neurospheres, remain in a relatively undifferentiated state throughout, their rate of expansion and their numbers, as mentioned previously, is enhanced if these two mitogens are used simultaneously. Nonetheless, pure EGF is routinely used for embryonic mouse CNS culture, while a combination of bFGF and EGF is commonly used for culturing adult mouse subventricular zone cells, embryonic rat, and fetal human CNS cells (Louis et al., 2013). It is worth adding that early embryonic NSCs respond only to bFGF, while late embryonic and adult NSCs are responsive to both bFGF and EGF (Reynolds and Weiss, 1992b), (Kilpatrick and Bartlett, 1995; V et al., 1999; Arsenijevic et al., 2001).

bFGF is a prominent component of the medium since it is a part of a large cytokine family responsible for maintaining several biological processes involving embryonic cells' proliferation and differentiation (Nurcombe et al., 1993). It is a single-chain heparin-binding polypeptide synthesized by several cell types. It has been shown that bFGF is „a notoriously unstable protein" (Estapé et al., 1998), especially when stored at room temperature, in an alkaline pH or incubated in the presence of Cu^{2+} ions (Caccia et al., 1992). Additionally, human FGF, when stored longer than allowed, starts to form aggregates and degrades. Its instability is known to be linked with the presence of four cysteines in the primary sequence of this polypeptide – the two exposed ones can form disulfide bonds leading to multimerization. The stability of the human FGF can be improved by exchanging two exposed cysteines for serine (Sauer et al., 2021), (Sauer et al., 2019). Thus, bFGF should be used immediately after thawing and stored cooled. All things considered, we suggest using its modified cysteine-to-serine version.

While the benefits of combined use of bFGF and EGF are well

documented, it is crucial to carefully consider the necessary concentrations of the aforementioned mitogens with respect to the desired outcome (Fig. 3). Still, there is little to no consensus within the literature as to the most optimal bFGF and EGF concentrations. Regarding the concentration of the growth factors, controlled experiments in which various concentrations of EGF and FGF2 would be compared are still missing. Even though our research suggests that the most commonly used concentration is 20 ng/ml of EGF and 20 ng/ml of bFGF, there is a plethora of experiments using 20 ng/ml of bFGF and 10 ng/ml of EGF, as well as 10 ng/ml of both growth factors. Interestingly, an experiment performed on mouse embryonic NSCs by the Qian group has shown that, when exposed to low concentrations of bFGF, NSCs can differentiate into neurons. On the other hand, higher concentrations of bFGF make the cells susceptible to differentiation into both neurons and oligodendrocytes (Qian et al., 1997). As such, one needs to be aware that concentrations of growth factors should also be adapted to the concentration (total number) of cells and their exposure to the medium. This is important since large numbers of neurospheres with a small diameter will have more cells directly exposed to the growth factors, as opposed to smaller numbers of very large neurospheres.

Additionally, a few researchers, starting with the Carpenter group, performing their experiments on hNSCs, have suggested a significant benefit in utilizing a combination of bFGF, EGF, and LIF (leukemia inhibitory factor). Throughout their research, the effect of LIF, as well as bFGF, was shown to facilitate long-term expansion and preservation of cells' multipotency. Human neural progenitor cells, when exposed to these three factors in combination, remained multipotent for at least 1 year *in vitro* (Carpenter et al., *1999b*). Moreover, the same group has also demonstrated that both factors affect the progenitor cells' ratio within the population (Carpenter et al., 1999a). Morten et al. have also shown that the addition of LIF can facilitate better long-term propagation of hNSC cultures when compared to the use of only bFGF and EGF (Andersen et al., 2009). Other experiments on hNSCs are supplementing this knowledge by showing that EGF-responsive cells require LIF to avert senescence, strengthen their expansion and initiate self-renewal (Wright et al., 2003), (Shimazaki et al., 2001). Moreover, the addition of both LIF and bFGF was shown to better stimulate the initial cell population of NSCs which were newly thawed in high passages. On the other hand, the use of bFGF alone was also satisfactory for the short-term propagation of hNSCs (Teng, 2019), (Bjugstad et al., 2008). With all that being said, LIF seems to be a good choice for boosting the expansion rate and multipotency of human cells.

1.6.2. The role of heparin

On top of bFGF, which was shown to be one of the crucial factors necessary for the optimal cultivation of neural cells, one must not forget heparin. The critical importance of heparin can be explained by the fact that the bioactivity of bFGF is highly controlled by the mandatory cofactor, heparan sulfate proteoglycan (HSPG), which forms a complex with the fibroblasts growth factor receptor (FGFR) (Belov and Mohammadi, 2013; Yayon et al., 1991; Rusnati et al., 1994). As such, it has been shown that this component demonstrates a strong interaction with bFGF, being responsible for its stabilization (Caldwell et al., 2004). In other words, when establishing a culture of neural cells with a desire to have a high yield, bFGF needs to be combined with heparin. This is additionally supported by the fact that proteoglycans are one of the most important survival factors for both NSCs and neural progenitors (Nurcombe et al., 1993).

1.6.3. Glutamine

Although glutamine, the most abundant and naturally occurring amino acid is nonessential, it plays a critical role in a variety of metabolic processes as well as regulates early embryonic development (Stein and Moore, 1954), (Ryu et al., 2015). Even though its role as a source of energy has been revealed many years ago (DeBerardinis et al., 2007), there still exist studies where researchers did not use or describe its addition to the medium.

Glutamine can contribute to many metabolic tasks of proliferating cells by engaging bioenergetics or replacing glucose in macromolecule production (Deberardinis and Cheng, 2010). In adult mice neural stem/progenitor cells, glutamine was shown to be a necessary component for maintaining redox homeostasis (Yeo et al., 2013). Moreover, studies on mouse embryonic stem cells demonstrated that glutamine has a critical role in self-renewal, proliferation, and maintaining the undifferentiated status of cells (Ryu et al., 2015). Another study has shown that glutamine regulates the activity of mTOR signaling and, as a result, impacts coordinated tumor stem cell proliferation and growth (Nicklin et al., 2009). Taken together, glutamine seems to play an important role in controlling the stem cells' fate and, as such, comes highly recommended for the maintenance of NSC cultures.

1.6.4. Supplements

To achieve acceptable viability of cell culture, hormones, proteins, vitamins, and other nutrients need to be used (Fig. 2). Since the composition of supplements added to the basal media (predominantly DMEM/F12 or Neurobasal) exhibits great variability, this leads to different effects on cell cultures (Table 1). When it comes to these commonly used supplements (Table 2, Table 3 and Table 5) they include N-2, B-27, N21, and a variety of hormone mixes. Since each of them possesses distinct advantages, their function and use will be described in

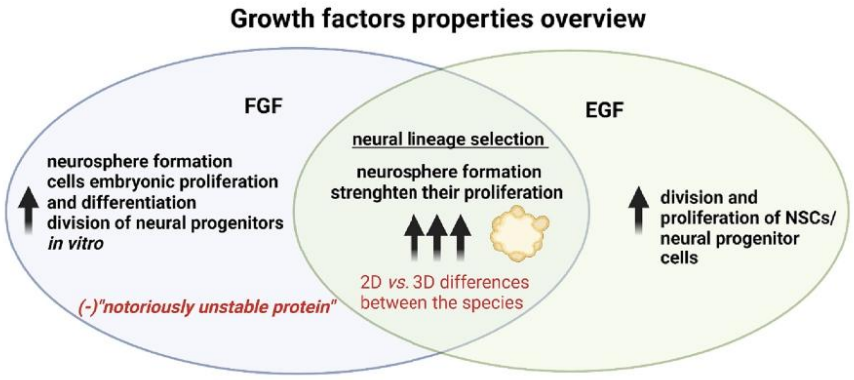

Fig. 3. A general overview of the properties of main growth factors depending on single VS. a combination use.

Table 1
The comparison of different supplements' composition.

Supplements composition

	B27	N2	N21	Hormone mix
Proteins	1. BSA, fatty acid free Fraction V 2. Superoxide Dismutase 3. Catalase **4. Human Transferrin**	**1. Human Transferrin**	1. Albumin (bovine) 2. Superoxide dismutase 3. Catalase **4. Holo-Transferrin**	**1. Transferin**
Hormones	**5. Human Recombinant Insulin** 6. T3 (triodo-I-thyronine) **7. Progesterone** 8. Corticosterone	**2. Recombinant Human Insulin** **3. Progesterone**	5. **Insulin** 6. Triiodo-L-thyronine 7. **Progesterone** 8. Corticosterone	**2. Insulin** **3. Progesterone**
Vitamins	9. Tocopherol (DL Alpha) 10. Tocopherol Acetate (DL Alpha) 11. Vitamin A (acetate) 12. Biotin		9. D,L-alpha-Tocopherol 10. D,L-alpha-Tocopherol acetate 11. Retinyl acetate 12. Retinol	
Other components	13. Ethanolamine HCl 14. Glutathione (reduced) 15. Galactose (D) 16. Linoleic Acid 17. Linolenic Acid **18. Putrescine 2HCl** **19. Sodium Selenite** 20. Carnitine L HCl	**4. Putrescine** **5. Selenite**	13. Ethanolamine 14. Glutathione 15. Galactose 16. Linoleic Acid 17. Linolenic Acid 18. Lipoic Acid **19. Putrescine** **20. Selenite** 21. L-Carnitine	**4. Putrescine** **5. Selenite salt**

*The components which are the same for each supplement are **bolded**.*

detail in the upcoming sections.

1.6.4.1. N-2 supplement. Out of all of them, the N-2 supplement is by far the most used one when it comes to the cultivation of NSCs. First developed in 1979 by Bottenstein et al., N-2 is a serum-free 11-ingredient supplement used for the proliferation of neural cells and enhancement of their differentiation into a variety of subtypes. It can be made from scratch or bought from manufacturers as a ready-to-use agent. However, the Bottenstein formulation and the commercially available N-2 supplements differ in one key ingredient – transferrin. While holotransferin, present in commercial N-2, has been described as a factor which stimulates cell proliferation, that effect is not seen in Bottenstein's formulation which contains apo-transferrin (iron-free) (Bottenstein, 1985; Rudland et al., 1977; Fletcher and Huehns, 1968). Thus, depending on the formulation used, differences in observed cell growth might occur (Kan and Yamane, 1984), (Silvestroff et al., 2013).

1.6.4.2. B-27 supplement. In 1993., right after Bottenstein developed N2, Brewer et al. developed the B27 supplement. It contains all of the ingredients of N2, as well as 15 additional components such as vitamins, hormones and proteins (Table 1) (Brewer et al., 1993). Upon performing experiments with this supplement, two groups have observed a higher survival rate of neurons cultured with N2 following 4 days in culture (Brewer et al., 1993), (Brewer, 1995). Additional experiments on rodent adult NSCs cultured in a Neurobasal medium have demonstrated that a combination of B27 and N2, in comparison to a singular use of N2, provides a 15 times higher expansion rate 1 week after the passage (Wachs et al., 2003a). Interestingly, it was also noted that retinyl acetate and triiodo-L-thyronine included in B27 could promote the differentiation of various precursor cells. However, since the redox state of these precursors could regulate their differentiation status, some scientists deem this a confounding factor influencing the ease of maintenance of neuronal cultures (Chojnacki and Weiss, 2008; Noble et al., 2005; Cressey, 2009).

1.6.4.3. N21 supplement. N21 is a relatively new supplement developed for the isolation and expansion of neurons from mouse and rat hippocampus, appropriately called a "redefined and modified B27 supplement". Like B27 and N2, N21 has also been validated in a plethora of experiments using mouse embryonic stem cells and human embryonic-derived neural progenitor cells. Nevertheless, when using different sources of bovine serum albumin, some differences between B27 and N21 supplements still exist. Additionally, some groups have also shown that the N21 supplement is very useful for establishing and maintaining hippocampal cultures for the purpose of studying the effects of oxygen and glucose deprivation (Chen et al., 2008).

1.6.4.4. Hormone mix. Developed by Di Porzio et al., the hormone mix can be prepared to supplement a serum-free medium. It contains 25μg/ml insulin, 100μg/ml transferrin, 2×10^{-8}M progesterone, 6×10^{-5}M putrescine, and 3×10^{-8}M selenite salt. As such, it is quite similar to Bottenstein's N2 (di Porzio et al., 1980). Even though the main differences between these two reportedly exist in their concentration of insulin and putrescine, we found it difficult to compare with commercially available N2 supplements currently on the market since they exhibit great variability amongst each other. Still, the hormone mix is being widely used to generate murine neural stem cells (Reynolds and Weiss, 1992a), (V et al., 1999), (Chojnacki and Weiss, 2008), (BA et al., 1992).

With all that being said, all of the listed supplements share groups of common ingredients. These include progesterone, insulin, transferrin, putrescine and selenite. Each of these ingredients has a different effect on neurosphere or monolayer culture. Progesterone, a sex steroid hormone mainly synthesized in ovaries and placenta, is also produced by the adrenal cortex and in the CNS of mammals (Tuckey, 2005). It is already present during fetal development and it is involved in neurogenesis, regeneration, neuronal plasticity, neuroprotection, and neuromodulation (Schumacher et al., 2004; Schumacher et al., 2014; González-Orozco and Camacho-Arroyo, 2019; Stein, 2001). Although some rodent studies have shown that progesterone decreases the proliferation of cells in the subventricular zone (SVZ) (Giachino et al.,

Table 2
Comparison of protocols for culturing of neurospheres – human cell culture Medium composition.

	Source	Application	Medium	FGF	EGF	Supplements	Antibiotics	Heparin	Other	Ref
Human	Brain (unknown developmental stage)	CRISPR/Cas9 Genome Engineering in Engraftable Human Brain-Derived Neural Stem Cells	X-VIVO 15 (Lonza)	+ 20 ng/ml	+ 20 ng/ml	N2(Invitrogen) 1:100	NI	+ 2ug/ml	LIF (10 ng/ml) N-acetylocysteine (63ug/ml)	(Dever et al., 2019)
	Fetal tissue	characterization of hfNSCs, grown as neurospheres, obtained from different developmental stages and brain areas	DMEM/F-12	+20 ng/ml	+20 ng/ml (Sigma)	N2(Invitrogen) 1:100	1% PSF antibiotic-antimycotic		LIF 0,1% (Sigma-Aldrich)	(Martín-Ibáñez et al., 2017)
	Embryonic stem cells (H9-hNSCs) (Gibco)	investigation of the beta-adrenoceptor pathway on mitochondrial function in human neural stem cells via rotary cell culture system	StemPro® NSC SFM (containing 1X KnockoutTM D-MEM/F-12 (GIBCO, USA) cGMP manufactured	+20 ng/ml (Gibco)	+20 ng/ml (Gibco)	2% StemPro® Neural Supplement (GIBCO, USA)	-		2 mM GlutaMAXTM–I Supplement (GIBCO, USA)	(Kang et al., 2019) (Chung et al., 2017) (Chiang et al., 2017) (Chiang et al., 2012)
	hNSCs were donated by Shandong Cell-Tissue Bank	investigating the therapeutic mechanism of human neural stem cell-derived extracellular vesicles against hypoxia-reperfusion injury *in vitro*	DMEM/F12 basic medium (1×, Gibco)	20 μg/mL (R&D)	20 μg/mL (R&D)	2% B27 (Gibco)	-	5 μg/ml (Sigma)	2 mmol/L GlutaMAX™ Supplement (Gibco)	(Liu et al., 2020a)
	H9 hESCs (cat. No. WA09; WiCell Research Institute)	the analysis of human embryonic stem cell-derived neural stem cells as an *in vitro* human model	DMEM/F12	20 ng/ml (R&D Systems, Inc., Minneapolis, MN, USA)	20 ng/ml (PeproTech, Inc., Rocky Hill, NJ, USA)	1X N2/B27 (Invitrogen; Thermo Fisher Scientific, Inc.)	-		10 ng/ml LIF (PeproTech, Inc., Rocky Hill, NJ, USA)	(Oh et al., 2018)
	hNS1 cells, v-myc immortalized, non-transformed, human fetal forebrain-derived	the analysis of the Amyloid Precursor Protein (APP) Levels role in Neuronal and Glial Differentiation of Human Neural Stem Cells	DMEM:F-12 (1:1)	20 ng/ml (PetroPech)	20 ng/ml (PetroPech)	N2	-		1% bovine serum albumin	(Coronel et al., 2019)
	NSC cell line K048 cortical fetal brain tissue (human)	investigating the effect of growth factors on proliferation and phenotypic differentiation of human fetal neural stem cells	DMEM/F12	10 ng/ml recombinant human bFGF (R&D Systems)	20 ng/ml recombinant human EGF (R&D Systems, Minneapolis, MN)	-	-		LIF	(Wang et al., 2018) From (Tarasenko et al., 2004)
	Fetal tissue	human neural stem cells' transplantation in delayed tissue plasminogen activator-treated aged stroke brains	DMEM/F12 high glucose (Invitrogen)	20 ng/mL (Invitrogen)	20 ng/mL (Invitrogen, Carlsbad, CA, USA)	-	antibiotics (penicillin, amphotericin, and streptomycin)	heparin (8 μg/mL, Sigma, , USA)	l-glutamine (Invitrogen), LIF 10 ng/mL, Millipore, Billerica, MA, USA)	(Boese et al., 2020)
	H9 (WA09) hESC	obtaining a method that provides an effective neural culture system with structurally and neurochemically mature cell populations and neural networks, suitable for studying a range of neurological diseases from a human perspective	Neurobasal Medium (NBM, components listed in Supplementary material, Table 2)	20 ng/mL (Invitrogen, 13256029,).	20 ng/mL (Invitrogen, PHG0314)	-	-		-	(Liu et al., 2018b)
	Primary fetal hNPCs (GW 16–18) and hiPSC-NPCs	Comparative performance analysis of human iPSC-derived and primary neural progenitor cells (NPC) grown as neurospheres *in vitro*	DMEM (Life Technologies, USA) and Hams F12 (Life Technologies, USA; 3:1)	20 ng/mL (R&D Systems, Germany)	20 ng/mL (Biosource, Germany)	1 × B27 (Invitrogen GmbH, Germany)	-		-	(Hofrichter et al., 2017)
	Human fetal NSCs	investigating the role of exosomes (hNSC-Exo) derived from human	DMEM/F12	20 ng/mL (PeproTech	20 ng/mL (PeproTech)	2% B27 (Gibco)			2 nM L-glutamine (Invitrogen)	(Zhang et al., 2020)

(*continued on next page*)

Table 2 (*continued*)

Source	Application	Medium	FGF	EGF	Supplements	Antibiotics	Heparin	Other	Ref
	neural stem cells stimulated by interferon-gamma in the ischemic stroke model					1% penicillin-streptomycin (Gibco)	2 μg/ml Heparin sodium		
GIBCO® hNSCs were originally obtained from National Institutes of Health (NIH) approved H9 (WA09) human embryonic stem cells.	the role of resveratrol in human neural stem cells on amyloid-beta-induced inflammation and oxidative stress	KnockOut™ D-MEM/F-12	20 ng/mL	20 ng/mL	2% StemPro® Neural Supplement			2 mM of GlutaMAX™-I.	(Chiang et al., 2018)
hNSCs were donated by Shandong Cell-Tissue Bank.	exploring the human neural stem cell-derived extracellular vesicles (hNSC-EVs) therapeutic effect on neuronal hypoxia-reperfusion (H/R) injured neurons	DMEM/F12 basic medium (1 ×, Gibco)	20 μg/mL (R&D),	20 μg/mL (R&D)	2% B27 (Gibco)		5 μg/ml heparin (Sigma)	2 mmol/L GlutaMAX™ Supplement (Gibco)	(Liu et al., 2020b)
Neural precursor cells (NPCs) derived from the human embryonic stem cell (hESC) line H9	the role of SEPT7 in the regulation of Ca^{2+} entry through Orai channels in human neural progenitor cells and neurons	1:1 DMEM F-12/ Neurobasal medium			1:200 N2, 1:100 B27			3 μM CHIR99021, 0.5 mM Purmorphamine 150 μM ascorbic acid (Sigma, catalog no: A92902)	(Deb et al., 2020)

2004), other studies demonstrated that progesterone increases the proliferation of adult rNPCs in a dose-dependent manner (Liu et al., 2009).

Insulin has neurotrophic effects and, like progesterone in some studies, acts by increasing the proliferation of progenitor cells. However, special attention should be paid to its use in human NSCs cultures, since it has been shown that these types of cells, unlike the rodent NSCs, are extremely sensitive to insulin. As such, they can maintain a healthy state only when exposed to relatively low insulin concentrations (Rhee et al., 2013).

On the other hand, micronutrients, such as transferrin, are responsible for extracellular iron storage and transport. To accomplish that, transferrin acts as an antioxidant, binding to iron and preventing it from catalyzing free radicals production (Chaichana et al., 2006). While putrescine has a neuroprotective effect, being involved in cellular survival and growth by stabilizing cellular membranes and nuclear material (Schipper et al., 2000), selenite possesses antitumor properties by inducing apoptosis (Yeo and Kang, 2007), (Cao et al., 2006).

To sum up, since the aforementioned supplements vary greatly in their effects on cell culture, we suggest using only those which have been shown to align with your experimental needs. Finally, an important consideration to keep in mind while choosing appropriate supplements is to utilize only those for which you have access to the full chemical composition, be it either online or upon request from the manufacturer.

1.6.5. Antibiotics and antimycotics

Antibiotics and antimycotics are not the determinants/critical factors in NSC culture as they are not used in cell preparation for clinical therapy. Therefore, since their use might affect the cells in a multitude of ways, it is not recommended to use them in preclinical research.

Since microbial contamination can change the cellular phenotype, genome, functioning, and survival, some cell culture experiments do involve the use of antibiotics. When it comes to NSCs these, in most cases, include 1% penicillin/streptomycin with or without amphotericin B. Although the addition of antibiotics seems to become a routine, there have been several reports throughout the years demonstrating that they can affect cell function (Llobet et al., 2015; Kagiwada et al., 2008; Varghese et al., 2017; Cohen et al., 2006). This is, in part, due to aminoglycosides, including streptomycin, which can interfere with protein synthesis and, therefore, have a toxic effect on cultured cells (El Mouedden et al., 2000), (Laurent et al., 1990). Moreover, penicillin G also exhibits an inhibitory effect on cardiomyocyte, osteogenic, chondrogenic, and neural murine ESCs' differentiation (Zur Nieden et al., 2004). Experiments performed on the same types of cell culture also demonstrate that both gentamicin, as well as the combination of penicillin and streptomycin, can affect cellular proliferation and differentiation (Cohen et al., 2006). Similar results were also obtained from experiments on human mesenchymal stem cells, such as those derived from the adipose tissue, which were treated with penicillin/streptomycin with or without amphotericin B (Skubis et al., 2017).

Apart from their antimicrobial effects, the use of antibiotics brings forth a plethora of benefits to any cell culture experiment. For example, rat NSCs preconditioned with minocycline *in vitro*, and then transplanted into rat brains 6 hours following ischemic injury (middle cerebral artery occlusion, MCAO), were shown to be protected in host tissue via upregulation of Nrf2 and Nrf2-regulated antioxidant genes. Moreover, the same study has shown that minocycline induced the paracrine abilities of NSCs, including the secretion of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) (Sakata et al., 2012). This suggests that the aforementioned preconditioning could enhance the efficiency of transplantation therapy in ischemic stroke. However, extensive research on the influence of antibiotics and antimycotics on human stem cell cultures is still lacking. A recent review of a few studies done on human ESCs, iPSCs and MSCs, written by Farzaneh, advises to use antibiotics only for the initial step of isolation and short-term culturing (Farzaneh, 2021). As such, it is clear

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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All figures were made in BioRender.

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Table 3
Comparison of protocols for culturing of a monolayer – human cell culture.

Source	Application	Medium	FGF	EGF	Supplements	Antibiotics	Heparin	Other	Ref
Human NSCs (ReNcell VM; EMD Millipore, Burlington, MA, USA)	high-throughput *in vitro* assessment of proliferation and differentiation of human neural stem cells	ReNcell medium (ReNcell NSC maintenance medium, EMD Millipore)	20 ng/mL (, EMD Millipore)	20 ng/mL (EMD Millipore)	-	1% (v/v) penicillin/ streptomycin (Thermo Fisher, Waltham, MA, USA),	-	laminin-coated, tissue culture-treated, T-75 flasks	(Joshi et al., 2018)
Human neural stem/progenitor cells (hNSCs) obtained from Millipore (Billerica, MA). The cells were derived from the ventral mesencephalon region of human fetal brain and immortalized by retroviral transduction with the v-myc oncogene.	promoting neuronal differentiation and maturation of human fetal tissue-derived neural stem cells (hNSCs) in a brain lesion site of a rat traumatic brain injury model using a biodegradable nanoparticle-mediated transfection method	ReNcell Neural Stem Cell Medium (Millipore)	20 ng/mL	20 ng/mL				laminin (20 μg/ mL)	(Li et al., 2016)
hNSC, a primary dissociated neural cell suspension cultured from the periventricular region of the telencephalon from a 13-week human fetal cadaver	investigating human neural stem cells migration along the nigrostriatal pathway in a primate model of Parkinson's disease	Dulbecco's Modified Eagles Medium (DMEM) + F12 (1:1)	20 ng/ml	20 ng/ml	N2 medium (Gibco, Grand Island, NY),		8 μg/ml heparin	10 ng/ml LIF	(Bjugstad et al., 2008)
Neural precursor cells (NPCs) derived from the human embryonic stem cell (hESC) line H9	the role of SEPT7 in regulation of the Ca^{2+} entry through Orai channels in human neural progenitor cells and neurons	1:1 DMEM F-12/ Neurobasal medium			1:200 N2, 1:100 B27			0.5% Matrigel 3 μM CHIR99021, 0.5 mM Purmorphamine and 150 μM ascorbic acid (Sigma, catalog no: A92902)	(Deb et al., 2020)
Fetal NSC	the impact of regional identity of human neural stem cells on oncogenic responses to histone H3.3 mutants	serum-free basal medium supplemented	10 ng/ml (Peprotech,)	10 ng/ml Peprotech)	N2 and B27 (Life Technologies)			laminin (Sigma, 1 μg/ml) added to the medium (no need for pre-coating of flasks)	(Bressan et al., 2017), (Bressan et al., 2021)
hESC-NS were obtained from H6 hESC line (Royan Stem Cell Bank, Tehran, Iran).	investigating the encapsulated in hyaluronic acid NSC regenerative potential in a contusion spinal cord injured rat	Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco)	40 ng/ml (Royan biotech)	20 ng/ml (Royan biotech)	Knockout serum replacement (5%, Gibco), N2 supplement (1%, Gibco), and B27 supplement (0.1%, Gibco)			poly-L-ornithine/ laminin-coated	(Zarei-Kheirabadi et al., 2020)
HNSCs (American Tissue Culture Collection, USA)	developing thiolated gellan gum hydrogels as a peptide delivery system for 3D neural stem cell culture	ReNcell neural stem cell medium (Millipore)	20 ng/mL	20 ng/mL				laminin (20 mg/ mL)	(Yu et al., 2020)

that additional testing of culture conditions with and without antibiotics is necessary.

1.6.6. *Obtaining NSC and neuronal lineages from pluripotent stem cells*

In addition to isolation from the fetal brain, human NSCs can also be obtained from pluripotent stem cells (PSC, i.e. ESC and iPSC). This is accomplished by understanding of the signaling pathways involved in the development of the nervous system. Although there are many protocols for obtaining NSCs from PSCs, the majority of them rely on the formation of neural rosettes – self-organized clusters of neuroepithelial-

Table 4
Comparison of protocols for culturing of human cell cultures - other conditions.

	Source	Density	Expansion time	Dissociation method	Ref
human	Brain	100 000/cm2	-	-	(Dever et al., 2019)
	Fetal tissue	80 000/cm2	-	-	(Martín-Ibáñez et al., 2017)
	Embryonic stem cells (H9-hNSCs) (Gibco)	100 000/cm2	-	-	(Kang et al., 2019)
	Human NSCs (ReNcell VM; EMD Millipore, Burlington, MA, USA)	1.5×10^6 cells	-	accutase ® (EMD Millipore),	(Joshi et al., 2018)
	hNSCs were donated by Shandong Cell-Tissue Bank			accutase ®	(Liu et al., 2020b)
	Primary dissociated neural cell suspension cultured from the periventricular region of the telencephalon from a 13-week human fetal cadaver	5×10^5 cells/ml		trypsin-EDTA (0.05%)	(Bjugstad et al., 2008)
	Neural precursor cells (NPCs) derived from the human embryonic stem cell (hESC) line H9			accutase ®	(Deb et al., 2020)

like cells that resemble an embryonic neural tube (Barak et al., 2022), (Galiakberova and Dashinimaev, 2020). These rosettes are then isolated from surrounding cells and propagated in culture to obtain a homogenous multipotent NSC line. In addition to the isolation of NSCs from PSCs, there are also protocols for forming specific neural lineages, most often neurons, without going through a stage of rosettes/NSCs (Barak et al., 2022), (Linaro et al., 2019), (McComish and Caldwell, 2018). This existence of a myriad of protocols, when combined with a detailed characterization of the generated populations of cells, gives additional insight into the processes involved in the development of neural cells. Independent of the protocol used, and before considering the translation of research into the clinic, cells must be characterized in great detail to give insight into the presence of specific cell types in a given population. Namely, a differentiation must be made between NSCs and other specific types of neural cells. Each study on the generation of neural stem cells from iPSC requires revisions, as there is very often a lack of definition of what type of neural cells the authors obtained and what's in the mixture called "neural stem cells". When NSCs or specific neural lineages are obtained from PSCs *in vitro*, one has to bear in mind the risk of remaining residual pluripotent cells which could form teratomas when transplanted *in vivo* (Daadi et al., *2008)*, (Daadi and Steinberg, 2009). Therefore, in addition to extensive *in vitro* characterization, a given population of cells must be tested *in vivo* for a potential beneficial effect in a specific disease model in order to eliminate a possible formation of teratomas.

1.6.7. Small molecule-based protocols for neural stem cell induction

As mentioned above, induced pluripotent stem cells (iPSCs) have brought great hope for cellular therapies, even though their use still presents a risks for potential viral infection-induced instability, incomplete differentiation or mutagenesis, and tumor formation (Patel and Yang, 2010), (Thier et al., 2012). In comparison to iPSCs, induced neural stem cells (iNSCs) maintain self-renewal capabilities and can be easily differentiated into neurons, astrocytes and oligodendrocytes. As such, cell differentiation following the delivery of small molecules seems to be a relatively safe and secure method (Yu et al., 2014). Although a great improvement in *in vitro* small molecule-based protocols regarding NSCs derivation has been made in the last 20 years, researchers still have many problems in comparing these. This is the case because many of the readily available protocols use different PSC lines, different methods and markers for NSC characterization, different methods for calculation of their effectiveness and have different purposes behind NSC derivation (Galiakberova and Dashinimaev, 2020).

Small molecules can manipulate cell fate by playing a variety of different roles: a) epigenetic modifiers (valproic acid, sodium butyrate, tranylcypromine, Bix01294, RG108, azacytidine, ascorbic acid, retinoic acid), b) signaling pathway regulators (transforming growth factor (TGF)- β family inhibitors, A83-01, Repsox, SB431542, LDN193189, c) glycogen synthase kinase-3β inhibitors or agonists (GSK-3 β inhibitors, CHIR99021, MAPK signaling pathway inhibitors, PD0325901, SP600125, Sonic Hedgehog (SHH) pathway agonists, ROCK inhibitors), d) metabolic modulators, or e) nuclear receptor agonists and antagonists (Liu et al., 2018a). According to Galiakberova and Dashinimaev, the most popular, reliable and easy-to-use method is Dual SMAD inhibition. The aforementioned protocol could be used for both 2D and 3D culture induction of NSCs (Galiakberova and Dashinimaev, 2020). Developed in 2009 by the Chambers group, this protocol uses two SMAD signaling inhibitors, namely Noggin and SB431542, to induce neural conversion of non-human embryonic stem cells (ESCs) and human ESCs (hESCs) as well as hIPSCs (Chambers et al., 2009). Considering all that was mentioned here, researchers emphasize that selecting the standardized protocols and criteria that have been most widely accepted/used based on published data for evaluating NSC phenotypes and methods plays a crucial role in facilitating their tangible translation into clinical settings. Undoubtedly, when discussing the therapeutic use of NSCs, parameters such as their homogeneity, self-renewal ability or well-described characteristics of these cells must be assured.

2. The influence of various factors on differentiation of NSCs

Since every *in vitro* cell model has its benefits and drawbacks, it is equally important to discuss the influence of a variety of factors that could impact the differentiation of NSCs. Additionally, within this chapter we also present a detailed overview of options that could lead to protocol optimization and refinement of experimental conditions.

2.1. Characterization of cells obtained by NSC differentiation

To confirm their identity *in vitro*, NSCs are tested for the expression of their characteristic markers like Nestin and SOX2 (Coronel et al., 2019), (Chen et al., 2016). In addition, these cells have to be able to self-renew and differentiate into neurons, astrocytes, and oligodendrocytes when exposed to a specific type of differentiation media. The most widely used methods for verifying NSCs and their differentiation outcomes include immunocytochemistry (ICC), quantitative real-time PCR, and western blot (WB) (Coronel et al., 2019), (Li et al., 2014b), (Lee et al., 2020), (Horie et al., 2008), (Chen et al., 2016), (Gao et al., 2017b), (Mcgrath et al., 2018). Immunocytochemistry allows for the counting of cells that express a specific marker and thus facilitates the calculation of their percentage within the culture. On the other hand, by using real-time PCR and western blot analysis one can show the change in the relative expression of a specific marker normalized to a marker that represents the endogenous control. Some of the markers used to verify the differentiation outcomes include MAP2, TUBB3, and NeuN for neurons (Li et al., 2014b), (Lee et al., 2020), (Chen et al., 2016), (Gao et al., 2017b); GFAP and S100B for astrocytes (Li et al., 2014b), (Lee et al., 2020), (Chen et al., 2016), (Gao et al., 2017b); and MBP for oligodendrocytes (Li et al., 2014b), (Lee et al., 2020). In addition to these, one can also

Table 5
Comparison of protocols for culturing of neurospheres – mouse cell culture.

	source	application	medium	bFGF	EGF	supplements	antibiotics	heparin	glucose	glutamine	ref
mouse	E14, Ganglionic eminences	transplantation in a controlled cortical impact (CCI) model of adult mouse somatosensory cortex	F-12-DMEM		20 ng/ml	3% B27, 0.5% N2, 1% BSA	1% ABAM			1% Glutamax	(Nasser et al., 2018)
	P11, SVZs	investigating the role of HIF-1α p in the maintenance of adult NSCs and stabilization of the SVZ vascular niche using conditional, tamoxifen-inducible Hif1a knock-out mice	Neurobasal	20 ng/ml	10 ng/ml	2 % B27	100 U/ml Pen, 100 ug/ml Strep			2.0 mM glutamine	(Li et al., 2014a)
	E14, cerebrum	the influence of nardosinone on the proliferation, migration, and selective differentiation	NeuroCult™ Proliferation Medium	10 ng/ml	20 ng/ml			2 μg/ml			(Li et al., 2014b)
	E14, whole brain	investigating the effects of histone deacetylation inhibition on neuronal differentiation of embryonic mouse neural stem cells	Neurobasal	20 ng/ml	20 ng/ml	B27	Pen/strep 1% (Sigma)			1 % L-glutamine, 1% glutamax	(Balasubramaniyan et al., 2006)
	E13, cerebral cortex	investigating the role of NSC transplantation at critical period on learning and memory in Alzheimer's disease mouse model	DMEM/F12 (1:1)	10 ng/ml	20 ng/ml	5 μg/ml insulin, 100 μg/ml apotransferrin, 30 nmol/l sodium selenite, 100 nmol/l putresine, and 20 nmol/l progesterone			0,6 % glucose	2mmol/l l-glutamine	(Kim et al., 2015)
	E16.5 whole telencephali	investigating the mechanism by which Notch2 promotes resistance to apoptosis of NSCs to cytotoxic insults.	DMEM/F12 (1:1)	10 ng/ml	20 ng/ml	2% B27	Pen/Strep 1 %	2 μg/ml heparin		0.2 mg/ml L-glutamine	(Tomé et al., 2019)
	SVZ, adult male C57BL/6, approximately 9 weeks of age	investigating the role of Runx1 in NSPCs	Neurocult complete culture media (Stem Cell Technologies, WA, 05702)	20 ng/mL	20 ng/mL			2 μg/mL heparin			(Logan et al., 2015)
	Brain, Kun Ming mice at embryonic day 12.5 (E12.5).	investigating the precise roles of TIGAR in NSCs and the possible involvement of metabolic reprogramming in the TIGAR regulatory network	DMEM/F12 medium	20 ng/ml (R&D, USA)	20 ng/ml (Invitrogen, USA)	2% B27 (Gibco, USA)	1% penicillin/ streptomycin solution				(Zhou et al., 2019)
	Striata of embryonic mouse brain at E14.5 or SVZ of adult mouse brains	evaluating the effects of several putative bioactive Aβs and gangliosides on neural stem cells isolated from embryonic mouse brains or the subventricular zone of adult mouse brains	Neurobasal A medium (Invitrogen, Carlsbad, CA)	20 ng/ml (Peprotech, Rocky Hill, NJ)	20 ng/ml (Peprotech).	B27 (Invitrogen),					(Itokazu et al., 2013)
											(Ohta et al., 2016)

(continued on next page)

Table 5 (*continued*)

source	application	medium	bFGF	EGF	supplements	antibiotics	heparin	glucose	glutamine	ref
NSPCs from mouse E14.5 GE	exploring the role of CHD7 in proliferation promotion of neural stem cells mediated by MIF	neurobasal medium (Thermo Fisher Scientific,)	10 ng/ml (Peprotech)	20 ng/ml (Peprotech)	B27 (Thermo Fisher Scientific)					
Cerebral cortical tissues from E12.5 C57BL/6 mice (KOATECH) or Tau-GFP mice	establishing a three-dimensional (3D) differentiation model of neurospheres, which produce unique neuronal clusters, termed NeuroCore (NC)	DMEM/F- 12 (Life Technologies, 11320033)	20 ng/ml (R&D systems, 233-FB)	20 ng/ml (Invitrogen, PHG0313)	1% N2 (Life Technologies, 17502048), 2% B27 (Life Technologies, 17504044)	1% penicillin/ streptomycin (P/S; Life Technologies, 15140122).				(Lee et al., 2020)
forebrains of E15 embryos C57/BL6J	the characterization of gene expression during mouse neural stem cell differentiation *in vitro*	DMEM/F12,1:1	20 ng/ml (Invitrogen)		2% B-27 (Invitrogen, CA, USA)	penicillin (100 U/ml)/ streptomycin (100 μ g/ml)	5 μ g/ml heparin			(Park et al., 2012)
E15.5 ICR mice ganglionic eminence	investigating the effects of oxygen concentration on the proliferation and differentiation of mouse neural stem cells *in vitro*	DMEM/F-12		20 ng/ml (Invitrogen Corp., Carlsbad, CA, USA	23 μg/ml insulin, 92 μ g/ml transferrin, 55 μ M putrescine, 27.5 nM sodium selenite, 20 nM progesterone,	50 U/ml penicillin-streptomycin		36.4 mM glucose		(Horie et al., 2008)
E14 forbrains	investigating the mechanism by which integrin β4 modulates mouse neural stem cell differentiation *in vitro*	supplemented culture medium	20ng/mL (EssexBioGroup, China)		, 0.4 mM HEPES, 2% B27			4 mM D-glucose	0.1 mM l-glutamine	(Su et al., 2009)
E12.5, cerebral cortex of Kun Ming mouse (KM strain)	mouse NSCs were used as a model system and cell behavior was monitored in the presence of the protein palmitoylation inhibitor 2-bromopalmitate (2BRO)	DMEM/F12 (1:1) (Gibco-BRL, Carlsbad, CA, USA)	20-ng/mL (Invitrogen, Carlsbad, CA, USA)	20-ng/mL (Invitrogen, Carlsbad, CA, USA)	2 % B27 (Gibco-BRL, Carlsbad, CA, USA),	100 U/ mL penicillin, and 100 μg/mL streptomycin				(Chen et al., 2016)
E15.5 brains	investigating the role of miR-342-5p in the regulation of Neural Stem Cell Proliferation and Differentiation Downstream to Notch Signaling in Mice	DMEM/F12	20 ng/ml (R&D Systems),	20 ng/ml (R&D Systems)	N-2 supplement (Gibco)					(Gao et al., 2017a)
E14, ganglionic eminences of C57-BL6 mice	purifying Immature Neurons from Differentiating Neural Stem Cell Progeny Using a Simple Shaking Method	mouse NeuroCult basal NSC Medium (Stem Cells Technologies, Vancouver, Canada)	10 ng/ml (R&D system, Minneapolis, MN, USA)	20 ng/ml	mouse Neurocult NSC Proliferation Supplements (Stem Cells Technologies, Vancouver, Canada)		2 μg/ml heparin (Sigma)			(Azari et al., 2014)
E12.5 cortices	determining the effect of formyl peptide receptors (FPRs) on differentiation of NSCs	DMEM/F12	20 ng/mL	20 ng/ mL	B27					(Zhang et al., 2017)

Table 6
Comparison of protocols for culturing of mouse cell cultures – other conditions.

	Source	Density	Expansion time	Dissociation method	Ref
Mouse	E14, Ganglionic eminences	200 000 cells in 5 ml (T25)	6-7 days	trypsin-EDTA (0.05%)	(Nasser et al., 2018)
	P11, SVZs	1.4 x10^4 cells/cm^2 in 6wp	8 days	Accutase®	(Li et al., 2014a)
	E14, cerebrums	8x10^4 cells/cm^2 when isolating, 2x10^4 cells/cm^2 when passaging	When they reached 100-150 μm in size	NeuroCult™ Chemical Dissociation Kit	(Li et al., 2014b)
	E14 whole brain		5-7 days after isolation, 3-4 days for passaging	mechanical dissociation with a fire-polished Pasteur pipette every 3–4 days	(Balasubramaniyan et al., 2006)
	E16.5 whole telencephali	5x10^4 cells/ml both in isolation and passaging	5-6 days	mechanical dissociation with a pipette in the presence of 0.25% trypsin (Gibco) for 2 min	(Tomé et al., 2019)
	SVZ, adult male C57BL/6, approximately 9 weeks of age	For treatment with Ro5-3335, cells were plated at a density of 6 × 10^5 cells/ 100mm dish. For lentiviral transduction experiments, cells were plated in 48well culture dishes at a density of 2000 cells/ well.	These primary cultures were grown initially for 7 days. Cells were then passaged every 5 days for subsequent passage numbers.	Tissue was homogenized (Miltenyi neural tissue dissociation kit, Miltenyi Biotec, MA)	(Logan et al., 2015)
	Brain, Kun Ming mice at embryonic day 12.5 (E12.5).	2×10^5/ml	3-5 days	The brains were cut mechanically and digested with 0.25% trypsin (Gibco, USA) for 5 min at 37 °C. Dissociation was performed with trypsin and EDTA.	(Zhou et al., 2019)
	Striata of embryonic mouse brain at E14.5 or SVZ of adult mouse brains		Neurospheres formed after 1 week were collected for passage or further analyses.	Trypsinization and mechanical trituration	(Ohta et al., 2016)
	Cerebral cortical tissues from E12.5 C57BL/6 mice (KOATECH) or Tau-GFP mice.	5 × 10^5 cells per well in an Ultra-Low Attachment 6-well Plate		Accutase (Innovation Cell Technology, AT104) for 5 min at 37 °C	(Lee et al., 2020)
	forebrains of E15 embryos C57/BL6J mice		subcultured once per week	0.25% trypsin/EDTA	(Park et al., 2012)
	E15.5 ICR mice ganglionic eminence	25 x 10^4 cells/5 ml in T25 culture flask	Cultures were incubated for 5 days to form enough primary neurospheres	mechanical dissociation	(Horie et al., 2008)
	E14, forebrains		7 days	tissue dissociated with papain (Worthington Biochemical Lakewood, NJ) at 37 °C for 45min neurospheres mechanically dissociated	(Su et al., 2009)
	E12.5, cerebral cortex of Kun Ming mouse (KM strain)	2×10^5 cells/mL	3-5 days	Tissue trypsinized for 5 min at 37 °C neurospheres dissociated using trypsin and EDTA (Sigma-Aldrich Co. LLC, St. Louis, MO, USA),	(Chen et al., 2016)
	E15.5 brains	1 × 10^5 cells/ml in 24-well plates (0.4 ml/ well), for the adherent culture, cells were plated at a density of 5 × 10^4 cells/ml in poly-L-lysin (PLL)-coated 24-well plates (0.4 ml/ well)		mechanical dissociation of brain tissue	(Gao et al., 2017a)
	E14, ganglionic eminences of C57-BL6 mice	2×10^5 cells/ml initial seeding, subsequent passages - 5×10^4 cells/ml	5-7 days	0.05% trypsin-EDTA (Gibco, USA)	(Azari et al., 2014)
	E12.5 cortices	Initial seeding - 250,000 cells/mL (density of ~65,000 cells/cm^2) in a 100-mm culture dish	7 days	Tissue: 0.25% trypsin−EDTA and 250 U/mL DNase I at 37 °C for 30 min Neurospheres: StemProAccutase Cell Dissociation Reagent	(Zhang et al., 2017)

evaluate neuronal subtypes by analyzing markers such as GABA, glutamate, tyrosine hydroxylase (TH), and serotonin (Horie et al., 2008). Another approach for characterization of NSCs and their differentiation outcomes includes single-cell transcriptome analysis (Burke et al., 2020), (Lam et al., 2019). This approach offers a detailed characterization which is very important when considering the use of these cells in clinical settings.

2.2. Medium composition

2.2.1. Supplements and growth factors

As described in the previous section, one of the major elements needed for NSCs' self-renewal is the presence of mitogens, EGF and bFGF. Their removal leads to a significant decrease in the proliferation potential of the cells and onsets the differentiation process. Still, these are not the only effects associated with the presence of mitogens in the cellular medium. For example, although bFGF is a mitogen, it can also be

Table 7
Comparison of protocols for differentiation (human cell culture).

Medium composition								
	Source	Application	Medium	Serum	FGF	Supplements	Other	Ref
Human	Fetal tissue	characterization of hfNSCs, grown as neurospheres, obtained from different developmental stages and brain areas	Complete serum	1% fetal calf serum		2% B27		(Martín-Ibánez et al., 2017)
		exploring that the human neural stem cell-derived extracellular vesicles (hNSC-EVs) have therapeutic effect on neuronal hypoxia-reperfusion (H/R) injured neurons *in vitro* by mediating the nuclear translocation of NF-E2-related factor 2 (Nrf2) to regulate the expression of downstream oxidative kinases	Neurobasal medium (Gibco) For 7 days				accutase	(Liu et al., 2020a)
	The hESCs (H9, passages 25–55; WiCell Research Institute)	investigating the therapeutic effects and mechanisms of combined human neural stem cells with rTMS in a middle cerebral artery occlusion (MCAO) rat model	neurobasal medium (Gibco)			1 × B_{27} (Gibco, 12587010)	10 ng/ml BDNF (PeproTech, 450-02), 10 ng/ml glial-derived neurotrophic factor (GDNF; PeproTech, 450-10), 10 ng/ml insulin-like growth factor 1 (IGF1; PeproTech, 100-11), and 1 μM cAMP (Sigma-Aldrich, D-0260)	(Peng et al., 2019)
	hNS1 cells, v-myc immortalized, non-transformed, human fetal forebrain-derived	investigating the potential function that APP plays in cell fate specification and differentiation of hNS1 cells	DMEM:F-12 (1:1)	0.5% heat-inactivated fetal bovine serum (FBS)		N2	1%bovine serum albumin	(Coronel et al., 2019)
	Fetal tissue	The culture of fetal human neural precursors in the absence of serum as neurospheres	1× DMEM:F12 (1:1)		+/-		0.66 % glucose (w/v), 2 mM glutamine, 14.6 mM $NaHCO_3$, 5 mM HEPES buffer, 23 μg/ml insulin, 93 μg/ml apo-transferrin,19 nM progesterone, 56 μM putrescine, 21 nm sodium selenite.	(Chojnacki and Weiss, 2013)
	Fetal tissue	the establishment of a stable human neural stem cell line (immortalized human NSCs [IhNSCs]) by v-myc-mediated immortalization of previously derived wild-type hNSCs	NS-A control medium (CM)	2% fetal calf serum	presence of 20 ng/ml FGF2		After 72 hours, cultures were shifted to NS-A control medium (CM) containing 2% fetal calf serum and grown for 2 weeks.	(De Filippis et al., 2007)
	H9 (WA09) hESC	Derivation of phenotypically diverse neural culture from hESC by combining adherent and dissociation methods	NBM					(Liu et al., 2018b)
	hiPSC, Primary fetal hNPCs (GW 16–18)	comparing two hiPSC neural induction protocols resulting in 3D neurospheres: one using noggin and one cultivating cells in neural induction medium (NIM protocol)	DMEM (Life Technologies, USA) and Hams F12 (Life Technologies, USA; 3:1)			1 × B27 (Invitrogen GmBH, Germany) 1 × N2 supplement (Invitrogen, Germany)		(Hofrichter et al., 2017)
	Human fetal NSC	exploring the role of exosomes (hNSC-Exo) derived from human neural stem cells (hNSCs)	DMEM/F12	2% FBS			1% Penicillin-Streptomycin	(Zhang et al., 2020)
	hNSCs were donated by Shandong Cell-Tissue Bank.	exploring that the human neural stem cell-derived extracellular vesicles (hNSC-EVs) have therapeutic effect on	Neurobasal medium (Gibco)					(Liu et al., 2020b)

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Table 7 (*continued*)

Medium composition								
	Source	Application	Medium	Serum	FGF	Supplements	Other	Ref
		neuronal hypoxia-reperfusion (H/R) injured neurons *in vitro* by mediating the nuclear translocation of NF-E2-related factor 2 (Nrf2) to regulate the expression of downstream oxidative kinases						
	(hNSCs) were obtained from Millipore (Billerica, MA). The cells were derived from the ventral mesencephalon region of human fetal brain and immortalized by retroviral transduction with the v-myc oncogene.	promoting neuronal differentiation and maturation of human fetal tissue-derived neural stem cells in a brain lesion site of a rat traumatic brain injury model using biodegradable nanoparticle-mediated transfection method	ReNcell Neural Stem Cell Medium (Millipore)					(Li et al., 2016)
	Human induced pluripotent stem cell (hiPSC)-derived neural stem cells	investigating the impact of POPs upon brain development and neurodevelopmental disorders	Neurobasal Medium			N2 Supplements, B-27 Supplements	Penicillin/ Streptomycin (50 U/ mL) BDNF (2.5 ng/mL) and GDNF (1 ng/mL)	(Davidsen et al., 2021)

Table 8
Comparison of protocols for differentiation (human cell culture) – other conditions.

	Source	Density	Coating	Duration of the diff stages	Ref
Human	Fetal tissue	25 000/cm^2	Poly-L-lysine 01 mg/ml		(Martín-Ibáñez et al., 2017)
	The hESCs (H9, passages 25–55; WiCell Research Institute)		poly-ornithine/laminin		(Peng et al., 2019)
	hNS1 cells, v-myc immortalized, non-transformed, human fetal forebrain-derived		poly-L-lysine- (10–30 μg/ml; Sigma)		(Coronel et al., 2019)
	Fetal tissue	100,000 cells/ ml	poly- L -ornithine-coated		(Chojnacki and Weiss, 2013)
	Fetal tissue	2.5 × 104 cells per cm2	Matrigel (BD Biosciences, San Diego)	2 weeks	(De Filippis et al., 2007)
	H9 (WA09) hESC		poly-d-lysine (PDL) and laminin (10 μg/mL, Sigma, P6407, Life Tech, 23017015).	28 days	(Liu et al., 2018b)
	Human induced pluripotent stem cell (hiPSC)-derived neural stem cells (NSCs)	21.000 cells/ cm^2	polystyrene Poly-d-Lysine coated 96-well (flat bottom) plates (ThermoFisher Scientific (Rietze and Reynolds, 2006)) coated with reduced growth factor matrigel	28 days *in vitro*	(Davidsen et al., 2021)

used in the NSC differentiation medium to obtain more neurons (Meng et al., 2020). In addition to bFGF, other factors like BDNF, NGF and IGF-I, can also be used for the same purpose (Choi et al., 2008), (Chen et al., 2014). Interestingly, this effect is more pronounced if two or more factors are used. As such, a combination of three neurogenic factors (NGF+BDNF+bFGF) was shown to generate more neurons than combinations of only two factors (NGF+BDNF, NGF+bFGF or BDNF+bFGF). Additionally, two-factor groups generated more neurons than one-factor groups (NGF, BDNF or bFGF) (Chen et al., 2014). When comparing single factor groups, cultures grown with an addition of NGF or BDNF yielded more neurons than those using bFGF alone. Among two-factor groups, a combination of NGF with BDNF resulted in more neurons than appeared in the culture using a combination of NGF with bFGF or BDNF with bFGF. (Chen et al., 2014) Contrastingly, a study performed by Choi et al. demonstrated that, in single factor groups, bFGF-supplemented cultures yielded more neurons than those supplemented with just NGF (Choi et al., 2008). These contrary results could be due to the differences in concentration, wherein a five times smaller concentration of bFGF compared to NGF was used in the first study (Chen et al., 2014), while the second study used the same concentration of both (Choi et al., 2008). In addition, the second study also evaluated the effects of combining BDNF and IGF-I, alongside bFGF and NGF. Interestingly, when considering two-factor combinations, the combination of bFGF with IGF-I resulted in the highest percentage of neurons in the cell culture (Choi et al., 2008). LIF used in some protocols for NSC expansion, is often omitted from the culture medium for differentiation (Tarasenko et al., 2004), (Mcgrath et al., 2018). Still, some studies have shown that the presence of LIF in the differentiation medium can increase the number of neurons present within the culture (Majumder et al., 2012) .In addition to different factors which can enhance NSCs' differentiation into neurons, there also exist alternative methods to facilitate their differentiation into astrocytes and oligodendrocytes. This can be seen in a study performed by Pous et al. wherein the differentiation of adult subventricular zone-derived and hippocampal-derived neural stem/progenitor cells (NSPC) into astrocytes was induced with fibrinogen treatment, as revealed by an increased abundance of GFAP+, Aldh1l1+, and Aqp4+ cells. An increase in *GFAP*, *Aqp4*, and *Aldoc* mRNA and protein expression in astrocytes, with a decrease in the fraction of Tuj-1+ neurons present in the cell culture, was also observed (Pous et al., 2020). Furthermore, differentiation toward desired cell

Table 9
Comparison of protocols for differentiation (mouse cell culture).

Medium composition									
	Source	Desirable lineage	Application	Medium	Serum	FGF	Supplements	Other	Ref
Mouse	E14, cerebrum		exploring the role of nardosinone in Proliferation, Migration and Selective Differentiation of Mouse Embryonic Neural Stem Cells	Complete NeuroCultTM Differentiation Medium					(Li et al., 2014b)
	E14 whole brain		investigating the effects of the HDAC inhibitor trichostatin A (TSA) on the differentiation pattern of embryonic mouse NSCs during culture in a minimal, serum-free medium, lacking any induction or growth factor	Neurobasal, glutamine, glutamax, B27, P/S					(Balasubramaniyan et al., 2006)
	Brain, Kun Ming mice at embryonic day 12.5 (E12.5).	DMEM/F12 medium	investigating the precise roles of TIGAR in NSCs and the possible involvement of metabolic reprogramming in the TIGAR regulatory network	stimulated with differentiation medium containing no growth factors, and cultured for 1–9 days	2% fetal bovine serum (Gibco, USA)			dissociated 1st passage neurospheres used	(Zhou et al., 2019)
	E12.5 C57BL/6 mice (KOATECH) or Tau-GFP mice		establishment of a three-dimensional (3D) differentiation model of neurospheres, which produce unique neuronal clusters, termed NeuroCore (NC)	For differentiation, the growth factors were removed at three days after cell seeding. On day 7 of differentiation, the neurospheres were grown in a neuronal maturation medium: 1:1 mixture of DMEM/F-12 and neurobasal medium (Life Technologies, 21103049)			0.5% N2, 2% B27, 1% P/S, and 1% GlutaMAX (Life Technologies, 35050061). From day 14 of differentiation, neuronal maturation medium containing 1 μM cyclic AMP (Sigma, D0260), 200 μM ascorbic acid (Sigma, A4403), 10 ng/ml brain-derived neurotrophic factor (BDNF, PeproTech, 450-02), and 10 ng/ml Insulin-like growth factor 1 (IGF-1; PeproTech, 250-19) were used for long-term culture	The cell medium was replaced every 3 days	(Lee et al., 2020)
	forebrains of E15 embryos C57/BL6J mice		The characterization of gene expression during mouse neural stem cell differentiation *in vitro*	DMEM	1% FBS		P/S, 2% B-27	Neurospheres from the 4th passage were used for the following experiments	(Park et al., 2012)
	E15.5 ICR mice ganglionic eminence	Neurons and astrocytes	investigating the effects of oxygen concentration on the proliferation and differentiation of mouse neural stem cells *in vitro*	DMEM/F- 12	1%) of FBS		36.4 mM glucose, 23 lg/ml insulin, 92 lg/ml transferrin, 55 lM putrescine, 27.5 nM sodium selenite, 20 nM		(Horie et al., 2008)

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Table 9 (*continued*)

Medium composition								
Source	Desirable lineage	Application	Medium	Serum	FGF	Supplements	Other	Ref
						progesterone, and 50 U/ml penicillin-streptomycin		
E12.5, cerebral cortex of Kun Ming mouse (KM strain)		mouse NSCs were used as a model system and cell behavior was monitored in the presence of the protein palmitoylation inhibitor 2-bromo-palmitate (2BRO)		2 % FBS (Gibco-BRL, Carlsbad, CA, USA)			All experimental procedures were carried out using NSCs dissociated from P1 neurospheres	(Chen et al., 2016)
E15.5 brains		investigating the role of miR-342-5p in regulation of Neural Stem Cell Proliferation and Differentiation Downstream to Notch Signaling in Mice	DMEM/F12	5% of FBS (Gibco)	5 ng/ml bFGF, 5 ng/ml EGF	0.5 × N2		(Gao et al., 2017a)
E14, ganglionic eminences of C57-BL6 mice		purifying Immature Neurons from Differentiating Neural Stem Cell Progeny Using a Simple Shaking Method		5% fetal calf serum (FCS) (Gibco)			Neurospheres harvested from passages one to three were dissociated into single cells using 0.05% trypsin-EDTA (Gibco, USA) and plated at a density of 3×10^5 cells/ml in mouse NSC supplemented by 5% fetal calf serum (FCS) (Gibco), 20 ng/ml EGF, 10 ng/ml bFGF and 2 μg/ ml heparin (Sigma) for 3-4 days (Proliferation Stage). When the culture reached 90-95% confluency, the medium was changed to the growth-factor-free medium containing 5% FCS so that neuronal progenitor cells could divide and generate clusters of immature neuronal cells (Differentiation Stage)	(Azari et al., 2014)
E12.5 cortices of fetal mouse pups		application determining whether formyl peptide receptors (FPRs) regulated the differentiation of neural stem cells	DMEM/F12	1% FBS				(Zhang et al., 2017)

types can also be achieved with specific transcription factors. Ehrlich et al. showed that the induction of the transcription factors SOX10, OLIG2, and NKX6.2 in iPSC-derived neural progenitors accelerates oligodendroglial differentiation and results in up to 70% of O4+ oligodendrocytes within 28 days (Ehrlich et al., 2017).

Serum

FBS contains various factors and other components which support the survival and differentiation of NSCs, mainly toward astrocytes (Fig. 4) (Pollard et al., 2006). In most of the studies (listed in Table 7 and Table 9), FBS is used directly in the differentiation medium. From our

Table 10
Comparison of protocols for differentiation (mouse cell culture) – other conditions.

	Source	Density	Coating	Duration of the diff stages	Ref
Mouse	E14, cerebrum	$5x10^4$ cells cm^2	poly-D-lysine and laminin (does not say concentration)		(Li et al., 2014b)
	E14 whole brain	15,000-20,000 cells in 12 mm coverslips (does not say 24 wp but I suppose it is)	poly-L-lysine (10 ug/ml, Sigma)/laminin (5 ug/ml, Sigma)		(Balasubramaniyan et al., 2006)
	NSPCs were isolated from mouse E14.5 GE	single dissociated cells of cultured neurospheres were plated on $2x10^5$ cells/cm^2 in NSP medium without growth factors for 4 days, and then subjected to qPCR analysis	poly-L-lysine coated glass coverslips		(Ohta et al., 2016)
	forebrains of E15 embryos C57/BL6J mice		poly-l-lysine-coated culture dishes	1 or 2 days	(Park et al., 2012)
	E12.5, cerebral cortex of Kun Ming mouse (KM strain)			3–9 days	(Chen et al., 2016)
	E15.5 brains		PLL-coated 35-cm^2 culture dishes at a very low density (Molofsky et al., 2003) (fewer than 100 clones per dish for a 7-day culture)		(Gao et al., 2017a)
	E14, ganglionic eminences of C57-BL6 mice		polyornithine-coated dishes		(Azari et al., 2014)

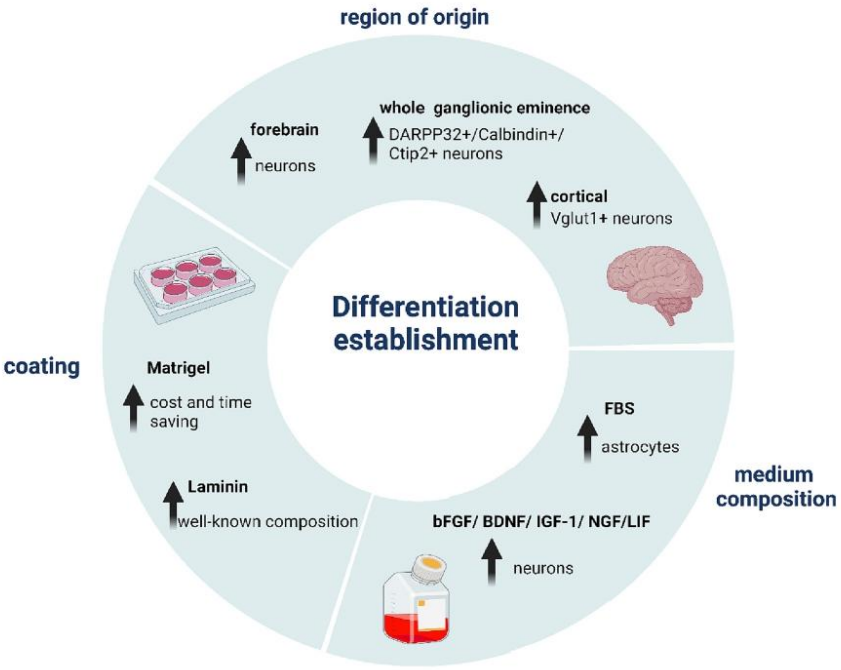

Fig. 4. Critical points at NSC culture differentiation establishment.

experience, the use of 1% of FBS in the differentiation medium significantly improves the viability/survival of differentiating mouse embryonic NSCs as well as leads to obtaining both neurons and astrocytes within the cell culture. Even though the use of FBS is accepted for conducting *in vitro* experiments, its addition to cells that are being cultured for clinical application is not approved. This is due to its animal origin and partly unknown composition. Therefore, other viable alternatives to FBS for clinical use are currently being developed (Witzeneder et al., 2013) (Chelladurai et al., 2017).

2.2.2. Region of origin

Since the anatomical origin and time of isolation can influence the features of obtained NSCs, it is crucial to consider these elements to facilitate a more optimal translation toward clinical application. Although it was shown that both embryonic cortical and spinal cord NSCs have similar self-renewing properties and exhibit multipotency,

they still retain gene expression patterns indicative of the region of origin through multiple passages (Kelly et al., 2009). Additionally, neurospheres generated from single cells that were isolated from CNS of human fetal cadavers at 13 weeks of gestation were shown to give rise to significantly more neurons if they were isolated from the forebrain as opposed to those isolated from the midbrain or hindbrain (Kim et al., 2006). Martín-Ibáñez et al. showed that NSCs originating from both cortical and whole ganglionic eminence (WGE) give rise to a high percentage of MAP2 mature neurons (Martín-Ibáñez et al., 2017). Yet, the phenotypes of these neurons were slightly different. While WGE cultures showed a predominantly neuronal phenotype positive for DARPP32, Calbindin and Ctip2, the cortical cultures were more prone to differentiate to Vglut1 positive neurons. This is indicative of cells maintaining their region-specific characteristics (Martín-Ibáñez et al., 2017). In addition, the same study observed a diminished number of neural precursors and an increase in post-mitotic cells with advanced fetal brain development stages. Even though the whole brain showed cells positive for Nestin and Ki67 at 5 wpc (weeks post-conception), these proliferative cells were restricted to the germinal zone adjacent to the ventricle at 10 wpc. Moreover, an increase in expression of the neuronal markers β-III tubulin, Enkephalin, Substance P, Calbindin and vesicular glutamate transporter 1 were observed in later stages of the forebrain development (Martín-Ibáñez et al., 2017). All of this suggests that, before considering NSCs for any clinical use, their capacity to achieve the desired effect must be checked *in vitro*.

2.3. Coating

On top of supplements, growth factors and serum, another key target for the optimization of cell culture protocols is the coating. Being a crucial part of the cell's microenvironment, the coating of the culture plate plays a vital role in determining NSCs behavior. The most common compounds which are used for coating are primarily based on the extracellular matrix (ECM), which interacts with the cells through the integrin family of receptors. Thus, it is crucial to provide a suitable coating material that promotes proliferation and differentiation of cells. Even though plate coatings are more commonly used in a 2D culture, several of them can be used in a 3D model. One of the more common coating agents is laminin (Table 3, Table 8, Table 10) since it improves cell proliferation and differentiation. Other often used coatings include Matrigel, polylysine and poly-l-ornithine. Still, it should be noted that each of the materials presented above has different features. These affect cell proliferation and differentiation and should be the main criterion when choosing the perfect coating.

While laminin is known for promoting NSCs/NPCs adhesion and proliferation, as well as neuronal and oligodendrocytic differentiation, Matrigel mainly promotes their expansion and differentiation (Christopherson et al., 2009; Flanagan et al., 2006; Hughes et al., 2010; Lee et al., 2015). Even though it has been shown that 0,02% of Matrigel can support NSCs differentiation into neurons and glial cells similarily to laminin, the method is more costly and time-consuming (Lee et al., 2015). Moreover, when laminin and Matrigel are made from mouse sarcoma cells, their clinical application is forbidden. Comparatively, the use of recombinant/human laminin is more costly (Liu et al., 2020c). Additionally, since the composition of Matrigel remains somewhat ambiguous, its use might also yield inconsistent results.

On the other hand, alfa-polylysine and poly-l-ornithine represent relatively affordable and freely available options since they can be synthesized by polycondensation reactions. However, since these might be toxic for the cells, it is necessary to do ample research in order to ascertain a viable concentration (Lee et al., 2015; Liu et al., 2020c; Ge et al., 2015).

3. Cryopreservation

The long-term preservation of cells and tissues is made possible by their reduced biochemical reactions as a response to low temperatures. Nevertheless, the process of freezing is rather fatal for most organisms because of the formation of intra- and extracellular crystals which, in turn, cause chemical changes in cells and leads to mechanical injuries, including damage of the plasma membrane and the membranes of intracellular organelles (Karlsson and Toner, 1996), (Fujikawa, 1980). Due to changes in solute concentrations, such as salts, which can become more concentrated, freezing can also induce osmotic stress (Lovelock, 1953). As such, one of the biggest challenges for cells at low temperatures is to overcome the intermediate zone of temperature (-15 to -60°C), something that cells must go through twice – once during their cooling and the second time during their thawing (Mazur, 1970). The process through which biological material is placed in a suspended animation state, allowing for the preservation of the cell structure at low temperatures, is called cryopreservation (Mazur, 1970).

3.1. Methods of cryopreservation

A standardized cryopreservation protocol is a prerequisite for an uninterrupted supply of a reproducible NSCs population. Although the methods of NSCs cryopreservation seem to be well-optimized, they remain somewhat ambiguous. For example, the plethora of protocols in the literature pertaining to cell freezing, still vary from each other even for cells of the same origin. This results in different cellular responses throughout. As such, researchers are still on the lookout for other solutions that could increase the survival of cells after thawing.

One possible alternative to the deep-freezing method is vitrification (Fig. 5). Several studies have shown that freezing and vitrification do not significantly impact the stemness, proliferation or differentiation abilities of human and murine NPCs, however, results do slightly vary, depending on the conditions used in the protocols (Kuleshova et al., 2009; Chong et al., 2009; Pegg, 2007). While freezing facilitates reduced cell damage since crystals are formed extracellularly, vitrification pertains a glass-like solidification process in order to completely avoid crystal formation (Kuleshova et al., 2009), (Hunt, 2017). Still, since freezing is mainly performed by rapid or slow-cooling protocols, it has been shown to be harmful to some sensitive cells, e.g. embryonic stem cells (Katkov et al., 2006; Reubinoff et al., 2001; Trounson and Pera, 2001). Importantly, freezing can also significantly reduce the viability and structural integrity of cells or neurospheres (Tan et al., 2007). Another disadvantage of the freezing method is that it, potentially, facilitates further cell differentiation following the use of DMSO as a cryoprotectant or following the addition of any animal sera (Jacob and Herschler, 1986), (Mallon et al., 2006). Although many attempts to replace animal proteins with human examples in xeno-free cryopreservation protocols have been made throughout the years, the ideal solution would be a complete elimination of sera (Richards et al., 2004). This change would also decrease the chance of contamination. More so, the use of any protein is not required in the vitrification method, which has already been demonstrated to be effective for the cryopreservation of neurospheres (Kuleshova et al., 2009). Furthermore, there also exist reports suggesting the use of a „straw-in-straw" method which is meant to prevent contamination and sustain proper NSCs differentiation and proliferation rates (Kuleshova et al., 2009). Another interesting alternative to traditional methods of cryopreservation was presented by Nishiyama et al. They developed a technique of slow-freezing using a programmed freezer with adjunct magnetic field application and examined its effects on hiPSCs-derived NPCs. Their results have shown an improved cell survival following thawing as well as demonstrated that the thawed cells were comparable to non-frozen ones on a transcriptome level (Nishiyama et al., 2016).

3.2. Cryopreserving medium composition

Apart from various methods of cryopreservation, another element which influences the efficacy of the procedure is the composition of the

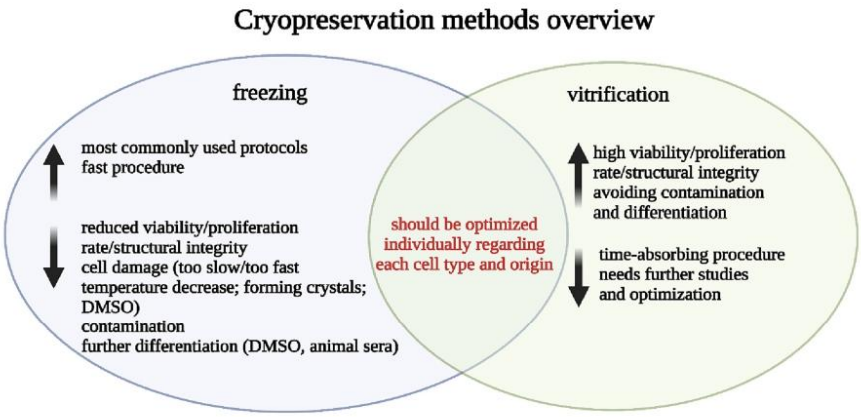

Fig. 5. General overview of main cryopreservation methods properties.

medium in which the cells are cryopreserved. Most commonly, researchers use media with 10% of DMSO along with a slow-cooling protocol, regardless of the species. For neurospheres, the concentration of DMSO used reaches around 10-15% (Paynter, 2008). Interestingly, in one of our yet unpublished studies, we have observed that cryoprotectants and different reagents added to the media have a significant impact on the cells' structural and functional abilities following the thawing process. Therefore, the rate of success in removing DMSO from the dish remains a critical element in ensuring increased survival and viability of cells derived from human fetal brain tissue (Dong et al., 1993). Although current reports on mNSCs are not demonstrating any significant impact of cryopreservation with DMSO or glycerol , either with or without serum (Milosevic et al., 2005), this does not hold true for sensitive cells of human origin, such as fhNSCs and NSCs with iPSC origin. Nevertheless, problems like these are beginning to be addressed nowadays, with Niles and Snyder publishing a protocol pertaining to a computational tool for cryomedium composition wherein the cryoprotectant concentration meets the criteria for vitrification. This protocol facilitates relatively high cell viability and reproducibility (Niles and Snyder, 2021). In order to optimize the protocol even further, the viability of cells (mNSCs) following thawing can also be strengthened by adding some caspase inhibitors (Milosevic et al., 2005).

3.3. 3D vs. 2D

When studying the distinct conditions present in 2D and 3D cultures, no significant differences between the effects of freezing or thawing of neurospheres and enzymatically dissociated murine NPCs have been observed (Milosevic et al., 2005). Nevertheless, a study performed on rat NSCs showed a higher survival rate for cryopreserved spheres (Ma et al., 2010). This study also pointed to the importance of the sphere size, with 80-100 μm diameter being the optimal one ensuring their survival. Since these large spheres are not easy to permeate by cryoprotectants and, conversely, water from the cells inside the sphere is unable to permeate out, inner crystallization and necrotic cores develop. On the other hand, spheres that are too small could result in a lower cell survival rate following thawing as intracellular connections are not optimal. It has additionally been shown that, similar to NSCs and spherical formations occurring when cultured as a suspension *in vitro*, a close association and the activation of the Notch signaling pathway is necessary to maintain cells' high proliferation abilities and maintain their undifferentiated state (Ma et al., 2010; Capela and Temple, 2002; Grandbarbe et al., 2003; Woo et al., 2009). Since much is yet unknown regarding human NSCs and the impact of the spherical structure on cellular proliferation during cryopreservation, more studies are necessary to further elucidate these mechanisms (Mori et al., 2006).

3.4. Cooling rate

The maintenance and upkeep of stem cell cultures could be ensured through 3 distinct steps, including: i) culturing at 37°C; ii) hibernation at 4°C; and iii) cryopreservation. When it comes to long-term cell culturing at 37 °C, a decrease in proliferation and culture senescence can be observed. This leads to changes in the properties of the cell line. Similarly, the process of hibernation also presents limitations, wherein hibernation at 4°C has been shown to facilitate optimal survival of nerve tissue for only up to 8 days. For longer cell storage, the only acceptable option is to keep it below -130°C. As such, in most studies, the cells are stored at -80°C or in liquid nitrogen (at -196°C) (Paynter, 2008), (Frodl et al., 1995; Gage et al., 1985; Nikkhah et al., 1995). Still, when the temperature drops too rapidly, and if the protocol is not performed adequately, the quantity of water leaving the cell osmotically is insufficient. This leads to internal impairments and apoptosis. However, when the cooling process is faster, the formed ice crystals are, indeed, smaller, causing less damage to the cell.

At the most rapid cooling rate, crystallization can even be completely avoided altogether when the aforementioned cell solutions are getting vitrified. Still, since vitrification requires a relatively high dose of cryoprotectants, it is used only for small cells and samples. With that being said, the most commonly used protocols still include slow cooling (~1°C/min). Yet, if the cells are cooled slowly, the ice formation occurs outside of the cell, with their internal content getting more concentrated. In turn, such intracellular increase of solutes could become toxic and cause cell damage (Mazur, 1970), (Bajerski et al., 2018).

3.5. Warming process

As previously mentioned, the second biggest challenge for cells after the cooling process is the subsequent process of warming. Since warming could also exert similar effects on the cells' survival (Mazur, 1984), the warming rate plays a crucial role in cell recovery (Gao and Critser, 2000). This is especially important if some intracellular ice was formed during cooling (Mazur, 1984), (Karlsson, 2001). While those samples that have been slowly cooled can be warmed both slowly and rapidly, the cells that have undergone the process of vitrification must be warmed as fast as possible (Paynter, 2008). Following the warming

process, and due to the risk of toxicity and the osmotic effects, the cryoprotectants must be removed from the cells. This can be done stepwise in order to reduce the osmotic stress (Paynter, 2008).

When it comes to fetal brain tissue, the warming/freezing processes have been shown to cause a decrease in the cell yield from 10 to 88% (Frodl et al., 1995), (Collier et al., 1993; Mattson and Rychlik, 1990; Swett et al., 1994). However, cell ultrastructure has been shown to remain unchanged throughout (Silani et al., 1988). On the other hand, the most commonly used method for primary neural cultures is rapid warming. In studies which used rat tissue, rapid warming in DMSO (30-40s in a 37°C water bath) was preferred over slow warming (in ambient temperature for 5-7 min) (Das et al., 1983), (Fang and Zhang, 1992). Interestingly, vitrified rat hippocampal tissue ultrastructure has also been shown to be very well preserved after thawing (Pichugin et al., 2006). Moreover, NSCs susceptibility to cryopreservation injuries depends on the cell type, wherein it has been reported that human tissue is more sensitive to cryopreservation than for example rat striatal tissue (Frodl et al., 1995). Additionally, the size of the neurospheres has also been seen to impact the result of cryopreservation. If the formed sphere was too small, the intercellular connection and cell vitality led to reduced cell survival following thawing (Ma et al., 2010). When the sphere is too large, the penetration of the cryoprotectants into their core as well as the removal of intracellular water could be hampered, leading to NSC crystallization and causing necrosis. This could also presumably affect the close interaction of NSCs in neurospheres, vital for Notch signaling activation and maintaining NSC activity, their undifferentiated state and cryopreservation processes.

Even though there exists a great diversity in cellular responses to cryopreservation and thawing of NSCs, there are some general principles related to every cell type which should be followed. Namely, the cells should be cryopreserved in a way that allows them to avoid intracellular freezing and warmed in a way that any unfrozen intracellular water remains unfrozen (Mazur, 1984).

4. Conclusions

In conclusion, each year brings forth novel methods and sources for obtaining NSCs. However, due to inconsistencies in the protocols used, these cells may differ in their genetic stability and their ability to maintain phenotypes or immunophenotypes. As such, NSCs derived from both ESCs and fetal stem cells cannot be used in an autologous system. However, cells isolated from patients suffering from neurodegenerative diseases could help identify abnormalities in myelin production and nerve conduction, helping to better diagnose diseases and develop patient-specific therapies. Moreover, these cells could also be used to study the pathogenesis of the disease, as well as provide a platform for drug screening. Several groups isolate NSCs from somatic tissues such as DRG, bone marrow, skin, hair, heart, or intestines cells with neural crest-like features, boasting a similar transcriptional profile and the potential to differentiate into neural phenotypes. These multipotent stem cells with features shared with crest-derived cells (NClSCs) probably originate from embryonic neural crest and remain dormant in a multipotent state under specific culture conditions. These cells can be applied in an autologous system, but there is still no convincing evidence of functional differentiation in the *in vivo* model. Recently, most NSCs are seen to be derived from iPSCs. Even though they could potentially be used in an autologous system, the issue of the immune response is still contentious. Additionally, the question of NSCs immunogenicity still remains; and so does the question whether NSCs obtained in such a way can even differentiate into distinct functional forms in appropriate quantities as well as whether these cells can properly form synapses. Answers to all these questions can only be achieved through line-tracing experiments as well as tracking of the survival, migration and differentiation of derived NSCs.

In this review, we presented the importance of standardization of the culture conditions for neural stem cells, regardless of their origin. In addition to observing significant differences in protocols pertaining to distinct species, we specifically highlight the fact that even the comparison of results from experiments performed on the same cell population might become challenging if one of the components of the culture medium is changed – be it the changes occurring from 2D to 3D cell culture systems, or the type of the surface coating used. As such, several crucial points must be considered, and further investigated, in order to obtain a more homogenous and comparable outcome. These can be divided into three key points which are as follows:

- Ensuring a high proliferation rate: being mindful of bFGF, glutamine, 3D culture, type of coating (for 2D);
- Facilitating further, well-controlled differentiation of NSCs: carefully choosing the necessary supplements, growth factors, serum, coatings;
- Maintaining appropriate cryopreservation conditions: choosing freezing vs. vitrification, medium components vs. temperature, cooling rate.

On top of challenges in facilitating optimal cell proliferation and differentiation, another obstacle in optimizing neural cultures and facilitating their translation into clinics is the insufficient number of studies on both animal and human models which are assessing the properties of cryopreserved NPCs before and after thawing. Since the mere thawing process can greatly alter NPCs' properties, a detailed quantification of the effects of such a process on cell culture viability is a crucial step in enabling safe and effective use on a clinical level (Tan et al., 2007), (Ma et al., 2010), (Hancock et al., 2000). As such, a detailed validation of the cells' properties should be performed at many given time points to avoid possible adverse events, including engrafting failure. A better understanding of the cell cryopreservation processes should facilitate protocol modification and optimization to improve the survival rate of sensitive cells following thawing.

Taken altogether, there are still several unresolved issues obstructing the translation of this research into clinical settings. Therefore, for any future applications, we should carefully evaluate each parameter which has been „uniquely optimized" by an experimenter and its impact on the validity of the result. Moreover, special attention should also be paid to the presence of unavoidable differences between the species. This means that the conditions used for rodent NSC cultures should not be directly implemented for human ones since just the mere factors used throughout the cultivation process have a different impact on the functional properties of the cells. Even though the available data suggests that there is no "ideal" method for cultivation of NSCs, further investigation into the mechanisms involved in cell proliferation and differentiation as well as the cryopreservation processes modulated by aforementioned crucial points, will facilitate easier standardization of basic protocols for each species, bringing us closer to reliably comparing, interpreting and improving the results. For future therapeutic applications, we should optimize NSC preparation in a way that allows us to obtain the most homogenous, stable, self-renewable and expandable culture with clearly defined multipotent stem cell characteristics. The establishment of NSC lines is also critical for their use as models for neurodevelopment, as well as the ones that carry CNS disease mutations that would provide a precious resource for further investigations. That also rises hope regarding the generation of patient-specific cell lines using induced pluripotency actions.

Author contributions

Conceptualization: KR, Data curation: KR and VH, Formal analysis: KR and VH, Funding acquisition: AS, Supervision: DM and AS, Visualisation: KR, Writing- original draft: KR, VH and JI, Writing- review & editing: DM and AS. All authors have read and approved the manuscript.

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Article

Understanding Intra- and Inter-Species Variability in Neural Stem Cells' Biology Is Key to Their Successful Cryopreservation, Culture, and Propagation

Klaudia Radoszkiewicz [1], Katarzyna Jezierska-Woźniak [2], Tomasz Waśniewski [3] and Anna Sarnowska [1,*]

[1] Translational Platform for Regenerative Medicine, Mossakowski Medical Research Institute, Polish Academy of Sciences, 02-106 Warsaw, Poland
[2] Department of Neurosurgery, Laboratory for Regenerative Medicine, Stem Cells Bank, University of Warmia and Mazury in Olsztyn, 10-720 Olsztyn, Poland
[3] Department of Obstetrics and Gynaecology, School of Medicine, Collegium Medicum, University of Warmia and Mazury, 10-561 Olsztyn, Poland
* Correspondence: asarnowska@imdik.pan.pl; Tel.: +48-22-608-6598

Abstract: Although clinical trials on human neural stem cells (hNSCs) have already been implemented in the treatment of neurological diseases and they have demonstrated their therapeutic effects, many questions remain in the field of preclinical research regarding the biology of these cells, their therapeutic properties, and their neurorestorative potential. Unfortunately, scientific reports are inconsistent and much of the NSCs research has been conducted on rodents rather than human cells for ethical reasons or due to insufficient cell material. Therefore, a question arises as to whether or which conclusions drawn on the isolation, cell survival, proliferation, or cell fate observed *in vitro* in rodent NSCs can be introduced into clinical applications. This paper presents the effects of different spatial, nutritional, and dissociation conditions on NSCs' functional properties, which are highly species-dependent. Our study confirmed that the discrepancies in the available literature on NSCs survival, proliferation, and fate did not only depend on intra-species factors and applied environmental conditions, but they were also affected by significant inter-species variability. Human and rodent NSCs share one feature, i.e., the necessity to be cultured immediately after isolation, which significantly maintains their survival. Additionally, in the absence of experiments on human cells, rat NSCs biology (neurosphere formation potential and neural differentiation stage) seems closer to that of humans rather than mice in response to environmental factors.

Keywords: neural stem cells; intra-species variability; inter-species variability; cryopreservation; long-term culture; human; rodents; medium composition; growth factors

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1. Introduction

Over the last four decades of studies on neural stem cells' (NSCs) clinical applicability, researchers from different laboratories have optimized culture conditions which facilitate long-term culture and further cell differentiation. A considerable interest in NSCs is mainly aroused by promising results obtained in patients with neuropsychiatric disorders and a variety of neurological diseases, such as amyotrophic lateral sclerosis, multiple sclerosis, glioma, Parkinson's disease, and ischemic stroke, which in most cases still need successful treatment [1]. This is caused by a variety of beneficial NSC properties (Figure 1). In comparison to other cell types, NSCs demonstrate a restricted ability to differentiate into mature, functional cells which can replace the injured ones, and they show a minimal risk of tumorigenesis [2–4]. What is more, they can migrate into the damaged areas and promote tissue repair by secreting pro-neurogenic and pro-angiogenic factors [5–13]. Human neural stem cells' (hNSCs) potential has not only been detected in trophic support but also in a cell replacement strategy (neurorestoration) after damage [12]. Moreover, hNSCs derived from

the human olfactory bulb (hOBNSCs) seem to represent a new approach for anticancer therapy within the central nervous system, as their potential to be used as a carrier/vehicle of anticancer agents has been indicated [14]. In addition, it has been observed that the use of some factors could enhance their therapeutic properties. For example, recent suggestive results of the Alzheimer's disease rat model study indicated that transplanted NSCs' further differentiation and integration with the host tissue could be enhanced by the previous administration of rosemary oil [15].

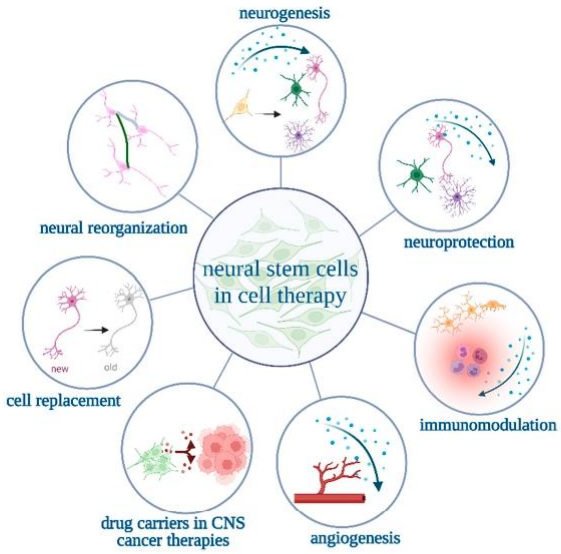


Figure 1. Possible benefits of neural stem cells' (NSCs) use in clinical trials suggest the need to conduct further NSC studies at the preclinical level.

Despite the impressive variety of the aforementioned benefits, the attempts to carry out cell-based therapy with NSCs have revealed several limitations [12]. Even at the preclinical level, the interpretation of the results obtained by different laboratories could be hampered because of several variables, such as different medium composition, 2D (in monolayer) or 3D (as neurospheres) spatial culture conditions, dissociation methods, and/or the species used for analyses. Moreover, it is difficult to assess which NSC origin type would be the most efficient for cell therapy [16,17]. The question also arises as to which conclusions regarding the isolation, cell survival, proliferation, or cell fate observed *in vitro* in rodent NSCs are appropriate and sufficient to be introduced into clinical applications.

In our study, we compared intra-species and inter-species variability in NSCs' biology as a response to different cryopreservative, spatial, nutritional, and dissociation conditions. For the analysis, fetal hNSCs obtained from the University of Warmia and Mazury in Olsztyn were used [18]. Rodent NSCs were derived from the most commonly used Wistar rats and C57BL/6J-type mice.

Throughout our research, we focused on the differences in the cell fate, which might depend even on negligible environmental variables, and we underlined the problems with the translation of the results from rodent into human cells (Figure 2).

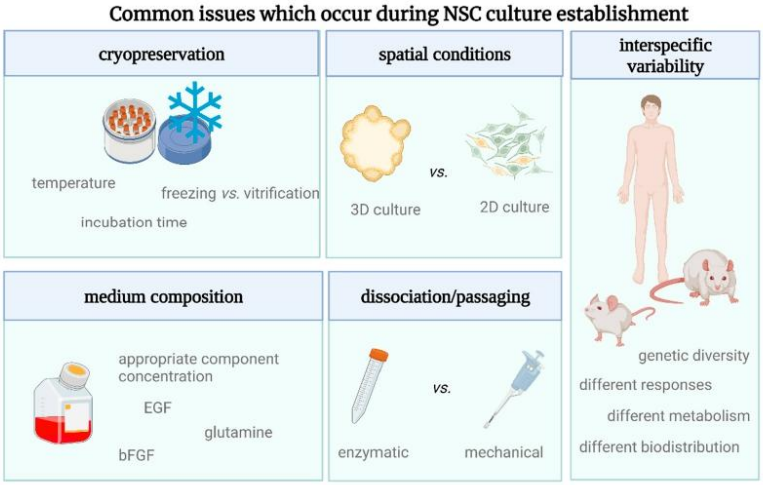


Figure 2. A variety of culture establishment options regarding NSCs' cultivation.

2. Materials and Methods

2.1. NSC Isolation

2.1.1. hNSCs' Isolation

hNSCs were obtained from the Stem Cell Research Laboratory, Department of Neurosurgery, University of Warmia and Mazury in Olsztyn, Poland, where the material was collected and prepared according to the protocol developed by Prof. Vescovi's group [19–21], slightly modified for dissociation by means of Accutase Cell Detachment Solution (Beckton Dickinson, Franklin Lakes, NJ, USA). The neural tissue was obtained from the whole brain in 2020, from fetuses after spontaneous miscarriages from 10 and 18 weeks of gestation (mixed XX and XY). The study was approved by the Bioethical Committee of the School of Medicine, University of Warmia and Mazury in Olsztyn, Poland (ethical approval No. 15/2018 and No. 19/2018, both in May 2018). One part of the cells was cultured before cryopreservation and the other part was cryopreserved directly after the isolation. After isolation, to identify the undifferentiated state of NSCs, immunocytochemical staining of anti-Nestin and anti-SOX-2 was performed. To study the multipotentiality of NSCs, their differentiation potential into glial or neuronal progenies was analyzed after growth factor removal. The cells were plated into 24-well plates in the medium supplemented without epidermal growth factor (EGF) and basic fibroblastic growth factor (bFGF) for 7 days. The presence of the astrocytic marker GFAP, neuronal marker β-tubulin III, and oligodendrocytic marker O4 were analyzed by immunofluorescence (IF).

2.1.2. Mouse Neural Stem Cells (mNSCs) and Rat Neural Stem Cells (rNSCs) Isolation

For this experiment, newborn Wistar rats and C57BL/6J-type mice from the Mossakowski Medicine Research Institute Animal Breeding House were used.

All macro- and micro-dissection procedures were performed on ice in a careful and swift manner (Figure 3). After decapitation, murine/rat brains were extracted, and the area around the subventricular zone (SVZ) was isolated. To remove the pia mater and blood vessels, an additional set of microdissection instruments was used under a binocular. The samples were mechanically defragmented using a razor blade, collected in a 15 mL centrifuge tube, and centrifugated (5 min, 200× *g*). Then, the pellet was digested in Accutase® solution (Beckton Dickinson) for 20 min, at 37 °C. After every 5 min of digestion, the sample was gently triturated using a Pasteur Pipette. Next, the samples were centrifugated again and the pellets were resuspended in a freshly prepared culture medium, filtered

in a 70 µm cell filter, and 10 µL of suspension aliquots were mixed with 10 µL of Trypan blue (Sigma-Aldrich; Merck; Irvine; United Kingdom) in a continuous manner at room temperature (RT) to calculate cell viability. The obtained cells were seeded in non-adhesive T75 flasks (Nunc, Thermo Fischer; Rochester, NY, USA) at a density of 2×10^5 cells/mL and incubated at 37 °C with 5% CO_2 and 5% O_2 or cryopreserved in 1.5 mL cryovials, at high density in a medium consisting of 50% basal medium (cells+culture medium), 20% fresh culture medium, 20% platelet lysate (Macopharma, Tourcoing, France), and 10% DMSO (Sigma-Aldrich, St Louis, MO, USA). The vials placed in a freezing container (Mr. Frosty, Thermo Fisher Scientific, Rochester, NY, USA) and were transferred to a −80 °C freezer for at least 4 h in our laboratory, where they were stored in liquid nitrogen. The same identification of the undifferentiated state and multipotentiality of rodent NSCs as was shown for hNSCs was performed.

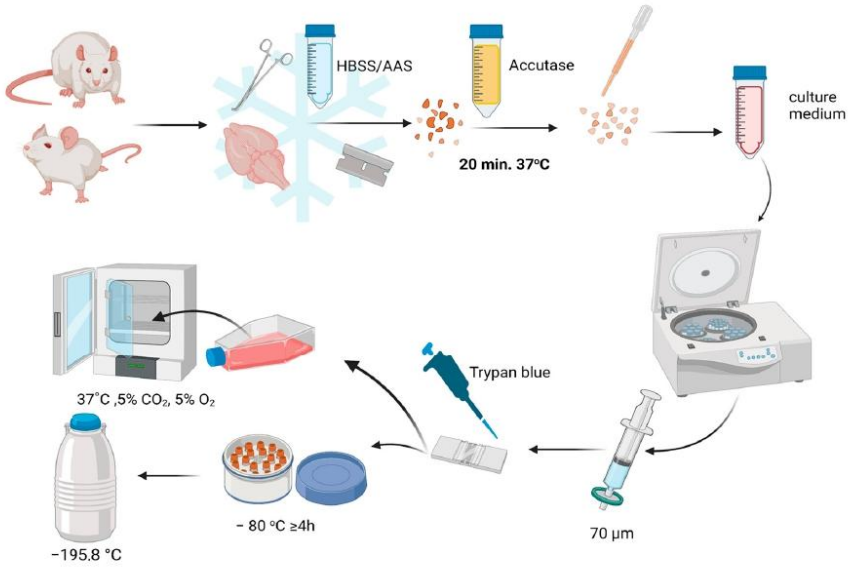


Figure 3. rNSCs and mNSCs isolation procedure.

2.2. Cell Culture

2.2.1. Thawing

The thawing procedure included immediate warming of cryotubes (maximum 2 min at 37 °C, in a water bath), the cells' suspension in a standard culture medium, and centrifugation (to eliminate the toxic impact of DMSO (Sigma-Aldrich)). Then, the cells from the pellet were suspended in a fresh standard culture medium. Next, the number of cells was counted using the Bruker chamber.

2.2.2. Basic Culture

The optimal medium composition and seeding density for NSCs was determined on the basis of the latest relevant literature (Table 1).

Table 1. The medium composition, coating, and seeding density used in the following experiments.

	2D Culture	3D Culture
Medium composition	DMEM/F12 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) GlutaMAX® (1%, Gibco) Penicillin/streptomycin (1%, Gibco) Heparin (0.1%, Sigma-Aldrich, Saint Louis, MO, USA) N2 supplement ® (1%, Gibco) B27 supplement® (2%, Gibco) EGF (20 ng/mL, Gibco) bFGF (20 ng/mL, Gibco)	
Coating	poli-L-lysine + laminin	The non-adhesive coating on NunclonSphera® plates and t75 flasks (Nunc, Thermo Scientific™)
Seeding density	40,000 cells/cm^2	10×10^4 cells/cm^2

Next, the obtained cultures were placed in humidified incubators under 5% O_2 and 5% CO_2 conditions at 37 °C. The medium was replaced every 3 days. When the cell culture was subconfluent (in 2D culture) or the neurospheres (3D) gained the desired diameter, the cells were detached (2D) or dissociated (3D) with Accutase® (Accutase Cell Detachment Solution, Beckton Dickinson, Franklin Lakes, NJ, USA). In case of further proliferation, lactate dehydrogenase (LDH) and senescence were used for NSCs' differentiation state analysis (Results Sections 3.2–3.5), and the cultures were sub-cultured, from four to six passages, before starting the aforementioned experiments, in order to enrich the culture in NSCs.

2.3. Live/Dead Assay

A live–dead assay (Invitrogen™) was used according to the manufacturer's instructions. Briefly, the medium was removed, and the cells were washed with phosphate buffer saline (PBS, Sigma-Aldrich) followed by incubation with the dyes. Live cells (stained with calcein-AM) and dead cells (stained with ethidium homodimer-1 (EthD-1)) were visualized using a Zeiss Axiovert A.1 fluorescent microscope (Carl Zeiss, Oberkochen, Germany). The percentage of living and dead cells was calculated by determining the percentage of calcein-AM/EthD-1-positive cells in the total number of cells.

2.4. Proliferation Assay

To estimate the cell proliferation rate, PrestoBlue™ Cell Viability Reagent (Invitrogen, Thermo Fischer, Rochester, NY, USA) was used according to the manufacturer's protocol. The cells were incubated with this reagent for 2 h in the dark, on Day 1, Day 5, and Day 7 of 2D culture performed on a 96-well poly-L-lysine/laminin-coated plate. The cells were seeded at a density of 1×10^4 cells/well. The starting point (Day 1 measurement) was assumed to equal 100%. Absorbance was measured using a microplate reader, Omega Plate Reader (BMG LABTECH), at a 590–600 nm wavelength.

2.5. Lactate Dehydrogenase Assay

Cell viability was measured using the LDH assay and trypan blue (Sigma-Aldrich) staining was used at the cell count stage (Takara). A colorimetric assay kit (Takara Bio Inc., Kusatsu, Japan) was used to quantify LDH release from cultured NSCs, in line with the producer's instructions. Absorbance was measured using a microplate reader, Omega Plate Reader (BMG LABTECH), at a 490–690 nm wavelength. The final LDH release was calculated according to the following formula:

LDH release (%) = 100 × (experimental LDH release-culture medium background)/(maximum LDH release-culture medium background).

2.6. Senescence Assay

The senescence analysis was performed with a CellEvent™ Senescence Green Detection Kit (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's protocol. In brief, the cells were seeded on 96-well plates (Nunc, Thermo Fischer Scientific), washed with PBS (Sigma-Aldrich), fixed with a fixation solution (2% PFA (Sigma-Aldrich) in PBS (Sigma-Aldrich)), and incubated in a working solution without CO_2 at 37 °C for 2 h. Then, the cells were washed with PBS. The fluorescence intensity was measured using a microplate reader, Omega Plate Reader (BMG LABTECH).

2.7. Immunocytochemistry 2D

For immunocytochemical analysis, the cells cultured in 2D and 3D conditions were seeded on 24-well plates (Nunc, Thermo Fischer Scientific). The seeding number of cells was 7×10^4 per each 13 mm-diameter poly-L-lysine/laminin-coated coverslip. The 2D culture cells were gently washed in PBS (Sigma-Aldrich), fixed with 4% PFA (Sigma-Aldrich) in PBS (Sigma-Aldrich) for 15 min at RT, and again washed in PBS (Sigma-Aldrich). To detect intracellular proteins and to permeabilize cell membranes, 0.2% Triton X-100 (Sigma-Aldrich) was used. Subsequently, a mixture of 10% goat serum (Gibco) and 1% bovine serum albumin (Sigma-Aldrich) was applied for one hour to block nonspecific binding. Next, the samples were washed in PBS (Sigma-Aldrich) and incubated with primary antibodies overnight at 4 °C. For each variant, a negative control was performed. The cells were washed in PBS (Sigma-Aldrich), and the incubation with secondary antibodies was performed in the dark for 2 h. After the cells were washed again in PBS (Sigma- Aldrich), the nuclei were stained using Hoechst 33342 (Sigma-Aldrich) for 15 min. All the samples were analyzed with the LSM 780 confocal laser scanning system and ZEN software (Carl Zeiss). Quantitative analysis was performed as a relation of positive cells to a total cell number (50 cells per repetition). Each variant had 3 repetitions.

2.8. Developing a New Procedure of Immunocytochemical Staining for Better hNSCs Neurosphere (3D) Immunofluorescent Visualization

The next step was to develop a protocol for neurosphere staining (3D conditions) which would allow for the cell staining and visualization in the core of the neurosphere, thus enabling the analysis to be performed without the need for cryosection. The neurospheres were fixed in 4% PFA (Sigma-Aldrich) in PBS (Sigma-Aldrich) for 15 min at RT, then washed with 0.1% Triton X-100 (Sigma-Aldrich) in PBS (Sigma-Aldrich). The next step involved blocking non-specific bonds for at least 12 h using a blocking mixture (made with 6% BSA solution (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich) in PBS (Sigma-Aldrich) at RT. The neurospheres were washed with 0.1% Triton X-100 (Sigma-Aldrich) in PBS (Sigma-Aldrich) and 24 h incubation with primary antibodies (Table 2) was performed at 2–8 °C. After the 0.1% Triton X-100 (Sigma-Aldrich) wash in PBS (Sigma-Aldrich), 24 h incubation with secondary antibodies (Table 3) at RT was carried out in the dark. Next, the neurospheres were washed again with 0.1% Triton X-100 (Sigma-Aldrich) in PBS (Sigma-Aldrich) and stained with 1% Hoechst 33342 (Sigma-Aldrich) solution for 30 min to visualize the nuclei. After washing the neurospheres with 0.1% Triton X-100 (Sigma-Aldrich) in PBS (Sigma-Aldrich), 20 µL of the Triton/PBS solution with the stained neurospheres was transferred to the surface of the slide, drawing off the solution with due care so as not to pull the spheres away. A single drop of the mounting medium was applied and closing with a 14 mm-diameter coverslip was performed. The slides were left at RT for overnight drying.

Table 2. Primary antibodies used for the experiments.

Antibody	Catalog Number	Source	Isotype	Dilution	Manufacturer
anti-Nestin	SAB4200347	Rabbit polyclonal	IgG	1:500	Sigma-Aldrich
anti-NeuN	MAB377	Mouse monoclonal	IgM	1:50	Millipore
anti-NF200	N0142	Mouse monoclonal	IgG1	1:800	Sigma-Aldrich
anti-SOX2	SAB5700644	Rabbit polyclonal	IgG	1:150	Sigma-Aldrich

Table 3. Secondary antibodies used for the experiments.

Antibody	Fluorochrome	Catalog Number	Isotype	Dilution	Manufacturer
Alexa Fluor Goat (anti-rabbit)	Alexa 488	A11034	IgG	1:1000	Life Technologies
Alexa FluorGoat (anti-mouse)	Alexa 546	A21123	IgG1	1:1000	Life Technologies
Alexa FluorGoat (anti-mouse)	Alexa 546	A21045	IgM	1:1000	Life Technologies

2.9. Statistical Analysis

Raw data statistical analysis was performed with GraphPad Prism 7 software. The results are presented as mean and standard deviation. To conduct multi-group comparisons, a one-way analysis of variance (ANOVA) was used, followed by the Tukey test as a post hoc statistical analysis for each group. The values were considered significant at $p < 0.05$.

3. Results

3.1. The Effect of Direct Cryopreservation on Human and Rodent NSCs' Viability and Growth Potential

3.1.1. Intra-Species Variability

hNSCs which were cryopreserved directly after the isolation showed a reduced viability (higher LDH release in the first 24 h of culture) and growth potential after thawing than the cultivated ones (Figure 4c), regardless of the type of culture (2D or 3D). These cells needed a much longer timespan to form spheres or adhere to plastic than the cells cultivated directly after isolation. The defrosted hNSCs began to form neurospheres after 10–15 days, eventually reaching a diameter of 100–120 µm, as opposed to cultivated hNSC which needed 7–10 days to demonstrate the same effect (Figure 4a).

Despite the optimization of the thawing protocol, such as: omitted centrifugation of cells before their resuspension in the basic medium (to avoid possible mechanical damage to the cells), the first change of the medium performed after the second day of culture (to reduce stress after thawing), and a higher concentration of glutamine in the medium (to possibly increase their proliferative potential), it was not possible to obtain the sufficient number of cells to establish long-term cultivation and expansion (Figure 4b).

NSCs isolated from both mice and rats and immediately cryopreserved showed a significantly reduced viability after thawing, regardless of the type of culture (2D or 3D), which is consistent with the observations made on hNSC culture (Figure 4c) that needed approximately 7–10 days longer to reach the stage of development observed on Day 7 in freshly-isolated culture (Figure 4a).

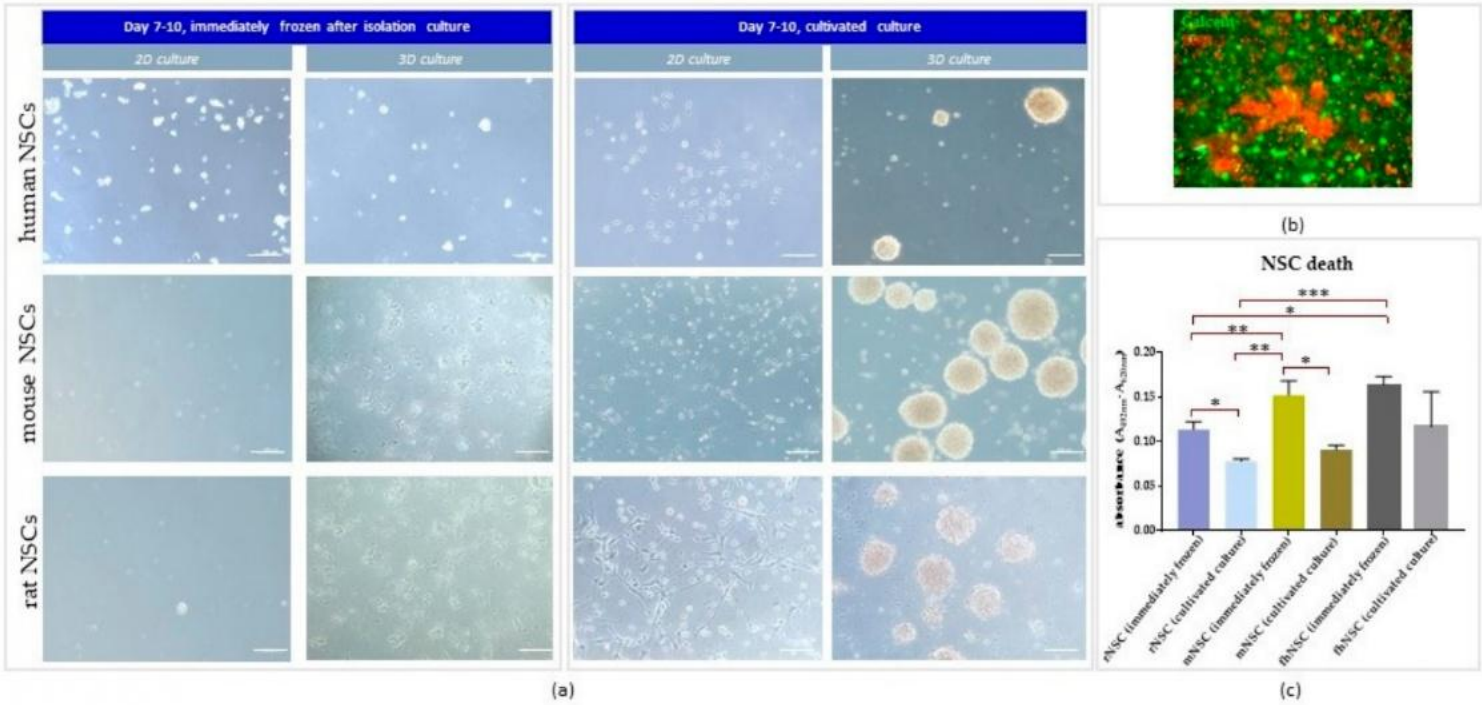


Figure 4. The effect of freshly isolated NSCs' cryopreservation on their viability and growth potential. (**a**) NSCs on days 7–10 of 2D and 3D culture after the isolation: NSCs frozen right after isolation (on the left) and NSCs after the standard cultivation procedure (on the right). (**b**) Immunofluorescent analysis of dead (stained with red Edth-1) and live (stained with green calcein) immediately frozen hNSCs. (**c**) Immediately frozen and cultivated NSCs' death depending on the species after 24 h of 3D culture. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C. N = 3. The results of all experiments presented above are expressed as mean values of three experiments ± SEM. The differences were considered statistically significant when the *p*-value < 0.05. Statistical significance level: * for $0.01 < p < 0.05$, ** for $0.001 < p < 0.01$, and *** for $0.0001 < p < 0.001$.

3.1.2. Inter-Species Variability

No differences between rNSCs and mNSCs in cell fate were observed with regard to the post-isolation protocol. Despite no statistically significant difference, the tendency observed in human NSCs seemed to be similar in rodent NSCs (Figure 4).

3.2. The Influence of the Medium Composition (Growth Factors, Glutamine) on the Viability, Proliferation, and Senescence of Human and Rodent NSCs

The viability, proliferation, and cell senescence were analyzed after 1, 3, and 7 days of cell culture using the following medium variants:

- **Full medium**—the medium with 20 ng/mL of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), which is a control for our experiments.
- **FGF**—standard medium, with bFGF as the only growth factor (without EGF).
- **EGF**—standard medium, with EGF as the only growth factor (without bFGF).
- **FGF20/EGF10**—standard medium, with 20 ng/mL of bFGF and 10 ng/mL of EGF.
- **-GFs**—standard medium without bFGF and EGF.
- **-GFs, Gln**—standard medium without bFGF, EGF, and glutamine.
- **-Gln**—standard medium without glutamine.

3.2.1. Intra-Species Variability in 2D

Only in the full medium was hNSC proliferation potential maintained at a constant level (117 ± 12%) (Figure 5). In each incomplete variant of the medium, the proliferation significantly decreased, especially in the cells cultured in the media without GFs, GFs and Gln (90 ± 10 %), and without glutamine (up to 59 ± 12%).

The medium composition did not influence hNSCs' viability in a significant manner (LDH test) (Figure 6a).

Next, the senescence in hNSCs was analyzed (Figure 6b) and no significant changes were observed between the presented culture variants.

mNSC culture was found less demanding and the cells were also able to proliferate in the incomplete medium (Figure 5). After 7 days, a significant decrease in proliferation was noted only for the cells cultured in the medium without growth factors and glutamine (81.5 ± 1%).

On Day 1 of culture, the most substantial changes in cell viability were seen for the -Gln culture medium variant. The cell viability was more elevated (9 ± 3% of total LDH release) than in the remaining variants, especially in comparison to the full medium (13 ± 1% of total LDH release), FGF (13 ± 1% of total LDH release), and the -GFs, Gln (15 ± 2% of total LDH release) variant (Figure 6a). However, the most significant differences in cell viability were seen after 7 days of culture—the lowest viability was observed in the glutamine-free medium (15 ± 4% of total LDH release) and in FGF medium (12 ± 0.1% of total LDH release).

The senescence observed in mNSCs did not significantly differ between the variants (Figure 6b).

After 7 days of culture, rNSCs' proliferation decreased in the medium without glutamine and without growth factors (78 ± 15%) (Figure 5).

The lowest viability was recorded for rNSCs cultured in the medium without glutamine, both after Day 1 (15 ± 4% of total LDH release) and Day 7 (30 ± 1% of total LDH release) of culture (Figure 6a). For both time points, low cell viability was observed for the FGF20/EGF10 variant (15 ± 8% and 9 ± 0.5% of total LDH release for Days 1 and 7, respectively) (Figure 6a).

Significantly lower cell senescence was observed in the EGF and -Gln media than in the FGF medium (Figure 6b).

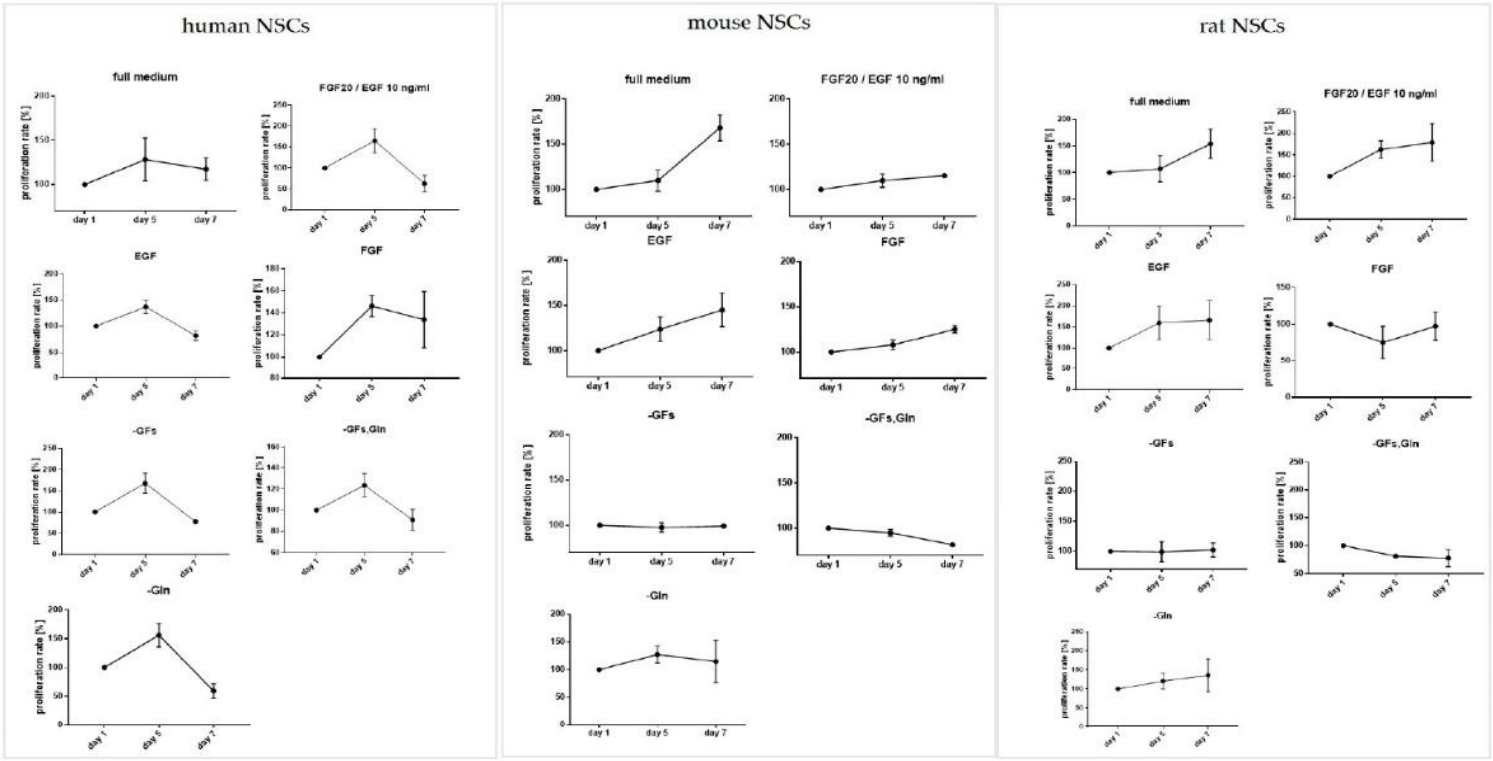


Figure 5. The influence of culture medium composition on the cell proliferation rate on Days 1, 5, and 7 in relation to the cells' origin. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C. N = 3. The results are presented as mean values of three experiments ± SEM.

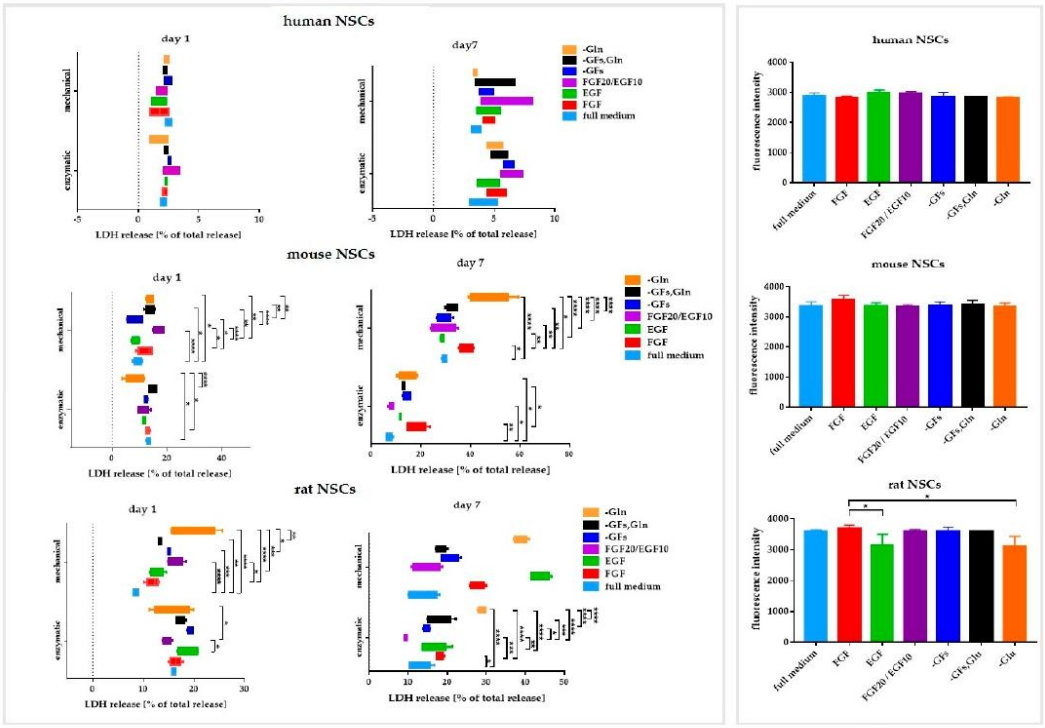


Figure 6. The influence of the medium composition on the viability and senescence of NSCs. (**a**) LDH release of NSCs on Day 1 and Day 7 of culture after enzymatic/mechanical dissociation. (**b**) Senescence activity of NSCs after Day 7 of culture. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C, N = 3. The results of all presented experiments are expressed as mean values of three experiments ± SEM. The differences were considered statistically significant when the *p*-value < 0.05. Statistical significance level: * for $0.01 < p < 0.05$, ** for $0.001 < p < 0.01$, *** for $0.0001 < p < 0.001$, and **** for $p < 0.0001$.

3.2.2. Inter-Species Variability in 2D

The proliferative potential of mNSCs was significantly greater in the media with EGF, FGF20/EGF10, and without glutamine in comparison to hNSCs and rNSCs (Figure 7a). The hNSC proliferation potential was the lowest in comparison to all tested species in most medium variants, except for the FGF medium and -GFs/Gln medium.

However, hNSC demonstrated the best viability regardless of the medium composition, whereas the lowest viability was observed in rNSC culture (Figure 7b). The cell death was positively correlated with the cell senescence for all NSC cultures.

The senescence activity was significantly elevated in all culture variants of rodent NSCs in comparison to hNSCs (Figure 7c).

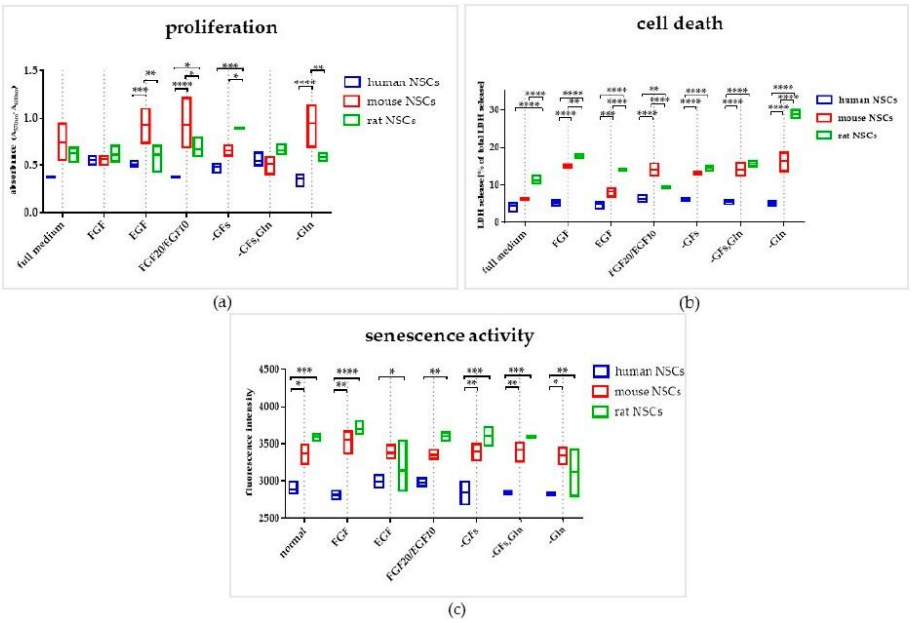


Figure 7. The influence of the medium composition on proliferation, viability, and senescence of 2D NSCs. (**a**) Proliferation of NSCs on Day 7 of culture. (**b**) Cell death of NSCs after Day 7 of culture. (**c**) Senescence activity of NSCs after Day 7 of culture. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C, n = 3. The results of all presented experiments are expressed as mean values of three experiments ± SEM. The differences were considered statistically significant when the *p*-value < 0.05. Statistical significance level: * for $0.01 < p < 0.05$, ** for $0.001 < p < 0.01$, *** for $0.0001 < p < 0.001$, and **** for $p < 0.0001$.

3.2.3. 3D Cells' Response to Medium Conditions

In 3D culture, hNSCs were observed to create neurospheres in all medium variants. Their growth was slower and the final diameter was smaller in the medium without FGF, without both growth factors, as well as without growth factors and glutamine (Figure 8 and Figure 13). This observation was shown to be repeated even more markedly with regard to both rodent cultures. A strong disability to form neurospheres cultured in a medium without growth factors and glutamine was recorded in both mNSCs and rNSCs. Furthermore, we noticed a reduced number of neurospheres formed by mNSCs treated with the medium without FGF. However, the impact of FGF absence was not as pronounced in rNSCs.

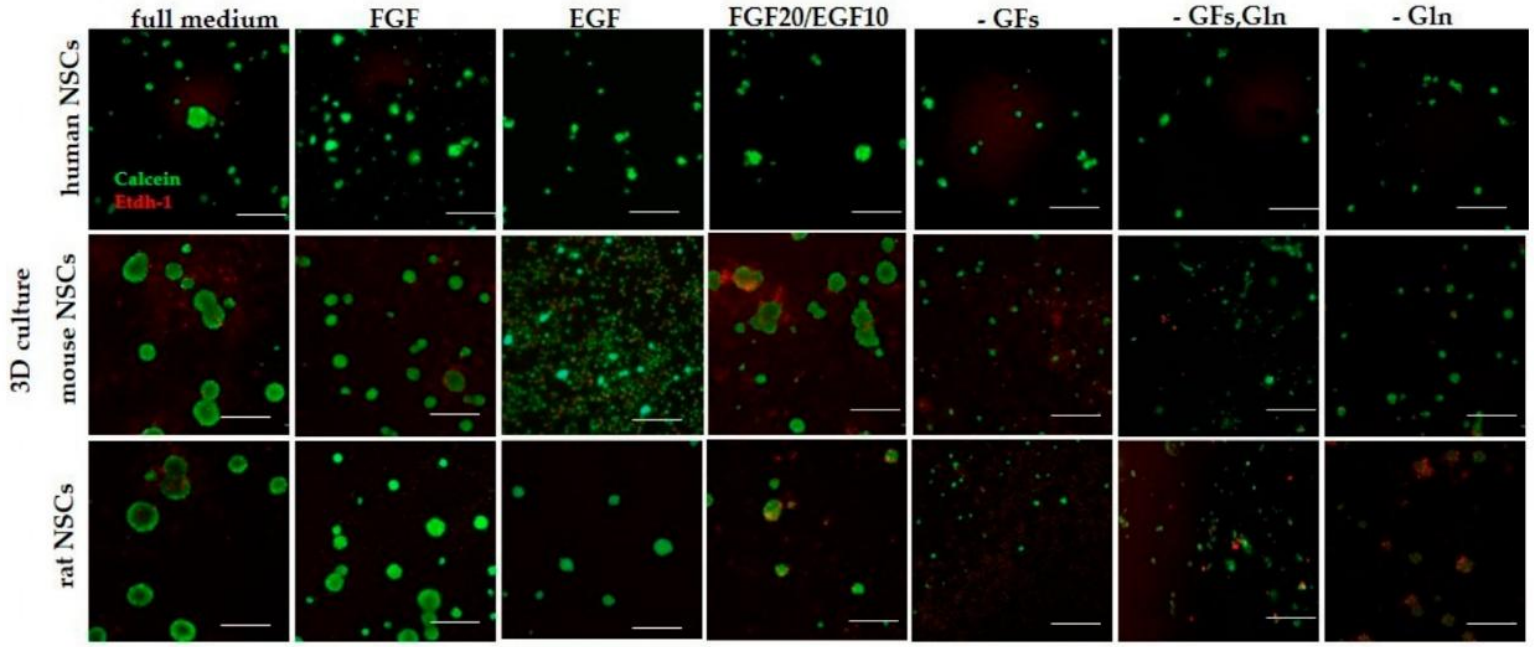


Figure 8. Fluorescence microscopic images of 3D NSCs cultured in different media and spatial conditions, on Day 7 of culture. Cells were stained with calcein (green) and EthD-1 (red) staining. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C. Scale: 100 μm.

3.3. Comparison of Dissociation Methods—Enzymatic vs. Mechanical

To expand the 3D culture, the neurospheres were dissociated after reaching approximately 120 µm in diameter. To analyze the impact of the dissociation method on each NSC culture, in the next step of this study, two methods of dissociation were compared: enzymatic, known as the most commonly used, *vs.* the mechanical one. The viability was checked with two techniques: the calcein/Etdh-1 test as the live/dead assay and the LDH test as an intact/impaired indicator (Figures 9a and 10a). First, we applied dissociation as the only factor in the standard culture medium (full medium) (Figures 9b and 10b). Next, we correlated the method of dissociation with all culture medium composition variants (Figure 6a).

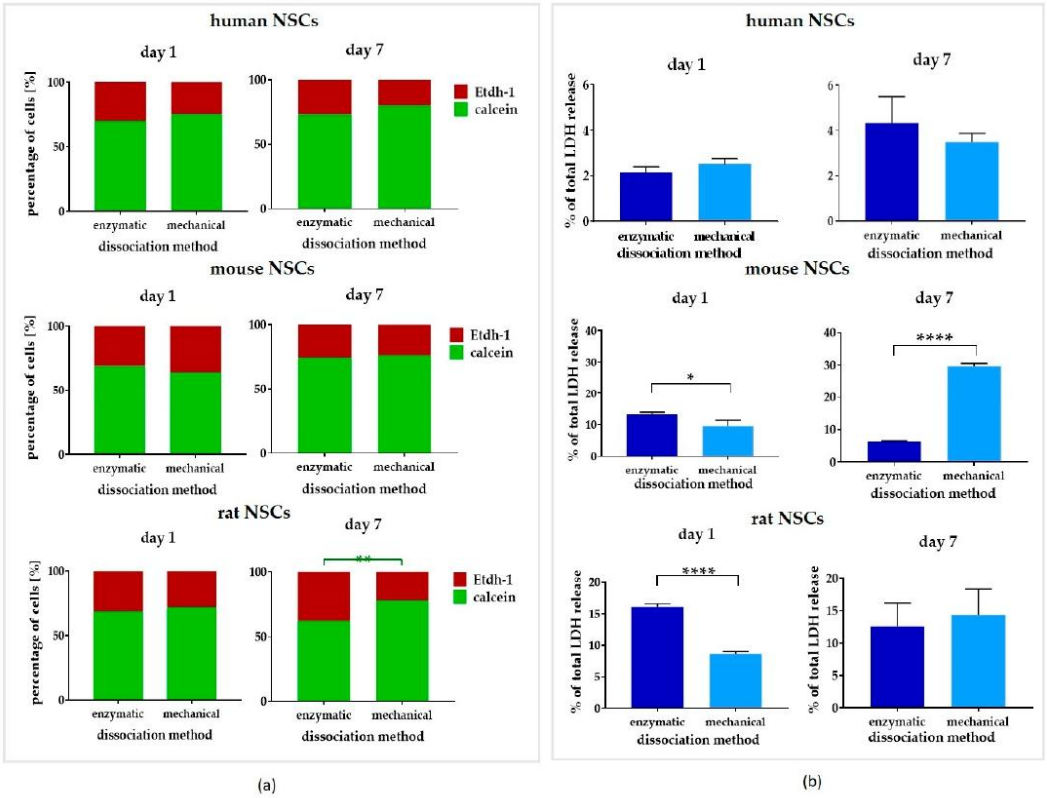


Figure 9. The influence of dissociation methods—enzymatic and mechanical—(**a**) on NSCs' calcein+ and Etdh-1+ cells' presence on Day 1 and Day 7 of culture, and (**b**) on NSCs' LDH release on culture Days 1 and 7. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C, N = 3. The results of all presented experiments are expressed as mean values of three experiments ± SEM. The differences were considered statistically significant when the *p*-value < 0.05. Statistical significance level: * for $0.01 < p < 0.05$, ** for $0.001 < p < 0.01$, and **** for $p < 0.0001$.

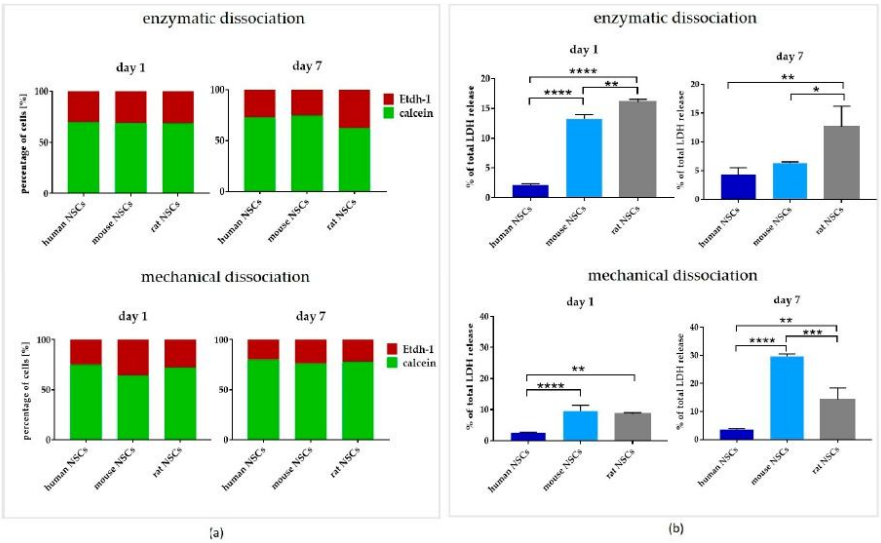


Figure 10. The influence of dissociation methods—enzymatic and mechanical—(**a**) on NSCs' calcein+ and Etdh-1+ cells' presence on Day 1 and Day 7 of culture, and (**b**) on NSCs' LDH release on culture Days 1 and 7. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C, n = 3. The results of all presented experiments are expressed as mean values of three experiments ± SEM. The differences were considered statistically significant when the *p*-value < 0.05. Statistical significance level: * for $0.01 < p < 0.05$, ** for $0.001 < p < 0.01$, *** for $0.0001 < p < 0.001$, and **** for $p < 0.0001$.

3.3.1. Intra-Species Variability

In the live/dead assay, the cells cultured in a full medium demonstrated a significantly lower number (around 62%) of live rNSCs which were dissociated with the enzymatic method (Figure 9). However, no other significant differences were recorded either for rNSCs or for hNSCs and mNSCs.

In the LDH test, no significant changes in cells' viability were observed in hNSCs when Day 1 and Day 7 of cultures were compared (Figure 9b).

The most remarkable differences were noted for mNSCs. On Day 1 of culture, LDH release was significantly more elevated in the cells dissociated enzymatically (13 ± 0.7% of total LDH release) than mechanically (9 ± 2% of total LDH release); however, the values changed dramatically after a week of culture, when the same cells' viability seemed to decrease (7 ± 0.3% of total LDH release) and was much lower in enzymatically dissociated cells than in mechanically dissociated ones (30 ± 1% of total LDH release).

On rNSCs' culture Day 1, we also observed a significantly elevated level of LDH release for enzymatically dissociated cells (7 ± 0.5%), however this effect was not visible after Day 7, and there were no remarkable differences between both presented dissociation methods.

When correlating different culture mediums with dissociation methods (Figure 6a), no significant changes were identified in hNSCs. However, in rodent cultures, the viability was found to significantly decrease after mechanical dissociation in the cells cultured in the glutamine-free medium (15 ± 4% and 30 ± 1% of total LDH release, respectively), and the effect was most pronounced on Day 7. Interestingly, 7 days after mechanical dissociation, mNSCs' viability significantly decreased in the FGF medium (29 ± 0.6% of total LDH release), while the same observation was made for rNSCs in the EGF medium (42 ± 1% of total LDH release).

3.3.2. Inter-Species Variability

There were no significant differences between the species in relation to the dissociation method and the day of culture recorded in the calcein/Ethd-1 test. However, dissociation method-dependent differences in LDH release during the culture were noted for all the analyzed species. The highest viability was noted for hNSCs regardless of the method and time of observation. The most significant differences were seen in enzymatic dissociation on Day 1 when the release of LDH was considerably more elevated in rodent NSCs than in hNSCs, and in rat more than in mouse cells. The relation was maintained after Day 7, however, the LDH release was less expressed. Conversely, a negative impact of mechanical dissociation was recorded most evidently in mNSCs.

3.4. hNSCs' Characteristics

To analyze the neural differentiation stage of NSCs, the presence of selected neural (Nestin and SOX2) and neuronal (NeuN and NF200) markers was assessed using immunofluorescence staining. The quantitative analysis was performed in the 2D cell culture (Figures 11 and 12). The IF-stained neurospheres treated with different culture media revealed their distinct impact on the presence of the aforementioned markers. Since a precise method of cell counting in the neurosphere is yet to be designed, we were only able to perform the qualitative analysis (Figure 13).

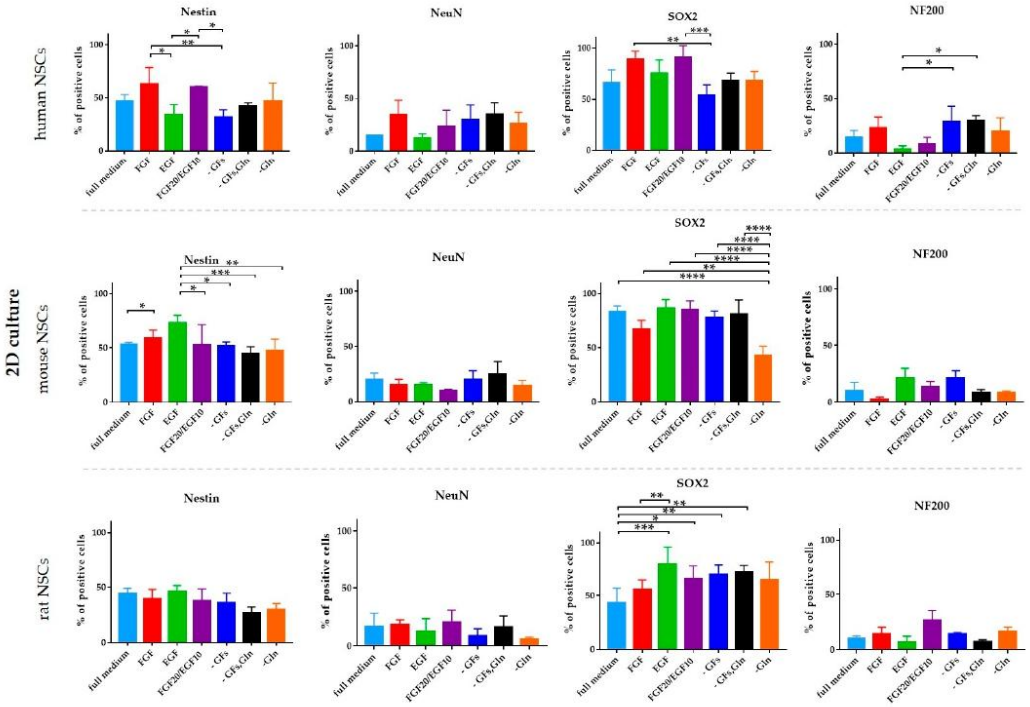


Figure 11. Neural differentiation of human, mouse, and rat NSCs cultured in 2D conditions. Quantitative analysis of neural (Nestin, SOX2) and neuronal (NeuN, NF200) markers. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C. The results are presented as mean values of three experiments ± SEM. The differences were considered statistically significant when the *p*-value < 0.05. Statistical significance level: * for 0.01 < *p* < 0.05, ** for 0.001 < *p* < 0.01, *** for 0.0001 < *p* < 0.001, and **** for *p* < 0.0001.

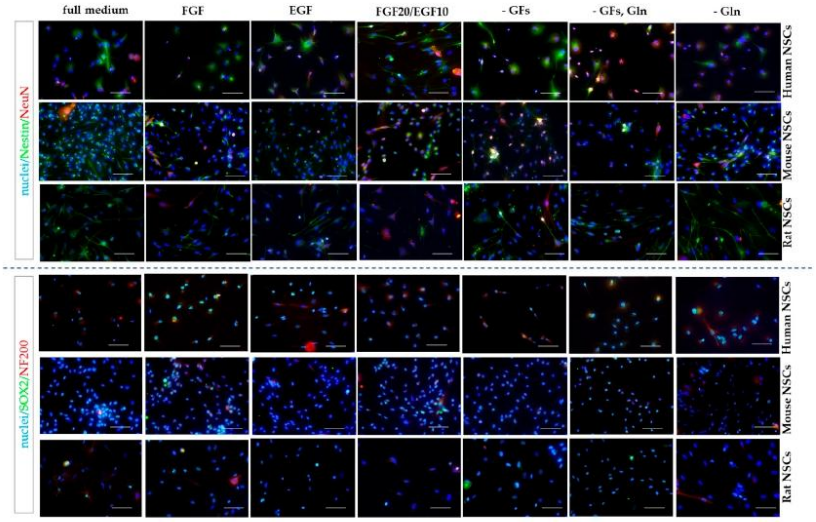


Figure 12. Neural differentiation of human, mouse, and rat NSCs in 2D conditions. Nestin, NeuN, SOX2, and NF200 immunofluorescence analysis. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C. Scale: 100 µm.

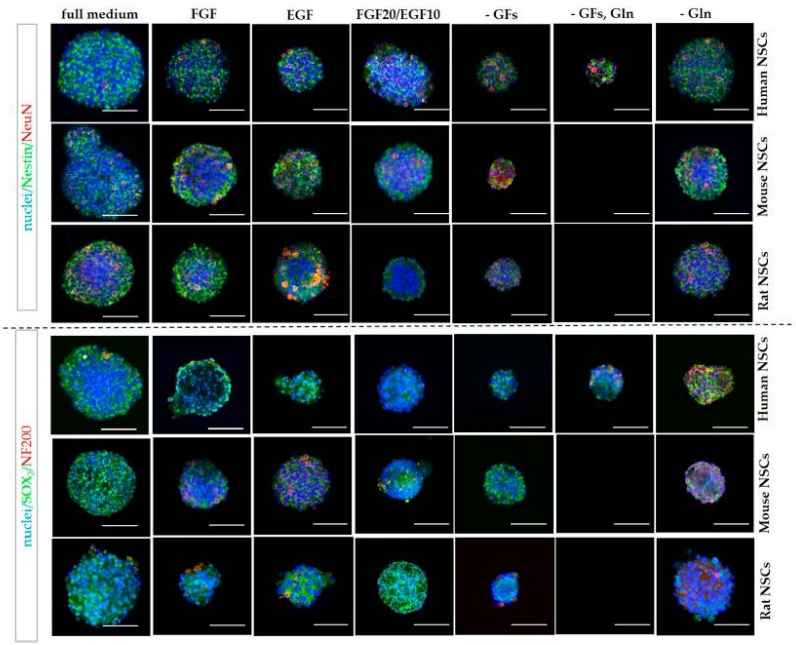


Figure 13. Neural differentiation of human, mouse, and rat NSCs in 3D conditions. Nestin, NeuN, SOX2, and NF200 immunofluorescence analysis. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C. Scale: 50 µm.

3.4.1. Intra-Species Variability

In 2D culture of human NSCs, the most significant changes in the expression of Nestin and SOX2 markers were observed for all medium variants (Figures 11 and 12). The highest expression of Nestin in NSCs was detected in hNSCs treated with FGF medium (62 ± 15%) and FGF10/EGF20 medium (60 ± 1%). The expression was significantly lower for the cells cultured in EGF (34 ± 10%) or without any growth factors (31 ± 7%). These results positively correlated with the expression of SOX2+ cells. The expression of neuronal markers was significantly lower in all tested variants, but the cells responded to the medium supplements in a similar pattern.

In the mNSCs' 2D culture, a significant difference in Nestin presence was recorded between mNSCs cultured in FGF (59 ± 7%) and FGF10/EGF20 (52 ± 18%) medium, the medium without GFs (51 ± 4%), the GFs and glutamine variant (45 ± 6%), and glutamine-free medium (47 ± 10%). The highest expression of Nestin was observed in EGF medium (73 ± 7%). The most significant changes were seen for SOX2 expression, which was the lowest in the glutamine-free medium (42 ± 8%).

We did not observe any significant differences in Nestin, NeuN, and NF200 expression in the rNSC monolayer culture. The highest expression of SOX2 was noted in the EGF-treated cell medium (79 ± 16%). Interestingly, a significantly lower level of that marker was seen in rNSCs cultured in the full medium (43% ± 14%).

3.4.2. Inter-Species Variability

The lowest expression of early neural markers was recorded in rNSCs. The oscillation in marker expression initiated by different supplements was mostly noted for SOX2 (Figure 14). This marker's presence was significantly lower in rNSCs cultured in full medium (43 ± 14%) than in hNSCs and mNSCs (respectively, 66 ± 12% and 83 ± 5%). Similarly, the lowest percentage of SOX2+ cells was observed in rNSCs in FGF-treated medium, where the percentage of these cells oscillated up to 55 ± 9%. Conversely, in glutamine-free medium, a significantly lower expression of SOX2+ was seen in mNSCs (43 ± 9%) than in hNSCs (68 ± 9%) and rNSCs (65 ± 16%). Moreover, the SOX2+ level was significantly lower in rNSCs' FGF20/EGF10 medium (65 ± 11%) than in hNSCs (91 ± 11%). The level of the medium without growth factors was more elevated in mNSCs (78 ± 6%) than in hNSCs (54 ± 10%). Moreover, clear differences in the presence of Nestin were identified between the species, with the most significant change being noted for the EGF medium variant. The highest percentage of positive cells was observed in mNSCs (73 ± 7%) and the value was significantly lower for both hNSCs (34 ± 10%) and rNSCs (46 ± 5%). A similar relation of Nestin's presence was observed in FGF, FGF20/EGF10, and -GFs variants when compared with the changes in the SOX2 level between the species. We did not notice any significant changes between the species with regard to the presence of NeuN and NF200.

3.5. The Assessment of Migration of NSCs Grown as Neurospheres and Transferred to 2D Conditions

To evaluate the functional properties of NSCs grown as neurospheres after changing the culture conditions into the monolayer, the neurospheres were transferred onto the coverslips coated with poly-L-lysine and laminin once they had reached the desired diameter (approximately 120–150 µm). After about 10–12 h, single cells began to migrate from the spheres. The migration of cells from the spheres was counted using the AxioVert A1 microscope, measuring the diameter of the field occupied by cells migrating from the spheres (Figure 15). Within the first 12 h of the start of the experiment, the cells were seen to adapt to the change in the spatial conditions, showing a high potential for migration. The migration rate changes during the culture were similar to those observed in human and mouse NSCs. The rate was the highest on Day 5 of culture, while in the rat NSCs the highest values were recorded on Day 3 (Figure 15b,c).

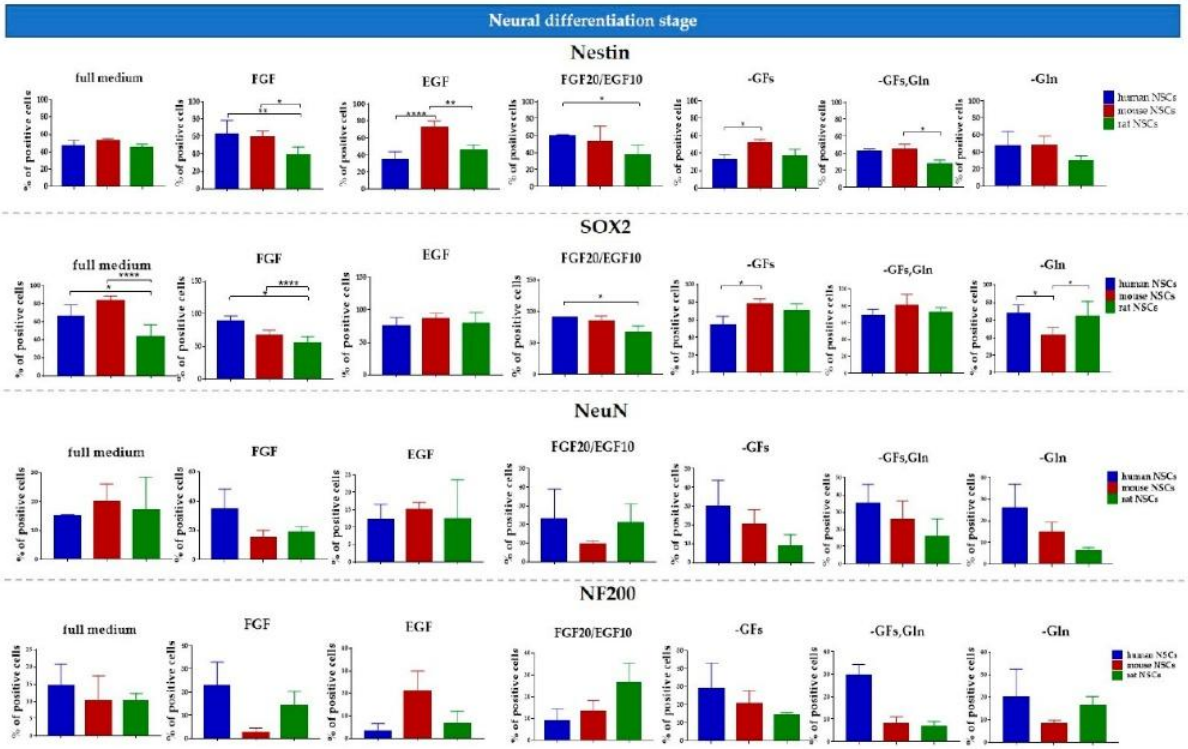


Figure 14. Neural differentiation of human NSCs in 2D culture. Quantitative analysis of neural (Nestin, SOX2) and neuronal (NeuN, NF200) markers. Cell culture conditions: 5% O_2, 5% CO_2, 37 °C; N = 3. The results are presented as mean values of three experiments ± SEM. The differences were considered statistically significant when the *p*-value < 0.05. Statistical significance level: * for $0.01 < p < 0.05$, ** for $0.001 < p < 0.01$ and **** for $p < 0.0001$.

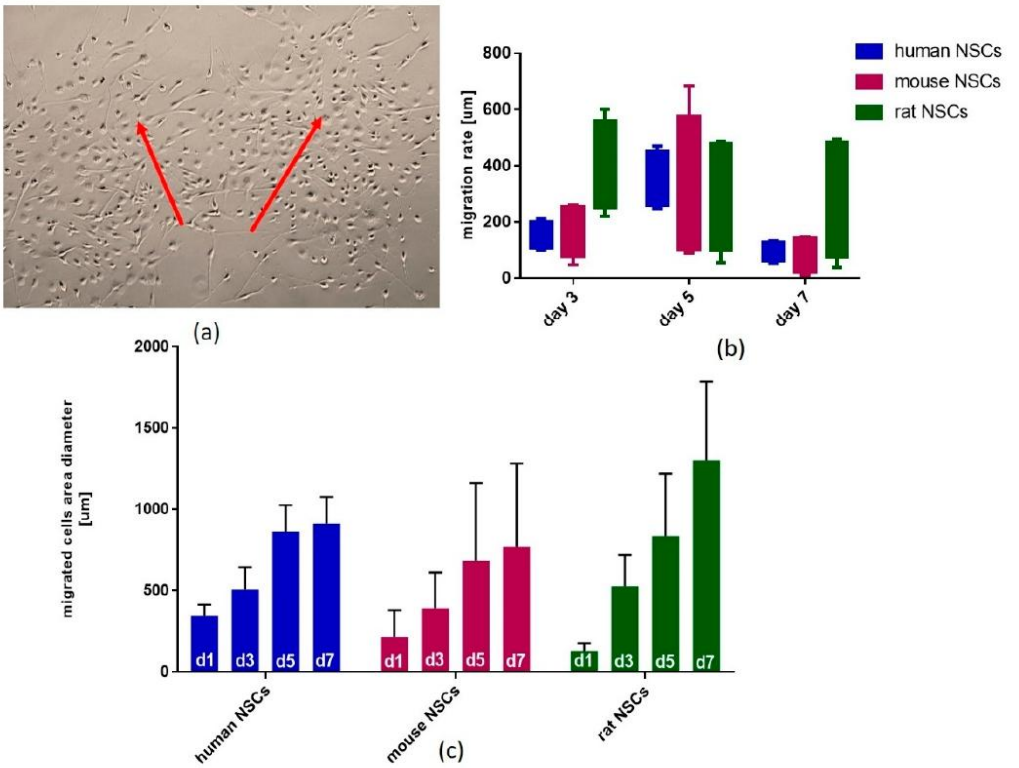


Figure 15. The migratory properties of the human, mouse, and rat NSCs. (**a**) Microscopic view of migrating human NSCs from 2 neurospheres (red arrows). (**b**) Migration rate of human, mouse, and rat NSCs on Days 3 (d3), 5 (d5), and 7 (d7) of culture. (**c**) Migrated cells' area on Days 1, 3, 5, and 7 (d1, d3, d5, and d7, respectively). Cell culture conditions: 5% O_2, 5% CO_2, 37 °C. N = 3.

4. Discussion

Current treatments of some neurological diseases are still unsatisfactory. Even though the self-repair process is induced by the endogenously activated quiescent NSCs immediately after the brain injury, in most cases, it still proves to be insufficient. Therefore, a plethora of studies have searched how to enhance endogenous neuroprotection and neurorestoration or how to apply exogenous NSC treatment [16]. Some *in vivo* research on rats has shown that exogenous hNSCs do not replace the injured tissue but they actually recreate the microenvironment which can induce endogenous neurogenesis [22,23]. The benefits of cell therapies have already been revealed, however, over the years, scientists have been grappling with the issue of results' interpretation as the reported outcomes seem to be laboratory-dependent and could have been affected at numerous levels, including a preclinical stage, for example, with regard to different species' origin, or intra-specifically, due to different methods of isolation, banking, cryopreservation, expansion, long-term culture, or differentiation.

4.1. The Effect of Direct Cryopreservation on Human and Rodent NSCs' Viability and Growth Potential

As mentioned above, the differences can be noticed in the variety of isolation protocols from the very beginning of the cell culture establishment. One of most important steps

in further cell banking involves the use of an appropriate freezing/vitrifaction procedure. A well-optimized cryopreservation protocol is a prelude to a stable, intact supply of a reproducible NSC population. Even though the currently used methods of NSCs' cryopreservation seem to be well-characterized, they still remain ambiguous. The protocols for cell freezing vary even intra-specifically, which can result in different cellular responses. Researchers are still striving to discover a better way to increase post-thawing cell survival. It has also been disputable whether shortening the isolation procedure by cell cryopreservation without previous culture cultivation could be as efficient as after standard protocol steps. Such an isolation method could be beneficial, especially in the establishment of primary cell cultures in which the isolation is performed fully/partially in hospital conditions. Nevertheless, our results confirmed the reports by Vescovi's group [19,21], as we can also conclude that to perform successful derivation of NSC culture (regardless of cell origin), the cells should be cultured immediately after the isolation. Despite several thawing procedure verifications, the survival of hNSCs which were freshly cryopreserved after the isolation still seemed to be insufficient to continue the culture. The same effect was observed in rodent NSCs. The rNSCs and mNSCs that were freshly cryopreserved after isolation were characterized by a significantly lower cell viability than the cultivated ones. Thus, we recommend previous cell cultivation prior to their cryopreservation, even though it entails an immediate tissue transfer to the laboratory and the staff's 24 h standby.

4.2. The Influence of the Medium Composition (Growth Factors, Glutamine) on the Viability, Proliferation, and Senescence of Human and Rodent NSCs

Nowadays, the establishment of physiological niche-like conditions seems to be the main goal in preclinical research as they are one of the major components is culture medium composition. An unquestionable significance of two main mitogens—bFGF and EGF—has been reported throughout the last 30 years, particularly in regard to NSC culture maintenance [24]. First and foremost, the mitogens have been proven to promote neurosphere formation [25]. However, some attention should be paid while interpreting their function individually or when comparing them with different species, as some studies have reported that they produce various effects depending on the type of culture (2D *vs.* 3D). Although neurosphere culture seems to be easier to perform, it has some limitations that can result in alterations of neurosphere frequency, which depends on many factors, e.g., medium composition, isolation method, dissociation process, or even the density of cultured cells [26–29]. We observed that the culture in EGF medium (without bFGF) decreased neurosphere formation, in mNSCs in particular, while in 2D culture this medium variant seemed to even strengthen their proliferation potential and maintained early neural markers' presence (SOX2 and Nestin) in comparison to hNSCs and rNSCs. Reportedly, EGF induces cell division and increases the *Notch-1* intracellular domain level in neural progenitor cells, which was shown to be involved in the promotion of NSC survival and self-renewal in CNS development [24,30,31]. Moreover, embryonic mouse NSCs were shown to respond only to bFGF, while late embryonic and adult NSCs were found to be responsive to both bFGF and EGF [24,32–34]. Although our NSCs were not isolated in the early embryonic gestation stage, we observed slightly limited responsiveness to EGF in 3D culture. In addition, apart from the formed neurospheres, a monolayer of cells was observed in this variant and that could suggest a mix of EGF- and FGF-responsive cell populations in our culture. It is a combination of both bFGF and EGF that is most commonly used in culturing adult mouse subventricular zone cells, embryonic rat, and fetal human CNS cells; thus, it is difficult to compare such data with other groups [35]. On the other hand, limited formation of neurospheres was not seen in all our species in cell cultures whose medium lacked EGF. Thus, bFGF seemed to maintain this parameter for all presented species. It was previously shown that bFGF could promote the acquisition of EGF responsiveness in mNSCs [36]. However, the modulation of GFs concentration causes another difference to appear when compared to the most commonly used medium. We observed that the use of the FGF20/EGF10 variant of the medium produced a beneficial effect on cell proliferation, particularly in rodent NSCs.

Such a concentration allowed NSCs of all origins to form neurospheres whose diameters were close to those of the full medium-treated NSCs. Moreover, this variant maintained one of the highest percentages of cells that were SOX2-positive in comparison to the full medium-treated groups, which was clearly visible in our rNSC culture. Moreover, another study reported that when EGF and bFGF were removed, despite the induced differentiation, stronger expression of cell survival-promoting genes—*igf-1* and *pdgfb*—was observed in mouse neural precursor cells, which could result in more effective engraftment of such cells with the host tissue [24]. Our 2D culture results showed a decreased number of neural marker Nestin+ cells after removing both GFs from all species, which was also previously reported in the available literature [24]. This could potentially be associated with their further differentiation; however, it was not subsequently investigated by us as it was not the subject matter of our study. We also confirmed the negative impact of both GFs removal on neurosphere formation as well as inhibition of proliferation potential for all species [25]. However, a much stronger reduction in forming neurospheres was seen for cultures cultivated in the -GFs, Gln medium. This variant was found to result in limited proliferation in all the species. Glutamine is known as an important source of energy [37], however, in many NSCs studies, researchers omit to add it to the medium or fail to mention it in the materials and methods section. This, in turn, could lead to various consequences regarding NSCs, which are yet to be clarified. It has been observed that withdrawal of glutamine in hair follicle stem cells could even maintain specialized stem cell niches via TORC-Akt signaling [38]. By regulating mTOR activity, translation, and autophagy, glutamine also coordinates the proliferation and growth of tumor stem cells, which could explain why the -GFs, Gln medium variant was observed here to be stronger than the medium without the GFs proliferation rate inhibition only [39]. Moreover, by affecting the aspartate-malate shuttle, glutamine can increase the NADPH/NADP(+) ratio and suppress oxidative stress as a result [40]. Therefore, it can also affect cell viability, which was detected particularly in our study on rodent cultures. The critical importance of glutamine metabolism for NSC maintenance was reported in the study on FoxO3 signaling [41]. The study demonstrated that the impaired glucose and glutamine metabolism compromised the proliferative potential of NPCs. Furthermore, regarding the NSCs' differentiation stage, we observed that the -GFs, Gln medium variant led to a significantly higher percentage of Nestin+ mNSCs than rNSCs, but no differences in SOX2 were identified, which may suggest the impact of GFs and glutamine on further neural differentiation of mNSCs. We should also emphasize the drastic decrease which we observed in SOX2+ cells' presence in mNSC culture in the medium deprived of glutamine only. Glutamine's critical importance for the self-renewal and undifferentiated status maintenance of cells has already been demonstrated in mouse embryonic stem cells [42]. Thus, its loss could affect the expression of SOX2, which is known as a transcription factor that controls NSC long-term self-renewal and proliferation as well [43]. Our observation was followed by high-senescence formation and LDH release of mNSCs, which suggests the important role that glutamine played, especially in mouse-origin NSCs' fate. Interestingly, glutamine significantly reduced the proliferation rate but did not remarkably affect the viability and senescence or the differentiation stage in our study's hNSCs. Furthermore, the limitation of proliferation was stronger in this variant than in hNSCs cultured in a medium without both GFs and Glu, which indicates a possible interaction between glutamine activity and the presence of growth factors/supplements. However, this effect was not observed in our study's rNSCs and mNSCs. This also suggests a different metabolic response in each species and should be further studied using more specialized tests. To sum up, the composition of the culture medium, and the presence of glutamine and growth factors in particular, is species-dependent, and it proves important for the cell proliferation, senescence, and viability.

4.3. The Influence of Dissociation Methods (Enzymatic and Mechanical) on the Viability, Proliferation, and Senescence of Human and Rodent NSCs

Another culture parameter indicated in the relevant literature includes the method of neurosphere dissociation. The enzymatic method has been established as a golden standard nowadays, as it provides better cell viability [44,45]. However, the most commonly used enzymes—accutase or trypsin—can impact cell viability to a different degree. In some studies, trypsin was demonstrated to induce cell membrane damage and cell death, while accutase seemed to increase cells' survival, growth rate, and their viability [44,46,47]. In many studies, the enzymatic dissociation method was recommended to be applied in rodent and human NSC culture [44,48]. Nevertheless, benefits of the mechanical method of dissociation were also reported [49,50]. Even though this procedure seems to be quite aggressive, it was reported to provide a higher expansion rate in hNSCs when compared to the enzymatic method [51]. As the studies on the impact of mechanical dissociation on NSC fate are scarce, we decided to analyze basic parameters—the number of live cells and the LDH release. The assessment of the LDH release after 7 days of culture confirmed better cell viability in the enzymatically dissociated cells, especially in mNSCs. These data, however, differed from our results obtained after Day 1 of culture in mNSCs as well as in rNSCs, where the LDH release was significantly elevated after enzymatic dissociation, which suggests that although directly after 24 h of this procedure, the enzymatic method could affect the cells more drastically, the cell recovery after 7 days of culture is significantly higher than after the mechanical procedure. This also shows the importance of the day of the analysis performance. Moreover, the results appeared to be species-dependent. We observed the highest LDH release in rNSCs after enzymatic dissociation, however, the highest release was noted for mNSCs after the mechanical method. After the analysis was performed, yet another question arose: is it reliable to analyze the parameters individually? For example, no considerable differences in the number of calcein+ cells between the species were observed in response to different dissociation methods, which could suggest that the viability of cells is the same for both presented methods. However, we did observe statistically significant changes in LDH release, which may suggest more substantial cell membrane damage in some of the culture variants. We believe that the obtained results should be followed by further, more specified proliferation/senescence analyses, which will be our further issue of interest. Nevertheless, the dissociation method selection already seems to be species-dependent and to have a key impact on the parameters achieved in long-term culture.

4.4. The Influence of Spatial Conditions (3D and 2D Culture) on the Viability, Proliferation, and Senescence of Human and Rodent NSCs

NSCs can be cultured either as a monolayer (2D) or in a suspension (3D). Both methods have already shown several advantages and disadvantages, which should be analyzed with regard to the future NSCs' application. The 2D culture has been shown to provide conditions that allow for a more homogeneous population of NSCs, which also proliferate faster [19,20]. However, it does not adequately recreate the microenvironment of physiological 3D niche and cell-to-cell interactions; thus, it can lead to misleading results of *in vivo* responses [24]. Moreover, the growth potential and self-renewal of NCSs seem to be higher in 3D culture [52]. Thus, transplantation of NSCs as neurospheres to a damaged CNS seems to produce multiple benefits.

5. Conclusions

To summarize, we outlined the critical importance of the NSCs' culture conditions' optimization, and the following points seem to be of particular significance:

- Direct cultivation of NSCs before cryopreservation.
- Proper concentration of growth factors (bFGF and EGF) in the medium, which we estimated at 20 ng/mL for both bFGF and EGF.
- Presence of glutamine in the medium.

- Enzymatic method of neurosphere dissociation.

This would also simplify the comparison of the obtained results with a larger number of the available studies. We also showed that the migratory potential of each species changes over time, which could be useful while interpreting experiments *in vivo*.

It should be emphasized that possible reasons for the intra-species differences in proliferation, senescence, LDH activity, or the differentiation stage could be associated with the method, region, and time of NSC isolation. So far, NSCs have been derived from the dental gyrus of the hippocampi, the SVZ region, the olfactory bulb, the subcallosal zone underlying the corpus callosum, as well as the spinal cord of the embryonic, newborn, and adult rodent CNS. They have also been derived from developing and adult human brains [20,53,54]. Their further expansion *in vitro* is possible thanks to the use of mitogens [24,55] and propagating genes [56,57]. The NSCs isolated from embryonic, fetal, and adult brains are known to show different characteristics; moreover, they differ between rodent and human origin. However, the comparison of the three species analyzed here has only been scarcely discussed in the available literature so far. What appears to be a crucial step after NSC isolation is the verification of their stemness properties, including proliferation ability, self-renewal capacity, functional stability, and multipotentiality [53,58].

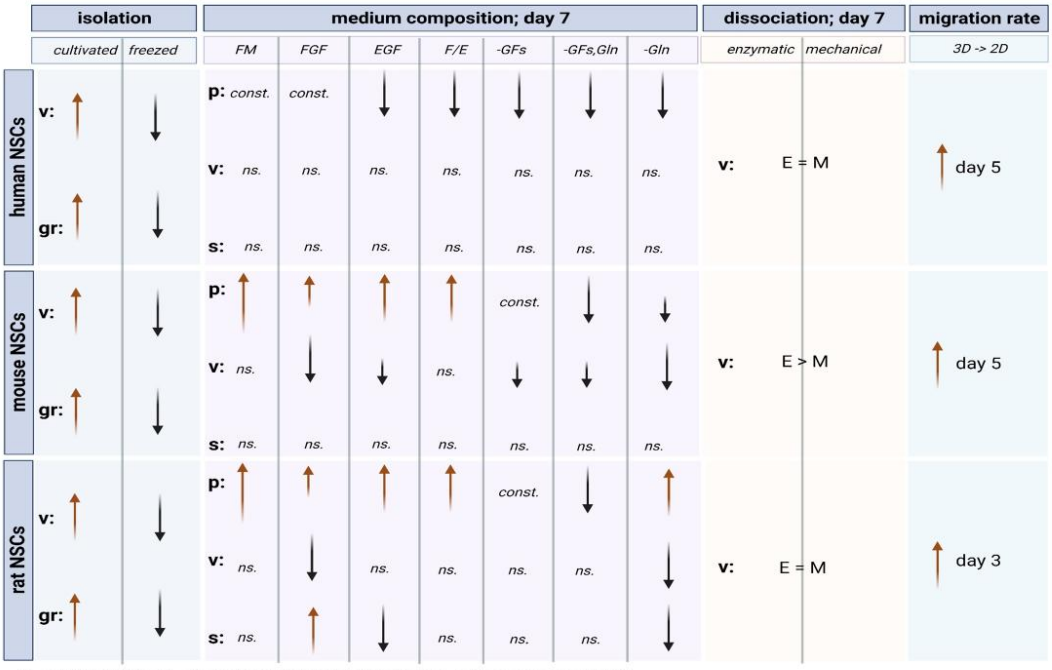
Moreover, with no available data on the medium composition's impact on human NSCs, we should not be directly inspired by the experimental results obtained on rodent NSCs as there is a multitude of inter-species differences. Although the hNSC proliferation rate was lower in comparison to rodent NSCs, they displayed better cell viability and decreased senescence (Figure 16). Moreover, hNSCs were shown to require a complete medium to maintain their proliferative abilities, while rodent NSCs appeared less demanding and responded to the lack of both growth factors and glutamine to decrease proliferation (Figure 17). According to our results obtained in the most commonly used medium, regarding neurosphere formation potential and the neural differentiation stage, more similarities were observed between hNSCs and rNSCs than between hNSCs and mNSCs; however, hNSCs were more similar to mNSCs with regard to senescence, cell viability, and migratory potential. Another issue regarding further studies using an *in vivo* model is that we should keep in mind the plethora of developmental and functional differences between present neurotraumatic and neurodegenerative animal models. Various expansions of the neocortex, neuronal subtypes, and human-specific aspects of gene regulation and expression affect the abilities of these models to recapitulate the human brain and the pathological mechanisms of neurological disorders, especially those influencing intellectual diseases [59].

medium composition; day 7						
	FM	*FGF*	*EGF*	*F/E*	*-GFs*	*-GFs,Gln*	*-Gln*
p:	m>r>h	r>h=m	m>r>h	m>r>h	r>m>h	r>h>m	m>r>h
v:	r<m<h	r<m<h	r<m<h	m<r<h	r<m<h	r<m<h	r<m<h
s:	r>m>h	r>m>h	r>m>h	m>r>h	r>m>h	r>m>h	r>m>h

isolation		dissociation; day 7		migration rate
cultivated	*freezed*	*enzymatic*	*mechanical*	*3D -> 2D*
v: h=m=r	h=m=r	**v:** h<m<r	h<m>r	h=m>r
gr: h=m=r	h=m=r			

h- hNSCs; m- mNSCs; r- rNSCs; v- cell viability; gr- growth rate; p-proliferation; s-senescence activity

Figure 16. The summary of selected inter-species variability in different culture conditions.



v- cell viability; gr- growth rate; p-proliferation; s-senescence activity

Figure 17. The summary of selected intra-species variability in different culture conditions.

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Abbreviations

NSCs	neural stem cells
hNSCs	human neural stem cells
rNSCs	rat neural stem cells
mNSCs	mouse neural stem cells
LDH	lactate dehydrogenase
HBSS	Hanks' Balanced Salt Solution
DMSO	dimethyl sulfoxide
CNS	central nervous system
bFGF	basic fibroblast growth factor
EGF	epidermal growth factor
NGF	nerve growth factor
BDNF	brain-derived neurotrophic factor
VEGF	vascular endothelial growth factor
IGF	insulin-like growth factor

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7.2.3. Article III

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EDITED BY
Paola Tognini,
University of Pisa, Italy

REVIEWED BY
Mohammad Saied Salehi,
Shiraz University of Medical Sciences, Iran
Pavla Jendelova,
Institute of Experimental Medicine (ASCR), Czechia
Yang (Ted) D. Teng,
Harvard Medical School, United States

*CORRESPONDENCE
Anna Sarnowska
asarnowska@imdik.pan.pl

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Deciphering the impact of cerebrospinal fluid on stem cell fate as a new mechanism to enhance clinical therapy development

Klaudia Radoszkiewicz, Aleksandra Bzinkowska, Magdalena Chodkowska, Paulina Rybkowska, Monika Sypecka, Ilona Zembrzuska-Kaska and Anna Sarnowska*

Translational Platform for Regenerative Medicine, Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

Neural stem cells (NSCs) hold a very significant promise as candidates for cell therapy due to their robust neuroprotective and regenerative properties. Preclinical studies using NSCs have shown enough encouraging results to perform deeper investigations into more potential clinical applications. Nevertheless, our knowledge regarding neurogenesis and its underlying mechanisms remains incomplete. To understand them better, it seems necessary to characterize all components of neural stem cell niche and discover their role in physiology and pathology. Using NSCs *in vivo* brings challenges including limited cell survival and still inadequate integration within host tissue. Identifying overlooked factors that might influence these outcomes becomes pivotal. In this review, we take a deeper examination of the influence of a fundamental element that is present in the brain, the cerebrospinal fluid (CSF), which still remains relatively unexplored. Its role in neurogenesis could be instrumental to help find novel therapeutic solutions for neurological disorders, eventually advancing our knowledge on central nervous system (CNS) regeneration and repair.

KEYWORDS

cerebrospinal fluid, artificial cerebrospinal fluid, embryonic cerebrospinal fluid, adult cerebrospinal fluid, neural stem cell, cell therapy, neurological disorders

1 Neural stem cells for treating neurological disorders – current challenges

Neural stem cells (NSCs) are known as the perfect candidates for cell therapy because of their rich neuroprotective and pro-regenerative properties. The promising outcome of neural stem cell-based preclinical studies in neurological diseases encourages researchers to further investigate their properties and responses, and move them to the clinical level. However, our knowledge regarding neurogenesis and the mechanisms accompanying this process is still limited. Due to the complexity of the central nervous system (CNS), neurological disorders still lack effective clinical outcomes, thus, there is a need to find a more successful therapeutic approach and target the factors which are involved in their pathogenesis. Currently, these cells cannot only be isolated from different regions of the fetal or adult brain and spinal cord but

can also be generated from other cell types, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and reprogrammed somatic cells (induced neural stem cells-iNSCs). In addition, neural-like cells can be obtained from mesenchymal stem/stromal cells (MSCs) (Tang et al., 2017; Willis et al., 2020). Such a wide variety of cell sources, by enriching the availability of NSCs, seems to provide safer and easier ways for preparing *in vitro* models of neurological diseases or using them for *in vivo* treatment but is their effect/response the same as for the native NSCs? What conditions would be able to mimic the natural brain environment? Despite several pros of NSC application *in vivo*, researchers are aware of many cons, including poor cell survival, poor integration into the host tissue as well as uncontrolled differentiation (Mothe et al., 2013; Wu et al., 2018). We wondered if there could be any particular factor that could have been overlooked. Although NSCs and neural niche components, including various cell types and matrix factors, are being extensively studied, there is limited data pertaining to the impact of the fundamental fluid on NSC which is present in the brain. Thus, here, we want to sum up our current knowledge in that aspect. It has been suggested previously that cerebrospinal fluid (CSF) plays a vital role not only in brain development, but also in neuroectodermal stem cells' survival, proliferation, and differentiation processes, thus, we find it a perfect model to study NSC behavior. In this review, we analyze the current knowledge on CSF's influence on stem cell fate *in vitro*, with the hope of finding out what still needs to be further investigated in order to better understand the processes that occur in the brain.

2 Cerebrospinal fluid – a conduit to further improve NSC therapy development

Cerebrospinal fluid (CSF) is a colorless liquid produced by the filtration of blood in the choroid plexus (ChP), which is an epithelial barrier that prevents free entry of toxic molecules or drugs in the circulation from reaching the brain. Together with the blood–brain barrier (BBB), the blood-spinal cord barrier (BSCB), and the blood-CSF barrier (B-CSF-B) make up the CNS barrier (Stoop et al., 2010). Humans produce approximately 500 mL of CSF a day and the total volume of CSF at a given time is usually about 150 mL (Spector et al., 2015). To obtain human CSF, the lumbar atraumatic needle is usually placed at the level of L3-L4 vertebrae, so that the introducing needle enters below the level at which the spinal cord ended. 10 to 15 mL of cerebrospinal fluid is collected into the polystyrene probes, centrifuged (2000 *g*, 10 min, RT) and biobank in-80C for further analysis (Liu and Duff, 2008). The methods used in animal models differ depending on the available instruments, animal model, and study group. Most often the animal is placed in the head-first prone position with the head remaining lowered due to approximation of the nasal clamp of the stereotactic frame at the head, just above the eyes. The hair around the cavity of the cisterna is shaved. Then the gingival needle connected to the microsyringe by a very thin and flexible tube or Hamilton syringe with needle or capillary is used. Once the dura over the cisterna magna is exposed, a needle or capillary is used to gently pierce it. Then CSF in amount of 40–70 µL is collected and frozen (Pegg et al., 2010; Lim et al., 2018).

2.1 CSF composition

Circulating CSF ensures homeostasis in the brain – it maintains metabolic clearance and also protects the CNS from mechanical shocks. The composition of CSF has been deeply investigated over the years. CSF contains ions including Na^+, Cl^-, $HCO3^-$, K^+, Ca^{++}, Mg^{++}, Mn^{++}; vitamins (e.g., Vitamin C, thiamine monophosphate, pyridoxal phosphate) and a great number of different proteins (Schilde et al., 2018). It is assumed that 80% of these proteins originate from blood and that the remaining 20% is released from the neural tissue. CSF of healthy people contains less than five cells per µL (Olsson et al., 2016). Due to its close proximity to the brain, CSF plays a crucial role when it comes to the diagnosis of various neurological disorders – the composition of CSF reflects biological processes that take place in the CNS. For example, Lepennetier and co-workers observed increased levels of multiple cytokines in CSF obtained from patients with neuroinflammatory diseases when compared to patients with non-inflammatory neurological diseases (Lepennetier et al., 2019). Thus, many studies concentrate on finding biomarkers characteristic of particular disorders that can be found in circulating CSF (Figure 1) (Olsson et al., 2016). Analyzing single-cell transcriptomics in CSF has revealed immune responses across a spectrum of neurological disorders, from inflammatory and degenerative to infectious and oncological CNS conditions. Despite the current lack of large CSF datasets, establishing a robust reference atlas demands collaborative efforts among multiple centers. Essential steps include optimizing CSF cell preservation, integrating existing datasets, and ensuring the resulting annotated datasets are publicly accessible, featuring interactive visualization (Heming et al., 2022). Relatively recent scientific evidence points to the role of CSF in regulating the sleep–wake cycle through its effect on prostaglandin synthesis, specifically prostaglandin D2 (Hayaishi, 2000). Moreover, different cells throughout the body, including cells making up the central nervous system (CNS), secrete extracellular vesicles (EVs). EVs are involved in intercellular communication via the transfer of numerous membrane receptors, proteins, lipids, RNA, and miRNA between neighbor and more distant cells (Jarmalavičiūtė and Pivoriūnas, 2016). EVs are secreted by neurons and glial cells into the CSF which remains in direct contact with the CNS. EVs play a critical role in bidirectional crosstalk between the CNS and the periphery, as they are able to cross the blood–brain barrier (BBB) (Honorato-Mauer et al., 2023; Kong et al., 2023). As EVs can cross the BBB and later circulate in the CSF, their contents can serve as biomarkers of the pathological processes that might be happening in the brain. One of the most promising biomarkers of different neurological disorders is miRNAs, which have been identified as the cargo of EVs (Mori et al., 2019; Šalamon Arčan et al., 2023).

Though CSF is an important diagnostic tool, its components and their role are one of the most under-explored areas of neuroscience. Previously, CSF was considered to be a fluid with basic physiological and mechanical functions. Nowadays, studies show that CSF plays a critical role in complicated brain physiology, especially during development, modulating the functions of neural stem cells (NSC) (Wichmann et al., 2022) and brain restoration.

2.2 Embryonic CSF (eCSF) vs. adult CSF (aCSF)

Depending on the stage of development, CSF changes its composition and function. Embryonic CSF (eCSF) produced during

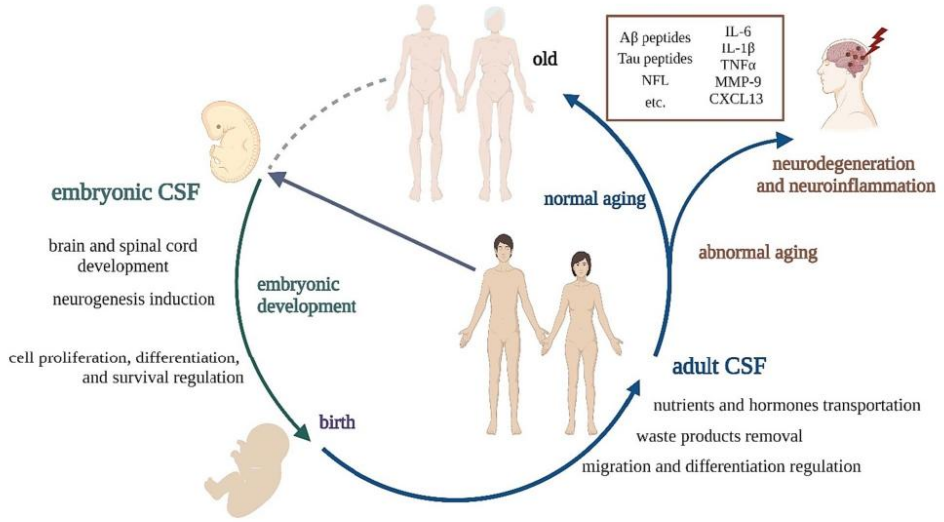

FIGURE 1
The scheme presenting the role of CSF at particular life stages.

embryogenesis is essential for proper development of the brain and spinal cord. It contains growth factors, cytokines, and other signaling molecules that regulate cell proliferation, differentiation, and survival in the developing nervous system. eCSF is also important for regulating the size and shape of the developing brain ventricles (Gato et al., 2014; Bueno and Garcia-Fernàndez, 2016; Chang et al., 2016).

The eCSF can support the fate of NSCs at any stage of their development, including the expansion of the undifferentiated NSC population or by inducing neuronal differentiation, migration, and final neuronal maturation (Alonso et al., 2017). Direct contact between eCSF and NSCs is necessary for their survival, replication, and neural differentiation and is considered a major source of signals in NSCs' niche regulation (Gato et al., 2005). Nevertheless, these inducive properties change suddenly during adulthood, where CSF has been described as NSCs' 'migratory guidance.' aCSF has mitogenic properties but has lost its ability to induce neurogenesis. Some studies have associated ontogenic changes in the composition of eCSF and aCSF with a dramatic reduction of the neurogenic potential of the adult brain (Alonso and Gato, 2018).

Due to the existence of technical and ethical constraints, no studies of eCSF have been conducted in humans to date. As it is crucial to learn more about the impact of eCSF on NSCs' neurogenesis, this matter might be studied with the use of animal models, especially mammals. This may pave the way for the preparation of art-CSF that could reflect the neurogenic potential of native CSF and therefore unlock the possibility of enhancing neuroregeneration in humans.

On the other hand, Pellegrini and her group created vascular plexus (ChP) organoids that reproduced key morphological and functional features of the human vascular plexus. This model allows the created ChP organoids to secrete a CSF-like fluid highly similar to *in vivo* CSF. Moreover, these organoids and CSF-like fluid also mature over time, reaching a state highly similar to postnatal stages and adulthood (Pellegrini et al., 2020).

Adult CSF (aCSF) is produced and circulated in the brain and spinal cord throughout adulthood. Its primary functions include cushioning the brain and spinal cord, removing waste products, and transporting nutrients and hormones. eCSF and aCSF differ considering their composition and function. Studies show that human eCSF has a higher concentration of total proteins than aCSF (Gato et al., 2005; Parada et al., 2005a) and a similar situation can be observed in other mammals, for example, in rat and sheep embryos. On the other hand, significant phylogenetic differences in CSF's maturation have also been identified, possibly reflecting the particularities of CNS development across species (Bueno et al., 2020).

2.3 CSF in-laboratory-models

These differences are useful to study the neurogenesis-related aspects. The type of CSF used may prove to be a key factor in potential cell therapies in the central nervous system.

Moreover, there are CSF in-laboratory-models that closely mimic the composition of CSF. They can be made using a variety of techniques, including mixing various biochemical components in specific proportions, culturing cells in media that contain CSF, or using specialized microfluidic devices to create artificial CSF-like (art-CSF) environments.

One of the most advanced models in brain research seems to be the microfluid system. These systems can be designed to mimic the complex microenvironment of the brain, allowing for the study of the transport and diffusion of molecules within the CSF and their effects on brain cells. Furthermore, microfluidic systems can also

be used to study the interactions between CSF and brain cells, such as neurons and glial cells (Cliver et al., 2021). In order to closer mimic the brain environment, Cho and his group developed a brain-mimetic 3D organoid culture system by combining two basic elements: human brain tissue-derived ECM and a microfluidic chamber device.

CSF models in the *in vitro* research might help to understand the underlying mechanisms of brain function and disease, and may ultimately lead to the development of new therapeutic interventions for neurological disorders (Table 1).

3 Impact of CSF on stem cell fate

3.1 Effect on native NSCs

As mentioned before, NSCs transplantation is a promising tool for stem cell therapy development what is especially important for neurological diseases in which endogenous NSCs are not able to supply enough cells to repair the injured neural tissue (Arvidsson et al., 2002; Pluchino et al., 2008; Willis et al., 2020). These cells are self-renewing and multipotent, capable of differentiating into three major cell types of CNS, astrocytes, oligodendrocytes, and neurons, which makes them a perfect candidate for neurodegenerative disorders therapy. During embryonic development, they give rise to neurons and glia, while in adulthood, they are found mostly within the subventricular zone (SVZ) and in the subgranular zone (SGZ) of the hippocampus. Through the last two decades, researchers have found that CSF factors impact NSC activity, playing a significant role both in physiological and pathological processes in the brain with adult neurogenesis included (Alcázar et al., 1998; Redzic et al., 2005; Lehtinen et al., 2011; Silva-Vargas et al., 2016).

The study findings regarding the role of CSF on NSCs seem to be inconsistent. In the study of Ma and coworkers, it has been observed that human aCSF did not support the survival of newborn rats' neurons. Moreover, it inhibited neurogenesis. Similarly, NSCs derived from rat fetuses, when exposed to human aCSF, differentiated only into glial cells (Ma Y. et al., 2013). These results suggest a potential inhibitory effect of human CSF on neuronal development and a bias toward glial cell differentiation. However, in a study of another group performed on HTX rats, it was revealed that rat aCSF (obtained from newborn rats) stimulated neurosphere differentiation into neurons, glial cells and ependymal cells as well (Henzi et al., 2018). These results suggest that specific experimental conditions including different species origin of CSF may strongly contribute to its final effect on presented cells.

The effect of CSF derived from patients with neurological diseases was also investigated. Buddensiek's group reported that adult human leptomeningeal CSF promotes survival, and astrogliogenesis of fetal NSCs obtained from rats (Buddensiek et al., 2009). They have continuously observed such effects on human NSCs. Moreover, such cell culture conditions lead to stronger cell extension outgrowth and loss of cell proliferation potential measured with Ki67 and BrdU (Buddensiek et al., 2010). These results seem to provide evidence for the presence of factors regulating NSC proliferation and differentiation in adult CSF. The authors proposed that the CSF factors that presumably influenced NSC differentiation could be bone morphogenetic proteins (BMPs), such as BMP7 or BMP4. The first one is expressed in the choroid plexus and secreted into the CSF. It was shown to inhibit the CSF-induced neuronal dendritic outgrowth, while the second one promoted the differentiation via ERK pathway activation and GSK3β inhibition (Dattatreyamurty et al., 2001; Moon et al., 2009; Buddensiek et al., 2010). In addition, BMP2, BMP3, BMP4, BMP5 and BMP6 are also expressed in the choroid plexus but it is not known if they are present in CSF (Jensen et al., 2021). It has been observed by Zveik and his group that CSF from patients with relapsing multiple sclerosis (rMS) and progressive MS (pMS) enhance the ability of mice oligodendrocyte progenitor cells (OPCs) to differentiate into mature oligodendrocytes and to express immune functions. OPCs exposed to CSF from rMS patients were more morphologically mature compared to cultures exposed to CSF from pMS. After MS-patients-derived CSF exposure, OPCs become immune activated by NFκB activation, processing and presenting antigens to T cells, and secreting anti-or pro-inflammatory cytokines (Zveik et al., 2022). It is known that in case of a brain damage, the injured brain releases specific factors to stimulate endogenous neurogenesis and these factors might appear in CSF. Another study examined the relationship between neurogenesis and subarachnoid hemorrhage (SAH) features using CSF. The group observed that CSF from SAH patients enhanced the proliferation capacity of cultured rat NSCs (Chen et al., 2018). They showed that the capacity to proliferate of these cells is correlated with the severity and functional outcome of the disease. Another explanation for these observations may be the presence of blood in CSF obtained from patients with SAH. The presence of platelets (one of the main morphotic components of the blood) and their subsequent degranulation results in the release of trophic factors such as platelet-derived growth factor (PDGF), epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), endothelial cell growth factor (ECGF), fibroblast growth factor (FGF), transforming growth factor beta (TGFβ) and insulin-like growth factor (IGF). Which affect stem cell behavior by inducing proliferation and differentiation (Masoudi et al., 2016).

Overall, the role of aCSF appears to involve providing a supportive microenvironment for NSCs, promoting their survival, influencing their differentiation into specific cell types, and potentially containing factors like BMPs that modulate NSC behavior.

It has been also examined whether using CSF obtained from different developmental stages would impact the NSC culture. The

TABLE 1 The comparison of embryonic cerebrospinal fluid (eCSF) and adult cerebrospinal fluid (aCSF).

	eCSF	aCSF
Stage of development	Before the formation of the choroid plexus	After the formation of the choroid plexus
Main functions	Neurogenesis induction; brain and spinal cord development	Waste products removal; nutrients and hormones transportation
CSF composition	Rich in protein – high eCSF concentration of proteins such as albumin, fetuin, alpha-fetoprotein transferrin, and lipoproteins	Lack of protein (the protein concentration after birth falls dramatically)

group of Peirouvi research investigated the differentiation of the hippocampal neural stem/progenitor cells isolated from 3-month-old Wistar rats in response to the embryonic cerebrospinal fluid (eCSF) including E13.5, E17-CSF and the adult cerebrospinal fluid (aCSF), all extracted from rats. In their study they observed that hipp-NSC react differently to the eCSF and aCSF and that the effect depends on the concentration of CSF in the medium. They reported that hipp-NS/PCs were highly neurogenic in response to E13.5 and E17-CSF, but eCSF had no significant effect on astrogliogenesis. Hipp-NS/PCs exposed to aCSF increased GFAP+ expression and decreased MAP2 expressions, which indicates that aCSF promotes differentiation to glial cells (Peirouvi et al., 2015).

In addition, CSF benefits, such as NSC survival enhancement, have been observed *in vivo*. One of the possible administration routes for cell graft is intrathecal (Suzuki et al., 2022). Many studies on NSC transplantation in neurological diseases models used rodent cells and rodent hosts. For example, in the study of Wu and coworkers, rat neurospheres injected into the CSF of a rat with a spinal cord injury migrated into the lesion site and integrated into the spinal cord tissue (Wu et al., 2002). Good survival of NSCs, migration and integration with the injured spinal cord of rat NSCs has been seen also by Bai et al. (2003). There are also studies where human NSCs are transplanted into the rodent models, including transgenic mice, which can mimic the pathological and behavioral mechanisms occurring in neurodegenerative diseases. Such studies are crucial in the case of following the hNSCs reactions and further future transplantations into a human (Willis et al., 2020). However, it seems like there is a lack of studies that would be concentrated on investigating the exact CSF influence in the aspect of the final study outcome.

3.2 Effect of CSF on iPSCs-derived NSCs

The multiplicity of ethical issues and concerns regarding the usage of primary NSCs derived from human fetal or embryonic tissues may limit the scope of future research in the field of stem cell-based therapies in neurological diseases. Induced pluripotent stem cells (iPSCs), which are induced from autologous or non-autologous (e.g., homologous/allogenic) cell sources, may be the answer for the future. Their favorable properties can be exploited in many ways, firstly, iPSCs, by the use of commercially available protocols, can be placed in an environment rich in stimulus factors that promote iPSCs' differentiation into the specified direction such as NSCs, mature neurons or other cells of the nervous system (Galiakberova and Dashinimaev, 2020).

The iPSCs derived-NSCs have been used in Izsak's group *in vitro* research in 2020. The unique methodological approach allowed the team to obtain iPSCs-derived NSCs cultured as 3D aggregates, which they decided to culture in a healthy adult human cerebrospinal fluid (aCSF) environment. Their results showed that aCSF can be the physiological equivalent of the human healthy brain environment and can enhance the maturation of the functional neuronal network compared to the standard used medium. The Izsak's team showed that aCSF cells' incubation may also trigger several processes responsible for neural circuit development in the iPSCs-NSCs 3D model including electrophysiological activity, neuro and astrogenesis, and synapse formation. Interestingly, at the same time, they demonstrated suppression of cell proliferation which may be related to the plethora of factors contained in human cerebrospinal fluid derived from healthy volunteers (Izsak et al., 2020). Such positive effect of aCSF on the neurogenic, astrogenic, proliferation, and synapse formation potential of these cells is related to the one observed by aforementioned groups working on native NSCs.

Due to the lack of relevant research related to the influence of aCSF on iPSCs-derived NSCs, we still have limited information about the behavior and differentiation pattern of iPSCs-derived NSC cells in both *in vitro* and *in vivo* studies.

3.3 Effect of CSF on ESCs-derived NSCs

Another approach to studying the effects of cerebrospinal fluid on the therapeutic properties of stem cells, and NSCs in particular, in neurodegenerative diseases may involve the use of NSCs derived from embryonic stem cells (ESCs). ESCs isolated from human embryos are characterized by major ethical concerns, but their superior biological properties related to the ability to pluripotency and differentiation can potentially provide a good source for obtaining NSCs.

The properties of human embryonic stem cells have been employed by Kiiskii et al. group *in vitro* research in which they described the influence of adult healthy human cerebrospinal fluid (aCSF) on hESC-derived neural crest cell properties. Their research revealed that culturing hESCs-derived neural crest cells in the environment of aCSF may change the differentiation potential of NSCs and redirect their expansion toward glial cells at the expense of neuronal differentiation. However, after 4 weeks of hESCs-NSCs cultivation in aCSF the expression of genes related to neural precursors and neurons, astrocytes, and oligodendrocytes phenotypes has been observed by researchers. What is more, the aCSF microenvironment has been revealed to be enriched in FGF2, B-NGF, PDGF, and VEGF-A proteins, but also reduced hESCs-derived NSCs proliferation rate at the same time. Interestingly, despite the glial vs. neuronal differentiation pattern switch and proliferation ability reduction of NSCs, the researchers did not observe changes in neuronal network activity (Kiiski et al., 2013).

In other scientific reports, authors attempted to investigate the behavior of hESC-derived NSC under the alteration of aCSF obtained from neurologically disordered individuals, such as Cristofanilli's group, whose study used aCSF from volunteers diagnosed with progressive multiple sclerosis disease. This is another confirmation that factors produced during neurological diseases, such as multiple sclerosis can influence NSC survival and therapeutical properties after transplantation. The Cristofanilli et al. group discovered in their *in vitro* studies, that neural progenitor cells (NPCs) differentiated from hESCs, treated with multiple sclerosis patients-derived aCSF may be characterized by reduced proliferation potential with no affection of cells survival. Moreover, researchers found that CSF-treated NPSs significantly upregulate genes associated with differentiation into neurons and oligodendrocytes, but not astrocytes. Acquired outcome has been verified, by other techniques analysis, in which the enhanced astrocytic differentiation potential was not confirmed. The authors suggested that soluble factors dispensed in multiple sclerosis patients-derived CSF may play an important role during the redirection of NPCs from proliferation to differentiation state in order to rebuild the pool of the lost neural cells during the course of the disease (Cristofanilli et al., 2013).

To investigate how aCSF affects the functional activities of nervous system cells, the group of Sumitha, used hESCs-derived motor neurons that were incubated in aCSF derived from sporadic Amyotrophic Lateral Sclerosis (ALS). Authors observed a number of toxic effects of ALS-derived aCSF on ESC-derived motor neurons and noticed early signs of neurodegeneration such as lower viability, increased apoptotic proteins, impaired mitochondrial complex activities, hyperexcitability, organelles alterations and downregulation of BDNF expression (Sumitha et al., 2019). Presented studies are in accordance with the Brauer group findings, in which they also demonstrated the harmful effect of ALS-derived aCSF on iPSCs-derived motor neurons (Bräuer et al., 2020).

All of the presented studies lead us to the belief that the origin of aCSF itself plays an important role in terms of modifying ESC phenotype and function. Differences in composition of CSF might determine a multitude of processes taking place in cells, including neural differentiation or proliferation.

3.4 Effect of CSF on other cell types

In this review, we also decided to analyze the impact of CSF on other stem cells due to the large data provided. As the derivation of native NSCs from the primary tissues can be problematic and invasive, and the performed procedure can raise ethical issues, the researchers try to find other effective sources of these cells. So far, most studies on CSF have been performed on mesenchymal stem/stromal cells (MSCs). Using MSCs in regenerative medicine seems to bring many benefits. Autologous MSCs can be easily isolated, cultured, and directed into cells expressing neural markers and secreting altered, responsible for neuroprotection cytokines, chemokines, and morphogenes (Nivet et al., 2011; Tang et al., 2017; Zhang et al., 2017; Kaminska et al., 2022). However, it should be emphasized that mostly, their neurogenic abilities are presented in the increased expression of neural markers, which could be linked not with actual differentiation. For example, it has been suggested, that βIII-tubulin expression can be induced due to phenotypic changes in passaging and may be related to reorganization in the cytoskeleton (Słysz et al., 2022), and was proposed to be a common feature of MSCs (Drela et al., 2014).

It has been observed that short-term culture in artificial CSF (art-CSF) did not affect the morphology of adipose-derived mesenchymal stem/stromal cells (AD-MSCs) (Krull et al., 2021), however, after 9 days of such culture in adult human CSF (derived from benign intracranial hypertension (BIH) patient), morphological changes toward neural-like cells were observed (Elgamal et al., 2019). Furthermore, culture in adult human CSF increased the proliferation and viability of AD-MSCs (Zhu et al., 2015), whereas the culture in art-CSF decreased both features (Zhu et al., 2015; Krull et al., 2021). Regarding adult human CSF, it was shown to elevate the gene expression of migratory marker CXCR4 (C-X-C Motif Chemokine Receptor 4) (Zhu et al., 2015) and Nestin, with no changes of MAP2 (microtubule-associated protein 2) (Elgamal et al., 2019). Interestingly, the use of a lower concentration of adult human CSF resulted in a decreased gene expression of GFAP (glial fibrillary acidic protein) while a higher concentration increased its expression (Elgamal et al., 2019). AD-MSCs cultured in art-CSF did not express the pluripotent genes and Ki67 (a marker of proliferation) but expressed PCNA (proliferating cell nuclear antigen) (Krull et al., 2021). In another study, adult human CSF increased the number of $Ki67^+$ cells, while art-CSF decreased the amount of $Ki67^+$ cells (Zhu et al., 2015). These studies showed that art-CSF should not be treated like a substitute for adult human CSF in studying the effects of CSF on MSCs.

Moreover, in the study where AD-MSCs were administered via intrathecal injection, a group of Kuzma-Kozakiewicz observed that TNF-alpha (tumor necrosis factor alpha) level was decreased while FGF basic (basic fibroblast growth factor), IL-6 (interleukin 6) and MMP-6 (Matrix Metallopeptidase 6) levels were increased in CSF after the treatment with AD-MSCs over patients with amyotrophic lateral sclerosis (ALS) (Kuzma-Kozakiewicz et al., 2018). Another group pointed out that the treatment of AD-MSCs in Autoimmune Refractory Epilepsy resulted in increased angiogenin, CXCL12/SF1alfa I and IL-10 (interleukin 10) and decreased osteopontin levels (Szczepanik et al., 2020).

Culturing bone marrow mesenchymal stem/stromal cells (BM-MSCs) in CSF resulted in morphological changes to present astrocyte dendrite and axon-like cells [human CSF and human BM-MSCs; (Ye et al., 2011; Ge et al., 2015)] neurite length [rat CSF and murine BM-MSCs; (Shokohi et al., 2017)]; long spindle-shaped cells [rat CSF and rat BM-MSCs; (Mafikandi et al., 2019)]; mature neurons and dendrite-like cells presenting Nissl bodies [rabbit CSF and rabbit BM-MSCs; (Otify et al., 2014)]. Moreover, CSF from newborn rats of healthy mothers (N-CSF, normal-CSF) has been shown to undergo more rapid morphological change into long spindle-shaped cells than CSF from newborn rats of mothers with hypothyroidism (HTH-CSF, hypothyroidism-cerebrospinal fluid) (Mafikandi et al., 2019). The presence of CSF (human and rat) increased protein expression of β-III-tubulin in human (Ye et al., 2011; Ge et al., 2015) and murine BM-MSCs (Shokohi et al., 2017). Moreover, an increased GFAP expression has been observed [in human and rabbit CSF; (Ye et al., 2011; Otify et al., 2014; Ge et al., 2015)]. Different effect of CSF on stem cells has been also observed depending on their origin. Shokohi et al. (2017) observed that CSF affected the expression of marker MAP2 (increase) in murine BM-MSCs, but did not affect MSCs derived from dental pulp (Haratizadeh et al., 2017). In addition, rat CSF increased the expression of the neural marker Nestin in dental pulp MSCs (Haratizadeh et al., 2017) and rat BM-MSCs (Mafikandi et al., 2019). Rat BM-MSCs maintained higher viability in N-CSF than in HTH-CSF (Mafikandi et al., 2019). In addition, viability was increased when the concentration of rat CSF in the medium was increased to 10%. However, viability was reduced at 20% rat CSF in the medium (Haratizadeh et al., 2017). The culture of human BM-MSCs in aCSF showed they secreted BDNF (brain-derived neurotrophic factor), CNTF (ciliary neurotrophic factor), TGF-β (transforming growth factor β) and possess antioxidant properties. Interestingly, aCSF used for BM-MSCs culture increased the viability of PC12 and SH-SY5Y (Valitsky et al., 2019). BM-MSCs are also used in clinical trials of neurodegenerative diseases using intrathecal injection. The administration of BM-MSCs to patients with active progressive multiple sclerosis resulted in NF-L (neurofilament light chains) and CXCL13 (chemokine receptor) decrease in CSF (Petrou et al., 2022). In patients with ALS, increased levels of TGF-β1-3, IL-6, IL-10 (Oh et al., 2015, 2018), TGF-β2, TGF-β3 (Oh et al., 2015), VEGF (vascular endothelial growth factor), HGF (hepatocyte growth factor), LIF (leukemia inhibitory factor) (Berry et al., 2019) and reduced levels of MCP-1 (monocyte chemoattractant protein-1) (Oh et al., 2018; Berry

et al., 2019), SDF-1 (stromal cell-derived factor-1), CHIT-1 (chitotriosidase-1) (Berry et al., 2019) were observed.

It has been proven that CSF also has a considerable impact on WJ-MSCs (mesenchymal stem cells derived from Wharton's Jelly of umbilical cord). When cultured in the presence of CSF (100%), changes in cell morphology and proliferation rate have been observed over time. While WJ-MSCs usually represent a typical fibroblast-like morphology, in the presence of CSF the cells became elongated and formed axon-like protrusions. WJ-MSCs exhibited a high proliferation rate for the first 3 days of *in vitro* culture, which then slowed down between 3 and 5 days of culture. The cells stopped proliferating after 5 days of culture (Sypecka et al., 2022). In the aforementioned study, the authors also investigated whether WJ-MSCs cultured in CSF undergo neural differentiation. It turned out that WJ-MSCs cultured in CSF expressed higher levels of specific neural markers such as: Nestin, β-III-tubulin, S-100-β, GFAP, and doublecortin than those cultured in standard culture medium. Moreover, RT-qPCR analysis revealed that WJ-MSCs cultured in CSF expressed higher levels of MAP2 and NeuN than those cultured in standard culture medium. However, when it comes to NG2, the expression level of this gene was decreased in WJ-MSCs cultured in CSF. Based on these results, the authors suggest that WJ-MSCs undergo neural differentiation in the presence of CSF (Ge et al., 2015). Other studies report that even a small addition of CSF (10 µL/2 mL of DMEM or 100–200 µL/mL of DMEM, without platelet lysate) to the culture medium triggers changes in WJ-MSCs' phenotype. Changes in cell morphology were observed as before – after 3 days of culture they became irregular, and many were triangle-shaped; after 7 days of culture the cells formed characteristic, axon-like protrusions. It was also observed that cells cultured in standard culture medium did not express GFAP, MAP2 and β-III-tubulin. However, the addition of CSF to the medium triggered the expression of these markers (Farivar et al., 2015; Pellegrini et al., 2020).

The aforementioned studies reveal a crucial impact of CSF on MSC morphology, differentiation, and protein expression. Notably, CSF from different sources, such as human, rat, mouse, and rabbit, exhibits various effects on MSC behavior, underlining the complexity of their interactions. In addition, the results obtained with the use of artificial vs. healthy vs. diseased patient-derived CSF differ from each other.

The influence of CSF was also studied on other types of stem cells. For example, it has been investigated whether CSF can stimulate the therapeutic potential of multipotent stem cells residing in the bulge of hair follicles – epidermal neural crest stem cells (EPI-NCSCs) for their further use in neurodegenerative disorders treatment (Pandamooz et al., 2013). To do so, a group of Pandamooz investigated the fate of mouse EPI-NCSs cultured in adult rat CSF. The researchers observed a decrease in cell proliferation and differentiation inhibition in such conditions, which in their opinion could be beneficial for transplanted cells because of a possible direct differentiation as a response to the signals of the target injured site, making CSF a great route of cell administration to CNS. However, it is noteworthy that the used CSF was obtained from healthy donors of different species (Wistar rats) than the used cells (mice). The same group has recently published a letter suggesting the potential of using neural crest-derived stem cells from hair follicles in Parkinson's disease treatment, thanks to their ability to generate dopaminergic neurons as a response to eCSF presence (Pandamooz et al., 2022). A similar effect on neuronal differentiation was presented in a study in which human dental pulp stem cells (hDPSCs) were investigated (Goudarzi et al., 2020). This research showed, that CSF isolated from the *cisterna magna* of 19-day-old Wistar rat embryos added to the culture even for 2 days can induce final differentiation to neuron-like cells from hDPSCs, resulting in expression of Nestin, MAP2 and the presence of Nissl bodies in the cytoplasm (Table 2).

4 Potential roles of CSF in future cell therapy development for neurological disorders

NSCs play a pivotal role in the formation and maintenance of the entire nervous system. Their defects or damage lead to serious neurological consequences, thus, more and more studies are performed in order to understand the mechanisms involved in CNS dysfunctions. Along with the growing number of studies, there is a rising discussion in the aspect of some even contradictory findings. Previously, we stressed the importance of interpreting the results obtained after performing different culture conditions on the same cell type, as well as misleading findings using the same conditions regarding different species origin (Radoszkiewicz et al., 2023a,b). We pointed out that in many cases the protocols standardized on stem cells from animal models cannot be directly applied to human stem cells. We should also keep in mind that animal models of neurological disorders do not fully mimic the ones of human brain, as there are significant developmental differences between the species.

In this paper, we wanted to emphasize the significance of another factor which is still not well-studied *in vitro* and in our opinion, could be critical for striking the right balance between interpreting preclinical and clinical results. Nowadays, CSF is yet considered as a fluid with not only basic mechanical and chemical properties. Its crucial roles seem to change, starting from the complicated processes of neural development up to adulthood. A variety of complex changes in CSF occur in pathologies of neurological disorders. CSF, by transporting several necessary nutrients, hormones, and other factors around the CNS, provides a kind of highway for intricate cell signaling pathways that guarantee the right brain homeostasis. Thus, it is a perfect model to study endogenous as well as exogenous NSC behavior. Here, we analyzed the effect of CSF *in vitro* on different stem cell populations. In 26 of the analyzed studies, the significant influence of CSF on stem cell proliferation, differentiation and survival has been described (Figure 2).

The presented studies have shown that CSF has a great impact on the proliferation of not only native NSCs but also other stem cell populations. This effect is likely due to the presence of specific signaling molecules in the CSF that regulate cell growth and differentiation. Interestingly, the effect depends on the origin of the CSF. The results obtained with the use of artificial CSF differ from the ones obtained with human origin treatment, thus, such exchange is not recommended. Moreover, a significant difference between embryonic CSF and adult CSF has also been shown. It has been already proposed by embryonic CSF studies that diffusible factors in CSF can regulate neuroepithelial stem cell fate, influencing brain development *in vivo* (Gato et al., 2005). While the exact mechanism of its impact on neuroectodermal cells remains unclear, components like proteins, particles, amino acids, and FGF2 were seen to

TABLE 2 Comparison of studies of CSF effect on NSCs.

Primitive cell type		CSF source	% of CSF in the medium	CSF-induced effect	CSF-induced cell type	Proposed mechanism of action	*In vitro*/*in vivo* studies	References
NSCs	Mesencephalic NSCs from E14.5 rat embryonic brain	Adult human leptomeningeal CSF		NSC survival enhancement; glial differentiation stimulation; neurogenesis inhibition; proliferative and migratory potential decrease	Promoted glial differentiation; reduced proliferation	–	*in vitro*	Buddensiek et al. (2009)
	Adult human hippocampal tissue obtained from routine epilepsy surgery procedures	Adult human leptomeningeal CSF		NSC survival enhancement; glial differentiation stimulation; proliferative potential decrease	GFAP+ cells increase	BMP4 was shown to induce neuronal differentiation of NSCs by activating the ERK and inhibiting the GSK3b pathway. BMP effects could be blocked by BMP inhibitor - noggin. However, in the article authors did not find any inhibiting effects of noggin.	*in vitro*	Buddensiek et al. (2010)
	Rat sympathetic neurons	Adult bovine CSF		Increase in dendritic growth		BMP-7 is known to induce dendritic growth. To examine whether the dendritic growth induced by CSF was due to BMPs, cultures were treated with follistatin, a protein that binds and sequesters activin and some BMPs. Follistatin caused a significant reduction in CSF-induced dendritic growth, as did noggin.	*in vitro*	Dattatreyamurty et al. (2001)
	Primary OPC cultures isolated from naïve P0 to P1 neonatal C57/BL6 murine cortices	Adult human CSF from relapsing and progressive multiple sclerosis patients	10–20%	CSF from pMS impedes OPC differentiation to mature oligodendrocytes; OPCs exposed to CSF from rMS were more morphologically mature compared with CSF from pMS; enhanced immune activity	Mature O1+ oligodendrocytes	–	*in vitro*	Zveik et al. (2022)
	Neurospheres obtained from NSPCs from the ventricular and subventricular zones (VZ/SVZ) of PN1 normal, non-hydrocephalic HTx rats.	CSF collected from nHTx and hyHTx rats	5% CSF from nHTx rats or 5% CSF from hyHTx	CSF enhances the differentiation of NEs into neurons and astrocytes	Nestin+ (65% of the cells); GFAP+(22%) βIII-tub+ (20%)/ neurons, glial cells and ependymal cells		*in vitro*	Henzi et al. (2018)
	Mesencephalic NSCs from E14 rat fetuses	Human CSF	1 mL CSF	No neuronal differentiation, no NEs; CSF cannot support newborn neurons to survive	GFAP+/glial cells		*in vitro*	Ma T. et al. (2013)
	NSCs obtained from rat embryonic day 15 (E15) fetal brain	Human CSF from patients with subarachnoid hemorrhage (SAH)	0.5%	Enhancement of neurogenesis and proliferative potential of NSCs			*in vitro*	Chen et al. (2018)
	Adult rat hippocampal neural stem/ progenitor cells (hipp-NS/PCs)	Rat embryonic CSF (eCSF) and rat adult CSF (a-CSF)	15% or 20%	eCSF: enhancement of neuronal differentiation; no strong effect on astrogliogenesis aCSF: enhancement of Gliogenesis; neurogenesis inhibition	eCSF: MAP2+ neurons aCSF: GFAP+		*in vitro*	Peirouvi et al. (2015)

(Continued)

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TABLE 2 (Continued)

Primitive cell type		CSF source	% of CSF in the medium	CSF-induced effect	CSF-induced cell type	Proposed mechanism of action	*In vitro*/*in vivo* studies	References
ESCs	Human embryonic stem cells-derived NSCs	Human, healthy adult patients	100%	Glial cells	Promoted glial differentiation; reduced proliferation		*in vitro*	Kiiski et al. (2013)
	Human embryonic stem cells-derived NPCs	Human, multiple sclerosis patients	5%	Neurons and oligodendrocytes	Reduced proliferation; neuronal and oligodendrocytic differentiation enhancement; survival maintenance		*in vitro*	Cristofanilli et al. (2013)
	Human embryonic stem cells-derived motor neurons	Amyotrophic lateral sclerosis patients; intracranial hypertension patients	10%	Not applicable	Induced apoptosis; reduced viability; organelle alterations; decrease of BDNF expression		*in vitro*	Sumitha et al. (2019)
Other stem cells	Human AD-MSCs	31-year-old woman with BIH	0.5%	Neural-like cells, increased proliferation rate decreased gene expression of GFAP and Nestin	Inhibited neural differentiation		*in vitro*	Elgamal et al. (2019)
		31-year-old woman with BIH	2.5%	Neural-like cells, increased proliferation rate decreased gene expression of GFAP and Nestin	Inhibited neural differentiation		*in vitro*	
		31-year-old woman with BIH	5%	Neural-like cells, increased proliferation rate decreased gene expression of GFAP and Nestin	Inhibited neural differentiation		*in vitro*	
		31-year-old woman with BIH	10%	Neural-like cells, increased proliferation rate increased gene expression of GFAP decreased gene expression of Nestin	Inhibited neural differentiation, glial differentiation		*in vitro*	
	Human AD-MSC	Human	100%	No changes in morphology	Fibroblast-like		*in vitro*	Krull et al. (2021)
		Artificial	100%	Decreased metabolic activity, proliferation, and viability no expression of pluripotent gene, Ki67	–		*in vitro*	
	Human AD-MSC	Human	25%	Low proliferation low number of Ki67 high % of apoptosis	–	Neutralization of IGF-1 in human CSF decreased migration and proliferation cells compared to non-neutralized human CSF, in contrast to the cells cultured in standard medium; the rate of apoptosis in neutralized human CSF was decreased compared to the standard medium but increased in human CSF	*in vitro*	Zhu et al. (2015)
		Artificial	25%	High proliferation high number of Ki67 Low % of apoptosis High gene expression of CXCR4	–		*in vitro*	

(Continued)

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TABLE 2 (Continued)

Primitive cell type	CSF source	% of CSF in the medium	CSF-induced effect	CSF-induced cell type	Proposed mechanism of action	*In vitro*/*in vivo* studies	References
BM-MSCs	Artificial	–	Secrete BDNF, CNTF, TGF-β, and anti-oxidant capacity	–		*in vitro*	Valitsky et al. (2019)
Human dental pulp MSCs	Newborn rat race Sprague–Dawley	10%	Neuron-like cells high level of markers: nestin and GFAP	Neural progenitor, astrocyte cells		*in vitro*	Haratizadeh et al. (2017)
Human BM-MSCs	Healthy human	–	Increased markers of β-III-tubulin and GFAP	Astrocyte dendrite, axon-like cells		*in vitro*	Ge et al. (2015)
Rat BM-MSCs	Healthy newborn rats	5%	High gene expression of nestin Low gene expression of NEORD-1	–		*in vitro*	Mafikandi et al. (2019)
Rat BM-MSCs	Hypothyroid newborn rats	5%	High gene expression of nestin, NEORD-1, and NEUN	–		*in vitro*	Mafikandi et al. (2019)
Murine BM-MSCs	Rat fetuses	10%	Longer neurite length			*in vitro*	Shokohi et al. (2017)
Human BM-MSCs	Healthy human	10 µl every day for 7 days	Increased markers of β-III-tubulin and GFAP	Astrocyte dendrite, axon-like cells		*in vitro*	Ye et al. (2011)
Rabbit BM-MSCs	Rabbit	10 µl every day for 9 days	Appearance of Nissl bodies, glycogen granules	Mature neurons and dendrite-like cells, astrocyte		*in vitro*	Otify et al. (2014)
WJ-MSCs	Healthy patients	100%	Neural-like morphology, expression of neural markers and genes (Nestin, β-III-tubulin, S-100-β, GFAP, doublecortin, NeuN, MAP2)	Neural		*in vitro*	Sypecka et al. (2022)
Umbilical cord blood-derived MSCs	Healthy patients	10 µL per 2 mL of medium	Neural-like morphology, expression of GFAP, β-III-tubulin	Neural		*in vitro*	Ge et al. (2015)
WJ-MSCs	Healthy patients (children)	100 µL or 200 µL per mL of medium	Expression of Nestin, GFAP, MAP2	Neural		*in vitro*	Farivar et al. (2015)
EPI-NCSCs isolated from 3-week-old NMRI mice	CSF was collected from the cisterna magna (CM) of Wistar rats	10%	CSF maintained the expression of nestin, β–tubulin III (early neuronal marker), and glial fibrillary acidic protein (GFAP, glia marker)	Decreased cell proliferation rate, CSF does not promote cell differentiation toward any specific destiny		*in vitro*	Pandamooz et al. (2013)
Human dental pulp stem cells (hDPSCs) were	From the Cisterna magna of 19-day-old Wistar rat embryos	5%	Expression of a neural progenitor marker (Nestin) and a mature neural marker (MAP2) Nissl bodies in cell cytoplasm	Promoted neural and neuronal differentiation		*in vitro*	Goudarzi et al. (2020)

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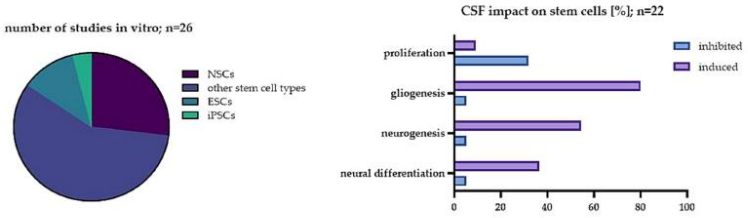

FIGURE 2
The impact of CSF on stem cells in the investigated studies. On the left: The percentage of studies analyzed *in vitro* in regard to different cell types; on the right: The most common effect of CSF on stem cells in the analyzed studies *in vitro*.

be implicated (Martín et al., 2006; Bachy et al., 2008; Huttner et al., 2008). Despite the complexity of embryonic CSF's composition compared to adult CSF, it retains the ability to influence the behavior of adult neural stem cells (NSCs) in the brain (Buddensiek et al., 2010). In the NSC studies, eCSF promoted neuronal differentiation, while adult CSF stimulated the cells into glial differentiation. It is worth noting that most of the analyzed studies were performed using adult CSF. Overall, these studies also showed that the proliferation capacity of stem cells seems to be lowered in the CSF obtained from healthy donors. One possible reason for such an inhibitory effect is that CSF contains factors that promote the differentiation of stem cells into specific types of cells, which in this case was mostly into glial cells. However, the CSF from patients with diseases like BIH or SAH enhanced the proliferation of NSCs. The possible explanation of this effect is that CSF from patients with these conditions contains higher levels of growth factors and cytokines that promote cell proliferation, such as those previously shown to be present during regeneration after brain injury-FGF, NGF, TGF-β, GDNF, BDNF, VEGF, which possess neuroprotective properties, improve the survival and proliferation rate of neurons (Lehtinen et al., 2011). It has been shown that CSF from patients with SAH contains elevated levels of VEGF or BDNF which are known to stimulate the growth of blood vessels, promote tissue repair and play a key role in post-SAH proliferation of NSCs (Sgubin et al., 2007; Chen et al., 2013, 2018).

In addition, in most of the described studies, the presence of CSF improved the differentiation into glial cells. The exact mechanisms of action are, however, not well-described. Buddensiek's group suggested that the enhanced differentiation is a result of BMP presence in CSF expressed by the choroid plexus (Buddensiek et al., 2010). It is not confirmed whether the choroid plexus expresses BMPs like BMP2, BMP3, BMP4, BMP5 and BMP6, however, it is known that BMP4 stimulates the differentiation via ERK pathway activation and GSK3β inhibition (Dattatreyamurty et al., 2001; Moon et al., 2009; Buddensiek et al., 2010; Jensen et al., 2021). Dattatreyamurty and coworkers also investigated BMP presence in CSF, by examination of BMP-7's known ability to induce dendritic growth in rat sympathetic neurons. To confirm that the dendritic growth induced by CSF was due to BMP-7, cultures were treated with BMP inhibitors – follistatin and noggin. Follistatin caused a significant reduction in CSF-induced dendritic growth, as did noggin. In addition, dendritic growth induced by bovine CSF was inhibited by function-blocking antibodies against BMP-7, i.e., 72% inhibition with 12G3 and 40% inhibition with 1B12. These observations suggest that a substantial portion of the dendrite-promoting activity in CSF is due to BMP-7 (Dattatreyamurty et al., 2001). In the study of the Zhu group, a noteworthy positive influence exerted by insulin-like growth factor-1 (IGF-1) was found in human CSF on the migration capacity and C-X-C chemokine receptor type 4 (CXCR4) expression in both human exogenous primary amniotic MSCs and fetal neural progenitor cells. The authors also confirmed the impact of CSF on the proliferation, migration, and viability of these stem cell types (Zhu et al., 2015). It has been also discovered that embryonic CSF, particularly CSF-insulin-like growth factor 2 (IGF-2), plays a vital role in providing factors that stimulate the growth and survival of the developing rodent cortex (Lehtinen et al., 2011). Moreover, in the developing chick brain, it has been seen that CSF contributes to the retention of midbrain markers such as Otx2 and Fgf8, while FGF2 in CSF seems to promote precursor proliferation (Parada et al., 2005b; Martín et al., 2006). In addition, within the mouse cerebellum, CSF-distributed Sonic Hedgehog (Shh) has been proposed to stimulate the proliferation of cerebellar granule neuron precursors (Huang et al., 2010). It has been also shown that CSF contains high levels of other cytokines, such as leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF), which promote the differentiation of NSCs into astrocytes (Nakanishi et al., 2007). Such effect could also be caused by TGF-β, which is known to play a key role in brain development, brain homeostasis during adulthood and neurological pathologies. TGF-β-1 signaling is one of the major pathways that regulate gliogenesis (Stipursky and Gomes, 2007; Diniz et al., 2018). What is more, the same factor seems to be responsible for neurogenesis inhibition, which also occurred in the analyzed studies. It has been presented as a molecule modulating the neural stem and progenitor cell proliferation in the CNS (Wachs et al., 2006). In mice hippocampal neurons, TGF-β induced cell cycle exit (Vogel et al., 2010). Moreover, it was shown to induce neurite growth, which was also observed in some of the investigated studies (Knöferle et al., 2010; Hiew et al., 2021).

So far, different effects of CSF on NSCs, even the ones of the same origin, have been described. The reasons for such discrepancies between groups can be traced not only to the different sources of the NSCs, but also to the amount of CSF used in cell culture (starting from 0.5% up to 100% in the culture medium), or finally, the biodiversity and origin of the CSF itself. However, those findings provide direct evidence that healthy cerebrospinal fluid, circulating through different parts of the CNS, may provide a beneficial environment for the administrated NSCs due to the presence of potentially stimulatory

factors for neural lineage differentiation at the expense of reducing proliferation.

In summary, the study analysis confirmed that further experiments to examine the fate of NSCs under specific influences of CSF are required. To understand the effects of CSF on these cells, it is important to further study the complex interactions between NSCs and CSF, including various growth factors, signaling molecules, and other factors present. Knowing the conditions in which CSF could support NSCs and promote regeneration and restoration could allow to improve targeted cellular therapy in CNS disorders. It's important to note that the effects of CSF on neurogenesis are complex and may depend on several different factors, including the age of the individual, the specific components of the CSF, and the specific brain region being studied. The CSF seems to be a potential vehicle for volume transmission of growth factors, implanted neural stem cells, and chemokines. As such, the CSF as a therapeutic vehicle to promote CNS homeostasis was described in animal models many times-however, the papers analyzing the effects of unaltered or naturally altered CSF on neurogenesis are very few. Thus, further research is needed to fully understand the relationship between CSF and neurogenesis.

Summary notes:

- CSF appears to provide a supportive microenvironment for NSCs, influencing their survival, and differentiation.
- Adult CSF environment influences iPSCs-derived NSCs, directing them toward neurogenesis or gliogenesis while suppressing proliferation. It promotes synapse formation, neurite outgrowth, and activation of neuronal circuits in these cells.
- ESCs-derived NSCs cultured in aCSF were seen to have changes in their differentiation potential, leading to the formation of glia, neural precursors, neurons, astrocytes, or oligodendrocytes. Additionally, there was an observed decrease in the proliferation rate of ESCs-derived NSCs during aCSF culture.
- ALS-derived aCSF negatively affects ESCs-derived NSCs. Harmful effects include lower viability, vacuolization, and the induction of apoptosis in ESCs-derived NSCs when exposed to ALS-derived aCSF.
- Irrespectively of the source, CSF affected MSCs properties such as morphology, proliferation, viability, neural gene expression and secretome which varied between the studies.
- The effects of CSF vary depending on its origin, with differences observed between artificial CSF and human-origin CSF.
- The effects of CSF vary based on factors such as cell sources, CSF concentration, and CSF origin.
- Adult CSF from healthy donors may inhibit stem cell proliferation, potentially due to factors promoting differentiation into specific cell types, mainly glial cells.
- Healthy CSF may provide a beneficial environment for NSCs, supporting neural lineage differentiation at the expense of reduced proliferation.
- Limited research on the effects of unaltered or naturally altered CSF on neurogenesis emphasizes the need for additional studies.
- This review underscores the importance of further research to comprehend the relationship between CSF and neurogenesis, with potential implications for improving targeted cellular therapy in CNS disorders.

Author contributions

KR: Conceptualization, Formal analysis, Writing – original draft. AB: Formal analysis, Writing – original draft. MC: Writing – original draft. PR: Writing – original draft. MS: Writing – original draft. IZ-K: Writing – original draft. AS: Supervision, Writing – review & editing.

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Figure 1 was made in BioRender. Figure 2 with all the provided data based on Table 2 was prepared with the use of GraphPad Prism 9.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

NSCs	neural stem cells
AD-MSCs	adipose tissue – mesenchymal stem/stromal cells
CSF	cerebrospinal fluid
aCSF	adult cerebrospinal fluid
art-CSF	artificial cerebrospinal fluid
eCSF	embryonic cerebrospinal fluid
CNS	central nervous system
BIH	benign intracranial hypertension
PRP	platelet-rich plasma
MAP2	Microtubule-associated protein 2
GFAP	Glial fibrillary acidic protein
KI67	marker of proliferation Ki67
PPARG	peroxisome proliferator activated gamma
hDPSCs	human dental pulp stem cells
EPI-NCSCs	epidermal neural crest stem cells
SOX9	SRY-Box transcription factor 9
ALPL	alkaline phosphatase
RUNX2	runt-related transcription factor 2
COL21A1	collagen type XXI alpha 1 chain
MGP	matrix glia protein
MSC	mesenchymal stem/stromal cells
OGN	osteoglycin
PODN	podocan
CXCR4	C-X-C Motif Chemokine Receptor 4
TNF-alpha	tumor necrosis factor alpha
FGF basic	basic fibroblast growth factor
IL-6	interleukin 6
MMP-6	Matrix Metallopeptidase 6
ALS	amyotrophic lateral sclerosis
IL-10	interleukin 10
BM-MSCs	bone marrow – mesenchymal stem/stromal cells
BDNF	brain-derived neurotrophic factor
CNTF	ciliary neurotrophic factor
TGF-β	transforming growth factor β
N-CSF	normal CSF
Shh	Sonic Hedgehog
HTH-CSF	hypothyroidism-cerebrospinal fluid
NEURD-1	neurogenic differentiation 1
NeuN	neuronal nuclei
NF-L	neurofilament light chains
CXCL13	chemokine receptor
VEGF	vascular endothelial growth factor
HGF	hepatocyte growth factor
LIF	leukemia inhibitory factor
MCP-1	monocyte chemoattractant protein-1
SDF-1	stromal cell-derived factor-1
CHIT-1	chitotriosidase-1
PCNA	Proliferating cell nuclear antigen

7.2.4. Article IV

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EDITED BY
Prasad S. Koka,
Biomedical Research Institute of Southern California, United States

REVIEWED BY
Armel Hervé Nwabo Kamdje,
University of Garoua, Cameroon
Birgitta Sundell-Ranby,
Wayne State University, United States

*CORRESPONDENCE
Anna Sarnowska,
asarnowska@imdik.pan.pl

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Unraveling the impact of human cerebrospinal fluid on human neural stem cell fate

Klaudia Radoszkiewicz[1], Aleksandra Bzinkowska[1], Monika Sypecka[1], Dorota Sulejczak[2], Daniela Ferrari[3], Maurizio Gelati[4], Angelo Luigi Vescovi[3,5] and Anna Sarnowska[1]*

[1]Translational Platform for Regenerative Medicine, Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland, [2]Department of Experimental Pharmacology, Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland, [3]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy, [4]UPTA Unit Fondazione IRCCS Casa Sollievo della Sofferenza, Foggia, Italy, [5]IRCCS Casa Sollievo della Sofferenza, Foggia, Italy

Human neural stem/progenitor cells (hNSCs) can potentially treat neurological diseases, but their low survival and proliferation rates after transplantation remain challenging. In our study, we preincubated hNSCs with the human cerebrospinal fluid (CSF) to obtain closer to the physiological brain environment and to assess NSC fate and their therapeutic abilities *in vitro*, *ex vivo*, and *in vivo*. We observed significant changes in the differentiation, migratory, and secretory potential of CSF-treated hNSCs, as well as their elevated neuroprotective potential after co-culture with ischemically damaged by oxygen-glucose deprivation (OGD) organotypic rat hippocampal slices culture (OHC) in comparison to the cells cultured in the standard conditions. Next, we investigated their survival and anti-inflammatory abilities in an *in vivo* ouabain-induced stroke model. This study highlighted and confirmed the critical importance of nutritional supplementation in maintaining NSC culture and enhancing its therapeutic properties.

KEYWORDS

neural stem cells, ischemic stroke, cerebrospinal fluid, neuroprotection, cell therapy

1 Introduction

After several decades, we still have not obtained sufficient clinical results that could significantly improve the lives of neurological patients (Grochowski et al., 2018). One of the most commonly occurring neurological diseases that has taken much interest is ischemic stroke. Its consequences often dramatically lower the life quality of patients, resulting in disability or even leading to death. Much hope is given to endogenous neural stem cells, which can, due to the activation, migrate and subsequently participate in restoring the damaged brain area functions after stroke. However, due to their relatively limited amount and survival, the recovery is still insufficient and the injury-induced neurogenesis is not successful (Kokaia and Lindvall, 2003; Liu et al., 2009; Kernie and Parent, 2010; Kokaia and Darsalia, 2018). Thus, using exogenous NSCs holds promise for the repair of ischemically damaged neural tissue (Park et al., 2002; Andres et al., 2008; Steinberg et al., 2016; Boese et al., 2018). In this case, exogenous NSCs, among other cell types, can further selectively differentiate into all neural lineages and present minimal tumorigenic risk (Rakic, 2009; Yan et al., 2013; Beattie and Hippenmeyer, 2017). Moreover, they were already seen to be very supportive serving as chaperone cells for the injured/dysfunctional tissue

(Hess and Borlongan, 2007). Despite all aforementioned properties suggesting a successful use in treating many diseases, achieving such a goal in clinical trials seems to be still elusive. To a great degree, this can be caused by their limited ability to regenerate. Low cell survival and proliferation rate after transplantation are one of the biggest issues (Lee et al., 2017; Hayashi et al., 2020). The mechanisms that occur after the cell injection are still not well-defined (Boese et al., 2018; Ottoboni et al., 2020). Along with still unsatisfying knowledge regarding numerous processes appearing in the accompaniment of specific factors of the brain niche, even at the preclinical level, the analysis of the results remains very difficult (Gil-Perotín et al., 2013). Reflecting the exact physicochemical and spatial conditions of the neural niche *in vitro* is currently debatable. We pointed out previously that the wide variation of medium composition used by each research group limits reliable comparison of the results and their interpretation (Radoszkiewicz et al., 2023c; 2023b). As a consequence, the mechanisms that are involved in *in vivo* cell application still need to be widely explored.

The behavior of NSCs is highly dependent on the surrounding microenvironment. The niche regulates its fate *via* biochemical *stimuli* (such as growth factors, hormones, or peptides), biophysical factors (pressure, shear stress, geometry), and cell-cell interactions. These components have a great influence on the adhesion, survival, proliferation, migration, morphology, and differentiation of stem cells. Through our exploration of factors influencing the poor fate of exogenous cells post-transplantation, especially after the intrathecal injection method, we turned our focus to the cerebrospinal fluid (CSF). While CSF has traditionally been seen as a fluid with basic physiological and mechanical functions, recent studies underlie its critical role in complex brain physiology, particularly during development, influencing the functions of NSCs and brain restoration (Wichmann et al., 2022). Despite its importance as a diagnostic tool, the components of CSF and their specific roles remain under-explored (Figure 1). What is more, after analyzing so far published papers on the effect of CSF on stem cells, we have seen that it varies between performed research, what hampers the ability to compare them, and coming to uniform conclusions (Radoszkiewicz et al., 2023a). Nonetheless, limited research on the effects and involved mechanisms of unaltered or naturally altered CSF on neurogenesis emphasizes the need for further studies. This, however, allows exploring still not well-known areas of human brain function and offers a glimpse of hope of creating a laboratory environment that closely mimics the physiological brain niche.

Abbreviations: hNSCs, human neural stem/progenitor cells; CSF, cerebrospinal fluid; OGD, oxygen-glucose deprivation; OHC, organotypic hippocampal slices culture; PI, propidium iodide; CNS, central nervous system; DMSO, dimethyl sulfoxide; CXCR4 C-X-C Motif Chemokine Receptor 4; ESCs, embryonic stem cells; NGF, nerve growth factor; PFA, paraformaldehyde; LIF, leukemia inhibitory factor; BDNF, brain-derived neurotrophic factor; HBSS, Hanks' Balanced Salt Solution; DMSO, dimethyl sulfoxide; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; MAP2, Microtubule-associated protein 2; GFAP, Glial fibrillary acidic protein; KI67, marker of proliferation Ki67; HGF, hepatocyte growth factor.

2 Materials and methods

2.1 Neural stem cell culture

Human fetal neural stem/progenitor cells (hNSCs) were obtained from the IRCCS Casa Sollievo della Sofferenza, Viale Cappuccini 1, San Giovanni Rotondo, 71,013 Foggia, Italy. The material was acquired in full compliance with legal and ethical standards, adhering to current local informed consent procedures. The cells were isolated from the fetal human brain following previously described protocols (Vescovi et al., 2009; Gelati et al., 2013). HNSCs were cultured in standard culture conditions in humidified incubators under 5% O_2 and 5% CO_2 conditions, at 37°C. The medium containing DMEM/F12 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), GlutaMAX® (1%, Gibco), Penicillin/streptomycin (1%, Gibco), Heparin (0.1%, Sigma-Aldrich, Saint Louis, MO, USA), N2 supplement® (1%, Gibco), B27 supplement® (2%, Gibco), EGF (20 ng/mL, Gibco), bFGF (20 ng/mL, Gibco) was replaced every 3 days. The neurospheres (3D culture) were mechanically dissociated when they gained the desired diameter. The cells in 2D culture were seeded into 24-well or 96-well plates covered with Poly-d-lysine and laminin and the standard culture medium without growth factors was used. Over the past years, the team members have comprehensively characterized used in the manuscript NSCs line regarding the markers, differentiation potential, and biological activity (Mazzini et al., 2015; Rosati et al., 2018; Profico et al., 2022).

2.1.1 Cerebrospinal fluid (CSF)

Human CSF samples were collected from 20 adult volunteers following routine diagnostic procedures conducted at the Department of Neurology, Medical University of Warsaw, Poland. The collection process adhered to the ethical guidelines outlined by the Medical University of Warsaw ethics committee (guideline AKBE/242/2022). These CSF samples were classified as leftovers, as they were no longer required for further medical diagnostics (neurological diseases were ruled out based on the CSF analysis). The leftover CSF samples were pooled, and selected analytes were analyzed using a Luminex assay. To assess the impact of CSF on NSCs, cells were cultured in a medium supplemented with 30% CSF, which was the maximal volume necessary for the planned experiments and their repetitions. For proliferation and migration assessments, cells were also cultured in 100% CSF. NSCs were cultured in the CSF-containing medium for up to 7 days.

2.1.2 NSC scratch injury

To evaluate the influence of CSF on NSC migration, a scratch assay was performed using culture inserts (ibidi). The experiment was conducted in triplicate, with 10,000 cells seeded into each chamber of the inserts. After 24 h, the inserts were removed, and each well was filled with one of the following media: standard culture medium without growth factors (control), medium supplemented with 30% CSF (30% CSF), or 100% CSF (100% CSF). Images of the scratch area were captured at 0, 2, 24, and 48 h after performing the scratch. At the end of the experiment, the cells were fixed with 4% paraformaldehyde (PFA; Sigma) and stained using a neurite outgrowth assay kit to visualize neurites. Three random pictures of

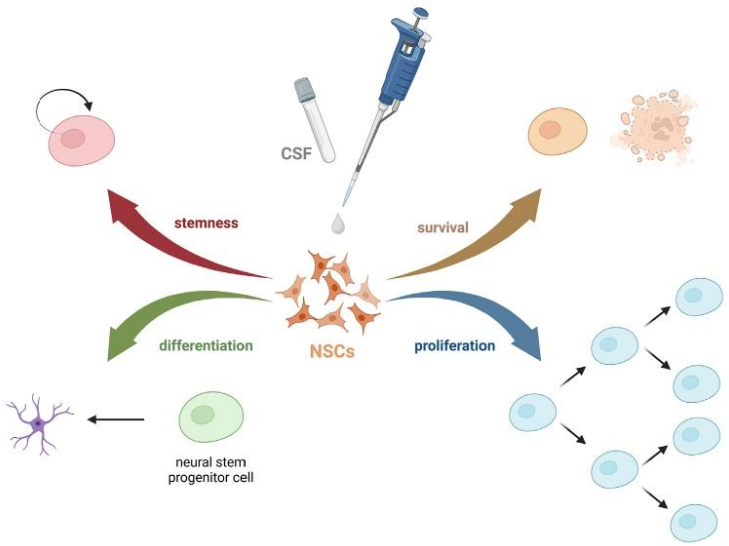

FIGURE 1
Possible effects of CSF on NSCs.

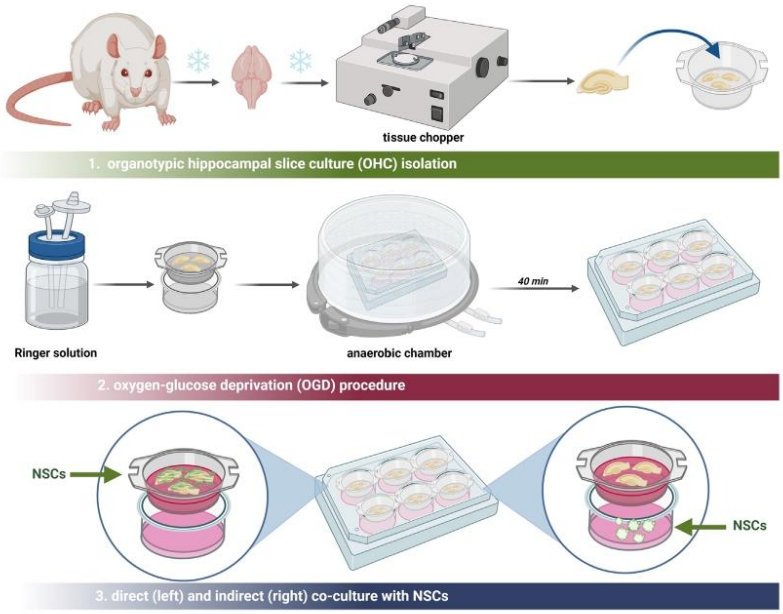

FIGURE 2
Schematic overview of all procedures executed in the *ex vivo* model.

each scratch injury were captured from each well of the 24-well plate using Cell Observer (Carl Zeiss, Oberkochen, Germany) inverted microscope and ZEN software (Carl Zeiss, Oberkochen, Germany). At least four wells were prepared for each variant.

2.1.3 Neurite outgrowth assay

To visualize the neurites in the scratch area, the cells were stained using a Neurite Outgrowth Kit (Thermo Fischer Scientific). Next, the neurites were analyzed using Fiji (formerly ImageJ) software. A region of interest (ROI) was marked around the scratch, excluding the edges and neuronal bodies, while the integrated density was used to calculate a sum of all pixels within the ROI. To obtain the percentage of ROI covered with neurites we divide the integrated density/area of ROI. These experiments were carried out using a 24-well plate. At least four wells were prepared for each variant.

2.1.4 Cell proliferation assay

The cell proliferation rate was assessed using the PrestoBlue™ Cell Viability Reagent (Invitrogen, Thermo Fisher, Rochester, NY, USA) following the manufacturer's protocol. The cells were cultured in a standard culture medium without growth factors. HNSCs were incubated with this reagent for 1 h in the dark, on Day 1, Day 3, and Day 7 of monolayer culture performed on a 96-well poly-L-lysine/laminin-coated plate. The cell density was 10,000 cells/well. For analysis, the starting point (Day 1) was set as the baseline at 100%. Absorbance was measured with the use of a microplate reader, Omega PlateReader (BMG LABTECH), at a 590–600 nm wavelength.

2.1.5 Organotypic rat hippocampal slice culture (OHC)

For *ex vivo* experiments, 7-day-old Wistar rats (12 animals) were obtained from the Mossakowski Medical Research Institute Breeding House. All experiments were approved by the Ethical Committee and conducted in accordance with ethical guidelines. The experiment was conducted following the Stoppini method that was previously modified in our lab (Stoppini et al., 1991; Figiel-Dabrowska et al., 2021). All procedures were performed on ice. In brief, following decapitation, the rat hippocampi were isolated and sectioned into 400 µm slices using a McIlwain tissue chopper (Ted Pella, Poznan, Poland) into 400 µm slices. The selected slices were transferred onto the organotypic culture membranes (Millipore, Concord Road Billerica, MA, USA), placed on 6-well plates (ThermoFischer Scientific) and filled with 960 µL of the medium consisting of DMEM/F12 (Gibco), 25% HBSS (Gibco), HEPES (Gibco), 5 mg/mL glucose (Sigma-Aldrich) and 1% of antibiotic-antimycotic solution (Gibco). The rat organotypic hippocampal slice cultures (OHC) were maintained for 7 days at 34°C, in an atmosphere of 5% O_2 and 5% CO_2. After the incubation period, the OHCs were utilized for co-culture experiments with various NSC culture conditions.

2.1.6 Oxygen-glucose deprivation (OGD)

The procedure of oxygen-glucose deprivation (OGD) was described previously (Figiel-Dabrowska et al., 2021) (Figure 2). After 7 days of organotypic hippocampal culture (OHC), preselection of the hippocampal slices was carried out using propidium iodide (PI; Thermo Fisher Scientific) to identify slices with damage in the CA1 region. Damaged slices were discarded. The culture medium was then replaced with deoxygenated Ringer's solution (Sigma-Aldrich) supplemented with mannitol (Sigma-Aldrich). The membranes with the selected slices were transferred into a hypoxic chamber for oxygen deprivation for 40 min at 34°C, 5% O_2, and 5% CO_2 conditions. Subsequently, the membranes were washed in PBS (ThermoFisher Scientific) and the co-culture with all cell variants was conducted. For indirect co-culture, the neurospheres were seeded into the 6-well plate, under the membrane with the slices. To perform the direct co-culture, the cells in the suspension were directly seeded onto the slice. To analyze the neuroprotective effect of hNSCs, 24 h after this procedure, the slices were stained with PI (ThermoFisher Scientific) for 30 min and CA1-region hippocampal cell death was investigated. The images of the CA1 region were taken using an LSM 510 confocal microscope (Zeiss).

2.1.7 Ouabain-induced brain injury and hNSC transplantation

For *in vivo* experiments, 3-month-old male 250g-weight Wistar rats from the Mossakowski Medical Research Institute Animal Breeding House were used (Figure 3). Approval for this study was granted by the Local Ethical Committee in Warsaw, Poland (No. WAW2/147/2022). For each experiment, six animals were used (n = 6).

To induce focal brain damage, the rats underwent ouabain-induced brain injury. The procedure involved anesthetizing the rats with ketamine (10%; 90 mg/kg body weight, administered intraperitoneally) and xylazine (2%; 10 mg/kg body weight, administered intraperitoneally). Subsequently, the rats were positioned in a stereotactic apparatus (Stoelting). A small hole was drilled in the cranium on the right hemisphere at the following coordinates (A 0.0, L 3.0, D 5.0 mm). Using a micro infusion pump (Stoelting) and a 10-µL Hamilton syringe (Hamilton) equipped with a 15-mm-long needle (gauge 33, Hamilton), 1 µL of 5 mmol ouabain (Sigma) was injected into the brain at a rate of 0.1 µL/min. To minimize brain shift, a 5-min delay was introduced between the needle insertion and the actual injection. Subsequently, the needle was carefully removed, and the incision in the skin was closed using sutures. Post-procedure, the rats received antibiotics and painkillers (Baytril and Metacam, 5 mg/kg, administered intraperitoneally) and were housed appropriately for recovery.

Two days following the ouabain-induced brain injury, the rats underwent a second anesthesia for cell transplantation. The stereotaxic surgery was conducted under the influence of ketamine (10%; 90 mg/kg body weight, administered intraperitoneally) and xylazine (2%; 10 mg/kg body weight, administered intraperitoneally). NSCs were transplanted using a 25-µL Hamilton syringe into the corpus callosum. The injection was controlled by a micro infusion pump at a rate of 0.5 µL/min. The coordinates employed were A 0.0, L 4.0, and V 3.0, with the bregma adjusted to the same horizontal plane, and ventral coordinates calculated from the dura. Upon removal of the needle, the skin was securely resealed with sutures (Braun). Subsequently, the rats received antibiotics and painkillers (Baytril and Metacam, 5 mg/kg body weight, administered intraperitoneally) and were housed.

Seven days post-transplantation, the animals were euthanized for immunohistochemical analysis. The rats were anesthetized and subsequently decapitated. Following decapitation, the brains

were isolated and fixed in 4% paraformaldehyde (PFA; Sigma). The fixed brains were then suspended in 30% sucrose for cryoprotection and stored at −80°C for cryopreservation. The day prior to cryosectioning, the brains were transferred to −20°C and subsequently sectioned into 20 µm slices using a cryostat. The sections were stored at −80°C until immunostaining procedures were performed. This approach facilitated the examination of the transplanted cell locations and their interactions with the damaged neural tissue.

2.2 Results analysis

2.2.1 Immunofluorescence (IF) staining

For IF staining, we used the previously described technique for 2D and 3D culture (Radoszkiewicz et al., 2023c). In 2D, after experiments, the cells were deprived of culture medium, washed twice in PBS (ThermoFisher Scientific), and fixed with 4% PFA (ThermoFisher Scientific) for 15 min, at room temperature (RT). Subsequently, the cells were permeabilized in 0.2% Triton X-100 (Sigma-Aldrich) for 30 min. To prevent nonspecific bindings, a mixture of 10% Goat Serum (GS, Gibco) in a 1% Bovine Serum Albumin (BSA, Sigma-Aldrich) solution was applied for 1 h at RT. Following this, the cells were washed again with PBS (ThermoFisher Scientific) and incubated with primary antibodies (Table 1) overnight at 4°C. Next, the cells were washed in PBS (ThermoFisher Scientific) and incubated with secondary antibodies (Table 2) in the dark for 1 h, at RT. The next step involved mounting the coverslips with Fluoromount-G with DAPI (ThermoFisher Scientific). The staining protocol for neurospheres and OHC slices was similar, but a blocking mixture was prepared using 6% BSA solution (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich) in PBS (Sigma-Aldrich). Moreover, the neurospheres were stained with 1% Hoechst 33,342 (Sigma-Aldrich) solution for 30 min to visualize the cell nuclei. The stained neurospheres were transferred to the surface of the microscope slide and a single drop of the mounting medium was applied to then close it with a 14 mm-diameter coverslip. The slides were left at RT for overnight drying. To assess cell survival, migration, differentiation, and local immune response *in vivo*, immunostaining procedures, as outlined above were performed. The presence of specific markers, such as Ki67 for proliferation, and Nestin, GFAP for neural differentiation, was determined. The host immune response was analyzed using goat polyclonal anti-ionized calcium-binding adapter molecule 1 (Iba1) antibody for microglia. Additionally, the presence of transplanted NSCs in rat brain tissue was investigated using anti-nuclear mitotic apparatus protein (anti-mitochondria antibody-a-mit). The brain sections were permeabilized and blocked using 6% BSA solution (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich) in PBS (Sigma-Aldrich) for 90 min at RT. After washing in PBS, the slices were incubated with primary antibodies overnight at 4°C. Then, they were washed in PBS again and secondary antibodies were incubated for 1 h. 1% of Hoechst 33,342 (Sigma-Aldrich) solution was used to visualize the cell nuclei. The slices were closed with the mounting medium (Dako).

For each variant, a negative control to eliminate non-specific reactions was executed. The results were analyzed using an LSM 780 confocal microscope (Carl Zeiss). For quantitative analysis, the calculation of positively stained 2D culture cells relative to the entire population was conducted using ZEN 2 Blue Edition software (Carl Zeiss). At least three repetitions were made for each experimental variant.

2.2.2 CMFDA (5-Chloromethylfluorescein diacetate) staining

NSC migration on the hippocampal slices *ex vivo* was visualized by a previous 30-min incubation at 37 °C with 10 mM of green CMFDA (abcam). Next, NSCs suspended in PBS were transferred onto the slices and analyzed after 7, 14, and 21 days using fluorescent microscopy with Zeiss Axiovert A.1 fluorescent microscope (Carl Zeiss) and LSM 780 confocal laser scanning system and ZEN software (Carl Zeiss).

2.2.3 qRT-PCR analysis

The gene expression analysis was performed with the use of qRT-PCR analysis. Total RNA was isolated from the cells using fenozol, according to the protocol instructions (A&A Biotechnology, Gdansk, Poland). The purity of each sample and RNA concentration was measured by NanoDrop ND-1000 (Thermo Scientific, Thermo Fischer Scientific). Then, the cDNA was obtained by reverse transcription conducted with a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Thermo Fischer Scientific). cDNA probes were diluted in RNase-free H_2O (Sigma-Aldrich) and stored at −20°C until further use. Quantitative RT-PCR reaction was performed on Fast 7,500 Thermocycler (Applied Biosystems) with 10 ng of cDNA in 15 µL with the use of 3-color RT HS-PCR SYBR Green Master Mix (Applied Biosystems) and specific primers for *KI67* (proliferation) *MAP2, GFAP, NESTIN, NG2,* and *β-TUBULIN-III (*neural differentiation) with *RPLP0* as a reference gene (Table 3). The final relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method which was described previously (Figiel-Dabrowska et al., 2021; Rybkowska et al., 2023).

2.2.4 Luminex cytokine and chemokine assay

To investigate the concentration of the selected analyte in the medium samples, Twelve-plex Human Magnetic Luminex Assays (R&D Systems, cat. no. LXSAHM-12) were used. We analyzed the following variants of the culture medium, at selected time points (24 h, 7 days, or 14 days of experiment).

- -control–NSCs cultured in the standard medium
- -CSF- NSCs cultured in standard medium with 30% of CSF addition
- -NSC OGD–NSCs in standard medium co-cultured with OHC after OGD
- -NSC CSF OGD- NSCs cultured in standard medium with 30% of CSF addition co-cultured with OHC after OGD

In addition, the media without cells were analyzed. The concentration of Angiogenin, BDNF, CCL2/JE/MCP-1, FGF basic/FGF2/bFGF, EGF, HGF, VEGF, VEGF-C, HGF, ICAM-1, LIF and beta-NGF was investigated using Luminex-based platform and Luminex 200 IS V2.1 Software (Bio-Rad, Hercules, CA, USA). Standard curves were obtained from the reference cytokine gradient concentrations. All the samples were stored at −80 °C before the analysis. All procedures were made on ice. Each sample was frozen/thawed only once.

TABLE 1 Primary antibodies used for IF staining.

Antigen	Source	Isotype	Dilution	Manufacturer	Catalog number
GFAP	Rabbit polyclonal	IgG	1:400	Dako	Z0334
Ki-67	Rabbit polyclonal	IgG	1:200	Abcam	AB15580
β-Tubulin III	Mouse monoclonal	IgG2b	1:1,000	Sigma-Aldrich	T8660
A2B5	Mouse monoclonal	IgM	1:200	Millipore	MAB312R
Nestin	Mouse monoclonal	IgG1	1:500	Millipore	MAB5326
NG2	Rabbit polyclonal	IgG	1:150	Millipore	AB5320
SOX2	Rabbit polyclonal	IgG	1:150	Sigma-Aldrich	SAB5700644
S100β	Rabbit polyclonal	IgG	1:100	Abcam	AB52642
a-mit	Mouse monoclonal	IgG1	1:100	Abcam	AB92824
MAP2	Mouse monoclonal	IgG1	1:1,000	Sigma-Aldrich	N4403
Iba-1	Goat Polyclonal	IgG	1:400	Abcam	AB5076

TABLE 2 Secondary antibodies used for IF staining. The presented dilutions were half-reduced for immunohistochemical stainings.

Antigen	Fluorochrome	Isotype	Dilution	Manufacturer	Catalog number
Alexa Fluor Goat (anti-rabbit)	Alexa 488	IgG	1:1,000	Life Technologies	A11034
Alexa Fluor Goat (anti-rabbit)	Alexa 546	IgG	1:1,000	Life Technologies	A11035
Alexa Fluor Goat (anti-mouse)	Alexa 546	IgM	1:1,000	Life Technologies	A21123
Alexa Fluor Goat (anti-mouse)	Alexa 546	IgG2b	1:1,000	Life Technologies	A21143
ALEXA FLUOR GOAT (ANTI-RABBIT)	Alexa 647	IgG	1:1,000	Life Technologies	A21245
ALEXA FLUOR (ANTI-GOAT)	Alexa 546	IgG	1:1,000	Life Technologies	A11056

2.3 Statistical analysis

The data are presented as the mean ± SD for n ≥ 3. One-way analysis of variance (ANOVA test) was used to conduct multi-group comparisons, followed by the Tukey test as *post hoc* statistical analysis for each group. The values were considered significant with $p < 0.05$. All statistical analyses of the raw data were performed using GraphPad Prism9.

3 Results

3.1 Analysis of neurogenic and secretory potential of hNSCs in response to the presence of CSF

To evaluate the proliferation rate of hNSCs, cells were cultured under standard 2D conditions (medium without growth factors, serving as the control) and treated with either 30% cerebrospinal fluid (30% CSF) or 100% CSF throughout the experiment (Figure 4a). The proliferation analysis revealed a decrease in the 100% CSF group after 7 days (183.8% ± 6.4%), whereas the proliferation rates for the control and 30% CSF groups remained similar (30% CSF: 199.4% ± 9.3%; control: 204.6% ± 12.1%). Thus, the CSF treatment can decrease the proliferation rate of hNSCs.

Next, we investigated whether CSF influences the migratory function of NSCs. Following the removal of the insert-made barrier, cell migration into the space was measured over 48 h (Figure 4e). The control group consistently showed the lowest scratch coverage at each time point (2 h: 9.8% ± 7.3%; 24 h: 42.9% ± 8%; 48 h: 43.6% ± 8.7%) (Figure 4b). Starting from the 2-h time point, the 100% CSF group demonstrated the highest coverage (21.3% ± 5.3%) compared to the 30% CSF group (19.8% ± 9.1%) and the control. This trend became more pronounced after 24 h (100% CSF: 63.8% ± 8.8%; 30% CSF: 54.7% ± 9.3%). Similarly, after 48 h, scratch coverage was approximately 60.8% ± 4.7% for the 30% CSF group and 76.8% ± 6.1% for the 100% CSF group.

TABLE 3 Primer sequences used with qRT-PCR.

Gene	NCBI reference Sequence	Product size	Primer sequence (5′ -> 3′)
RPLP0	NM_001002.4	1,229 bp	F: 5′AAAATCTCCAGGGGCACCATT3′ R: 5′GCTCCCACTTTGTCTCCAGT3′
KI67	NM_002417	12,507 bp	F: GAAAGAGTGGCAACCTGCCTTC R:GCACCAAGTTTTACTACATCTGCC
MAP2	NM_001375545.1	99 bp	F: TTGGTGCCGAGTGAGAAGA R: GTCTGGCAGTGGTTGGTTAA
GFAP	NM_001363846.2	100 bp	F: CCGACAGCAGGTCCATGT R: GTTGCTGGACGCCATTG
NESTIN	NM_006617.2	64 bp	F: GGGAAGAGGTGATGGAACCA R: AAGCCCTGAACCCTCTTTGC
NG2	NM_001897.5	118 bp	F: GTCTACACCATCGAGCAGCC R: TGTGTGAGAACAGCACGAGC
β-TUBULIN-III	NM_001197181.2	126 bp	F: GGAAGAGGGCGAGATGTACG R: GGGTTTAGACACTGCTGGCT

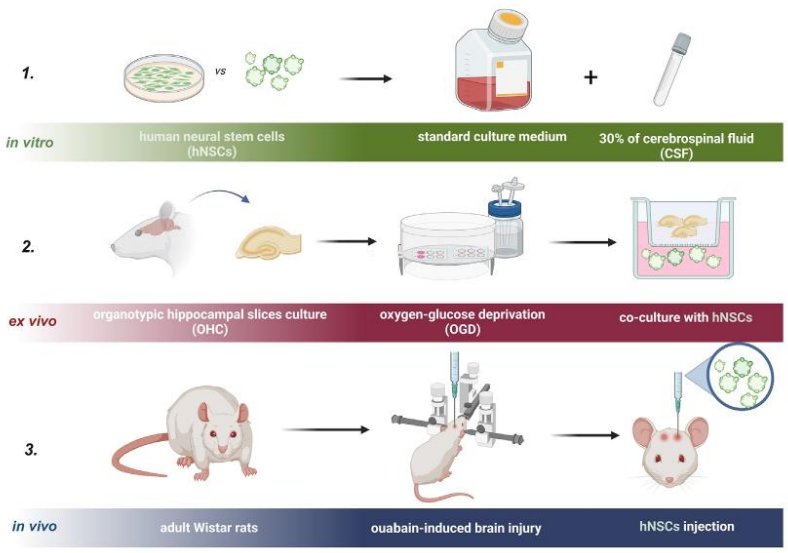

FIGURE 3
Experimental design of the study is presented in three steps. Step one: *in vitro*, step two: *ex vivo*, and step three: *in vivo* experiment.

Additionally, we examined neurite outgrowth in the scratch area at this time point, finding the highest neurite density in the 100% CSF group (100% CSF: 11.77 ± 3.85; 30% CSF: 9.1 ± 2.2; control: 7.23 ± 2.48) (Figures 4c, d). In summary, it seems that CSF can significantly enhance the migratory function and neurite outgrowth of neural stem cells, with the 100% CSF group showing the most visible effects. However, it's worth noting that the cells treated with 100% CSF started to strongly adhere to each other, prolonging their shape and detaching from the surface. Taken together with the worsened proliferation rate, consequently, the 100% CSF variant was eliminated and both the control and 30% CSF group (hereafter referred to as CSF) were further analyzed for neural and proliferation markers presence, migratory activity, and differences in their secretory profiles.

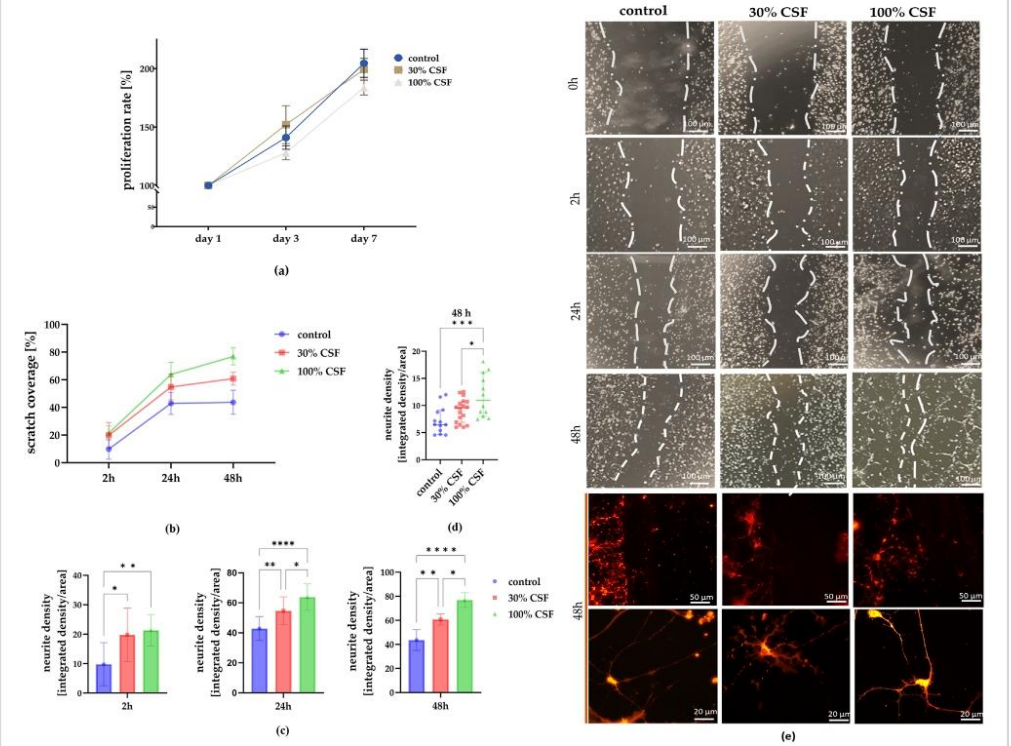

FIGURE 4
(a) The impact of CSF on hNSC proliferation rate (n = 5) after days 1, 3, and seven of culture. **(b)** Determination of neurite outgrowth from scratch injury after 0, 2, 24, and 48 h. Scratch coverage for all culture variants after 2, 24, and 48 h from the injury. **(c)** Changes in scratch coverage between the variants. **(d)** Neurite density after 48 h in all experimental variants. n = 12-18 **(e)** Representative images of NSCs cultured in standard medium (control), medium with 30% addition of CSF, and 100% of CSF. After 48h, the cells were stained using a Neurite Outgrowth Kit. The results of all presented experiments are expressed as mean values ±SD. The differences were considered statistically significant when p-value <0.05. Statistical significance level * for $0.01 < p < 0.05$;** for $0.001 < p < 0.01$;**** for $p < 0.0001$;*** for $0.0001 < p < 0.001$;**** for $p < 0.0001$.

On the protein level, in 2D culture, after 7 days, immunofluorescence staining revealed significant changes in marker expression (Figures 5a, c). The early neural marker NESTIN showed a significant decrease in the CSF group (70.6% ± 5%) compared to the control group (82.1% ± 6.6%). Similarily, we noticed a decrease in SOX2, neural stem and progenitor marker presence for the CSF culture (CSF: 41,5 ± 5.5%, control: 57% ± 10%). Conversely, the early neuronal marker β-TUBULIN III increased markedly in the CSF group (23.4% ± 6.1%) relative to the control (15.4% ± 3.1%). However, the neuronal marker MAP2 was significantly lower in the CSF group (9.3% ± 3.4%) compared to the control (17% ± 4.2%). Furthermore, NSC treatment with CSF resulted in a significant upregulation of GFAP, an astrocytic marker (CSF: 6.9% ± 5.8%; control: 11.2% ± 3.6%). No significant changes were observed in the presence of A2B5+ cells (a marker of NSCs able to differentiate into glia) or KI67+ cells (a marker of proliferation) between the treatment variants. CSF treatment in 2D culture significantly alters the expression of various neural markers, indicating further cell differentiation.

On the mRNA level, the qPCR evaluation of relative gene expression demonstrated results consistent with the observed protein level differences (Figure 5b). Compared to the control group, CSF treatment led to a significant increase in the expression of *GFAP* (52.17 ± 2.54 fold) and *β-TUBULIN III* (9.71 ± 0.93 fold) in hNSCs. Conversely, there was a notable decrease in the expression of *NESTIN* (0.22 ± 0.06 fold) and *MAP2* (0.49 ± 0.04 fold). Additionally, CSF treatment resulted in the downregulation of *NG2*, an oligodendrocyte marker (0.31 ± 0.02 fold), and a similar reduction was observed in *KI67* expression (0.28 ± 0.09 fold) compared to the control. The observation in the CSF group regarding the decreased proliferative potential based on *KI67* expression was also confirmed by their lowered proliferation rate. Thus, CSF significantly modifies the gene expression of hNSCs, enhancing astrocytic and early neuronal differentiation while

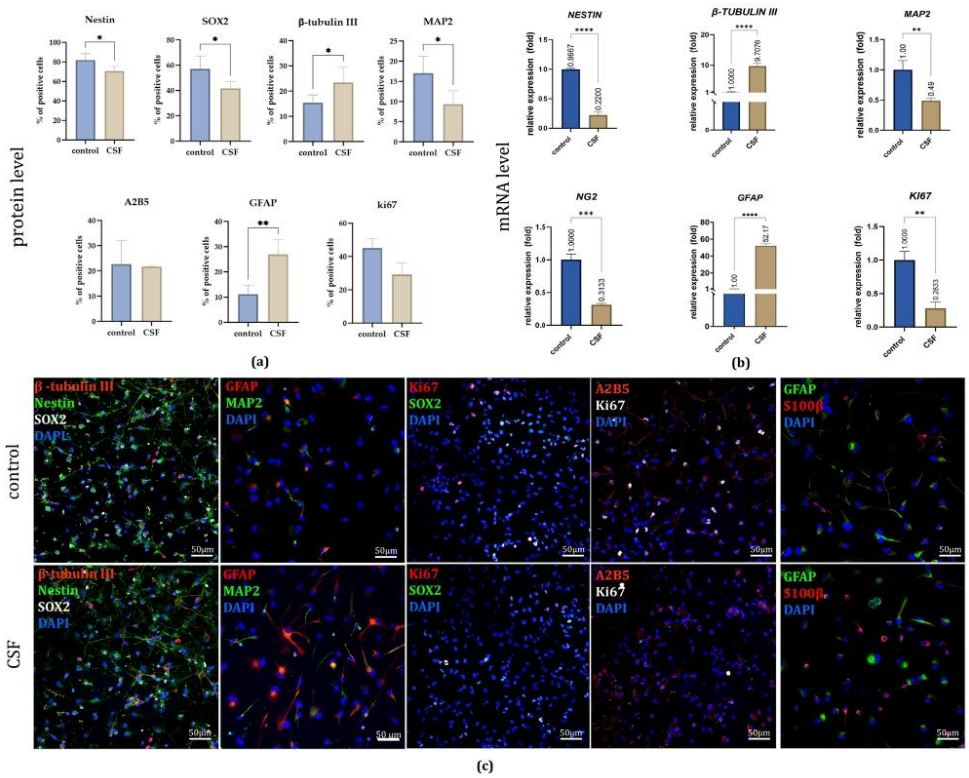

FIGURE 5
(a) The effect of CSF on NSC differentiation and proliferation on day 7. Quantitative analysis of neural lineages and proliferation markers presence. n = 5 **(b)** Relative expression of neural and proliferation genes with RPLP0 as a reference gene and NSC cultured in a standard medium without growth factors as the control group, considered as 1, on day 7. **(c)** Representative images of neural lineages and proliferation markers presence in all experimental variants. The results are presented as mean values of three experiments ±SD. Split-channel images are available in the Supplementary Files.*for $0.01 < p < 0.05$; **for $0.001 < p < 0.01$; ***for $0.0001 < p < 0.001$; ****for $p < 0.0001$.

reducing the expression of markers specific for oligodendrocytes, and proliferation.

To analyze the secretory potential of NSCs, we used cells cultured in 3D conditions, which are more representative of NSCs. This eliminates the impact of analytes present in the 2D coating. We investigated two culture variants (control and CSF) and performed an analysis of neural and proliferation markers expression (Figure 6).

The concentration of selected analytes in the pooled CSF and the media collected on the last day of the experiment were analyzed (Figures 6a, c). In the pooled CSF sample, we did not observe the presence of LIF, VEGF, or ICAM-1. However, there were remarkably high concentrations of angiogenin (9,242.67 pg/mL), CCL2 (519.12 pg/mL), VEGF-C (120.2 pg/mL), and HGF (130.42 pg/mL). Additionally, we detected bFGF (10.14 pg/mL), BDNF (12.51 pg/mL), EGF (3.22 pg/mL), GDNF (2.67 pg/mL), and beta-NGF (0.94 pg/mL).

Next, we analyzed the media collected after the experiment (Figure 6c). Comparative analysis of the investigated variants showed a significantly higher concentration of VEGF (2,635 ± 218.5 pg/mL) in the CSF-treated group compared to the control (1831 ± 227.7 pg/mL), as well as CCL2 (control: 2,189 ± 76.94 pg/mL; CSF: 5,482 ± 733.2 pg/mL). There were no significant changes in the concentration of angiogenin (control: 658.5 ± 44.84 pg/mL; CSF: 1,118 ± 283.3 pg/mL), VEGF-C (control: 34.02 pg/mL; CSF: 64.62 ± 17.89 pg/mL), beta-NGF (control: 0.14 ± 0.19 pg/mL; CSF: 0.5550 ± 0.78 pg/mL), BDNF (control: 73.76 ± 3.61 pg/mL; CSF: 111.5 ± 28.12 pg/mL), GDNF (control: 0.6400 ± 0.45 pg/mL; CSF: 1.915 ± 0.91 pg/mL), ICAM-1 (control: 13,341 ± 4,205 pg/mL; CSF: 10,361 ± 722.9 pg/mL), bFGF, EGF, and LIF.

In 3D culture, on the mRNA level, we found significant differences in *NESTIN* and *β-TUBULIN III* expression between the control and CSF groups (Figure 6b). Unlike in the 2D culture,

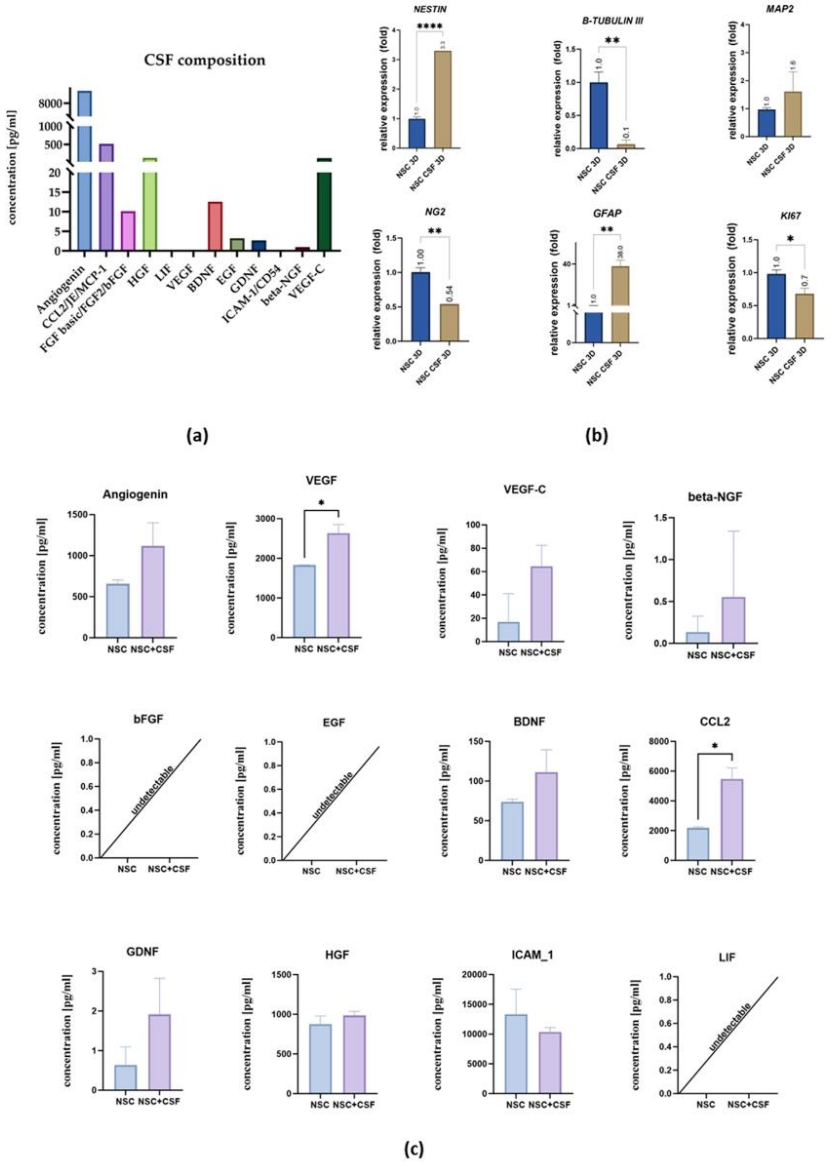

FIGURE 6
(a) CSF composition. **(b)** Relative expression of neural and proliferation on day 7 with RPLP0 as a reference gene and NSC cultured in a standard medium as the control group, considered as 1. **(c)** Cytokines concentration in the medium collected from control cell culture and CSF. The results of all presented experiments are expressed as mean values of three experiments ±SEM. The results are presented as mean values of three experiments ±SD,* for $0.01 < p < 0.05$; for $p < 0.05$;** for $0.001 < p < 0.01$;*** for $0.0001 < p < 0.001$;**** for $p < 0.0001$.

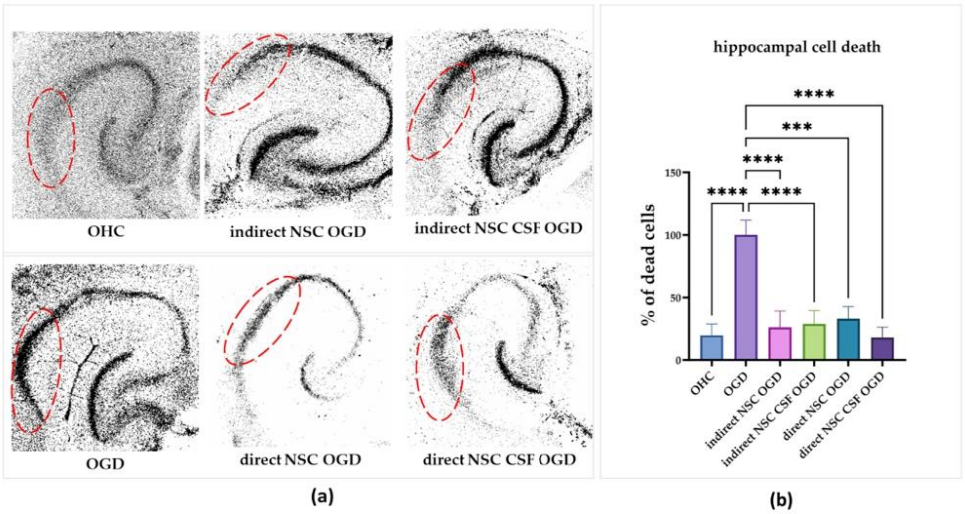

FIGURE 7
(a) The effect of indirect co-culture of hippocampal slices with NSC and NSC CSF on hippocampal cell death. PI incorporation into the CA1 region of the hippocampal slices (the region is marked red). **(b)** Quantitative analysis of effects of direct and indirect co-culture of hippocampal slices after OGD with NSC and NSC CSF on cell death in the CA1 zone. The results of all presented experiments are expressed as mean values ±SD. The differences were considered statistically significant when p-value <0.05. Statistical significance level ****for $p < 0.0001$.

NESTIN expression was upregulated in the CSF group (3.3-fold), whereas *β-TUBULIN III* was downregulated (0.065 ± 0.064 fold) compared to the control. For other markers, the trends were similar to those observed in the 2D culture. Most notably, *GFAP* expression was significantly higher in the CSF group (38.03 ± 5.49 fold) than in the control. Both *NG2* and *KI67* were downregulated in the CSF group (0.54 fold and 0.68 ± 0.085 fold, respectively) compared to the control. There were no significant differences in the expression of *MAP2* between the two variants. Thus, in 3D culture, CSF treatment induces changes in gene expression among hNSCs, including upregulation of neural and astrocytic markers, and downregulation of early neuronal, oligodendrocytic, and proliferation markers compared to the control conditions.

3.2 Analysis of neurogenic and neuroprotective potential of hNSCs pretreated with CSF in the presence of rat organotypic hippocampal slice culture

The neuroprotective potential of NSCs was investigated after 24 h for both direct and indirect co-culture of control and CSF-treated cells with OGD-treated OHC (Figure 7).

Initially, we assessed the CSF and control cell survival after OGD treatment in the CA1 region of the hippocampus. The hippocampi without cell co-culture which were OGD-treated revealed the mortality of most neurons in the CA1 region after 24 h, establishing 100% cell death as the baseline (Figures 7a, b). We discovered that the co-culture with all presented variants, whether it was direct or indirect, led to a significant reduction in cell death in the CA1 hippocampal region. The greatest decrease was observed with direct co-culture (29% ± 10.4%) and indirect co-culture (18% ± 8.3%) with both CSF-treated groups. For the control group, cell death was reduced to 26% ± 13.2% with direct co-culture and 33% ± 10% with indirect co-culture. Thus, co-culture with both cell variants significantly mitigated cell death in the CA1 hippocampal region after OGD treatment, with direct and indirect co-culture showing substantial neuroprotective effects.

Next, we analyzed the localization and survival of directly transplanted cells after 7, 14, and 21 days. The first one was assessed using CMFDA-stained cells. For both CSF and control groups, we noticed the migration and incorporation of cells to the most sensitive to damaged areas of the hippocampi through all experimental time points (Figure 8). Moreover, we investigated the colocalization of selective human-reactive anti-Nestin antibody with anti-ki67 antibody. A high presence of Nestin + Ki67+ cells, as well as hippocampal Ki67+ cells was observed for both variants up to 14 days of culture. Thus, previous CSF treatment facilitated the migration and integration of transplanted cells into damaged hippocampal regions and supported early proliferation up to 14 days, as evidenced by Nestin + Ki67+ cells presence. However, there was a notable loss of Ki67 expression on day 21, indicating a decrease in hippocampal and cell proliferation in both experimental groups. Consequently, the indirect co-culture experiments were conducted for up to 14 days.

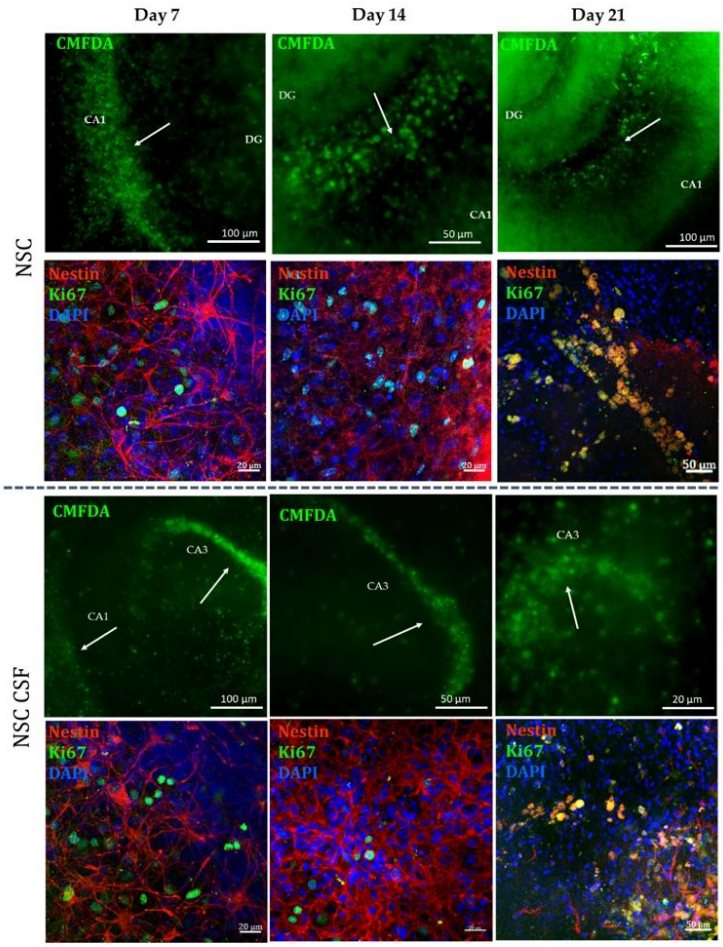

FIGURE 8
NSC localization in indirect co-culture with OGD-treated OHC after 7, 14 and 21 days of the experiment. NSC was stained with CMFDA (green) and human-reactive Nestin (red), while the proliferative cells were stained with KI67.

We investigated the concentrations of selected analytes in the media collected after 24 h, 7 days, and 14 days of indirect co-culture (Figure 9). Determining the exact secretory activity of cells cultured directly on hippocampal slices is currently beyond our technical capabilities, as the cells are incorporated into the hippocampal tissue. Therefore, the measured concentrations do not precisely reflect the cells' secretion.

Overall, for all analytes, the concentration was significantly higher in the CSF-treated groups compared to the control (Figure 9). After 24 h, the concentrations of bFGF and EGF were elevated, with significantly higher levels in the CSF group (bFGF: 341.4 ± 27 pg/mL; EGF: 161.2 ± 7.3 pg/mL) compared to the control (bFGF: 211.3 ± 4.5 pg/mL; EGF: 104.7 ± 7.5 pg/mL). A significant increase in angiogenin concentration was also observed in the CSF group (92.1 ± 4.8 pg/mL). This increase was even more pronounced after 7 days, with angiogenin levels rising to 324.2 ± 3.408 pg/mL in the CSF group compared to 72.58 ± 2.178 pg/mL in the control. While bFGF and EGF concentrations decreased after a week, we noticed significantly elevated levels of VEGF (control: 510.5 ± 15.85 pg/mL; CSF: 2,803 ± 140.1 pg/mL), BDNF (control: 14.98 ± 3.3 pg/mL; CSF: 107.8 ± 17.2 pg/mL), HGF (control: 202.5 ± 41.7 pg/mL; CSF: 780.2 ± 197.4 pg/mL), and ICAM-1 (control: undetectable; CSF: 9,987 ± 3,314 pg/mL). This trend continued after 14 days for angiogenin (control: 113.4 ± 12.4 pg/mL; CSF: 315 ± 3.4 pg/mL), VEGF

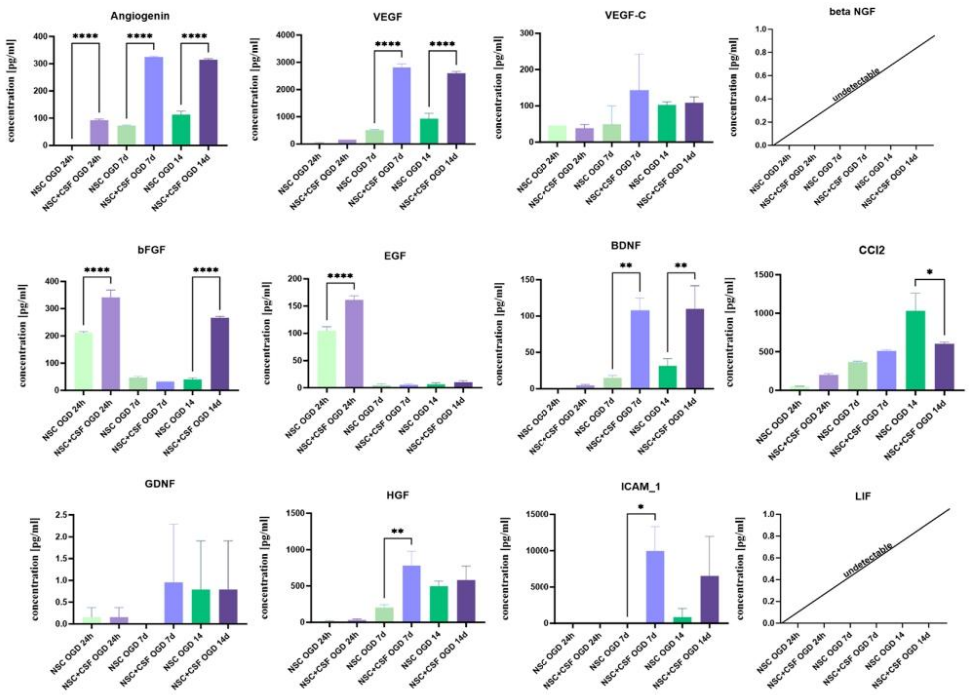

FIGURE 9
Cytokines concentration in the medium collected after co-culture of OGD-treated OHC with CSF-treated NSCs. The results are presented as mean values of three experiments ±SD. The differences were considered statistically significant when p-value <0.05. Statistical significance level * for $0.01 < p < 0.05$;** for $0.001 < p < 0.01$;**** for $p < 0.0001$.

(control: 936.2 ± 191.8 pg/mL; CSF: 2,597 ± 65.2 pg/mL), and BDNF (control: 31.69 ± 9.8 pg/mL; CSF: 110.0 ± 31.8 pg/mL). Additionally, CCL2 concentration was markedly higher in the control group (control: 1,031 ± 228.1 pg/mL; CSF: 602.3 ± 23.5 pg/mL), while bFGF was raised in the CSF group (control: 40.02 ± 6 pg/mL; CSF: 266.8 ± 4.7 pg/mL). The concentrations of both LIF and beta-NGF were undetectable. Thus, it seems that CSF can significantly increase the concentrations of various analytes at different time points, highlighting its regulatory effect on the secretion of neurotrophic and inflammatory cytokines.

At the mRNA level, we found contrary results for *NESTIN*, *GFAP*, and *KI67* expression between control and CSF-treated cells co-cultured with OGD-treated OHC (Figure 10b). *NESTIN* expression was upregulated in the NSC OGD group (1.42 ± 0.2 fold), but downregulated in the NSC CSF OGD culture (0.37 ± 0.04 fold). However, *β-TUBULIN III* was downregulated in NSC OGD (0.003 ± 0.006 fold), with no significant changes observed in the CSF-treated culture. Interestingly, *GFAP* expression was 41-fold higher in NSC co-cultured with OGD (40.55 ± 10.34 fold) with no remarkable change seen in CSF-treated NSCs. *KI67* expression was significantly upregulated in the NSC OGD group (8.08 ± 2.6 fold) while it was downregulated in the NSC CSF OGD group (0.16 ± 0.09 fold) compared to the control. There were no significant differences in *MAP2* expression between the variants. These observations were confirmed by immunofluorescence analysis of the same markers (Figure 10a). The results suggest that CSF may modulate certain gene expression related to neural differentiation and proliferation in NSCs, changing the final effect specific to overall experimental conditions.

3.3 Analysis of survival, proliferation, and anti-inflammatory potential of hNSCs pretreated with CSF after transplantation into focally injured rat brain

The *in vivo* step of this study was performed to assess the survival, migration, and differentiation of the hNSCs following the intracerebral injection after ouabain-induced stroke in rats. The ouabain injury model mimics the focal brain injury. Ouabain as an inhibitor of Na/K-ATPase, causes cellular energy deprivation upon intracerebral delivery, mimicking a stroke-like cascade of events [31-32] and neuroinflammation. After the cell injection, none of the examined rats displayed any adverse symptoms, such

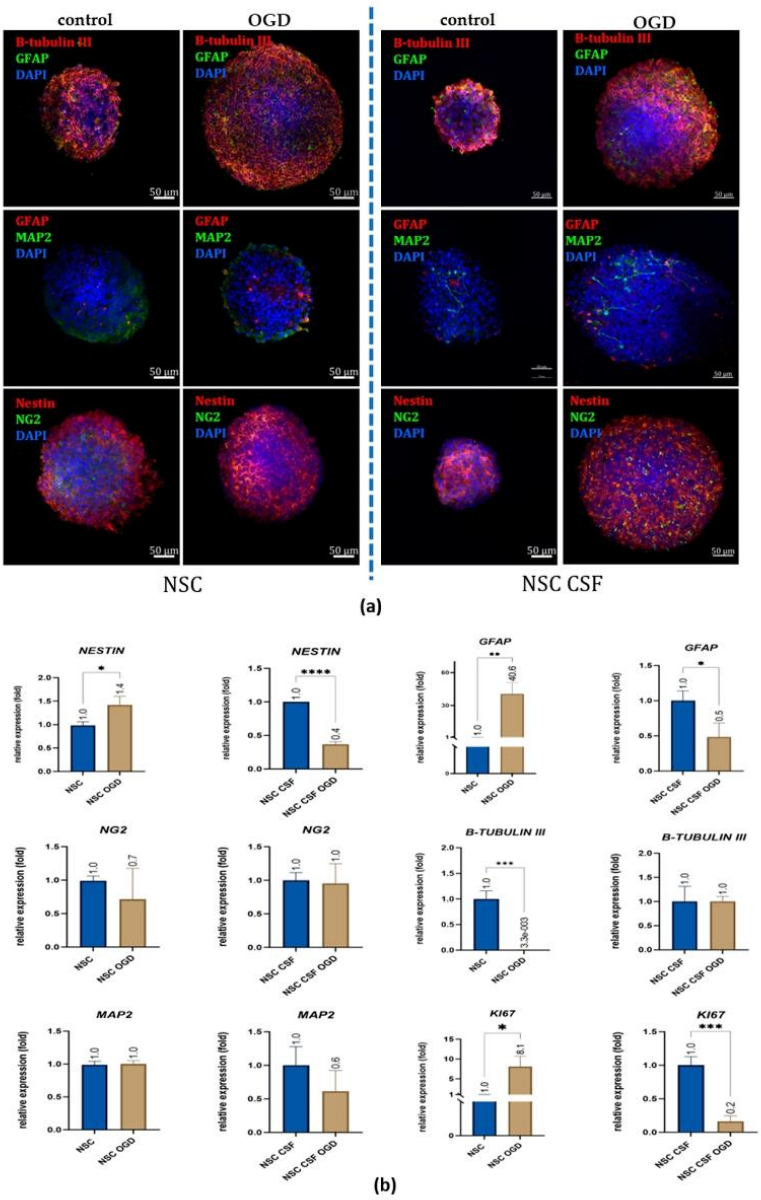

FIGURE 10
The effect of CSF and co-culture with OGD-treated OHC on NSC differentiation. **(a)** Representative images of IF staining analysis of neural markers presence. Split-channel images are available in the Supplementary Files. **(b)** Relative expression of neural and proliferation with RPLP0 as a reference gene and NSC cultured in a standard medium as the control group. The results are presented as mean values of three experiments ±SD,* for $0.01 < p < 0.05$; for $p < 0.05$;** for $0.001 < p < 0.01$;*** for $0.0001 < p < 0.001$;**** for $p < 0.0001$.

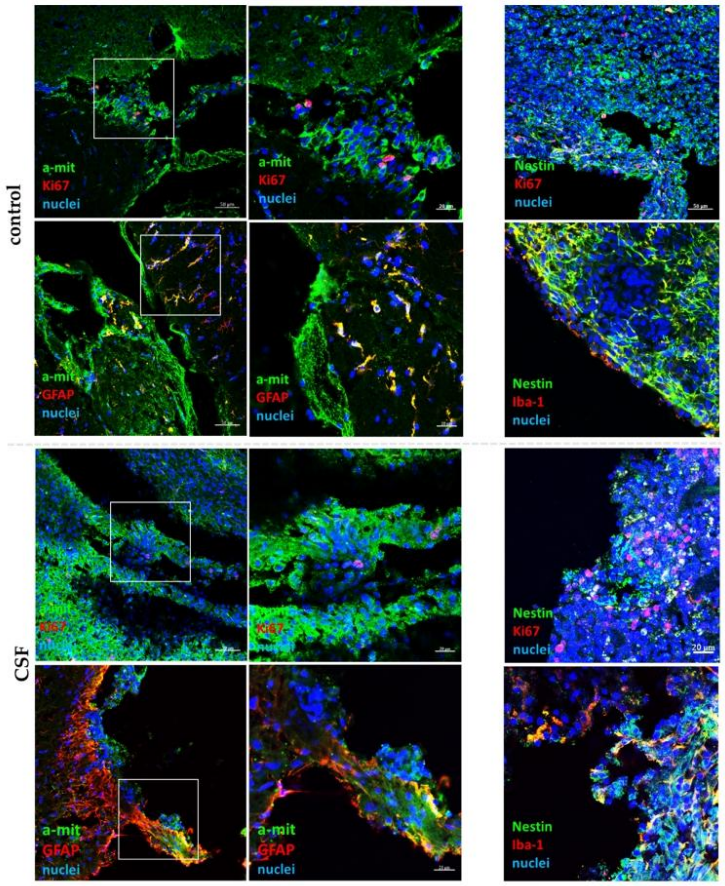

FIGURE 11
Confocal representative images of hNSC grafts *in vivo* (n = 6) in the damaged area after 7 days from transplantation. The insets present a magnification of a-mit-labeled hNSCs (green). HNSCs are stained with a-mit or Nestin (green), while the cell nuclei are labeled with Hoechst (blue).

as behavioral changes or signs of distress, indicating that the transplantation procedure was well tolerated and did not induce acute negative effects.

To track and identify the transplanted cells, we used immunofluorescence analysis with antibodies specifically targeting human mitochondria (a-mit) and neural stem cell marker (Nestin). This enabled us to distinguish the human cells from the rat cells and monitor their behavior post-transplantation. The graft core was identified at the delivery site, in the stroke area (Figure 11). This suggests that the cells remained localized to the targeted area.

After 7 days, the survival of transplanted cells was significantly reduced compared to the initial number of injected cells. This reduction is likely due to a stimulation of inflammatory response. Treatment with CSF did not improve cell survival; qualitatively, it even appeared to exacerbate this, potentially due to the human origin of the CSF. We observed a low presence of a-mit + KI67+ cells, indicating limited proliferation of the transplanted hNSCs in both the control and CSF-treated groups suggesting unfavorable conditions for these cells. Most of the a-mit + cells were also positive for GFAP, a marker for astrocytes, indicating that the transplanted hNSCs were differentiating predominantly into astrocytes. Despite the significantly improved differentiation of cells treated with CSF *in vitro*, the pre-treated hNSCs did not exhibit such differentiation potential *in vivo*, which was also observed *ex vivo*. Additionally, we noted a high expression of Iba-1+ cells, which indicates activated microglia. The presence of activated microglia suggests an ongoing immune response, likely due to the injury and the presence of human-origin cells.

Overall, these findings highlight several challenges in the survival, proliferation, and differentiation of transplanted cells, especially in the context of using CSF treatment. This underscores the complexity of translating *in vitro* outcomes to *in vivo* applications, particularly in the presence of inflammatory responses and other unfavorable conditions.

4 Discussion

Every year, hundreds of reports emerge detailing newly discovered properties and potential mechanisms of action of NSCs in various diseases, expanding the scope of using them in clinical applications. Recognizing the pivotal role of providing NSCs with proper nutrients and the neural niche-NSC interplay, this article shifts the focus to a step into the future perspective, i.e., what happens to intrathecally transplanted NSCs, when they come into contact with the CSF.

4.1 Analysis of neurogenic and secretory potential of hNSCs in response to the presence of CSF

Through recent years, it has been established that CSF composition and function undergo dynamic changes throughout development, reflecting the current needs of the CNS (Gato et al., 2014; Bueno and Garcia-Fernàndez, 2016). This plays a vital role both in neurogenesis and neuropathologies (Radoszkiewicz et al., 2023a). On the other hand, the effect of this fundamental element of the brain niche on transplanted NSCs is still relatively unexplored, especially when it comes to the studies on human CSF and hNSCs (Radoszkiewicz et al., 2023a). Due to our knowledge, there is no data regarding using such concentrations of human CSF on human NSCs. Thus, we took a deeper examination *in vitro.* Our findings underscore the impact of hCSF on hNSC differentiation. Previously, Ma and coworkers presented that in NSCs originated from rat fetuses, exposition to human adult CSF resulted in glial differentiation (Ma et al., 2013). We confirmed this observation on hNSCs for both 2D and 3D cultures, where the expression of GFAP was highly upregulated. Similar insights were noted in the studies on CSF from diseased patients. For example, Buddensiek and his group reported that the human CSF obtained from patients with idiopathic normal pressure hydrocephalus promoted astrogliogenesis of hNSCs (Buddensiek et al., 2010). Similarly to our results, the group noted low differentiation abilities into mature neuronal lineage based on MAP2 expression. Nevertheless, in 2D culture, we observed a significant decrease in the expression of the early neural marker *NESTIN* upon exposure to CSF, accompanied by a remarkable increase in the early neuronal marker *β-TUBULIN III*. This effect, along with the reduced presence of NESTIN+ and SOX2+ cells, suggests a potential shift towards differentiation into neuronal lineage under CSF influence. Such dependency was noted by Henzi's group on rat NSCs and rat CSF (Henzi et al., 2018). However, for 3D culture the expression of *NESTIN* raised after CSF treatment, while *β-TUBULIN III* was downregulated. As the expression of *NESTIN* is known to be greater in neurospheres than in the monolayer while β-TUBULIN III + cells are more frequent in 2D culture (Zheng et al., 2006), it suggests that CSF can remarkedly boost this effect and presumably strengthen the discrepancies between these two culture methods. The augmentation of *NESTIN* expression in neurospheres suggest the enhancement of NSC self-renewal and proliferative capacity in response to CSF signals (Bernal and Arranz, 2018). Indeed, we found a lower reduction of *KI67* expression in 3D culture than in the monolayer. To sum up, the differentiation effect of CSF on NSC behavior seem to be dependent not only of CSF itself, but also of cell culture methods, cell developmental origin, and microenvironmental conditions.

Moreover, CSF seems to have a great impact on NSC migratory capacity. We observed that while initial migration kinetics in scratch assay revealed enhanced scratch coverage in CSF-treated groups, this effect diminished over time and was weaker for lower CSF concentration, suggesting a dynamic modulation of NSC motion by CSF gradients. The analysis of neurite outgrowth in the scratch area further underscores this observation. Such effect has been reported previously on murine mesenchymal stromal/stem cells derived from bone marrow and rat CSF, even with using only 10% of CSF (Shokohi et al., 2017). It has also been shown that human CSF can influence fetal neural progenitor cell migration, supported by higher C-X-C chemokine receptor type 4 (CXCR4) expression (Zhu et al., 2015). The authors suggested this effect can be caused by insulin-like growth factor-1 (IGF-1), found in human CSF. Therefore, preincubation of hNSCs with CSF may augment their ability to migrate to pathological areas and increase their therapeutic potential, which is currently one of the limitations of neurological stem cell therapy.

Our investigation into the secretory potential of NSCs revealed a specific secretory profile of selected analytes in response to CSF exposure. While the pooled CSF exhibited high concentrations of both angiogenic and neurotrophic factors, CSF-treated NSCs displayed remarkably elevated secretion of VEGF and CCL2 compared to the control. This potentially suggests a role of CSF in modulating NSC secretome, which could be also correlated with migration capacity. It has been reported that the interaction between CCL2 and its receptor, CCR2, mediates the intravascular recruitment of NSC delivered through the vascular route to the ischemic brain, directing the migration of transplanted cells to the site of damage (Andres et al., 2011).

4.2 Analysis of hNSCs pretreated with CSF properties under *ex vivo* and *in vivo* conditions

The *in vitro* findings suggesting different therapeutic profiles of CSF-treated NSCs inspired us to check their *ex vivo* and *in vivo abilities*. To date, this is the first study that provides such insights using these two models.

The observed reduction in cell death in the CA1 hippocampal region following direct and indirect co-culture with both control and CSF-treated NSCs confirms the neuroprotective properties of these cells. Notably, the greatest reduction in cell death was observed for direct co-culture with CSF-treated NSCs, suggesting a potentiation of neuroprotective effects by CSF treatment. This exhibits the importance of the CSF in modulating NSC-mediated

neuroprotection, potentially through the secretion of trophic factors and cytokines.

Furthermore, the localization and survival analysis of directly transplanted NSCs revealed their migration and incorporation into the damaged areas of the hippocampus, indicating their regenerative potential. The sustained presence of Nestin + Ki67+ cells up to 14 days post-transplantation suggests ongoing NSC proliferation within the hippocampal niche. However, the observed loss of Ki67 expression and NSC death by day 21 should be further investigated-it can be caused by the transient nature of NSC-mediated neuroprotection or a response to the toxic environment of the dead nerve tissue that culture was prolonged too extensively.

The results obtained *ex vivo* more than those obtained in the *in vitro* model suggest that preincubation with CSF significantly increases the secretory activity of NSCs upon contact with the damaged neural tissue. The findings revealed that CSF significantly modulates the concentrations of various neurotrophic and inflammatory cytokines in neural progenitor cells and NSCs. This regulatory effect is evident across different time points and highlights its potential role in enhancing neuroprotective and regenerative processes following neural injury. The reduction in the concentration of several analytes after 7 days compared to 24 h suggests a dynamic modulation of NSC secretory activity over time. It was noticed that growth factors, bFGF and EGF, were secreted immediately after damage, while most of the investigated cytokines began to be released a week after damage. Both bFGF and EGF are crucial for cell proliferation and survival, and their elevated levels suggest that CSF may enhance the initial regenerative response in neural progenitors and NSCs. This is consistent with previous studies demonstrating the roles of these factors in promoting neurogenesis and tissue repair (Radoszkiewicz et al., 2023b). The observed in our study significant increase in angiogenin and BDNF concentration after 7 days highlights the role in sustaining long-term neurogenic and angiogenic processes. Angiogenin has been shown to activate neurogenesis in the subventricular zone and support neuronal survival, aligning with the elevated levels detected in CSF-treated cultures (Subramanian et al., 2008; Steidinger et al., 2011) while BDNF was reported to produce a neuroprotective effect, promoting neuronal survival, synaptic plasticity, and neurogenesis, crucial in post-injury phase (Schäbitz et al., 2004; Chen et al., 2013). Interestingly, CCL2 levels were higher in the control group compared to the CSF-treated group. Previous studies on the impact of CCL2 on the proliferative potential and neurogenesis of endogenous NSC in a mouse model of Niemann-Pick type C disease showed a significant increase in the ability for self-renewal, proliferation, and differentiation of neurons. Moreover, it was observed that the injection of CCL2 into the mouse brain resulted in a reduction of the inflammatory state in the nervous system (Hong et al., 2015). The delayed but sustained increase in HGF in CSF-treated cultures suggests a prolonged neuroprotective response. HGF is known for its anti-inflammatory and tissue-repairing properties, and its elevated levels align with our observed benefits of CSF treatment in promoting long-term recovery (Doeppner et al., 2011; Zhang et al., 2021). These results indicate that the contact of NSC with CSF may increase the chances of survival of endogenous NSCs and lead to a more effective regeneration of the nervous system. This confirms that preincubation of NSCs with CSF stimulates their neuroprotective nature.

Our study results also shed light on how CSF regulates gene expression in NSCs co-cultured with OGD-treated OHC. It revealed significant differences between the control and CSF-treated cells, particularly for markers such as *NESTIN, β-TUBULIN III, GFAP*, and *KI67*. The upregulation of *NESTIN* and downregulation of *β-TUBULIN III* in the CSF group compared to the control after indirect co-culture with OGD-treated OHC further support the notion of differential NSC response to the CSF. *NESTIN*, a marker for neural stem cells is typically associated with neural stem cell proliferation and undifferentiated state maintenance (Sakurada et al., 2008; Park et al., 2010; Brboric et al., 2019). Our findings indicate that *NESTIN* expression was upregulated in the NSC OGD group but downregulated in the CSF-treated NSC OGD group which suggests that while OGD conditions alone may promote a proliferative and undifferentiated state, the CSF could shift the balance towards differentiation and inhibit proliferation. This is followed by the downregulation of KI67 expression of CSF-treated NSCs co-cultured with OGD, indicating a CSF modulation on NSC proliferation. Moreover, *β-TUBULIN III*, a marker of early neuronal differentiation, was significantly downregulated in the NSC OGD group suggesting a suppression of neuronal differentiation under OGD conditions with no significant changes in its expression in the CSF-treated culture. However, the notable upregulation of GFAP expression in the NSC OGD group suggests potential astrocytic differentiation under CSF influence. Interestingly, CSF treatment did not result in a significant change in GFAP expression, indicating that CSF might help preventing excessive astrocytic proliferation and potentially detrimental gliosis. Thus, CSF can significantly alter the gene expression profile of NSCs under OGD conditions, promoting a possibly more favorable environment for neural repair and regeneration.

Furthermore, the *in vivo* experiments preliminarily demonstrated the efficacy of hNSC transplantation in a rat model of ouabain-induced stroke. The absence of adverse symptoms following cell injection, coupled with the successful identification of transplanted cells within the stroke area after 7 days, highlights the feasibility of utilizing hNSCs as a therapeutic intervention for stroke-induced brain injury in the future. Moreover, the ability of transplanted hNSCs to migrate to the targeted region underscores their potential for neural tissue repair and functional recovery. However, the *in vivo* environment also presented significant hurdles. One of the most notable observations was the markedly reduced survival rate of the transplanted cells, likely due to an inflammatory response triggered by the transplantation of human-origin cells to the rat brain. The inflammation created a hostile environment, leading to increased cell death. Indeed, the transplanted human cells were shown to have poor long-term survival in a xenobiotic environment, such as rat brains (Hovakimyan et al., 2012; Tierney et al., 2020). Additionally, both control cells and hNSCs pre-treated with CSF showed low proliferation. Moreover, while CSF treatment significantly enhanced cell differentiation *in vitro*, the pre-treated cells did not exhibit the same differentiation potential *in vivo*. However, the majority of transplanted, non-treated a-mit + hNSCs were also GFAP positive, indicating differentiation into astrocytes, which is consent with previous studies (Rota Nodari et al., 2010; Lee et al., 2015; De Gioia et al.,

2020). Astrocytes play several critical roles in the brain, including supporting neuronal function, maintaining the blood-brain barrier, and modulating synaptic activity (Siracusa et al., 2019; Gradisnik and Velnar, 2023). Thus, these cells might contribute to creating a supportive environment for neural repair and regeneration. However, they also elicit a microglial response, which needs to be carefully investigated. Future studies should focus on long-term tracking of these cells to better understand their potential for integration, proliferation, and differentiation of transplanted cells over time.

Taken together, this study highlights the potential of CSF to modulate NSC behavior, contributing to our understanding of brain function and disease mechanisms. *In vitro*, CSF treatment significantly influenced NSC proliferation, migration, and differentiation, with significant changes in protein and mRNA expression levels. *Ex vivo*, the analysis demonstrated that CSF maintained the neuroprotective potential of NSCs, influenced their proliferation and differentiation, and remarkably enhanced the secretion of various neurotrophic and inflammatory cytokines. *In vivo*, in a rat experimental stroke model, the transplanted NSCs showed targeted migration to the stroke-affected area without causing adverse effects. However, the study showed the limited survival and proliferation of these cells as well as the presence of activated microglia indicating an ongoing immune response. Further studies should focus on the extended survival, differentiation, and integration of these cells in the brain, as well as their impact on functional recovery and the host immune response over longer periods. This study lays the groundwork for developing new treatments for neurological disorders, potentially leveraging the unique properties of CSF to enhance NSC efficacy.

5 Conclusion

- CSF seems to have a vital role in inhibiting the proliferation and stemness properties while stimulating further differentiation of NSCs;
- CSF appears to change the secretory potential of NSCs, especially after contact with the damaged neural tissue;
- CSF contains factors that seem to modulate NSC behavior and support regeneration or even restoration of the damaged neural tissue;
- Following the ischemic injury, NSCs secrete factors that induce cell proliferation; after a longer period, they start releasing immunomodulatory, proangiogenic, and neuroprotective factors, which confirms our previous observations;
- NSCs exposed to human CSF presence exhibit decreased survival/proliferation and increased immunogenicity after intraparenchymal transplantation into the rat brain (xenograft) which is connected to additive immunogenic CSF impact;
- It is crucial to further study the interactions between NSCs and CSF and optimize the conditions in which CSF could support NSCs, promote regeneration and restoration, and improve targeted cellular therapy in CNS disorders.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Human fetal neural stem/progenitor cells (hNSCs) were obtained from the IRCCS Casa Sollievo della Sofferenza, Viale Cappuccini 1, San Giovanni Rotondo, 71,013 Foggia, Italy. The material was obtained legally and ethically, compliant with current local informed consent procedures. Ethical Committee Casa Sollievo della Sofferenza PROT.01/CE25/01/12. Human CSF leftovers were collected from 20 adult volunteers after proper CSF diagnostic analysis from the Department of Neurology, Medical University of Warsaw, Poland, according to the ethics committee of Warsaw Medical University, guideline AKBE/242/2022. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. The animal study was approved by the Approval for this study was granted by the Local Ethical Committee in Warsaw, Poland (No. WAW2/147/2022). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KR: Data curation, Formal Analysis, Investigation, Methodology, Software, Visualization, Writing–original draft. AB: Investigation, Software, Writing–original draft. MS: Investigation, Writing–original draft. DS: Writing–original draft, Methodology. DF: Writing–original draft, Validation. MG: Validation, Writing–original draft. AL: Writing–review and editing. AS: Writing–review and editing, Conceptualization, Project administration, Resources, Supervision.

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Conflict of interest

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

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7. Statements

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Warszawa, 16th April 2025

Klaudia Radoszkiewicz, MSc

kradoszkiewicz@imdik.pan.pl

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

I. **Radoszkiewicz K,** Hribljan V, Isakovic J, Mitrecic D, and Sarnowska A. Critical Points for Optimizing Long-Term Culture and Neural Differentiation Capacity of Rodent and Human Neural Stem Cells to Facilitate Translation into Clinical Settings. Experimental Neurology 2023, 114353. doi: 10.1016/J.EXPNEUROL.2023.114353.

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My contribution includes:

Conceptualization, data collection and formal analysis, visualization, writing the article draft and its final version.

II. **Radoszkiewicz K**, Jezierska-Woźniak K, Waśniewski T, Sarnowska A. Understanding Intra- and Inter-Species Variability in Neural Stem Cells' Biology Is Key to Their Successful Cryopreservation, Culture, and Propagation. Cells 2023; 12(3):488. doi: 10.3390/cells12030488.

IV. **Radoszkiewicz K**, Bzinkowska A, Sypecka M, Sulejczak D, Ferrari D, Gelati M, Vescovi AL, Sarnowska A. Unraveling the impact of human cerebrospinal fluid on human neural stem cell fate. Front Cell Dev Biol. 2025 13:1527557. doi: 10.3389/fcell.2025.1527557

My contribution includes:

Conceptualization, experimental design and performance, execution of the experimental work, data collection and formal analysis, visualization, writing the article draft, and its final version.

Radoszkiewicz Klaudia

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Warszawa, 7.05.2025

Prof. dr hab. n. med. Anna Sarnowska

asarnowska@imdik.pan.pl

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

I. Radoszkiewicz K, Hribljan V, Isakovic J, Mitrecic D, and **Sarnowska A**. Critical Points for Optimizing Long-Term Culture and Neural Differentiation Capacity of Rodent and Human Neural Stem Cells to Facilitate Translation into Clinical Settings. Experimental Neurology 2023, 114353. doi: 10.1016/J.EXPNEUROL.2023.114353.

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My contribution includes:

Conceptualization, coordination of international collaboration, writing the article draft, its editing, and final acceptance, resources, corresponding author responsibilities.

II. Radoszkiewicz K, Jezierska-Woźniak K, Waśniewski T, **Sarnowska A.** Understanding Intra- and Inter-Species Variability in Neural Stem Cells' Biology Is Key to Their Successful Cryopreservation, Culture, and Propagation. Cells 2023; 12(3):488. doi: 10.3390/cells12030488.

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My contribution includes:

Conceptualization, experimental design, resources, coordination of international collaboration, writing the article draft, its editing and final acceptance, funding, corresponding author responsibilities.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

.....

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Rijeka, April 17, 2025

Valentina Hribljan, PhD

valentina.hribljan@uniri.hr

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

I. Radoszkiewicz K, **Hribljan V**, Isakovic J, Mitrecic D, and Sarnowska A. *Critical Points for Optimizing Long-Term Culture and Neural Differentiation Capacity of Rodent and Human Neural Stem Cells to Facilitate Translation into Clinical Settings*. Experimental Neurology 2023, 114353. doi: 10.1016/J.EXPNEUROL.2023.114353

My contribution includes:

Participation in defining study objectives, specialist consultations, scientific collaboration, and manuscript writing and revision.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

Valentina Hribljan

(signature)

imdik.pan.pl/en

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Varazdin, 18th April 2025

Jasmina Isakovic, PhD

ji520@nyu.edu

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

I. Radoszkiewicz K, Hribljan V, **Isakovic J,** Mitrecic D, and Sarnowska A. *Critical Points for Optimizing Long-Term Culture and Neural Differentiation Capacity of Rodent and Human Neural Stem Cells to Facilitate Translation into Clinical Settings*. Experimental Neurology 2023, 114353. doi: 10.1016/J.EXPNEUROL.2023.114353

My contribution includes:

Scientific collaboration, manuscript revision and language correction.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

Jasmina Isakovic

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Zagreb, 17.04.2025.

Prof. Dinko Mitrecic, MD

Department for Regenerative Neuroscience, University of Zagreb School of Medicine

dinko.mitrecic@mef.hr

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

I. Radoszkiewicz K, Hribljan V, Isakovic J, **Mitrecic D**, and Sarnowska A. *Critical Points for Optimizing Long-Term Culture and Neural Differentiation Capacity of Rodent and Human Neural Stem Cells to Facilitate Translation into Clinical Settings*. Experimental Neurology 2023, 114353. doi: 10.1016/J.EXPNEUROL.2023.114353

My contribution includes:

Conceptual support, specialist consultations, discussions about the topic and manuscript revision.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

.....

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Olsztyn, 22.04.2025

dr n. med. Katarzyna Jezierska-Woźniak

katarzyna.jezierska@uwm.edu.pl

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

II. Radoszkiewicz K, **Jezierska-Woźniak K**, Waśniewski T, Sarnowska A. Understanding Intra- and Inter-Species Variability in Neural Stem Cells' Biology Is Key to Their Successful Cryopreservation, Culture, and Propagation. Cells 2023; 12(3):488. doi: 10.3390/cells12030488. 5-year IF: 6.0

My contribution includes:

Biological resources, methodology, and manuscript revision.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

K. Jezierska-Woźniak

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Olsztyn, 22.04.2025

Dr hab. n. med. Tomasz Waśniewski, prof. UWM

tomasz.wasniewski@uwm.edu.pl

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

II. Radoszkiewicz K, Jezierska-Woźniak K, **Waśniewski T**, Sarnowska A. Understanding Intra- and Inter-Species Variability in Neural Stem Cells' Biology Is Key to Their Successful Cryopreservation, Culture, and Propagation. Cells 2023; 12(3):488. doi: 10.3390/cells12030488.

My contribution includes:

Material resources, scientific collaboration, and manuscript revision.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

Tomasz Waśniewski

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Warszawa, 23.04.2025

mgr inż. Aleksandra Bzinkowska

abzinkowska@imdik.pan.pl

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

III. Radoszkiewicz K, **Bzinkowska A**, Chodkowska M, Rybkowska P, Sypecka M, Zembrzuska-Kaska I and Sarnowska A, Deciphering the impact of cerebrospinal fluid on stem cell fate as a new mechanism to enhance clinical therapy development Front. Neurosci. 2024, 17:1332751. doi: 10.3389/fnins.2023.1332751.

My contribution includes:

Data analysis, writing draft and final version of the manuscript.

IV. Radoszkiewicz K, **Bzinkowska A**, Sypecka M, Sulejczak D, Ferrari D, Gelati M, Vescovi AL, Sarnowska A. Unraveling the impact of human cerebrospinal fluid on human neural stem cell fate. Front Cell Dev Biol. 2025 13:1527557. doi: 10.3389/fcell.2025.1527557. 5-year IF: 5.2

My contribution includes:

Assisting in *in vitro, ex vivo* experiments, data analysis, visualization, and writing the manuscript.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

.....

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Warszawa, 23.04.2025

mgr inż Magdalena Chodkowska

mchodkowska@imdik.pan.pl

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

III. Radoszkiewicz K, Bzinkowska A, **Chodkowska M**, Rybkowska P, Sypecka M, Zembrzuska-Kaska I and Sarnowska A, Deciphering the impact of cerebrospinal fluid on stem cell fate as a new mechanism to enhance clinical therapy development Front. Neurosci. 2024, 17:1332751. doi: 10.3389/fnins.2023.1332751.

My contribution includes:

Data analysis, writing draft and final version of the manuscript.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

Chodkowska Magdalena

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Warszawa, 15.04.2025

mgr Paulina Rybkowska

prybkowska@imdik.pan.pl

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

III. Radoszkiewicz K, Bzinkowska A, Chodkowska M, **Rybkowska P**, Sypecka M, Zembrzuska-Kaska I and Sarnowska A, Deciphering the impact of cerebrospinal fluid on stem cell fate as a new mechanism to enhance clinical therapy development Front. Neurosci. 2024, 17:1332751. doi: 10.3389/fnins.2023.1332751.

My contribution includes:

Data analysis, writing draft and final version of the manuscript.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

Paulina Rybkowska

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Warszawa, 23.04.2025

mgr Monika Sypecka

msypecka@imdik.pan.pl

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

III. Radoszkiewicz K, Bzinkowska A, Chodkowska M, Rybkowska P, **Sypecka M,** Zembrzuska-Kaska I and Sarnowska A, Deciphering the impact of cerebrospinal fluid on stem cell fate as a new mechanism to enhance clinical therapy development Front. Neurosci. 2024, 17:1332751. doi: 10.3389/fnins.2023.1332751.

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My contribution includes:

Assistance in *in vivo* experiments, data analysis.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

MSypecka

.....

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Warszawa, 24.04.2025

mgr Ilona Zembrzuska-Kaska

ilona.z.kaska@gmail.com

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

III. Radoszkiewicz K, Bzinkowska A, Chodkowska M, Rybkowska P, Sypecka M, **Zembrzuska-Kaska I** and Sarnowska A, Deciphering the impact of cerebrospinal fluid on stem cell fate as a new mechanism to enhance clinical therapy development Front. Neurosci. 2024, 17:1332751. doi: 10.3389/fnins.2023.1332751.

My contribution includes:

Data analysis, writing draft and final version of the manuscript.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

Zembrzuska-Kaska Ilona

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Warszawa, 24.04.2025

Dr hab. Dorota Gołąbek-Sulejczak, prof. IMDiK

dsulejczak@imdik.pan.pl

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

IV. Radoszkiewicz K, Bzinkowska A, Sypecka M, **Sulejczak D**, Ferrari D, Gelati M, Vescovi AL, Sarnowska A. Unraveling the impact of human cerebrospinal fluid on human neural stem cell fate. Front Cell Dev Biol. 2025 13:1527557. doi: 10.3389/fcell.2025.1527557.

My contribution includes:

Secretome analysis and data curation.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Milan, 6th May 2025

Prof. Daniela Ferrari

daniela.ferrari@unimib.it

Co-Author Statement

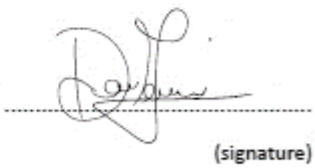
I hereby confirm my contribution to the following publication(s):

IV. Radoszkiewicz K, Bzinkowska A, Sypecka M, Sulejczak D, **Ferrari D**, Gelati M, Vescovi AL, Sarnowska A. Unraveling the impact of human cerebrospinal fluid on human neural stem cell fate. Front Cell Dev Biol. 2025 13:1527557. doi: 10.3389/fcell.2025.1527557.

My contribution includes:

Resources, methodology, specialist consultations, scientific collaboration, manuscript revision.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.



(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Milan, 6 May 2025

Dr. Maurizio Gelati

m.gelati@operapadrepio.it

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

IV. Radoszkiewicz K, Bzinkowska A, Sypecka M, Sulejczak D, Ferrari D, **Gelati M**, Vescovi AL, Sarnowska A. Unraveling the impact of human cerebrospinal fluid on human neural stem cell fate. Front Cell Dev Biol. 2025 13:1527557. doi: 10.3389/fcell.2025.1527557.

My contribution includes:

Methodology, specialist consultations, and scientific collaboration.

I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.

(signature)

Mossakowski
Medical Research Institute
Polish Academy of Sciences

Translational Platform for Regenerative Medicine
5 Pawińskiego Street, 02-106 Warsaw, Poland

Rome, 6th May 2025

Prof. Angelo Luigi Vescovi

angelo.vescovi@unimib.it

Co-Author Statement

I hereby confirm my contribution to the following publication(s):

IV. Radoszkiewicz K, Bzinkowska A, Sypecka M, Sulejczak D, Ferrari D, Gelati M, **Vescovi AL**, Sarnowska A. Unraveling the impact of human cerebrospinal fluid on human neural stem cell fate. Front Cell Dev Biol. 2025 13:1527557. doi: 10.3389/fcell.2025.1527557.

My contribution includes:

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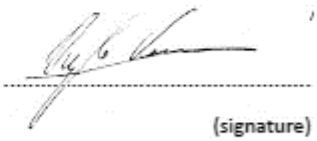
I give my consent for the above-listed publications to be used in the PhD proceedings of Ms. Klaudia Anna Radoszkiewicz.



(signature)