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Overexpressing BDNF in Neural Stem Cells using non-viral gene delivery strategies

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Abstract

The brain derived neurotrophic factor holds neuroprotective and neurogenic roles. The presence of this factor is believed to present highly beneficial effects on injured cells within several neurological disorders, where the levels of this neurotrophin are usually drastically decreased. Neural stem cells are multipotent, self-renewing cells with the ability to differentiate into the three main type of cells within the central nervous system - neurons, astrocytes and oligodendrocytes. The combination of gene therapy, through the introduction of therapeutic genes into the desired cells, with cell therapy aiming for the replacement of damaged cells within a given disorder, hold great promise in modern regenerative medicine.

The aim of this work was the overexpression of the brain derived neurotrophic factor in neural stem cells. For this, mouse and human neural stem cells were transfected with one of three non-viral techniques - microporation, lipid and cationic-polymer based strategies. Human NSC were efficiently transfected using the commercial lipid-based transfection reagent Lipofectamine 2000 with an efficiency of 35%, maintaining their differentiation potential. Cells within the differentiation process were efficiently transfected with 13% efficiency. Cell viability has always remained above 70% after the lipofection process. The transfection with the BDNF gene resulted in neurons with longer primary neurites, and more secondary neurites than control cells, which hints at the promotion of neurite outgrowth and ramification of neurons by this neurotrophin.

Finally, healthy cells were exposed to toxic concentrations of glutamate. Conditioned media containing secreted BDNF from transfected cells was able to protect these cells from glutamate-induced neurotoxicity, as well as reducing the levels of expression of the pro-apoptotic protein caspase7 to near-control levels.

Overall, this work provides the first evidences of the successful use of BDNF-overexpressing NSC, based on a non-viral gene delivery approach for decreasing neurotoxicity.

Keywords

Neural Stem Cells; Brain Derived Neurotrophic Factor; Non-viral gene delivery; Lipofection; Plasmid DNA; Neurotoxicity

Resumo

O trabalho proposto tinha por objectivo a sobre-expressão do “Brain derived neurotrophic factor” (BDNF) em células estaminais neurais. O BDNF é um factor neurotrófico com papéis neuroprotectores e neurogénicos. Este encontra-se drasticamente sub-expresso numa variedade de desordens neurológicas. Acredita-te que a sobre-expressão de BDNF seja benéfica para as células afectadas em determinado nicho *in vivo*.

São consideradas células estaminais neurais, aquelas que apresentam multipotência; a capacidade de auto-renovação; e o potencial para diferenciar em um dos três grandes tipos de células presentes no sistema nervoso central - neurónios, astrócitos e oligodendrócitos.

A simbiose entre terapia génica, através da introdução de genes em células de interesse, e a terapia celular, visando a substituição de células lesadas em consequência de determinada patologia, detém um enorme potencial e uma esperança renovada na área da medicina regenerativa moderna.

Células estaminais neurais de ratinho e humanas foram transfectadas através de um de 3 métodos não-virais - microporação, lipofeção e transfeção com base em polímeros catiónicos. A utilização do reagente de transfecção lipídico Lipofectamina 2000 permitiu notavelmente transfectar uma linha de células estaminais neurais humanas com uma eficiencia de 35%, mantendo o potencial de diferenciação da cultura intacto. Ainda, células durante o processo de diferenciação foram transfectadas com cerca de 13% de eficiência. Em todos os casos foi mantida uma viabilidade celular superior a 70% após o processo de lipofeção.

Um resultado promissor sugeriu que a presença de BDNF promoveu a ramificação de neurónios, potenciando ainda o crescimento de neuritos primários em relação ao controlo.

Foi ainda possível fazer uma análise dos efeitos neuroprotectores do BDNF, pela exposição de uma cultura diferenciada a concentrações tóxicas de glutamato. A presença de BDNF secretada pelas células transfectadas, permitiu a proteção da cultura por parte de neurotoxicidade induzida pelo glutamato, mantendo uma actividade celular superior ao controlo, e sendo ainda responsável pela redução apreciável do nível de actividade da proteína pro-apoptótica caspase7 para níveis semelhantes aos do controlo, sugerindo uma prevenção de eventos de morte celular programada.

Este trabalho fornece as primeiras evidências da utilização eficaz de células estaminais neurais a sobre-expressar BDNF, utilizando métodos não-virais de entrega de genes para a prevenção e redução dos mecanismos de neurotoxicidade induzidos pelo glutamato.

Palavras-chave

Células estaminais neurais; Brain Derived Neurotrophic Factor; Entrega não-viral de genes; Lipofeção; Plasmídeo; Neurotoxicidade

Index

List of figures

Figure 3.3.1 - CGR8-NS cells microporated using 1 pulse of 1500V for 20ms, and 0.5µg of pcDNA3.1-GFP. Cell viability for transfected and control cells (A), and cell recovery of transfected cells (B). Values were calculated based on mean ± SD of 4 to 6 countings of 2 replicates.

Figure 3.3.2 - CGR8-NS cells microporated using 1 pulse of 1500V for 20ms, and 0.5µg of pcDNA3.1-GFP or pcDNA3.1-BDNF. Cell viability of transfected and control cells (A) and cell recovery of control cells (B) on days 2 and 4. Values were calculated based on the mean ± SD of 4 to 6 cell countings of 2 replicates.

Fig. 3.3.3 - CGR8 cells transfected with TurboFect, using a 2:1 TF:DNA ratio (µL/µg) for each reaction. Cell viability, recovery and transfection efficiency is presented for each protocol performed. Cell viability and recovery were calculated based on the mean value ± SD of 2 cell coutings from 3 replicates. Percentage of GFP+ cells is the mean value ± SD of 3 replicates.

Fig 3.3.4 - CGR8-NS cells transfected with Lipofectamine® 2000 under different LF/DNA ratios. Cell viability is shown for each ratio and for LF quantities alone (A). Cell recovery and transfection efficiency (B). Cell viability and recovery were calculated based on the mean ± SD of 3 independent cell countings from 2 replicates each. Percentage of GFP^+ cells is the value of one experiment.

Figure 3.3.5 - Fluorescence images of CGR8 cells transfected with lipoplexes of several LF/DNA ratios. Brighfield images can be seen on the left. GFP-expressing cells can be seen (in green) on the right side of the panel.

Figure 3.4.1 - ReN cells microporated using 0.5µg of pcDNA3.1-GFP under several conditions. Cell viability and recovery are calculated based on the mean ± SD of 3 cell countings from 2 replicates. Percentage of GFP^+ are the value of one experiment for each condition. The conditions are characterized by the voltage (V), length of pulse(s) (ms) and number of pulses (p).

Fig 3.4.2 - ReN cells transfected with Lipofectamine® 2000 complexed with either pcDNA3.1-GFP or pcDNA3.1-BDNF (2µL/1µg). Cell viability, recovery and percentage of GFP^+ cells for day 1 (A) and day 3 (C). Fluorescence images of transfected ReN cells, by day 1 (B) and day 3 (D). Values are presented as the mean of 4 to 6 independent coutings from 2 replicates ± SD. Percentage of GFP+ cells is presented as the mean of 2 replicates ± SD.

Figure 3.4.3 - ReN cells transfected with Lipofectamine® 2000. One day after transfection, differentiation of the culture was induced. Cell viability, recovery and percentage of GFP+ cells on day 1 (A) and day 12 (B) of differentiation. Dot-plots of flow cytometry analysis of ReN cells

transfected with GFP, acquired on day 1 and 12 of differentiation, using a wider gate (C). Histograms based on the previously presented dot-plots (D). Cell viability and recovery was calculated based on the mean value ± SD of 3 independent countings from 3 replicates each. GFP+ cells is presented as the mean ± SD of 2 replicates.

Figure 3.4.4 - ReN cells transfected with Lipofectamine® 2000. One day after transfection, differentiation of the culture was induced. Cell viability, recovery and percentage of GFP+ cells on day 1 (A) and day 12 (B) of differentiation. Dot-plots of flow cytometry analysis of ReN cells transfected with GFP, acquired on day 1 and 12 of differentiation, using a smaller gate (C). Histograms based on the previously presented dot-plots (D). Cell viability and recovery was calculated based on the mean value ± SD of 3 independent countings from 3 replicates each. GFP+ cells is presented as the mean ± SD of 2 replicates.

Figure 3.4.5 - ReN cells within 4th of differentiation were transfected with Lipofectamine 2000. Cell viability, recovery and GFP+ cells (A). Cell viability and recovery was calculated based on the mean value ± SD of 3 independent countings from 3 replicates each. GFP+ cells is presented as the mean ± SD of 2 replicates. Fluorescence microscopy picture of differentiating transfected cells (B). Dot-plots of flow cytometry analysis of differentiating ReN cells transfected with GFP, acquired day 4 of differentiation, using a wide gate (C). Histograms based on the previously presented dot-plots (D).

Figure 3.4.6 - ReN cells within 4th of differentiation were transfected with Lipofectamine 2000. Cell viability, recovery and GFP+ cells (A). Cell viability and recovery was calculated based on the mean value ± SD of 3 independent countings from 3 replicates each. GFP+ cells is presented as the mean ± SD of 2 replicates. Fluorescence microscopy picture of differentiating transfected cells (B). Dot-plots of flow cytometry analysis of differentiating ReN cells transfected with GFP, acquired day 4 of differentiation, using a small gate (C). Histograms based on the previously presented dot-plots (D).

Figure 3.5.1 - Immunostaining of non-differentiated ReN cells. Neuronal cell marker Tuj1 and neural stem/progenitor cells marker Nestin are shown in red. The nuclear counter-stain DAPI in blue. Merged images are shown at the right. Scale bar is 50µm.

Figure 3.5.2 - Immunostaining of ReN cells transfected with BDNF and differentiated under standard conditions (A-D, M-O); non-transfected ReN cells differentiated with supplemented exogenous BDNF (E-H) and non-transfected ReN cells differentiated under standard conditions (I-L, P-R). The markers Tuj1 and Nestin are shown in red, GFAP in green and the nuclear counter-stain DAPI in blue. Scale bar is 50µm

Figure 3.6.1 - Differentiated ReN cells cultured in either normal differentiation conditions (control), with supplemented BDNF (exo-BDNF) or transfected ReN cells with BDNF (pcDNA-BDNF) were immunostained against neuronal-marker Tuj1. Fluorescence microscopy images were analyzed and mean length of primary neurites (A) and secondary neurites (B) were measured in each condition. Results are displayed as the mean ± SD of 19 independent measurements (n=19). Statistical differences are indicated with * for $p < 0.05$.

Figure 3.7.1 - Relative fluorescence intensity at different time-points of differentiated ReN cell cultures exposed to different glutamate concentrations (A), and to 4mM glutamate and 3-day old conditioned medium (B). Values are the mean ± SD of 2 replicates.

Figure 3.8.1 - Relative fold-increase in gene expression of several genes in BDNF-transfected cells (compared to non-transfected cells), on days 1 and 3. Results are displayed for cell markers Nestin, GFAP, β3-tubulin and Sox2 on day 1 (A) and day 3 (B), for BDNF (C) and IL-6 (D). Values are the mean relative gene expression normalized with respect to endogenous GAPDH. Duplicates were run on * marked samples.

Figure 3.8.2 - RT-PCR reaction products on days 1 (A) and 3 (B). The DNA ladders used were the 500bp ladder (Lonza®) and the HyperLadder 50bp (Bioline®), respectively.

Figure 3.8.3 - Relative fold-increase in gene expression of several genes in glutamate-exposed cells (Glu) and cells incubated with conditioned-media after glutamate exposure (Glu+BDNF) (compared to non-exposed control cells), at the 12th day of differentiation. Results are displayed for Caspase7 (A), BDNF (B) and β3-tubulin (C). Values are the mean relative gene expression normalized with respect to endogenous GAPDH. Duplicates were run on * marked samples.

Figure 3.8.4 - Agarose gel of the RT-PCR reaction products. The DNA ladder used was the HyperLadder 50 bp (Bioline®).

Supplementary figure S1 - Sequencing results, showing 738 matching nucleotides, confirming correct cloning of the desired gene into the pcDNA3.1 plasmid. BDNF nucleotide sequence is highlighted in blue. 23 nucleotides are missing in the sequencing results due to incomplete sequencing. By using a reverse primer with a sequence downstream of BDNF we were able to confirm the correct type of the initial base pairs. Both primers were designed by the company StabVida® according to the type of plasmid in use (pcDNA3.1). The obtained sequence perfectly matched with the sequence showed below that was provided by the supplier company (Origene®).

Supplementary figure S2 - Flow cytometry profiles of the ReN cells transfected with GFP at different time points, using the wider gate.

Supplementary figure S3 - Flow cytometry profiles of the ReN cells transfected with GFP at different time points, using the smaller gate.

Supplementary figure S4 - Fold-increase in BDNF expression at the 3rd day of culture, using a Ct of 23.3 (A) or a Ct of 18.8 (B). Both Ct values were returned in the same experiment within one sample, although none were used to prevent an inaccurate analysis.

Supplementary figure S5 - Fold-increase in IL-6 expression at the 1st day of culture, using a Ct of 38.3 (A) or a Ct of 32.6 (B). Both Ct values were returned in the same experiment within one sample, although none were used to prevent an inaccurate analysis.

List of tables

List of abbreviations

AD Alzheimer´s disease
ALS Amyotrophic lateral sclerosis
ASC Adult stem cells
BBB Blood brain barrier
BDNF Brain Derived Neurotrophic Factor
BGH Bovine Growth Hormone
BI BamHI
BLAST Basic Local Alignment Search Tool
BMP Bone morphogenetic protein
BPs Basal progenitors
bp Base pairs
cDNA Complementary DNA
CMV Cytomegalovirus immediate early promoter
CNS Central Nervous System
CNTF Cilliary neurotrophic factor
Ct Cycle threshold
D Digested
DA Dopaminergic
DAPI 4',6-diamidino-2-phenylindole
DMEM/F-12 Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO Dimethyl sulfoxide
EB Embryoid body
ECM Extracellular Matrix
EGF Epidermal Growth Factor
ERK Extracellular signal Regulated Kinase
exo-BDNF Exogenous BDNF
ESC Embryonic Stem cells
FACS Fluorescence Activated Cell Sorting
FCS forward-scattered light
FGF2 Fibroblast Growth Factor
GABA Gamma amino butyric acid
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GDNF Glial-cell-line-Derived Neurothrophic Factor
GFAP Glial Fibrillary Acidic Protein
GFP Green Fluorescent Protein
Glu Glutamate

hBDNF Human Brain Derived Neurotrophic Factor
hESC Human embryonic stem cells
hMSC Human mesenchymal stem cells
hNSC Human neural stem cells
HIII HindIII
HSC Hematopoietic stem cells
HT Huntington´s disease
ICM Inner cell mass
IL-6 Interleukin-6
ILF Insulin-like growth factor
iPSC Induced Pluripotent Stem Cells
JAK– STAT Janus-activated kinase–signal transducer and activator of transcription pathway
LB Luria Broth
LF Lipofectamine 2000
LIF Leukemia Inhibitor Factor
LII Ladder II
LIII Ladder III
MAPK Mitogen Activated Protein Kinase
mBDNF Mouse Brain Derived Neurotrophic Factor
MCS Multi-Cloning Site
MSC Mesenchymal stem cells
ND Non-Digested
NEPs Neuroepithelial progenitors
NGF Nerve Growth Factor
NGS Normal goat serum
NMDA *N*-methyl-D-aspartate
NPC Nuclear Pore Complexes
NSC Neural Stem Cells
NTs Neurothrophic Factors
NT-4/5 Neurotrophin-4/5
NV Non-Viable Cells
OB Olfactory bulb
PBS Phosphate-Buffered Saline
PD Parkinson´s disease
PDGF Platelet derived growth factor
PEG-**PEI** polyethylene glycol-polyethyleneimine
RMS Rostral migratory stream
PCR Polymerase Chain Reaction
PFA Paraformaldehyde
PILs PEGylated immunoliposomes

PolyA Polyadenilation signal
RB Resuspension Buffer
RG Radial glia
RT-PCR Reverse Transcription Polymerase Chain Reaction
S/MAR Scaffold/Matrix Attachment Region
SCID-X1 X-linked severe combined immunodeficiency
SD Standard deviation
SGZ Sub-Granular Zone
Shh Sonic hedgehog
siRNA Small interfering RNA
SSC Side-scattered light
SVZ Sub-Ventricular Zone
T3 3,3,5-tri-iodothyronine
TF TurboFect
TLR9 Toll-Like Receptor 9
Trks Tyrosine Kinases
TuJ1 Neuron-specific class III β-tubulin
VC Viable cells
WHO World Health Organization

1. Introduction

1.1 Stem Cells

A stem cell is an undifferentiated cell with the ability to self-renew, i.e. of reproducing itself, as well as the ability to give rise to specialized and functional cells through differentiation[1,7] (Figure 1.1)

This last property renders stem cells a great value in regenerative medicine, being the ultimate goal the possibility of providing sufficient specialized cells for the replacement of lost or damaged cells due to a certain injury or disease.

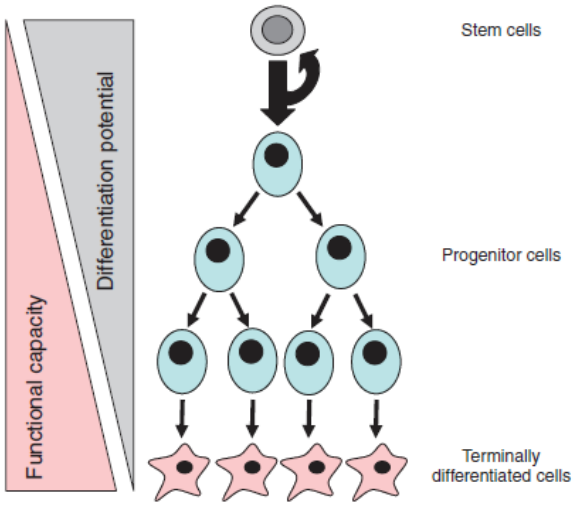


Figure 1.1 - Schematic representation of self-renewal and differentiation potential of stem cells. *Taken from* [1].

The self-renewal of stem cells can occur in two distinct ways: (1) through symmetric cell division, which is a proliferative type division in which one single cell originates two daughter cells genetically identical to the progenitor or (2) through assymetrical division where one cell originates an identical daughter cell and a cell of a different type with distinct genetic properties. The later cell division is a differentiative type division, giving rise to specialized cells as well as maintaining a pool of pluripotent cells[7,8].

1.1.1 - Embryonic stem cells

In humans, embryonic stem cells (ESC) can be found in the inner cell mass (ICM) of the pre-implantation blastocyst (4-5 days)[7,9]. This ICM is responsible for the development of the three embryonic germ layers conducting to the formation of the human body - the endoderm (responsible for generating pancreatic and liver cells); the mesoderm (leading to cardiomyocytes, skeletal muscle and smooth muscle) and the ectoderm (neurons, epithelial cells and glial cells)[1,7]. Unlike adult stem cells (ASC), ESC are considered pluripotent cells since they are able to give rise to all tissues of the adult organism[10]. Cultivation of these cells *in vitro* is a common procedure nowadays, and can be achieved through well-established culture conditions. This makes ESC tremendously helpful in the creation of disease models, through the study of specific drug-targeting systems, and from a basic developmental stem cell biology standpoint.

Some concerns still arise from the use of these cells for *in vivo* cell therapy, including that the proliferative properties required for proper expansion of these cells may trigger the formation of tumors, or give rise to cell types different than those required, or even triggering an immune response in the host[1]. Also, some drawbacks concerning the low efficiency and time required for cells to differentiate into the cell type required greatly hamper its broader use in cell therapy[10].

Immunogenic rejection of allogeneic cells led to extensive research aiming at fulfilling the need for successfully accepted cells by the host. In this sense, in 2005 adult fibroblasts were successfully reprogrammed to an ESC state, by fusion with ESC[11]. On another approach, Yamanaka and colleagues successfully reprogrammed adult fibroblasts into a pluripotent state, by introducing only four factors - Oct3/4, Sox2, c-Myc and Klf4[12]. These cells, then termed induced pluripotent stem cells (iPSC), are similar to human ESC in terms of gene expression and capacity of specific differentiation[9].

Following this approach, somatic cells from a certain individual could be subjected to *in vitro* reprogramming to a prior pluripotent state, and proceed to differentiation to the proper cell type required, avoiding the organism´s rejection of those same cells[13].

1.1.2 - Adult stem cells

Numerous types of stem cells exist in the adult organism. These are commonly referred to as adult stem cells. They comprise lineage committed cells residing in specific sites in the different tissues of the adult organism, and are able to give rise only to the cell type of the tissue or organ where they are localized, being responsible for the regeneration of those same tissues upon injury, or as the natural replacement of older cells, as reviewed elsewhere[1]. Mesenchymal stem

cells (MSC) are adult stem cells, which were first isolated from the bone marrow[14], although it is now known that these can also be obtained from other niches such as adipose tissue[15] and umbilical cord blood[16] and matrix[17]. MSC can differentiate into fibroblasts, chondrocytes, osteoblasts and adipocytes, being responsible for the regeneration of bone and cartilaginous tissues such as tendons and ligaments. Hematopoietic stem cells (HSC) are found in the bone marrow and umbilical cord blood, being responsible for the development of blood lineage cells. Cardiac stem cells support myocardial regeneration, and have been shown to differentiate into functional myocardium when injected into an ischemic heart[18]. Also in the liver, stable amounts of hepatic stem cells remain throughout life, giving rise to mature hepatocytes[19]. Neural stem cells are localized in well defined zones of the adult brain and are responsible for the generation of neurons, astrocytes and oligodendrocytes. The latter type of stem cells is the focus of this thesis, so these will be addressed in more detail later (section 1.3).

Adult stem cells present several clinical advantages regarding their lower potential of differentiation when compared to embryonic stem cells, originating a finite number of cell types, which ultimately decreases their malignant potential[20].

1.2 - Neural Stem Cells in the Central Nervous System

A neural stem cell can be defined as an uncommitted cell able to give rise to all three major cell types of the Central Nervous System (CNS) - neurons, astrocytes and oligodendrocytes, and it must have the capacity for self-renewal[9]. The former property renders NSC the concept of multipotent cells, suggesting the potential they hold for generating more than one possible cell type. They are radial astrocyte-like, nestin-positive cells[9,21].

The development of the vertebrate CNS begins with a neural plate consisting of a single layer of neuroepithelial progenitor cells (NEPs)[5,22,23]. Upon folding, this layer gives rise to a neural tube containing also NEPs, which later on give rise to both radial glia (RG) and basal progenitors (BPs)[5]. A significant amount of RG is present in primary cell populations from CNS tissue, and they are believed to be a good source of neural stem cells. Basal progenitors are neuronal-restricted cells, and can be generated both by RG and NEPs. In the mammalian brain, neural stem cells are believed to exist within two neurogenic niches - the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SVG) of the dentate gyrus[5,24]. Besides neural stem cells, those neurogenic niches comprise glial cells, ependymal cells, extracellular matrix (ECM) components and proteoglycans[9].

Oligodendrocytes are glial cells necessary to the proper development of the brain and neuronal functioning, through the myelin formation around the neurons axons[25] (figure 1.2). They are produced throughout the entire CNS[9,26,27]. Myelin is a membranous structure rich in lipids with low water content, which isolates the nerve electrical signals transmitted through axons. This structure is implicated in many neurological disorders, as in Multiple Sclerosis, caused by the demyelinization of neurons[25], i.e. loss of myelin in the neurons.
Oligodendrocytes and other glial cells are established early at the time of neurogenesis, although their maturation occurs mainly in the early post-natal stage.

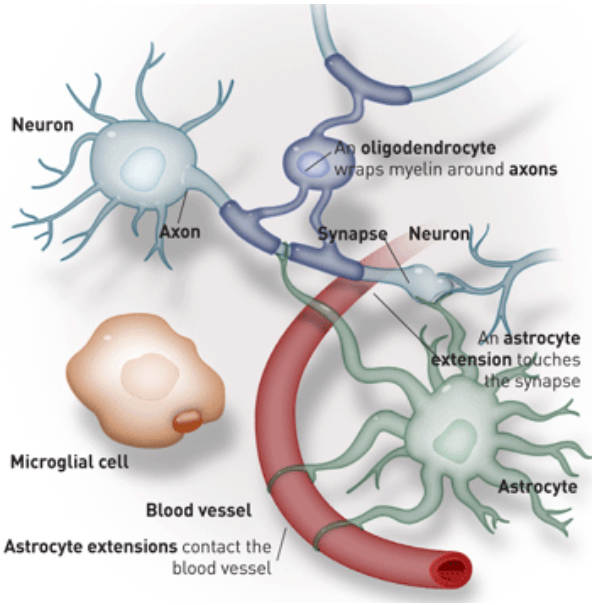


Figure 1.2 - Interactions between the three cell types of the CNS - neurons, astrocytes and oligodendrocytes. *Taken from* [2].

Astrocytes are the most abundant cells within the CNS, and are generated throughout the entire system[9,26,27]. These star-like cells are responsible for a wide variety of tasks including axonal guidance, synaptic support and regulation of the blood brain barrier and overall blood flow[28]. Through the secretion of growth factors important to proper axon growth and stable synaptic activity they modulate the microenvironment of adjacent neurons[28]. Glial cells, including astrocytes and oligodendrocytes constitute 90% of the total cells in the adult brain[25].

At last, the generation of new neurons is mainly restricted to the SVZ of the lateral ventricle and SVG of the dentate gyrus. Newly formed neuroblasts migrate through the rostral migratory stream (RMS) mainly composed of astrocytes, finally reaching the olfactory bulb (OB), where they migrate outwardly as isolated cells, later on maturating into differentiated neurons[8,24]. Neurons are highly differentiated cells responsible for the transmission of electrical signals throughout the entire nervous system that regulate every impulse and response in the organism. The brain contains a wide variety of neuronal cell types, such as motor neurons, responsible for making the connection between brain signals and muscle movement; sensory

neurons, which allow the body to perceive external stimuli from peripheral cells such as epithelial cells, and sending the signal to the spinal cord or the brain. Others tend to specialize in the generation and release of neurotransmitters for proper cell-cell communication. A cortical neuron makes up around 10,000 connections with other brain cells. This gives up the complexity of the neuronal network, and allows to realize the reason why the brain, especially the human cortex, is the core for the development of human learning, memory creation and overall rational thinking[29-31].

It is still unclear the extent to which multipotent stem cells exist in the CNS and what really defines them, so this renders the concept of neural stem cell quite an abstract meaning to it. Nonetheless, several stem/progenitor cells have been identified in the neurogenic niches in the brain, which, depending on the microenvironment signals, ultimately have the capacity to generate the supportive glial type of cells, or one of the neuronal lineages found in the adult intricate neuronal network[26]. So, a neural stem cell can be considered the one capable of self-renewal, although not necessarily for unlimited divisions, and of course, being able to give rise to neurons and glial cells as stated before[1,5].

1.2.1 - Sources of Neural Stem Cells

Neural stem cells can be found in several tissues or can derived through differentiation of other cells (figure1.3).

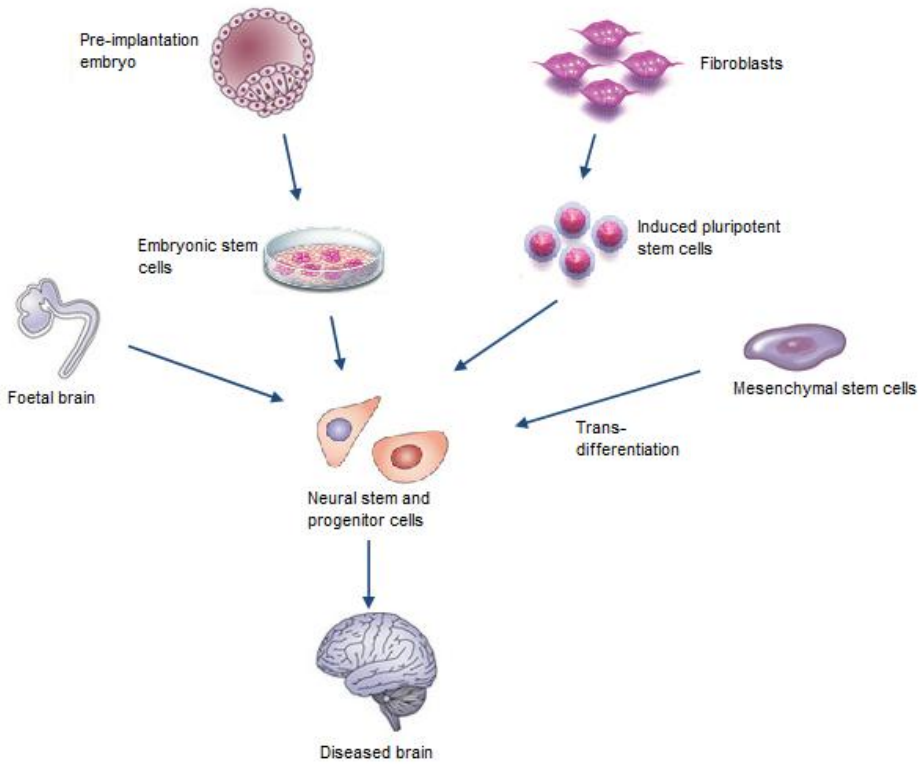


Figure 1.3 - Schematic representation of neural stem cell based therapy. *Adapted from* [3].

Since the work of Ramón y Cajal in 1928[32], it was believed that the brain did not have the ability to generate new neurons. Nevertheless, this theory has been proven wrong since neurogenesis is an active process that occurs throughout life[27,33]. The two main regions responsible for this phenomenon, which are as well the main sources of adult percursors are the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and in the subventricular zone (SVZ) of the lateral ventricles[26,27,33]. In 1992 the first adult and fetal NSC from the central nervous system of rodents were isolated[34]. A few years later, in 1998 the same was accomplished with human NSC[35,36].

The isolation procedure of NSC is based on the dissection and further dissociation of fetal tissue, following plating on the adequate conditions. Weiss and Reynolds managed to perform this by plating the cells from adult mice striata in culture-medium supplemented with epidermal growth factor (EGF)[34]. A few years later, the same was achieved with human cells. Flax and colleagues dissociated cells from the human fetal telencephalon, which were then cultured in medium containing fibroblast growth factor (FGF2) and/or EGF. This provided some insight about the nature of cells isolated from human fetal tissue, and the fact that in that case, those cells showed neural stem cell properties, being able to proliferate *in vitro*.

NSC can also be obtained by differentiation from other cell types, as described in the section 1.2.3 “Neural Stem Cells culture *in vitro*”.

1.2.2 - Phenotype and morphology

NSC are star-like cells with the ability to adhere to culture-grade plastic (figure 1.4).

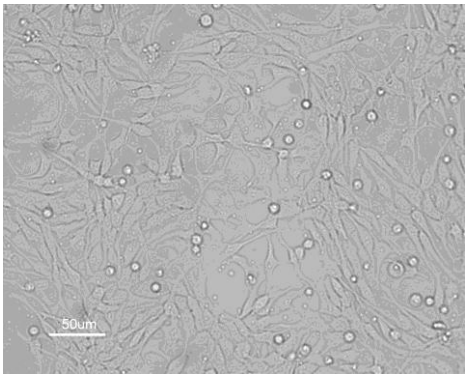


Figure 1.4 - Human immortalized neural progenitor cell population. Scale bar is 50µm.

NSC characterization remains a topic with no consensus within the scientific community. According to literature, there is not yet a single marker that characterizes NSC exclusively and so can be used to selectively identify and isolate these cells[4]. Considering this, usually the

characterization is achieved by the combination of several cell-specific markers such as nestin, Sox1/2, Pax6 and CD133[4] (figure 1.5).

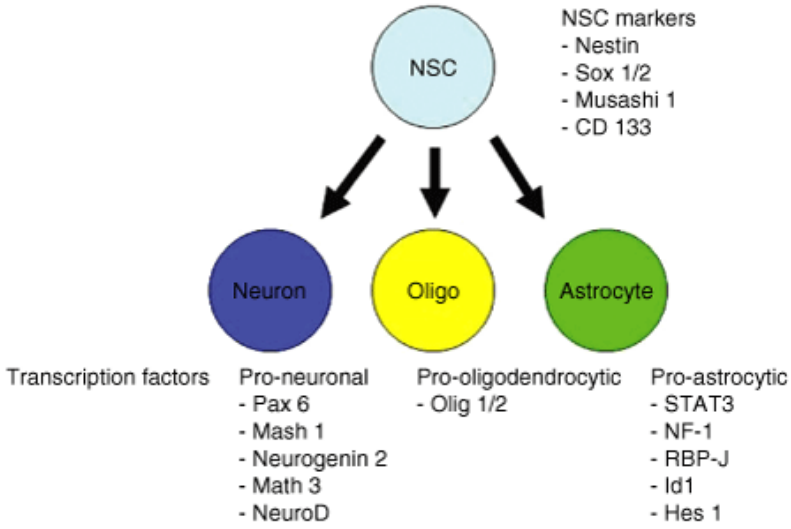


Figure 1.5 - Neural stem cells and lineage-commited cell markers. *Taken from*[4].

Nestin is an intermediate filament[8,34] that distinguishes neuroepithelial stem cells from other differentiated cells in the neural tube[25]. Sox2 is expressed in a variety of cells within the SVZ, being in part responsible for the maintenance of an undifferentiated proliferative state during development[37]. Pax6 is a transcription factor that also maintains the stemness of the culture, promoting asymmetric divisions[8]. CD133 is a marker for the transmembrane protein prominin-1, found in the apical plasma membrane of NSC[8]. The assessment of the expression of these markers is usually achieved through several techniques such as flow cytometry and quantitative real time PCR.

Upon assessment of differentiation capacity of NSC, usually cells which acquire neuron morphology are identified by the expression of several markers such as the microtubule associated protein 2 (MAP-2); β-tubulin III, a protein that is primarily expressed in neurons and is involved in axon guidance and maintenance[38]; substance P, a neurotransmitter that belongs to the small peptides group of neurokinins responsible for affective behavior, and the main inhibitory neurotransmitter GABA (gamma amino butyric acid) markers[34]. Cells with oligodendrocytic characteristics express cell-surface protein O4, and astrocytes are mainly distinguished through the expression of the glial fibrillary acidic protein[39].

1.2.3 - Neural Stem Cell culture *in vitro*

NSC can either proliferate *in vitro,* maintaining their multipotent state or differentiate into specific cell types. The maintenance of proliferative capacity and differentiative potential can be promoted by the addition of growth factors to the culture, such as EGF and fibroblast growth factor 2 (FGF2)[8,21,40]. The withdrawal of these growth factors leads to a mixed culture of neurons, astrocytes and oligodendrocytes[9]. But like any other stem cell, neural stem cells need the appropriate conditions to differentiate into the proper cell-type required, which is usually achieved by the withdrawal of mithogens or by the exposure to differentiative factors. EGF is normally used for astrocytic lineage commitment, although it plays a major role in maintaining the stemness properties of a population[39], with functions in the most important stages of the regulation of cell growth, proliferation and differentiation[41]. It is a proliferative factor not only for neural stem cells, but for mammalian cells in general, acting through binding to the EGF-receptor. It is involved in several signaling pathways such as the Mithogen activated protein kinases/Extracellular signal regulated kinases (MAPK/ERK) which ultimately promotes cell survival[42]. FGF-2 is one of the major players in neural development. It is implicated in the induction and patterning of mesoderm and neural tissues in vertebrate embryos[41]. It is also responsible for the proliferation and survival of early forebrain neural precursors, also by playing a role in the MAPK signaling pathway[40,41,43].

A culture of NSC can be maintained *in vitro* through the supplementation of culture medium with both EGF and FGF-2, or EGF alone. The withdrawal of EGF from the medium, leads to neuronal differentiation and apoptotic events, mediated by FGF-2[37].

One can distinguish two major *in vitro* culture methods for NSC: neurosphere formation or adherent culture[44]. Neurospheres can be understood as spherical clusters of undifferentiated neural stem cells[44,45] (figure 1.6). Weiss and Reynolds were the first to report culturing conditions to expand neural stem cells isolated from the adult mouse brain, using the neurosphere approach[46]. The cells from adult mice striata were dissociated enzymatically and plated in EGF containing medium without any other substrates or adhesive factors. After 6-8 days, clusters of cells could be observed. On a parallel assessment, it was observed that no proliferation events occurred upon EGF withdrawal, once more reinforcing its role in multipotent state maintenance. The cells from the cluster were dissociated and replated on polyornithine coated dishes, in order to assess the ability of these cells to generate new neurospheres again in the presence of EGF. Secondary neuroshperes were indeed observed, and the majority of cells within it expressed nestin. The withdrawal of EGF once more inhibited the proper expansion of cells[46]. Meanwhile other works reported the generation of neurospheres on low-attachment tissue culture plastic dishes[46], with medium containing FGF2 besides EGF, and by 6-8 days of culture, clusters of cells with 100-150μM are expected to form, although karyotypic instability is known to occur from the tenth passage and forth[5]. This approach has some advantages, as its tridimensional structure allows for relevant cell-cell and cell-matrix interactions, mimicking the *in vivo* niche where these cells are found, making it physiologically

interesting[44]. On the other hand, layers of cells are formed in a stratified manner, with differentiated cells at the core of the cluster under suboptimal conditions, leaving the precursors cells more exposed to the environment[5], being limiting in terms of diffusion of essential nutrients and oxygen[47]. Alterations in differentiative and proliferative patterns have also been identified, as well as chromosomal instability[44]. The three-dimensional structure of the clusters also makes it hard to identify the different cells, as the highly heterogeneous population greatly hampers the study of multipotentiality of single-cells. Another major drawback resides in the fact that neurospheres differentiate preferentially into astrocytes rather than neurons, *in vitro*, making it less interesting from the therapeutical standpoint[44].

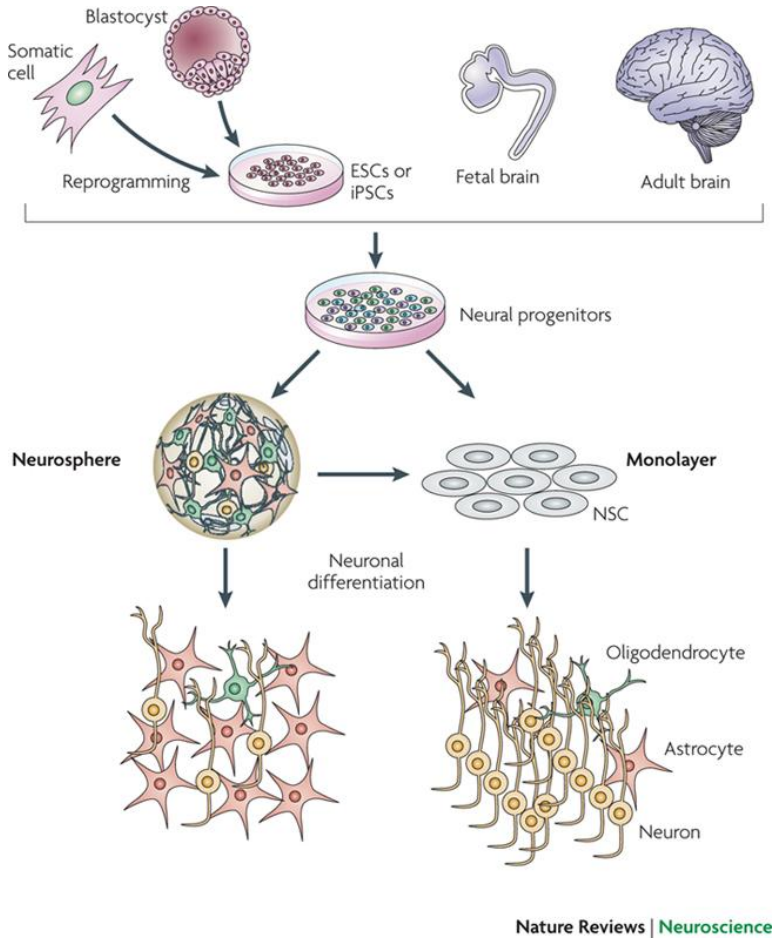


Figure 1.6 - Neural stem cell culture methods: neurosphere formation and adherent monolayer. *Taken from*[5].

Another method is based on the growth of cells on an adherent or monolayer culture, using ECM components[44] and growth factors such as EGF and FGF-2[5]. The culture flasks are usually coated with fibronectin, a component of various types of ECM suitable for cell adhesion and proliferation[41], or laminin, another substrate commonly used in the coating of culture dishes[41,48]. The latter interacts with the β1 integrins receptors present in NSC surface, which *in vivo* is responsible for the activation of a signaling pathway that maintains these cells in the CNS[41,44].

Conti and colleagues induced differentiation of neural precursors from ES-derived cells in an adherent monolayer. The precursors were plated in a basal medium containing supplementation with N2 (including insulin, which is known to promote differentiation of ESC as well as favoring differentiated cells survival), eliminating any commited or differentiating cell, with either FGF-2 alone, or combining FGF-2 and EGF. It was observed that FGF-2 alone did not allow for cell growth, but the combination of both mitogens led to proliferation of precursor cells expressing nestin, without expressing any glial or neuronal lineage markers[21].

Withdrawal from EGF resulted in death of a great number of cells as well as initiating differentiation events, even though observing that laminin would ensure cell viability upon EGF withdrawal. Removal of FGF-2 from the medium led to differentiation of 30-40% of the population to neuronal commited cells expressing type III β-tubulin, MAP2 and NeuN, while maintaining self-renewing NSC[21].

This system minimizes spontaneous differentiation, and its niche independence makes the cells more responsive to the specific factors in the media, which may lead to more homogeneous and defined cell populations[5].

Nonetheless, it seems that the specific roles of EGF and FGF-2 still remain somewhat unclear. A lot of evidence supports that long-term propagation of NSC requires both factors[5]. On the other hand, from the early work of Reynolds and Weiss, evidence suggests that EGF alone is capable of providing efficient renewal of this cells[34]. The more recent work of Conti and colleagues also supports this approach, stating that while both factors are required for derivation of NSC from ESC, only EGF is necessary to support the established ES-derived NSC line, playing the major roles mainly through the suppression of apoptosis[49].

Embryonic Stem Cell-derived NSC

As mentioned before, apart from the direct isolation from tissues, neural stem cells can also be obtained through the differentiation of other cell types.

For instance, NSC can be derived from ESC. As mentioned in section 1.2, embryonic stem cells are pluripotent cells derived from pre-implantation embryos, and can be maintained in that pluripotent state *in vitro*. ESC are prone to spontaneous differentiation upon withdrawal from the supportive pluripotency agents. However, withdrawal of these molecules often results in a heterogeneous culture of mixed cells[41]. For neural lineage restriction, the adequate growth factors must be provided. The neural differentiation of hESC can be achieved with 2 distinct models: as an embryoid body (EB) model, in which cells are allowed to grow freely on suspension promoting random differentiation, and then selecting required cells for plating on neural proliferation medium; or as an adherent culture, where cells are subjected to neural differentiation from the start, along with coating or co-culturing with feeder layers, which are later on removed and proliferation medium is added for continuous growth[50,51].

Some well-established culture conditions are available nowadays. For instance, Pollard *et al* have shown that ESC can be propagated *in vitro* by the addition of the leukemia-inhibition factor (LIF) and the bone morphogenetic protein (BMP) (known to block neural differentiation of ESC). Upon removal of these factors, some of these cells readily give rise to NEPs expressing Sox1. Finally the generation of Sox2-positive neural stem cells can be achieved by the addition of the mitogens EGF and FGF-2[49,52].

Conti and colleagues were able to derive neural precursors from several embryonic stem cell lines, including the CGR8 cell line[21], which has been first derived in the laboratory of Professor Austin Smith, Wellcome Trust Centre for Stem Cell Research (Cambridge, United Kingdom), deriving the CGR8-NS cell line also used in this work. Neural precursors were derived from ESC by culturing these cells in serum-free medium. After a week, cells were re-plated in basal medium in the presence of FGF-2 alone or FGF-2 plus EGF. This allowed for the selection of differentiating cells only. Clusters of cells were formed, which were then dissociated and re-plated on fresh medium. Bipolar cells were generated, able to proliferate in EGF and FGF-2-containing medium. These cells expressed immature neural marker nestin, among others, while lacking expression of astrocytic and neuronal specific markers and maintaining diploid chromosome content at late passages. This led the authors to suggest the generated cells present self-renewing neural stem cell behavior[21].

ESC can provide bigger quantities of fully defined populations of neural progenitors and differentiated cells. Hence, models of neurodegenerative diseases can be more readily created by the insertion of defined mutations on differentiated cells[50].

Induced-Pluripotent Stem Cell-derived NSC

Besides ESC, iPSCs can be used to obtain NSC *in vitro*. The derivation of iPSCs may circumvent some of the issues related to the rejection of cells upon allogeneic transplants[9]. Being a relatively new field little is known about the specific mechanisms of action, although some studies have aroused interest in the scientific community. Some well-characterized protocols are available to guide the differentiation of iPS cells to neural progenitors, neurons, and other CNS cells[41].

Once the generation of iPSC is achieved, the neural differentiation of ESC can usually be applied to these cells as well. Tipically, the cells are initially treated with BMP inhibitors[53] or they can be co-cultured with stromal cell lines. From this point, three possible methods are employed. One of them consists in neural induction by Noggin treatment. This molecule is the antagonist of BMP, and is known to increase neurogenesis by blocking gliogenesis through a negative feedback mechanism[26], being also responsible for the upregulation of expression of neural markers such as Pax6, Sox2 and Nestin[41]. Another method is based on co-culturing with

PA6 stromal cells. These are loose connective tissue cells found in several organs such as the bone marrow. They provide matrix-support for other cells[41]. The surface of these cells undergoes activities known to promote neural differentiation[54]. Another alternative carries out culturing of cells on a defined media with a laminin substrate[47,55,56]. All three methods are capable of generating early neural progenitors expressing neural stem cell markers, such as Pax6, Sox1 and/or Sox2. These progenitors can be maintained as neurospheres and then further differentiated to mature neurons or glia[41].

One of the well-characterized procedures for generation of iPSC-derived NSC has been described by Onorati and colleagues[57]. iPSC were first generated from mice fibroblasts as described elsewhere[13]. These cells were later on cultured on neural induction media supplemented with B27. After 12 days, mitogens EGF and FGF-2 were added. Aggregates were generated in the following days, from which were later on derived monolayers of the iPSC-derived neural stem cells, expressing the appropriate markers for NSC, presenting stability for more than 60 passages. Furthermore, successful differentiation was achieved, demonstrating the ability of these iPSC-derived NSC to give rise to neurons, astrocytes and oligodendrocytes[57].

The design of robust and well-characterized protocols for neural differentiation from iPS cells is of the utmost importance to a successful cell therapy approach. With this, somatic cells from the patient can be retrieved, and through the adequate reprogramming to a pluripotent state, the desired cell lineage can be generated in order to provide the CNS cells lost during the disease.

1.2.4 - Differentiation of Neural Stem Cells

The basis for neural stem cell differentiation seems to be the withdrawal of the proliferative factors EGF and FGF-2. This renders a rather mixed culture of several cell types as stated before. This is possible by the intrinsic capacity of NSC, and all stem cells for that matter, to start differentiating *in vitro* when the proliferative agents are removed from the medium. That approach can be advantageous for different cell-cell interactions study and for providing a more similar model of the real and diversified *in vivo* environment. Nonetheless, for fundamental stem cell biology, a rather pure population of the desired cell type is preferred.

For instance, Donato *et al* have described a differentiation protocol addressing the human neural stem cells used in this work (ReN cell VM)[58]. The differentiation is achieved by exposing a confluent population to medium without growth factors. Within as little as four days, rounded cell bodies possessing long neurites were achieved, reinforcing the belief that withdrawal of mitogens is the simplest method for generation of a differentiated neural stem cells culture[58].

Another optimized protocol for differentiation of ES-derived NSC into a homogenous neuronal population has been designed by Spiliotopoulos and colleagues[59], having used among others, the ES-derived NS cell line CGR8-NS, also used throughout this work. The approach is based on the culture of NSC in media containing FGF-2. The withdrawal of this factor causes widespread cell death, but promotes the survival of the differentiating cells. Later on, the cells are cultured in equal composition media with the addition of BDNF. From that point, a gradual increase in BDNF and decrease in FGF-2 concentration renders a homogeneous mature neuronal population[59]. Other reports state that a neuronal fate can be achieved firstly by removal of the proliferative factors. Then, the addition of the platelet-derived growth factor (PDGF) generates neuronal-lineage restricted cells, by increasing the expression of neuron-specific markers such as MAP2 and β3-tubulin[39] and by activating the MEK-RSK-C/EBP pathway, that is believed to be essential for neurogenesis[55]. Finally, it has been suggested that FGF2 and the neurotrophin-3 (NT3) lead to cell cycle exit and neuronal differentiation. Concerning neurons in particular, and the high degree of variability of neuronal types within the CNS, one must consider that different types of neurons require different factors for proper differentiation. For example, cortical pyramidal neurons usually require sonic hedgehog (Shh) signaling[53], a crucial molecule in the development and patterning of the nervous system[60] whereas interneurons (responsible for coordinating motor response[61]) seem to require retinoids[61]. The more suitable protocol will ultimately differ with the outcome pretended and overall work objectives.

Astrocytes can be obtained by the exposure of NSC to the cilliary neurotrophic factor (CNTF) in the presence of EGF, which will act via the JAK– STAT (Janus-activated kinase–signal transducer and activator of transcription) gliogenic pathway[55,62].

Although oligodendrocyte differentiation protocols are not as well established as neuronal and astrocytic lineages, a relatively recent work has provided new insights about this matter. A population containing 20% of mature myelinating oligodendrocytes has been achieved firstly by culturing cells in media containing growth factors FGF-2 and PDGF, and forskolin, after which cells with small condensed bodies and short processes could be observed. Subsequent factor withdrawal and addition of 3,3,5-tri-iodothyronine (T3) and ascorbic acid leads to a mixed population of oligodendrocytes (20%), astrocytes (40%) and neurons (10%). Functional validation of oligodendrocytes was performed, being these able to re-myelinate severely impaired mice models[63].

During differentiation, NSC reorganize their transcriptome, leading to the silencing of multipotency genes, while up-regulating genes involved in lineage-commitment[26]. However, this is not perfectly understood, and so, there seems not to be a singular approach in the differentiation of the desired type of cell. This may due to the high degree of uncertainty that still

exists regarding the specific roles of growth factors and differentiating agents, and the outcome resulting from their interaction.

1.2.5 - Applications of Neural Stem Cells

Neural stem cells find application in a wide variety of scientific areas ranging from the fundamentals of stem cell developmental biology, through its applications in the pharmaceutical industry as drug screening platforms, to the clinical applications where gene and cell therapy are used in modern regenerative medicine. Some relevant clinical applications in the field of neurological disorders therapy are described further in this section.

Besides the obvious tragic impact neurological disorders have on patients and their families, taking away nearly 7 million people every year, an outstanding economic impact arises from these diseases. In Europe only, almost 140 billion euros were spent on this matter, states a 2007 World Health Organization (WHO) report[64]. From this striking data, it is easy to acknowledge the imperative need to pursue more effective and accessible means to treat these types of illnesses, sparing millions from suffering and creating a healthier, well-informed community that will also save up great amounts of money in the long run.

Although the brain has capacity to regenerate damaged tissue, this phenomenon is quite limited[33]. For that reason, bioprocessing is required in order to provide additional sources of cells and tissue capable of restoring the damage caused by innumerous diseases[65]. A successful stem cell-based therapy for neurological disorders must aim at the replacement of the lost cells in the disease process, and in the integration of these cells into the cortical circuitries[66]. A great deal of neurological disorders such as the ones described below has already been addressed by stem-cell therapy, with promising outcomes.

Parkinson´s disease (PD) is responsible for the loss of dopaminergic neurons[66,67]. One of the therapeutical approaches involves the delivery and upregulation of neurotrophic factors, the generation of endogenous dopamine as well as implantation of supportive cells and dopaminergic neurons. An encouraging study provides proof of principle that nigrostriatal dopamine neurons can replace cells lost to this disease, and provide long-term recovery of motor function[68]. Amyotrophic Lateral Sclerosis (ALS) is a highly restrictive disease that results in the progressive disfunction and degeneration of motor neurons in cerebral cortex, brain stem and spinal cord. Stem cell therapy seems to have step into this disease with several good news. Transplanted NSC have reduced astrogliosis and inflammation events, ameliorating the overall symptoms of ALS[69]. This is believed to be due, in part, to the capacity of these cells to produce

and release trophic factors[69]. Another finding suggests that fetal motor neurons grafted to the adult rat spinal cord migrate to the correct zone and generate functional connection with skeletal muscle [70,71]. Some of the consequences of Alzheimer´s disease (AD) include decreased synaptic integrity and widespread neuronal loss ultimately leading to cognitive and memory impairment[68]. A study from Esmaielzade and colleagues demonstrated that epidermal neural-crest neural stem cells were capable of migrating to the host tissue and survive, generating both neuron and astrocyte-like cells in an *in vivo* rat model of AD[72]. Huntington´s disease (HT) is characterized by the loss of a specific class of neurons - projection neurons, which results in progressive dementia in the affected persons[73]. Nerve growth factor (NGF)-producing neural stem cells have been shown to protect striatal neurons against a induced excitotoxic damage, demonstrating that the supply of neurotrophic factors by genetically modified NSC may be a viable option in the treatment of these illnesses[74].

Also in brain tumors, NSC seem to play a promising role. It has been demonstrated that neural stem cells implanted into the brain migrate towards the tumoral region. These cells can then be used as delivery agents, whereas the localized secretion of numerous molecules such as anti-tumoral drugs may provide a successful alternative to highly invasive treatments[75].

1.3 - Neurotrophic factors

The growth and survival of developing neurons in the CNS as well as the maintenance of mature neurons is dependent on the presence of the so-called neurotrophic factors, herein called neurotrophins (NTs). These molecules constitute a wide family of proteins with different signaling pathways with the ultimate goal of sustaining neurite outgrowth, neuronal cell differentiation and survival, and overall network construction[76]. There are 109 ongoing clinical trials with neurotrophic factors (as listed by *clinicaltrials.gov* on July 23rd 2013).

The neurotrophin family is mainly composed by the nerve growth factor (NGF) which is the best characterized member of all, the brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Two more members have been identified, NT-6 and NT-7 although only detected in fish and are believed not to have human homologues[77]. Moreover, a few polypeptides show neurotrophic properties, although not being considered members of the family for structural reasons[77]. These include ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor (ILF) and basic fibroblast growth factor (bFGF2)[78,79].

NTs play their roles through the interaction with two main types of receptors. One is the tyrosine kinase receptor family (Trks), activated by the mature neurotrophin forms, responsible for mediating receptor dimerization and transphosphorylation of activation loop kinases[62]. The most relevant members of this family are TrkA/B/C. TrkA is responsible for binding NGF, while BDNF and NT-4/5 bind tightly to TrkB and NT-3 interacts with TrkC[62,80]. Besides this, a lower-affinity receptor called p75 binds all neurotrophins and is implicated in neurotransmitter release and cell death[81,82] as well as being responsible for deciding which neurons survive during development, through complex signaling[77].
Binding of NTs triggers the activation of the Trk receptors firstly by ligand-induced receptor dimerization, and then by autophosphorilation of tyrosine residues inside the cells. The activation of certain binding proteins leads to activation of the MAPK pathway and phosphorilation of extracellular signal-regulated kinases (ERKs) which results in the promotion of survival, proliferation and differentiation of NSC[62].

NTs present clear clinical advantages. In certain neuronal disorders there seems to be a diminished expression of some viability and neuronal functionality markers. Neurotrophic factors can be of extreme importance in these cases, holding great promise in the therapy of neurodegenerative disorders, owing to their neuroprotective properties[79]. Nevertheless, despite all the invaluable effects that these molecules present, the use of neurotrophins for the treatment of CNS disorders still presents major issues due to the restricted pharmacokinetics

and bioavailability properties of the proteins and also to their inability to cross the blood-brain-barrier[83]. Cell-based therapy may provide a better route to deliver lower doses of neurotrophic factors, by maintaining a tight regulation of gene expression. Several successful cases are described in the next section, where genetically modified cells are able to migrate to injured sites and replace, regenerate or up-regulate certain genes in damaged cells.

1.3.1 - The role of the Brain derived neurotrophic factor

BDNF is a member of the NT family, and plays major roles in neuronal development through the interaction with TrkB as mentioned above. A total of 65 ongoing clinical trials regarding BDNF are listed (as in *clinicaltrials.gov* on July 23rd 2013), implicated in a wide variety of neurological disorders.

Neuronal plasticity refers to the capacity of the brain to adapt when faced with environmental stress, through the formation of new synapses or the generation of new neurons[76]. BDNF is highly expressed in regions of the brain responsible for neuronal plasticity, and is a crucial mediator in this process, showing a regulated expression when altered neuronal function occurs[76]. Ma and colleagues showed that the induction of BDNF and activation of its intracellular receptors can produce neural regeneration, reconnection, and dendritic sprouting, improving synaptic efficacy in conditions of traumatic brain injury[84]. In fact, a study on the localization of TrkB receptor suggests that the synapses are the main site of action of BDNF in the CNS [85]. However, BDNF alone has been shown to fail to act as a survival factor for neuronal precursors, and was not capable to prevent their death overtime[86], suggesting that more factors may be needed in order to maintain a proper neural circuitry. This is supported by the fact that BDNF along with NT-4/5 has been shown to influence the survival and differentiation of neurons, through a retrograde manner leading to the upregulation of Acetylcholine and neuregulin[82].

BDNF is regulated in multiple ways. Glial cells present truncated forms of TrkB receptors, modulating its availability through the regulated storage and release of the active protein[80]. Hormones also influence BDNF expression, where for example glutamate receptor agonists induce, whereas GABA receptor agonists inhibit its expression[80]. It exerts its neuroprotective properties through blocking of apoptosis. Lee and colleagues demonstrated that BDNF-overexpressing cells down-regulate the expression of proapoptotic proteins such as p53, p21 and caspase 3, as well as up-regulating the anti-apoptotic protein Bcl-2 and cell surviving-promoting protein Akt1[87]. It mediates differentiation and survival of NSC in neuroshperes through the activation of its receptors in the cells, and activating several pathways such as ERK-1/2 neurogenic pathway; AKT, which supports neuronal survival and prevents apoptosis, and STAT-3, associated with proliferation of NSC as well as directing its differentiation into

glia[62]. BDNF-overexpressing NSC have been shown to stimulate synaptic protein expression and promote functional recovery following transplantation in rat model of traumatic brain injury[84]. In another study, it was shown they significantly improve neurological motor function compared to native NSC upon transplantation after traumatic brain injury, although 4 weeks after the transplantation that difference was no longer observed[84].

It has also been demonstrated that BDNF is crucial in the recovery of AD and PD. Under stress conditions, BDNF mRNA seems to have a diminished expression[80]. For instance, NSC have been shown to improve certain complex neuronal circuitry through the action of BDNF, that showed reduced activity due to AD[88]. Increased levels of hippocampal BDNF were reported as the main reason for the regain of cognitive function, providing solid negative controls demonstrating that BDNF knockdown in transplanted NSC failed to improve the condition and even reduced synaptic density after transplantation[88]. In PD as well, decreased levels of expression of this neurotrophin are observed[89]. Nevertheless, this is not true for all conditions, since after seizures, BDNF mRNA expression is greatly hampered, with the same profile being detected in cases of patients with temporal lobe epilepsy[90].

An interesting finding hypothesizes its implication in neurotransmitter communication, through autocrine loops and paracrine interactions between neighbor cells and communication from dendrites to axon terminals[85]. Through these interactions, it may promote survival and recovery upon injury, as well as regulate the function of synapses and dendritic arbor modeling[80], which may be the basis of all of BDNF´s highly protective and regenerative properties.

Summing up, BDNF exerts highly beneficial and constructive effects owing to its essential regulatory role in the survival and differentiation of various neural cell types during brain development and after injury, being arguably the most promising of all neurotrophic agents in the management of brain traumatic injury[84]. This encourages its use as a therapeutic agent in CNS disorders, either through the functional protein form, although direct infusion of the protein has some major limitations[84], or by the *ex-vivo* modification of cells, which has shown to be a promising approach.

1.4 - Gene Therapy

Gene therapy functions a lot like conventional drug therapy, except in this case the “drug” administered is a therapeutic gene to be taken up by the cells, which will then express it. The gene introduced will code for a protein whose production was either completely lost, or dramatically decreased as a consequence of a certain metabolic disorder. Or in other cases, the gene produces a totally new protein in the target cells envisaging the bypassing of a certain metabolic pathway. The therapeutic molecule, such as the gene of interest is usually packaged into a bigger molecule generally called a vector, which presents properties that facilitate the modification of the target tissue or organ. There are numerous kinds of vectors, ranging from relatively simple plasmid vectors to complex macromolecular aggregates of polymers and nucleic acids. Those will be described in greater detail further in the text.

There are two main types of gene based therapy. One consists in the direct delivery of the therapeutic gene packaged within a vector, into the target tissue or organ through a variety of methods. Another type of genetic therapy consists in the *ex-vivo* modification of cells by the vector containing the therapeutic agent[6]. When appropriate expansion of the required modified cells is reached, the administration to the patient can finally be accomplished (figure 1.7).

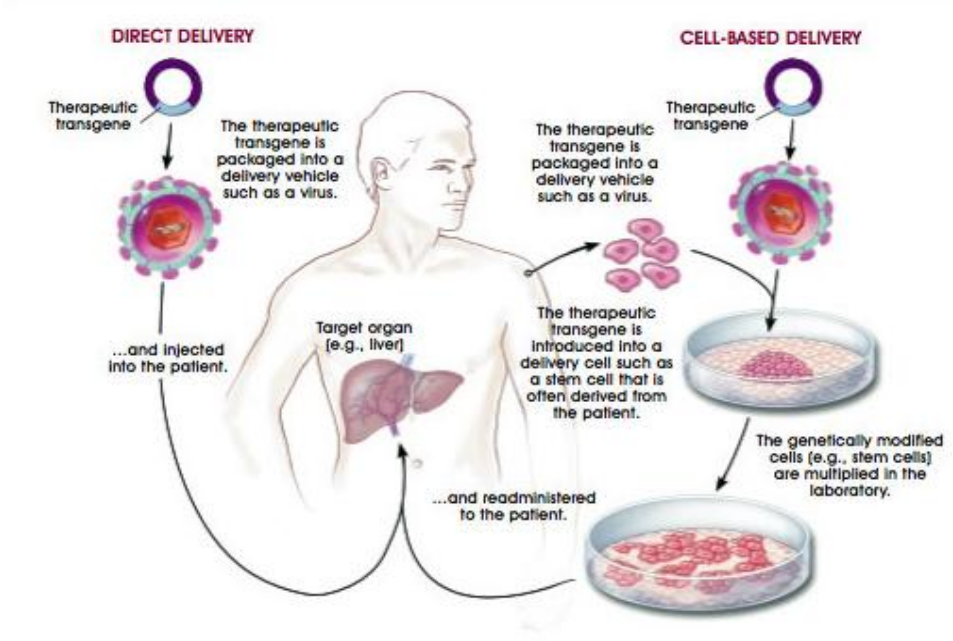


Figure 1.7 - Schematic representation of the two main types of gene based therapy: Direct delivery, and cell-based delivery. *Adapted from*[6].

A successful gene therapy approach must fulfill some fundamental criteria, circumventing several imposing barriers. Specifically, in cell-based delivery, intracellular barriers comprise the

overall mechanism of vector uptake by cells and gene expression, which includes the adequate interaction between the vector and the surface of the target cell, successful diffusion through the cytoplasm and finally entering the nucleus. The internalization into the target cell is a critical step, especially in the delivery of plasmid DNA (pDNA) since its negative overall charge will suffer electrostatic repulsion from the cell membrane[91]. In electroporation-based methods this is avoided, through the momentaneous depolarization of the membrane[92,93]. Upon internalization, the vectors that entered the cell by endocytosis must escape lysosomal degradation. Lysossomes are enclosed vesicles with a high nuclease content and low pH, being the cell compartment responsible for the degradation of internalized molecules[91]. Both liposomes and polymers have been shown to escape lysosomal degradation, through the disruption of the lysosome membrane[91]. The diffusion of pDNA through the cytosol may be troublesome due to the presence of nucleases that degrade unprotected DNA[92,93]. Finally, the nuclear envelope is interrupted by big proteic structures called nuclear pore complexes (NPC) that regulate the traffic of molecules into the nucleus. These allow ions to flow freely into the nucleus but will retain proteins larger than 60kDa and nucleic acids bigger than 300bp[92]. A relevant approach is based on the transfection of highly proliferative cells, taking advantage of the nuclear membrane reorganization events during mitosis. However, this can be particularly difficult in relatively quiescent tissues such as the brain, reason why transfection should be performed in more active cells such as NSC.

Vector genome persistence is another relevant aspect that should be considered[92]. A proper gene therapy method must be able to express the transgene for long periods of time, which in certain cases means the whole lifetime of the patient. This is especially important in the case of plasmids, since they usually exist as episomes and are, therefore, lost upon cell division[4]. Nonetheless this may not represent such a relevant problem in the case of relatively quiescent tissues such as the brain, as mentioned before, since the cell turnover is relatively slow, allowing periods of gene expression that may be sufficient for therapeutic activity.
Although the vector or gene may be sufficiently persistent, it must show sustained transcriptional expression. The overall level of transgene expression in the target cells tightly depends on the strength of the promoter (being the loss of its activity one of the main causes for gene silencing)[94] and the amount of pDNA used for transfection. In non-viral gene delivery strategies, strong promoter/enhancers are used, such as the Cytomegalovirus (CMV) immediate-early promoter and SV40 early promoter[4,94]. It is still relevant to state that despite a vector containing a strong promoter, its activity can be attenuated *in vivo*[94].

Finally, a successful gene therapy approach should not trigger an immune response in the host[91,95], except when DNA vaccines are being used for immunization. Specific immune response activating elements such as unmethylated CpG motifs are undesired, and can also decrease transgene expression[6,75]. This topic will be discussed in greater detail later.

There is not a *one-fits-all* approach to gene delivery in the CNS or any other kind of human health issues for that matter, considering the vast complexity of diseases. Rather, one must be able to realize the advantages of the different approaches available and refine them into their most suitable application.

1.4.1 - Viral methods

A viral vector is a modified viral particle, which takes advantage of the highly infectious capacity of viruses as a means to deliver genetic material of interest into mammalian cells[96]. The sequences needed to produce the actual viral particle are removed from the vector, so it won´t generate undesirable effects in the host. Through genetic engineering, transgenes can be expressed by heterologous plasmids, which when encapsulated by these modified viral particles are able to transduce the desired genetic information in target cells[96].

A variety of recombinant viral particles including adenovirus, retrovirus and lentivirus are available for gene delivery. Their powerful capacity for transfecting highly proliferative cells makes them very useful in such cases. However, they are unable to efficiently transduce mature cells of the CNS[4]. The biggest issue related to viral methods concerns their safety. These vectors can be toxic to cells, generating an immune response from the host and they have a limited transgene capacity in terms of the size allocated[89], although recently, some adenovirus vectors have shown to be able of transferring up to 30kb of genetic material, while mediating long-term gene expression, without presenting immunological issues[97].

Although viral methods remain the most efficient method to deliver genes into mammalian cells, with around 70% of the gene therapy-related clinical trials accounting for viral methods, the triggering of immunological responses, along their relative toxicity and overall genetic instability due to random integration[91],still remain serious issues that need further investigation in order to implement safer strategies. Besides, the inability to produce meaningful numbers of viral units through large-scale production holds back its broader use in gene therapy[68].

1.4.2 - Non-viral methods

Virtually all macromolecules and chemicals of non-viral origin that are capable of allocating genetic material and delivering it into target cells can be considered non-viral vectors.

Non-viral vectors usually show lower transfection efficiencies than their viral counterparts, but are perceived as much safer, and are more feasible in *ex vivo* applications[6] reason why much

attention has been given to the development of non-viral strategies. A more specific targeting of tissues or organs can be accomplished by the superficial functionalization of non-viral particles, in order to interact solely with certain antigens. They usually present easier production than viral vectors, which makes them suitable for larger scale approaches[92].

One of the oldest methods for transfection, first described in 1973[98] is based on precipitation with calcium phosphate, generating DNA-calcium phosphate complexes that are able to enter the cells through endocytosis. More recently, DNA has been complexed with a wide variety of chemicals including polymers such as polylysine, polyethylenimin, polysaccharides among others, although these pose some issues in the diffusion into cells and surviving endossomal degradation[6]. Lipossomes are spherical double-layer membranes enclosing the genetic material to be delivered[99]. These take advantage of its physico-chemical similarities to the cell membranes, being able to diffuse through them[99]. The transfection efficiencies achieved with this technique are relatively high, with authors reporting around approximately 74% mesenchymal stem cells being effectively transfected[100]. Electroporation uses an electric impulse capable of momentaneously disrupting the electric gradient between the two sides of the membrane, allowing the DNA (negatively charged as the phospholipids in the membrane) to freely pass through the membrane into the cell[92]. Up to 70% of transfection efficiencies have been achieved with this technique[101]. Microporation is a recent technology consisting of a modified type of electroporation, which uses a micropippete tip as a reaction chamber. A golden-coated electrode with a surface area of 0,33 mm^2 is responsible for the depolarization of cell membrane[102]. In our laboratory, as high as 60% transfection efficiency was achieved with mouse neural stem cells[103] and 40% with bone marrow-derived mesenchymal stem cells[104]. Some other recent technology has suggested the polyethylene glycol-polyethyleneimine (PEG-PEI) is capable of condensing small interfering RNA (siRNA) into spherical nanoparticles, capable of transfecting as high as 86% of neural stem cells[105].

Plasmids

Plasmids are circular or linear DNA molecules produced in bacteria. Often, the genes carried by plasmids confer bacteria some kind of genetic advantage, through the expression of antibiotic resistance markers[106]. A gene or set of genes of interest may be cloned into plasmids, and propagated by bacteria, in order to obtain a meaningful number of clones.

Plasmid DNA finds application in a very large spectrum of gene delivery tools, being the basis for vaccine development. They can also be delivered directly into the target tissue or cells by microinjection. Some successful cases report that the process of microinjecting plasmid DNA to cattle zygotes did not alter their morphology, with more than 70% of the injected cells expressing the reporter eGFP gene[107]. Direct injection into target tissues is an approach that

reports some successful number as well. On the other hand, plasmids can be packaged into the variety of vectors previously described.

There are some barriers that need to be surpassed in order to implement strong and reliable methods of gene delivery through plasmid vectors. Some of the issues are related to their weak transfection capacity of non-dividing cells; the silencing of transgene expression due to bacterial sequences; immunogenicity derived from unmethylated CpG motifs and insertional mutagenesis with potential for malign transformation and the overall toxicity of transfection agents[95]. The size of the molecule has also been proven a determining factor for efficient transfection. pDNA is greatly restricted by its size (usually no less than 2000 kDa), limiting its diffusion capacity and absorption by the tissue[94,108].

Unmethylated CpG dinucleotides are predominant in bacterial DNA but not in vertebrates[109]. These motifs trigger innate or acquired immune responses[103]. They are very important in the activation of plasmacytoid dendritic cells and B cells that protect the organism against a variety of pathogens. In fact, long and stable-expression viruses become resistant through the decrease in their CpG content[109]. The immunogenic response mechanism is achieved through the Toll-like receptor 9 (TLR9) which triggers a deregulation in redox balances of the cells, leading to the activation of MAPKs (Mithogen activated protein kinases) and NFkB[66,109]. The activation of NFkB and many other transcription factors, leads to upregulation of many cellular proto-oncogenes and proinflammatory cytokines. The pharmaceutical industry takes advantage of these unmethylated CpG motifs, in order to produce vaccines[109]. However, in gene therapy the opposite action is required. The elimination or drastic reduction of these motifs content from therapeutic plasmids must be achieved, in order to eliminate any inflammatory response from the host, and also to reduce transgene silencing[103].

Taking into the account the amount of vectors available, and the solid confirmation of the therapeutic value of cloning genes of interest into plasmids, creativity is the limiting step in the establishment of useful approaches that are able to surpass the abovementioned barriers.

1.4.3 - Gene delivery to the Central Nervous System

The brain´s access is highly limited due to the protection conferred by the cranium. Also, the blood-brain barrier (BBB) is impermeable to most of the molecules and proteins present in the blood stream which strongly restricts the brain´s access through the main circulatory system routes[83,110]. This is the main reason why most of the gene delivery techniques to the CNS are based on the direct infusion of vectors into the brain´s parenchyma (the functional tissue of the brain)[68].

Viral vectors remain the most prominent method to deliver genes into the CNS[68]. Namely, adeno-associated virus (AAV)-mediated transgene expression has been showed to hold a solid potential when applied into the brain parenchyma of Parkinson´s disease patients, showing considerable recovery while no relevant adverse effects were observed[111]. Still, besides some successful cases, viral vectors present major safety issues, reason why non-viral methods should be optimized for this purpose. An effective way to avoid BBB block is through PEGylated immunoliposomes (PILs), formulated with monoclonal antibodies that allow the crossing of the BBB, through the interaction with specific cell receptors[68]. Electroporation has also been used directly in the brain of a variety of adult animals, to deliver therapeutic plasmids, being stated as a safe and effective method to modify cells in the CNS, being arguably a robust method for treatment of Alzheimer´s and Parkinson´s diseases, as well as depression[92].

Another meaningful, relevant approach is the *ex-vivo* modification of cells such as NSC, HSC or MSC which are known to migrate to injured regions[68]. This approach is particularly interesting, and NSC have been shown to be great target-specific carriers of therapeutic agents to the CNS. NSC can be used as delivery agents, whereas the localized secretion of numerous molecules such as anti-tumoral drugs may provide a successful alternative to highly invasive treatments, with many of the approaches showing extended survival of animal subjects, and at times being responsible for total tumour regression[75]. For instance, *ex-vivo* genetically modified NSC have been shown to be able of delivering a cytosine deaminase to an intracranial tumor (clinical trial ID: NCT01172964). In the referring study, there was evidence of extensive migration of NSC, suggesting that even a systemic application of these cells preferentially repopulates an intracranial glioma[112]. Overexpressing neurotrophic factors in NSC has also been showing promising results from a clinical standpoint. Vascular endothelial growth factor (VEGF)-overexpressing NSC were transplanted into a rat spinal cord injury model. The genetically modified cells were able to increase gliogenesis and angionesis, ameliorating the severe symptoms of this condition[113]. GDNF has been overepxressed in NSC, as well. These have resulted in a decrease of infarct, yielding overall better behavioural results while protecting ischemic brain in rats from apoptosis[114].

NSC hold a tremendous potential of applications, even in the adult brain. The combination of NSC-based therapy with other techniques may provide more specific, effective and safer strategies to deliver genes in the CNS. There is a wide variety of cell and gene therapy approaches to the CNS, considering the high amount of neurogenic and neuroprotective agents available and the potential of NSC. Nonetheless, one of the main hurdles in neurological tissue regeneration is discovering the right combination and amount of each growth and/or neurotrophic factor to be delivered to cells. However, the *ex-vivo* modification of NSC can be of great value. It allows the control of gene expression, in a sense that both the timing and amount of transgene expression may be susceptible of control[115].

2. Materials and Methods

2.1 Bacterial strains and plasmid DNA

Escherichia coli XL-Gold (Stratagene, Santa Clara, CA, USA) was the chosen bacterial strain to produce the desired plasmid. Bacterial cells were cultivated at 37ºC in luria-bertani (LB) medium (20 g/L, Sigma-Aldrich®, St. Louis, MO, USA) with orbital agitation (250 rpm).

The parental plasmid MN530A-1, containing the BDNF gene (741 bp) was purchased from System Biosciences (SBI). The plasmid in which BDNF's and GFP's cloning was performed was the pcDNA3.1 plasmid (Invitrogen^{TM}, Carlsbad, CA, USA). Throughout this work the plasmid containing the BDNF gene (741 bp) will be called pcDNA3.1-BDNF, and the one harboring GFP sequence (720 bp) will be referred to as pcDNA3.1-GFP.

2.2 BDNF amplification by PCR

BDNF gene was amplified from the parental plasmid by the polymerase chain reaction (PCR), using the forward primer with the sequence 5´- GGG **AAG CTT** ATG ACC ATC CTT TTC 3´ , containing a BamHI restriction site (in bold) and the reverse primer 5´- AAA **GGA TCC** TCA TCT TCC CCT TTT 3´ harboring a HindIII restriction sequence (in bold).
The primers presented melting temperatures of 56ºC and 54ºC , and C+G contents of 46% and 42%, respectively. A series of PCR reactions were performed, containing 10 ng of template DNA, 1.5 µL of each primer at a 10 µM concentration, and 25 µL of KOD Hot Start Mix (Novagen®, Milipore®, Darmstadt, Germany) and adding miliQ water to a 50µL total reaction volume. The initial PCR step consisted in heating the mixture at 95ºC for 2 min. Then, the second step consisted in heating the mixture at 95ºC for 20 sec. The third step occurred for 10 sec at 51ºC for primers' annealing. Fourth step was performed at 70ºC for 15 sec for fragment elongation. The steps 2 to 4 were repeated as a cycle 40 times. For the final elongation step, the mixture stayed at 70ºC for 10 min. When the PCR reaction ended, all the volume was loaded into a 1% agarose gel for BDNF bands confirmation.

2.3 BDNF cloning into pcDNA3.1

After the right BDNF bands were confirmed, these were extracted from the gel, and the DNA was purified by NZYGelpure (NZYtech®, Lisbon, Portugal). This fragment was digested with both BamHI and HindIII as described earlier in section 2.2. The digested fragment was then again loaded into a 1% agarose gel and once more extracted into pure digested fragments.

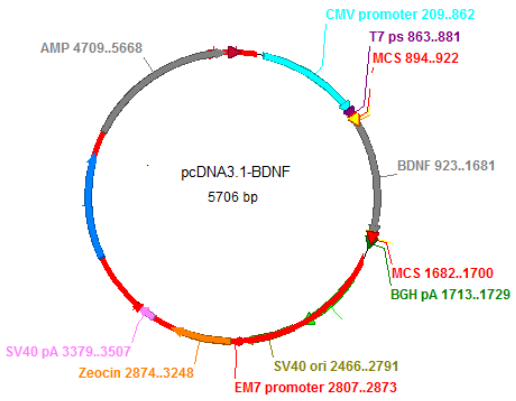
Both digested plasmid DNA and BDNF fragment samples were evaporated using Speed Vacuum (ThermoScientific™, Waltham, MA, USA) for 75 min at 60ºC, at medium speed. For concentration assessment, 1µL of each concentrated sample was loaded into a 1% agarose gel, and band intensity was visually compared to the Ladder III (200-10000 bp) (NZYtech®).

For plasmid cloning, a 1:1 and 3:1 ratio of insert/vector was used. 1µL of T4 DNA Ligase (Promega, Madison, WI, USA) was added to the mixtures, along with 1uL of T4 buffer (Promega), and either 1:1 ug of insert/vector or 3:1 µg of insert/vector. MiliQ water was added to a total 10 µL ligation reaction volume. The reaction occurred at room temperature overnight. The next day, competent cells were transformed as described earlier. A few colonies were picked and inoculated overnight in 5 mL LB containing 50 µg/mL ampicilin at 37ºC, 250 rpm. Purification of plasmid DNA and partial or double digestion was performed. The pcDNA3.1-GFP plasmid had been previously constructed. A confirmation on its correct construction was performed by digesting it with HindIII and ApaI restriction enzymes. The latter has a restriction recognition site in the sequence 5´ … GGGCCC … 3´.

When correct plasmid construct were confirmed (see figure 2.3.1), transformed cell banks were created. For this, a single colony was grown as described previously, and the next day, a new culture was initiated at a OD_{600nm} of 0.1. When OD_{600nm} reached a value of approximately 0.8, 800 µL of the cell suspension were loaded into cryovials containing 20% glycerol, and the aliquots were stored at -80ºC.

After the larger scale production, both pcDNA3.1-BDNF and pcDNA3.1-GFP were purified using Midiprep endotoxin free purification kit (Macherey-Nagel, Duren, Germany) following the high copy manufacturer's protocol. Concentration of purified plasmids was assessed by NanoDrop (ThermoScientific™) bioanalyser, by measuring absorbance at 260nm.

A



B

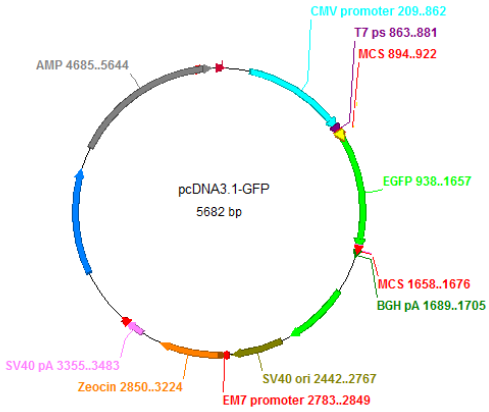


Figure 2.3.1 - pcDNA3.1-BDNF (A) and pcDNA3.1-GFP (B) plasmid maps, containing the respective BDNF and GFP genes. The most important elements of the plasmid are displayed: the CMV promoter; T7 promoter binding site; multiple cloning sites (MCS); BGH polyadenilation signals (BGH pA); SV40 promoter and origin; EM7 promoter; Zeocyn resistance marker; SV40 polyadenilation signal (SV40 pA) and ampicilin resistance marker.

2.4 Transformation of bacterial strains

Competent cells were transformed with the desired plasmid by the heat-shock protocol. In brief, 5 µL of pcDNA3.1 was added to 100 µL of *E.coli* competent cells and incubated on ice for 30 min. After that, the mixture was placed on a water bath at 42ºC for 45 sec to 1 min and then again on ice for 2 min. 900 µL of LB medium was added to the mixture and cells were incubated at 37ºC, 250 rpm for 1 hour. After that, cells were centrifuged at 4000 rpm for 5 min. 900 µL of the supernatant was discarded, and the pellet was ressuspended in the remaining 100 µL. The cellular suspension was afterwards spread on solid LB media with zeocyn (50 µg/mL) and incubated at 37ºC at 250 rpm overnight. Positive colonies were inoculated in 5 mL of LB media with zeocyn (50 µg/mL) and incubated at 37ºC with shaking at 250 rpm overnight. For large scale production of plasmids, selected colonies were grown as described, and when the desired OD_{600nm} was reached, adequate volume of the cellular suspension was inoculated on 400 mL total volume of LB medium, and incubated 37ºC, 250 rpm, overnight. Plasmids were purified by the DNA purification kit NZYMiniprep (NZYtech®). When the correct plasmid production was assessed by agarose gel electrophoresis, they were digested with BamHI and HindIII (Promega) restriction enzymes. For this, 10 µL of plasmid DNA is digested in 2 µL of Buffer E

(Promega) with 0.5 µL of each enzyme, and adding sterile water to the mix to a 20 µL total working volume and incubated at 37ºC for 1 hour. The whole sample was then loaded into an agarose gel, and then the correct bands were dissected from the gel and purified by NZYGelpure kit (NZYtech®).

2.5 Neural Stem Cell culture

2.5.1 Cell lines

Two cell lines were used throughout this work: the CGR8-NS cell line, derived from the mouse embryonic stem cell line CGR8 in the laboratory of Professor Austin Smith, Welcome Trust Centre for Stem Cell Research, Cambridge, United Kingdom; and the ReNcell VM (Millipore®) (herein abbreviated to "ReN" cells), an immortalized human neural progenitor cell line with the ability to readily differentiate into neurons and glial cells. "VM" stands for ventral mesencephalon, region of the brain from which the cells were isolated[116].

2.5.2 Expansion of Neural Stem Cells

The cells were thawed either from liquid nitrogen or -80ºC stock, containing cells ressuspended in 900 µL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) – GlutaMAX™ medium (Life Technologies™, Carlsbad, USA) and 100 µL of dimethyl sulfoxide (DMSO (Sigma®, St. Louis, USA)). The frozen vials were thawed by submerging the mixture in a 37ºC water bath, and by resuspending in DMEM/F12. The mixture was centrifuged for 3 min at 1000 rpm, and the supernatant was discarded in order to remove DMSO from the mixture. The appropriate medium was then added to the cells. CGR8-NS cells were ressuspended in DMEM/F12 supplemented with 10 ng/mL EGF and FGF2-2 (Peprotech, London, UK), 1% N-2 and 1 µl/mL B27® supplement (both from Life Technologies™), 20 µg/mL insulin (Sigma®) and 1% penicillin/streptomycin (Life Technologies™). The described medium composition will be herein termed expansion medium for this cell line.

ReN cells were ressuspended in DMEM/F12 supplemented with 20 ng/mL EGF, 10 ng/mL FGF2-2 (Peprotech), 1% N-2 and 20 µl/mL B27® supplement, 20 µg/mL insulin and 1% penicillin/streptomycin. This will be shortened to expansion medium for this cell line throughout the text.

Trypan blue dye exclusion test (Gibco®, Invitrogen™) was used to determine cell viability by counting cells in a hemocytometer under an optical microscope. Viability was described by equation (1):

$$\text{Viability } (\%) = \frac{VC}{VC + NVC} \text{ x } 100 \qquad (1)$$

where VC is the viable cell number and NVC the non-viable cell number.

An appropriate number of cells were then platted into either T-flasks, or tissue culture plates (Falcon®, BD Biosciences, San Jose, CA) under adherent conditions and incubated at 37ºC and 5% CO_2-humidified atmosphere.
CGR8-NS cells were expanded on untreated surfaces, although after transfection assays, fibronectin-coated surfaces were used. This was achieved by diluting the appropriate amount of fibronectin (3 µg of fibronectin/cm^2 surface area) in sterile phosphate buffered saline (PBS, Gibco®, Invitrogen™) and loading a sufficient amount of the solution to cover the culture plate surface. The plate was subsequently incubated at room temperature for 1 hour and the mixture was aspirated, just before cell inoculation.

ReN cells were expanded on polyornithine and laminin-coated surfaces. Coating with polyornithine/laminin was performed by pre-coating the desired surface with polyornithine (diluted in PBS) and incubating the mixture at 37ºC for at least 30 min. Then this solution was aspirated and a mixture containing the appropriate amount of laminin (10 µg of laminin/cm^2 surface area) was loaded to the surface and the mixture was incubated at 37ºC for at least 4h.

When cells reached sub-confluency they were harvested using Stempro® Accutase® (Life Technologies™) by incubation at 37ºC and 5% CO_2-humidified atmosphere for 3-5 min. Afterwards, the cell suspension was diluted 1:1 with DMEM/F12 and centrifuged at 1000 rpm for 3 min. The pellet was ressuspended in an appropriate volume of culture medium, and cell viability was assessed by trypan blue dye exclusion method, as described before in this section.

2.5.3 Differentiation of Neural Stem Cells

For differentiation purposes, only ReN cells were used throughout this work. For this, ReN cells were plated on polyortnithine/laminin-coated 24-well plates (Falcon®, BD Biosciences) at a density of 75,000 cells/cm^2. A 1:1 mixture of DMEM/F12 medium (Gibco®, Invitrogen™) and Neurobasal® Medium (1X) without Phenol Red (Gibco®, Invitrogen™) was used, along with 20 µl/mL B27® supplement (Gibco®, Invitrogen™), being incubated at 37ºC and 5% CO_2-humidified atmosphere. This will be described further in the text as the differentiation medium.

For positive control on BDNF effect on cell culture, 50 ng/mL of BDNF (Gibco®, Invitrogen™) was added to the mixture on selected cells. Culture conditions were maintained as described until differentiation was achieved. Differentiation was carried out for 12 days, adding fresh medium every 3 days.

2.6 Neural Stem Cell transfection

Neural stem cells were transfected using one of three different methods: an electro-physical method called microporation, and two chemical techniques - cationic polymer and lipid-based transfection reagents.

2.6.1 Microporation

CGR8-NS and ReN cells (150,000 – 200,000 CGR8-NS cells for each transfection) were resuspended in 10 μL of a resuspension buffer (RB), provided by the equipment manufacturer (Life Technologies™), and incubated with the adequate amount of plasmid DNA. The transfection reaction was carried out by the Microporator MP100 (Digital Bio/(Neon), Life Technologies™). After microporation, the mixture was plated into 24-well plates containing adequate pre-warmed culture medium without antibiotics, and incubated at 37ºC and 5% CO_2-humidified atmosphere for 24 hours. After this, fresh medium containing antibiotics was added. Cells could be visualized under the fluorescence optical microscope Leica DMI 3000B (Leica Microsystems GMbH, Heerbrugg, Switzerland), where GFP transfected cells could be easily identified using the appropriate fluorescence filter, allowing for recognition of efficiently transfected cells. Cells were then harvested at specific times for the different assays performed. Cell viability (equation 1), and cell recovery was assessed for microporated samples (equation 2):

$$\text{Recovery (\%)} = \frac{VC}{VCc}\ x\ 100 \qquad (2)$$

where VCc is the total viable cell number in the control (non-transfected cells). For quantitative assessment of transfection efficiency, Fluorescence Activated Cell Sorting (FACS) was performed, as will be described later.

2.6.2 Cationic polymer-based transfection

CGR8 cells were transfected with TurboFect (TF) (ThermoScientific™) at 150,000 – 200,000 cells per reaction. The process involved complexation of a polymer-based reagent complexed with pcDNA3.1-GFP, either through the standard protocol or through reverse transfection. The first is achieved by the addition of a 2:1 TF:DNA ratio to cells expanded for 24 hours in expansion medium, and incubated at 37ºC and 5% CO_2-humidified atmosphere on fibronectin-coated dishes for 5 hours, time after which, fresh media is added, and cells are allowed to grow for 24 hours.

In reverse transfection, the 2:1 TF:DNA ratio mix is added to the bottom of 2 cm^2 surface area wells. 200,000 cells in suspension are then added on top of the mixture, and incubated at 37ºC and 5% CO_2-humidified atmosphere on fibronectin-coated dishes. From this point, the cells receive the same treatment as mentioned above. Assessment of viability, recovery and percentage of GFP-expressing cells was performed as described earlier in section 2.6.1.

2.6.3 Lipid-based transfection

CGR8 and ReN cells (150,000 – 200,000 cells for each transfection) were transfected with Lipofectamine® 2000 (LF, Invitrogen™). The reaction involved complexation of the lipid-based reagent either with pcDNA3.1-GFP or pcDNA3.1-BDNF. After optimization of the protocol, a 2:1 LF/DNA ratio was chosen for the next experiments. Lipoplex formation was carried out by diluting the appropriate amount of LF in Opti-MEM® (Gibco®, Invitrogen™) medium, and mixing by vigorous vortex agitation for 15 sec. In parallel, the adequate amount of plasmid DNA was diluted in Opti-MEM® (Gibco®, Invitrogen™) medium, and mixed by pipetting up and down. The cationic-lipid was then mixed with plasmid DNA. The solution was mixed by pipetting up and down, and incubated for at least 20 min, at room temperature. Finally, the lipoplexes were added to cells expanded for 24 hours in appropriate expansion medium without antibiotics, and incubated at 37ºC and 5% CO_2-humidified atmosphere on differently coated dishes depending on the cells used, for 5 hours. After that, fresh media containing 1% penicillin/streptomycin (Gibco®, Invitrogen™) was added, and cells were allowed to grow for 24 hours. Assessment of viability, recovery and percentage of GFP-expressing cells was performed as described earlier in section 2.6.1.

2.7 Flow cytometry

GFP-transfected cells were harvested and counted. Cell viability and recovery were assessed as described previously in section 2.6.1. Cells were centrifuged at 1000 rpm for 5 min, and the pellet was ressuspended in 500 µL of a 2% paraformaldehyde (PFA) solution for cell fixation and the mixture was transferred to FACS tubes. The percentage of GFP^+ cells was then measured in the BD FACScalibur™ equipment (BD Biosciences). The results were analyzed with CellQuest software (BD Biosciences). Statistically significant results are considered when a minimum of 1000-gated cells are counted. Between one and three replicates were used for each assay.

2.8 Immunocytochemistry

For immunofluorescence analysis of cells, complete culture medium was removed from cells, and 4% PFA was added for 10 min at room temperature for cell fixation. The cells were then washed with PBS (Gibco®, Invitrogen™) and incubated for 1 hour with blocking solution (10% normal goat serum (NGS, Gibco®, Invitrogen™) and 0.1% Triton X-100 (Sigma-Aldrich®) in PBS). Afterwards, blocking solution was removed and cells were incubated with adequate primary antibody diluted in a staining solution (5% NGS and 0.1% Triton X-100 in PBS) overnight at 4ºC. The primary antibodies used were neuronal Class III β-Tubulin (TuJ1) (Covance, dilution 1:2000), Nestin (1:200, R&D Systems®), GFAP(1:200, Milipore). The next day, after washing with PBS, the cells were incubated with the appropriate secondary antibodies for 1 hour at room temperature. After this step, cells were once more washed with PBS and incubated with DAPI solution (Sigma-Aldrich®) (15:10000 in PBS) for 5 min at room temperature. Two final washes with PBS were performed, and the cells were ready for analysis under a fluorescence optical microscope - Leica DMI 3000B (Leica Microsystems GMbH). Photographs of the culture were taken with a Nikon® digital camera DXM1200F (Nikon, Tokyo, Japan).

2.9 Alamar blue assay

Alamar blue assay is based on a fluorometric/colorimetric cell growth indicator that is produced according to the detection of metabolic activity from a certain cell culture. This indicator is the dye resazurin, which is by itself weakly fluorescent. It is then reduced to the resorufin form, a highly red fluorescent compound, by means of proliferative activity within a given culture or by

other test-agents added to the medium[117]. It is a widely used method to assess the proliferation of a large spectrum of cell lines, and the cytotoxicity of agents within various chemical classes.

Briefly, selected cell populations were exposed to a certain test-agent, that will be highlighted later. After that, alamar blue was diluted 1:20 in differentiation medium, and cells were incubated with the agent for 1 hour. Then, fluorescence measurements were taken at pre-determined time-points (in this work 1 hour, 3 hours and 24 hours after the exposure to the test-agent), using the Plate Reader Tecan Infinite M200 Pro (Tecan®, Männedorf, Switzerland), by monitoring fluorescence excitation at 560nm and emission at 590nm. Absolute fluorescence intensity is returned by the equipment. Most of the time, relative fluorescence intensities are preferred since the purpose of the assay is to compare populations exposed to different test-agents. So, the relative fluorescence intensity was calculated according to equation 3:

$$\text{Relative fluorescence intensity sample} = \frac{Fluorescence\ instensity\ of\ sample}{Fluorescence\ intensity\ of\ control}\ x\ 100 \quad (3)$$

In order to test glutamate toxicity in a differentiated neuronal cell culture, ReN cells maintained in differentiation medium for 12 days were exposed to 100 µM, 500 µM, 2 mM and 4 mM of Glutamate (Sigma-Aldrich®) for 2 hours. Adequate volume of a 100 mM glutamate stock solution was added to the differentiation medium and then added to cells. Following the 2 hours of incubation, 3-day old differentiation medium was added to the cells. Glutamate toxicity was then assessed by the Alamar blue assay, at 1 hour, 3 hours and 24 hours after glutamate exposure.

To evaluate the potential protective activity from BDNF, the cells were exposed to 4 mM of glutamate for 2 hours. After that, 3-day old conditioned medium containing BDNF secreted by transfected cells was added to glutamate-exposed cells.

2.10 Qualitative analysis of neuronal neurites by ImageJ® software

Non-transfected ReN cells were differentiated using either the standard protocol, or by adding 50ng/mL of exogenous BDNF (Life Technologies™) to the medium, for positive-control on BDNF. Transfected ReN cells with BDNF were differentiated using the standard protocol. Fresh differentiation medium was added every 3 days. Differentiation was induced for 12 days. At the end of the process, the cells were fixed for immunostaining as described on section 2.8. Tuj1-stained cells were analyzed with ImageJ software. The plugin NeuronJ is a semi-quantitative method that allows the measurement of neurites of neurons as depicted on figure 2.10. Neurites

are herein understood as the processes that emerge from the soma (nucleus), which include axons and dendrites. First, the background fluorescence is subtracted with a rolling ball radius of 50 pixels. Then a color threshold is defined in order to enhance the contrast between neurites and other particles. The image is then binarized, rendering positively stained particles the maximum intensity (shown in dark) against zero intensity particles (in white). Through the NeuronJ plugin, tracings are created by placing the cursor at one end of the cell, stretching the tracing which is automatically calculated by the software, until the other end of the cell. The tracings are then automatically measured. From this, primary neurites and secondary neurites can be identified. The former are the neurites emerging directly from the soma. Secondary neurites emerge from primary neurites.

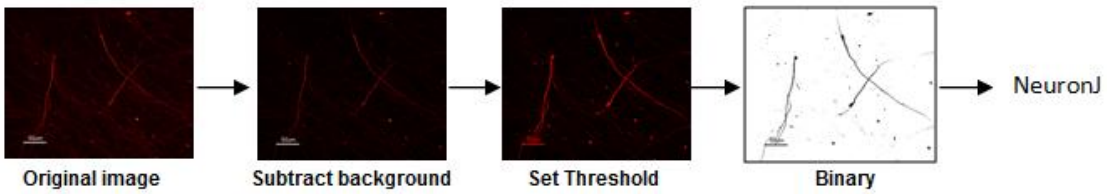


Figure 2.10 - Sequence of operations performed on ImageJ® until a final binarized image is achieved, following neurite measurement of NeuronJ plugin.

2.11 Quantification of mRNA extracts by real time PCR

In order to estimate the expression of specific gene transcripts, cells exposed to different conditions were plated into 6-well plates, then being harvested at specific times and the pellet was kept at -80ºC. Later, total mRNA extracts were isolated using High Pure RNA Isolation kit (Roche®), according to the manufacturer´s protocol. Then, mRNA was converted to cDNA using the Trancriptor First Strand cDNA Synthesis kit (Roche®). For this, 1µg of mRNA was combined with anchored-oligo$(dT)_{18}$ primers (final concentration 2.5 µM) and PCR-grade water until a volume of 13µL was reached. Then, Reverse Transcriptase Incubation Buffer was added, as well as Protector RNAse Inhibitor (final quantity of 20 units), deoxynucleotide mix (final concentration of 1mM of each) and Transcriptor Reverse Transcriptase (final quantity of 10 units), to a final volume of 20µL. cDNA was obtained by heating the mixture at 55ºC for 30min, followed by reverse transcriptase inactivation by heating the mixture to 85ºC for 5min. After obtaining cDNA, RT-PCR was performed, using several primers for the analysis of different genes including Nestin, Sox2, GFAP, β3-tubulin, IL-6, BDNF, Caspase7 and the housekeeping gene GAPDH (StabVida®, Portugal) (table I). The RT-PCR was performed using the manufacturer´s protocol (LightCycler®FastStart DNA Master SYBR Green I, version 18) from Roche®. Briefly, a set of 20µL solutions were prepared, which included 4mM $MgCl_2$, 2µL of template cDNA and a primer mix (final concentration was 0.5µM for each), filling the remaining volume with PCR-grade water. The control solutions contained 2µL of PCR-grade water

replacing cDNA. The reactions were performed using the equipment StepOnePlus™ (Life Technologies™). Between one and two replicates were used in the different assays.

Gene expression was assessed through the $2^{-\Delta\Delta CT}$ method described elsewhere[118], in which expression of each gene is displayed related to the control, assuming that both target and reference genes are amplified with a near 100% efficiency (equation 4).

$$\text{Relative gene expression} = 2^{-(\Delta Ct\ sample - \Delta Ct\ control)} \quad (4)$$

Two RT-PCR programs were run depending on the samples. Program 1: pre-incubation step at 95ºC for 10 min; denaturation step at 95ºC for 30 sec; annealing at 58ºC for 30 sec; elongation step at 72ºC for 30 sec. The last three cycles were repeated 40 times. Program 2: all steps were run at the same temperature for the same amount of time. The last three cycles were repeated 35 times.

For obtaining the melting curve, the temperature was increased to 95ºC for 15 min, then being decreased to 60ºC for 1 min, and then again increased to 95ºC through 0.2ºC increments, and remaining for 15 min at this last temperature.

Table I - Primers used in mRNA quantification by real-time PCR.

Gene	Primers 5´ -> 3´ (fwd/rev)	Tm (ºC)	Number of bases	Amplicon size (bp)
GAPDH	ACGACCCCTTCATTGACCTCAACT / ATATTTCTCGTGGTTCACACCCAT	60.2 / 56.4	24 / 24	324
Sox2	GTATCAAGGAGTTGTCAAGGCAGAG / TCCTAGTTCTTAAAGAGGCAGCAAAC	57.1 / 56.7	24 / 25	78
Nestin	GCCCTGACCACTCCAGTTTA / GGAGTCCTGGATTTCCTTCC	56.8 / 54.9	20 / 20	200
GFAP	CTGTTGCCAGAGATGGAGGTT / GGAGAACAACCCTCTGAGCTG	57.5 / 56.4	21 / 21	289
β3-tubulin	CCATCTTGCTGCCGACAC / CAATAAGACAGAGACAGGAG	56.7 / 49.6	18 / 20	126
IL-6	ATGAACTCCTTCTCCACAAGC / GTTTTCTGCCAGTGCCTCTTTG	21 / 22	21 / 22	264
BDNF	CATCCGAGGACAAGGTGGCTTG / GCCGAACTTTCTGGTCCTCATC	60.2 / 57.9	22 / 20	161
Caspase7	AAGAGCAGGGGGTTGAGGAT / TGAAGAGGGACGGTACAAACG	58.4 / 56.8	20 / 21	84

2.12 Statistical analysis

When appropriate, the Mann-Whitney-U test was used to evaluate the significance of the difference between two groups of independent samples that did not show a normal distribution and/or homogeneity of variances. Significant analyses ($p < 0.05$) are displayed as * on selected data.

3. Results & Discussion

3.1 - Amplification of BDNF by PCR

In order to construct the desired plasmids (see section 2.1), the BDNF gene was amplified by PCR as described in section 2.3. Figure 3.1 shows a unique band with less than 800bp (BDNF gene is 741bp long - sequence in appendix), confirming that the gene was efficiently amplified, without rendering non-specific amplification products.

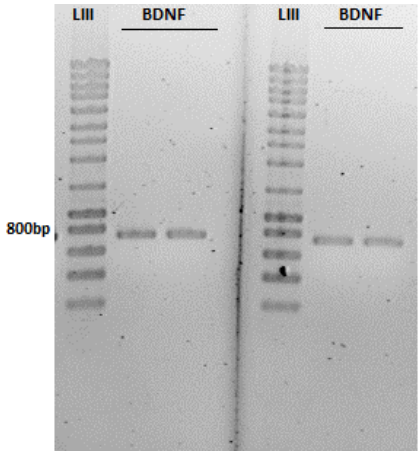


Figure 3.1 - Confirmation of efficient BDNF gene (741bp) amplification by PCR on a 1% agarose gel. LIII is the DNA Ladder III (NZYtech®).

3.2 - Cloning of BDNF into pcDNA3.1

The BDNF gene was extracted from the gel and cloned into the plasmid pcDNA3.1 (see section 2.3). In order to confirm the correct construction of the plasmid, a restriction analysis of the construct was performed (Figure 3.2.1). In parallel, another restriction analysis was performed in order to confirm the correct construction of the previously produced pcDNA3.1-GFP.

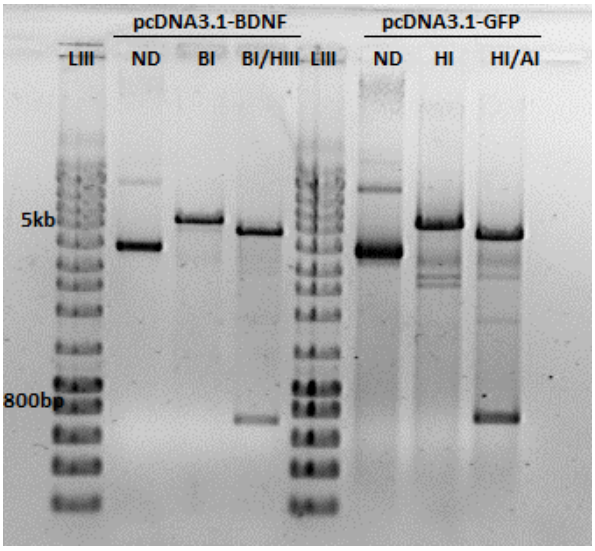


Figure 3.2.1 - Confirmation of the correct plasmid constructions on a 1% agarose gel. The samples are presented in their non-digested form (ND); single digestion with BamHI (BI) and HindIII (HI) and double digested with the pair BamHI/HindIII (BI/HIII) and HindIII/ApaI (HI/AI) for pcDNA3.1-BDNF and pcDNA3.1-GFP, respectively. LIII corresponds to the DNA ladder III (NZYtech®).

Supercoiled DNA migrates better through the agarose gel due its more compact structure, appearing further from the loading point in the gel, as it can been seen for both non-digested samples (ND) in Figure 3.2.1. A single cut in the double strand DNA leads to its linearization, which has a lower mobility than supercoiled DNA, reason why the single-digested bands appear slightly closer to the beginning of the gel (pcDNA3.1-BDNF restricted by BamHI (BI) and pcDNA3.1-GFP by HindIII). A double digestion by the enzymes previously used to achieve the construct, allows for the detection of the cloned gene. As it can be seen in Figure 3.2.1, double digestion of pcDNA3.1-BDNF by BamHI and HindIII results in two bands, one with approximately 5000bp corresponding to the linearized plasmid without the gene of interest, and another slightly shorter than 800bp, corresponding to the BDNF gene which is 760bp long. The same can be reasoned for pcDNA3.1-GFP, with the GFP gene band showing up below the 800bp reference band (GFP gene is 720 bp long).

The constructs were then produced in a larger scale, and purified by endotoxin-free purification kits (see section 2.4). The properties of the plasmids obtained are summarized in Table II.

Table II - Concentration of plasmids obtained after purification with the Endotoxin-free NucleoBond® Xtra Midi EF kit, and purity assessment through ratios of absorbance at different wavelengths. Concentration and purity of plasmid DNA is presented as the mean value ± SD of three independent measurements.

	Concentration (µg/µL)	**A_{260}/A_{280}**	**A_{260}/A_{230}**
pcDNA3.1-GFP	1.20 ± 0.01	1.88 ± 0.01	2.44 ± 0.17
pcDNA3.1-BDNF	1.11 ± 0.03	1.85 ± 0.01	2.41 ± 0.42

Based on the information shown in Table II, the plasmid DNA presented very low contamination with proteins, since a A_{260}/A_{280} ratio of 1.8 is usually related to pure DNA[119]. The ratio A_{260}/A_{230} is used to evaluate eventual contamination of DNA with compounds such as phenol and guanidine commonly used in DNA extraction kits. This value should be between 2.0 and 2.2[119]. Slightly higher values were obtained, meaning that the sample if virtually free of such contamination.

3.3 - Transfection of mouse Neural Stem Cells

The purified plasmids were then ready to use on neural stem cells. The following results refer to the transfection of the mouse ES-derived NSC line CGR8-NS.

3.3.1. Microporation

Microporation is a novel type of electroporation, which carries out the transfection reaction in a 10µL chamber, taking advantage of a 0,33mm^2 electrode surface[102]. Recently, mouse NSC were successfully microporated with plasmid DNA, with 75% of the transfected cells expressing a transgene[103].

CGR8 cells were first microporated using previously optimized conditions - 1 pulse of 1500V for 20ms (2 x 10^5 cells/microporation), and then plated on untreated surfaces. This resulted in cell death of all the population, not allowing for further analysis (data not shown). Surface coating with extracellular matrix components has been shown to provide a better support for cell attachment and to promote overall cell survival. Hence, the appropriate culture wells were coated with CELLStart™ (Gibco®, Invitrogen corporation, Carlsbad, CA), and microporation was carried out in the conditions stated before, using 0.5µg of pcDNA3.1-GFP. Cells survived in these conditions, allowing for continuous analysis of the cell culture for 4 days (figure 3.3.1).

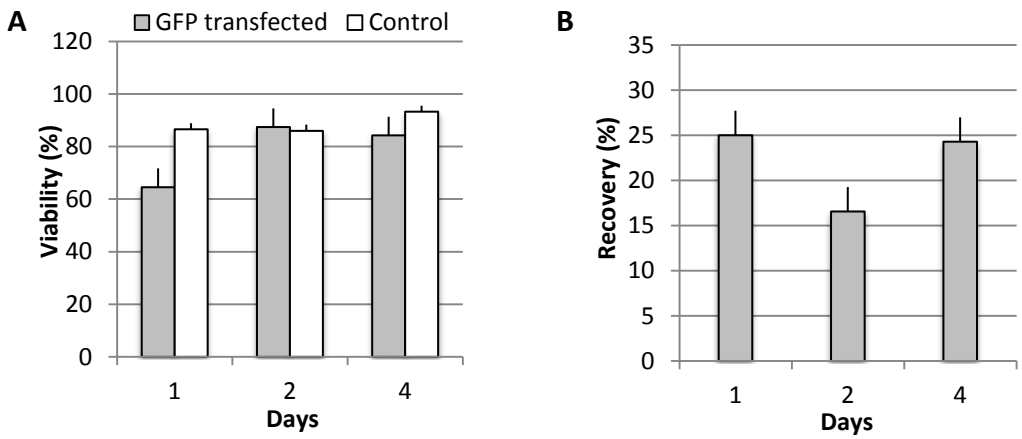


Figure 3.3.1 - CGR8-NS cells microporated using 1 pulse of 1500V for 20ms, and 0.5µg of pcDNA3.1-GFP. Cell viability for transfected and control cells (A), and cell recovery of transfected cells (B). Values were calculated based on mean ± SD of 4 to 6 countings of 2 replicates.

Coating with CELLStart™ seems to have made a difference in cell survival. This is a xeno-free substrate, widely used in the attachment of hNSC, hMSC and hESC[120]. The transfection process reduced cell viability in approximately 20% compared to the non-microporated control. Cell viability values quickly stabilized and are similar in both conditions for the remaining days. Cell recovery, on the other hand, is very low, never reaching more than 25%, which suggests that these conditions may still be very harsh for cells. Cell recovery is lower on the 2nd day than the day post-transfection. It is important to highlight that this is a value that represents the percentage of viable cells on a culture, in comparison to the percentage of viable cells on a control, reason why it is not directly related to the values of cell recovery on other days. This means the culture had a lower proliferative activity on that day, which could be related to their higher needs for nutrients while on a recovery state. One way to evaluate this would be provide fresh medium to the culture on the day after transfection.

In order to evaluate the effect of BDNF on cell recovery, CGR8 cells were microporated with pcDNA3.1-GFP and pcDNA3.1-BDNF, using 1 pulse of 1500V for 20ms, and 0.5µg of plasmid DNA. Days 2 and 4 were analyzed (figure 3.3.2)

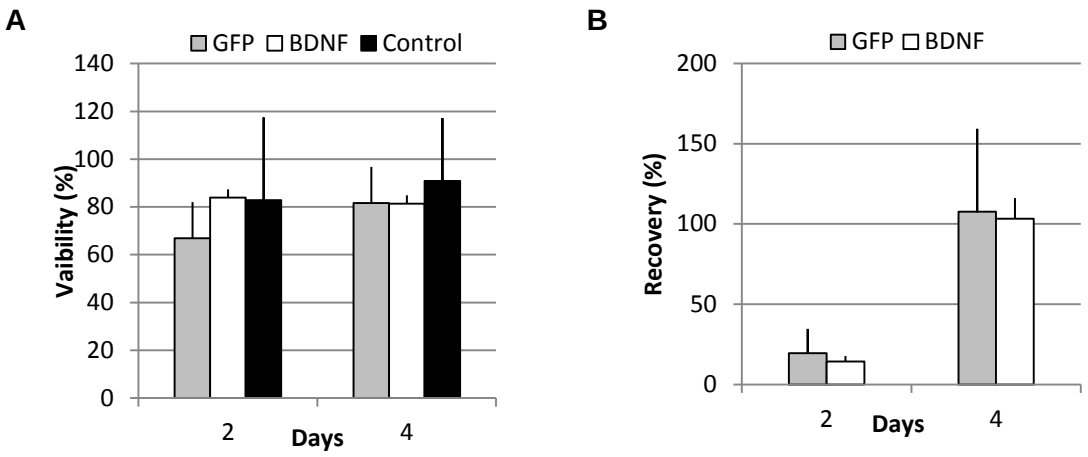


Figure 3.3.2 - CGR8-NS cells microporated using 1 pulse of 1500V for 20ms, and 0.5µg of pcDNA3.1-GFP or pcDNA3.1-BDNF. Cell viability of transfected and control cells (A) and cell recovery of control cells (B) on days 2 and 4. Values were calculated based on the mean ± SD of 4 to 6 cell countings of 2 replicates.

The transfection with a BDNF-containing plasmid did not improve cell recovery since no major differences were observed. Debilitated cells by the microporation process may be not so responsive to such signaling in a destabilizing environment. Higher values of cell recovery on the first few days post-transfection may be particularly important to address these questions, in order to draw conclusions on a healthy culture. These results may suggest that the transfection was performed under sub-optimal conditions. The parameters used were previously optimized for much smaller plasmids (as little as 2.2kb, as compared to 5.7kb in this work)[103]. The size of plasmids is an important factor that interferes with the efficiency of the transfection process. In fact, a reduction in plasmid size is directly related to an increased transfection efficiency of stem cells[108,121]. Unfortunately, in these assays, it was not possible to harvest enough number of cells to evaluate the transfection efficiency by flow cytometry.

3.3.2. Cationic polymer-based transfection

Cationic polymers are widely used transfection agents for gene delivery (forming polymer-DNA complexes usually called polyplexes[122]). Their versatility arises from the possibility to synthesize polymers with the desired properties, allowing for tunning of its length, geometry, ramification and functionalization[122] among other parameters.

CGR8 cells were transfected with the commercial cationic polymer-based reagent TurboFect (ThermoScientific®), complexed with pcDNA3.1-GFP.

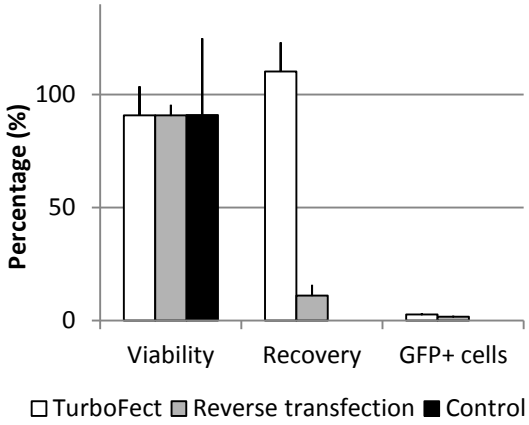


Fig. 3.3.3 - CGR8 cells transfected with TurboFect, using a 2:1 TF:DNA ratio (µL/µg) for each reaction. Cell viability, recovery and transfection efficiency is presented for each protocol performed. Cell viability and recovery were calculated based on the mean value ± SD of 2 cell coutings from 3 replicates. Percentage of GFP+ cells is the mean value ± SD of 3 replicates.

TurboFect (TF) is a polymer-based transfection reagent. Both normal and reverse transfection protocol yielded negligible percentage of transfected cells - around 3% and 2%, respectively. This can be explained by the use of polyplexes with incorrect TF/DNA ratio. An incorrect ratio can lead to the formation of an overall negatively charged complex, which is incapable of efficiently interacting with negatively charged cell membranes, and therefore incapable of penetrating cells.

Cell viability is virtually the same for both transfection protocols and for the non-transfected control cells. This may be corroborated by the fact that such a low number of cells were successfully transfected, so the overall process did not affect most of the cells in culture. On the other hand, only around 10% of cells recovered from reverse transfection. In this approach, the TF/DNA mixture is placed on the bottom of the cell culture wells (see section 2.6.2), and the cellular suspension is then added to the mixture. Hypothesizing the formed complexes present an overall negative charge, the deposition of these particles in the bottom of the well may have resulted in electrostatic repulsions that prevented cells from reaching the fibronectin coating. This would lead to a critical decrease in cell adhesion causing extensive cell death. Overall polyplex charge is one of the parameters that highly influences the interaction with living cells[91,94]. A more detailed and time consuming optimization of this transfection protocol would be required , in order to evaluate the best TF/DNA ratio to use within NSC. However, as the goal of this work was not the optimization of novel transfection reagents we decided to proceed using a well-established method with higher number of cell lines, and both somatic and stem cells tested.

3.3.3. Lipofection

Lipofection is throughout this work referred to as the transfection process through the lipid-based reagent Lipofectamine® 2000 (Invitrogen™). This reagent has been complexed with either the pcDNA3.1-GFP or pcDNA3.1-BDNF plasmids, forming lipoplexes.

3.3.3 a. Assessment of the optimal LF/DNA ratio

As in cationic polymer-based transfections, also in lipid-based reactions the overall charge of the lipoplex is important. In order to assess which Lipofectamine® 2000/DNA ratio (herein termed LF/DNA) provides the best transfection efficiency of CGR8 cells, four different ratios were tested - 1, 2, 2.5 and 3 (µL of LF/µg of DNA) (figure 3.3.4).

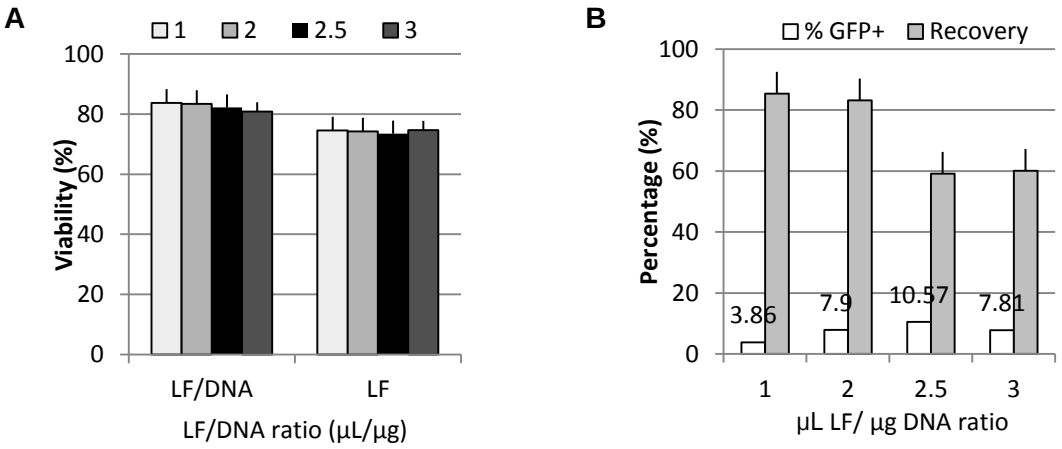


Fig 3.3.4 - CGR8-NS cells transfected with Lipofectamine® 2000 under different LF/DNA ratios. Cell viability is shown for each ratio and for LF quantities alone (A). Cell recovery and transfection efficiency (B). Cell viability and recovery were calculated based on the mean ± SD of 3 independent cell countings from 2 replicates each. Percentage of GFP^+ cells is the value of one experiment.

A decrease in total cell number (data not shown) and viability was observed with an increasing LF/DNA ratio, although with very slight differences between different ratios, achieving a cell viability of ~80%. A control test with no pDNA was performed in order to evaluate possible harmful effects from LF alone in cell viability. All LF quantities rendered more than 70% cell viability, which means that the transfection reagent *per se*, does not present significant toxicity to cells. This is in agreement with other reports, stating that LF provided less than 20% toxicity in MSC[123], which is considered suitable for most applications[124].

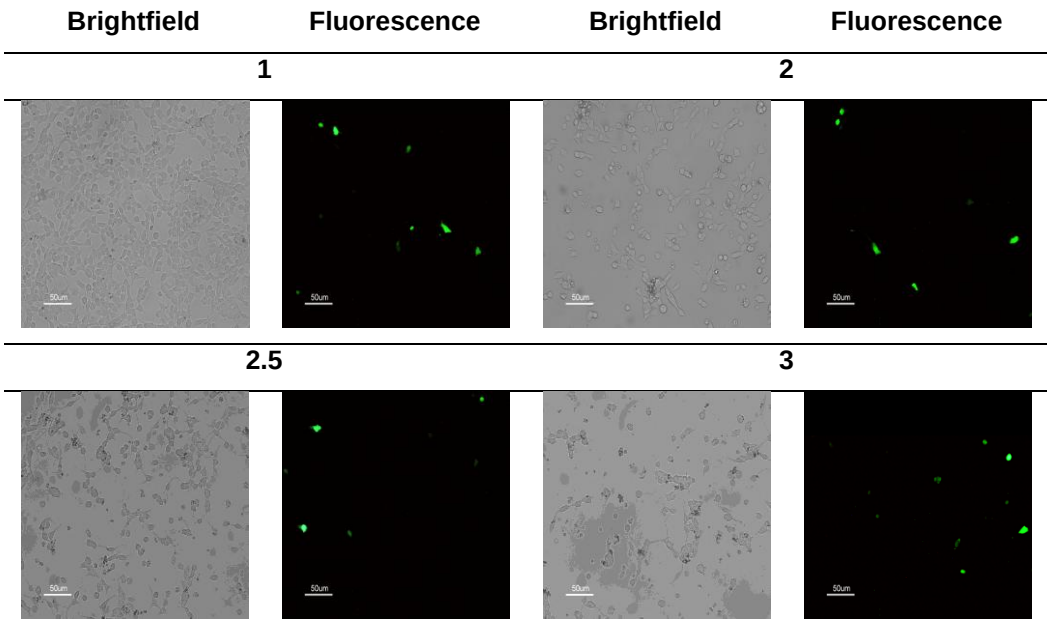


Figure 3.3.5 - Fluorescence images of CGR8 cells transfected with lipoplexes of several LF/DNA ratios. Brighfield images can be seen on the left. GFP-expressing cells can be seen (in green) on the right side of the panel.

A good balance between both molecules charges must be achieved in order to get the best electrostatic interactions between complexes and cell surfaces[99]. Ratios of 2, 2.5 and 3 provided the higher percentage of GFP-expressing cells, around 8%, 11% and 8% respectively. Nonetheless, a LF/DNA ratio of 2 was chosen for the upcoming assays, for resulting in more than 80% of cell recovery, contrasting with the 60% of the other ratios mentioned, while showing similar percentage of GFP expressing cells.

3.4. Transfection of human neural progenitor cells

Considering the low transfection efficiency achieved with mouse ES-derived NSC, the human ReN cell VM line was chosen for the remaining experiments.

3.4.1. Microporation

In order to optimize the microporation protocol for this type of cells, ReN cells were microporated with 0.5µg of pcDNA3.1-GFP ($2x10^5$ cells/microporation) using several different conditions varying the number of pulses, length of the pulse(s) and voltage. The cells from each

condition were then plated on laminin-coated wells of a 24-well plate. The results are displayed on figure 3.4.1.

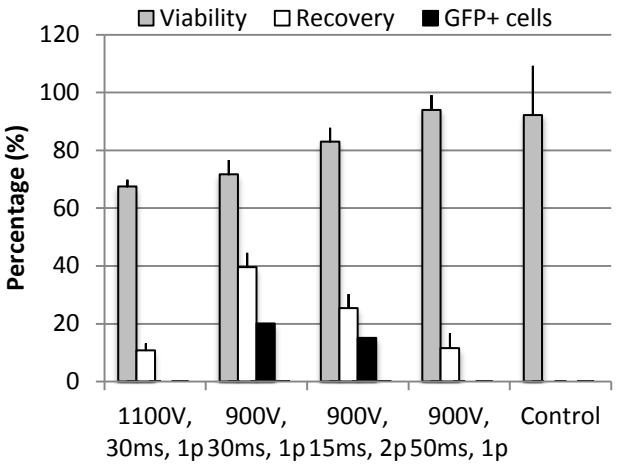


Figure 3.4.1 - ReN cells microporated using 0.5µg of pcDNA3.1-GFP under several conditions. Cell viability and recovery are calculated based on the mean ± SD of 3 cell countings from 2 replicates. Percentage of GFP^+ are the value of one experiment for each condition. The conditions are characterized by the voltage (V), length of pulse(s) (ms) and number of pulses (p).

Beyond the displayed settings, an additional condition was performed using 1 pulse of 1500V for 30ms which resulted in extensive cell death of the culture, not allowing for further analysis. Cell viability seems to increase when lower voltages are used, which may be due to the harmful effect of the electrical discharge during the process. In fact, microporation has exhibited cytotoxicity in human adipose tissue-derived stem cells, directly related to increase in voltage and pulse number[125]. Cell recovery is very low in all the conditions, with the best condition yielding only 40% of recovered cells, which may indicate that the microporation process may need further optimization. This value was obtained using what it seems to be the milder condition of all (1 pulse of 900V for 30ms). Comparing this condition with 2 pulses of 900 V for 15ms, it is possible to observe that the latter presents lower cell recovery, while the overall voltage is the same, which again may support the fact that the microporation process itself is detrimental to these cells.

Special attention must be given to the quantity of GFP^+ cells. Only using 1 pulse of 900V for 30ms or 2 pulses of 900V for 15ms, it was possible to obtain sufficient gated-cells to perform a statistically significative analysis (approximately 2800 and 2200 gated-cells were obtained, respectively). While this is below the recommended values (minimum of 10000 gated-cells), it suggests a tendency, in which the first condition may provide the best overall results. The remaining conditions resulted in less than one thousand gated-cells, not allowing for further analysis.

3.4.2. Lipofection

3.4.2 a. Transfection of ReN cells with GFP and BDNF-gene carrying plasmids

Considering that the chosen LF/DNA ratio of 2 was the most efficient condition on CGR8 cells lipofection, the same was attempted on ReN cells. In order to evaluate the validity of the assumption, ReN cells were plated at 75.000 cells/cm^2 on laminin-coated wells, and transfected the next day with a 2:1 LF/DNA ratio. Cells were harvested either one or three days after the transfection (figure 3.4.2).

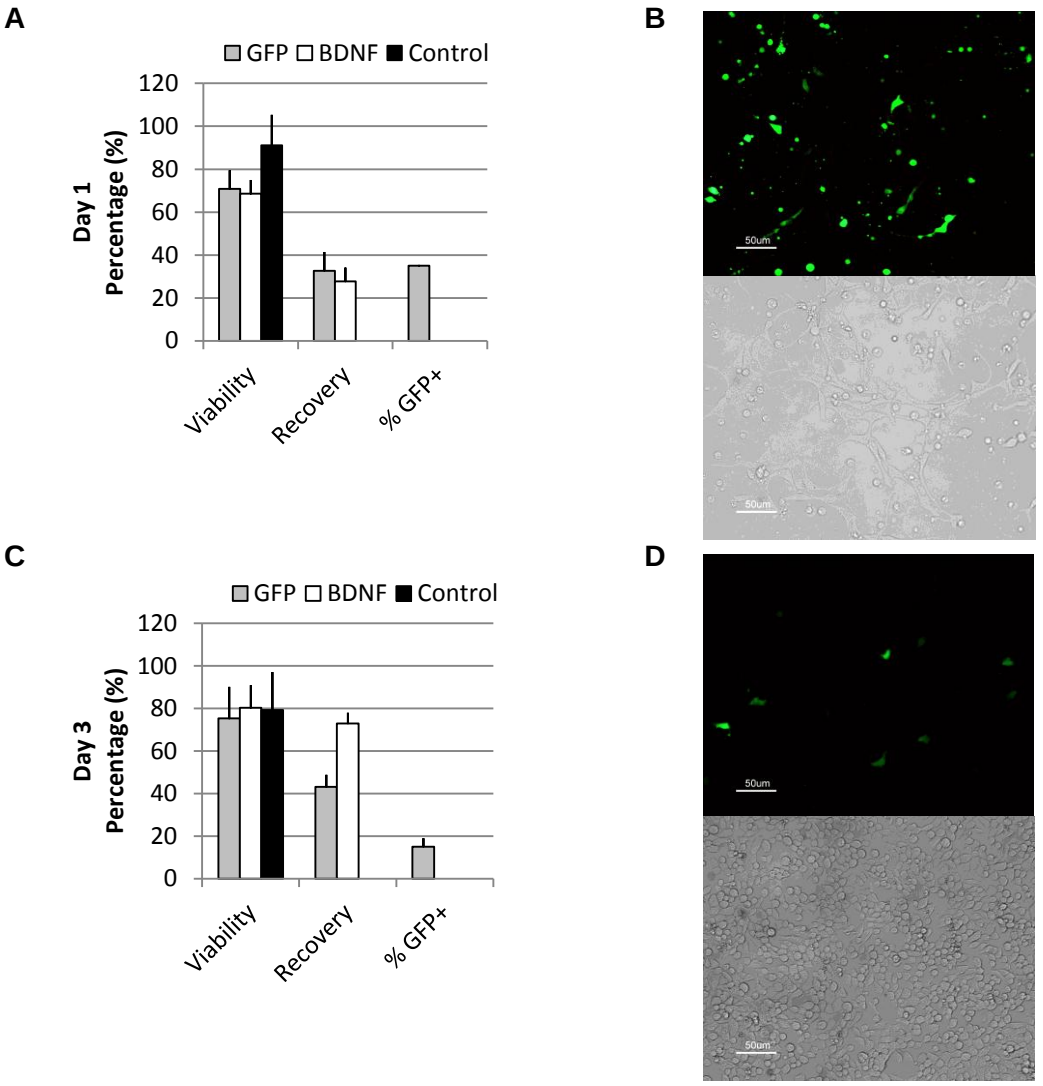


Fig 3.4.2 - ReN cells transfected with Lipofectamine® 2000 complexed with either pcDNA3.1-GFP or pcDNA3.1-BDNF (2µL/1µg). Cell viability, recovery and percentage of GFP$^+$ cells for day 1 (A) and day 3 (C). Fluorescence images of transfected ReN cells, by day 1 (B) and day 3 (D). Values are presented as the mean of 4 to 6 independent coutings from 2 replicates ± SD. Percentage of GFP+ cells is presented as the mean of 2 replicates ± SD.

On day 1, transfected cells present cell viabilities around 70% which is relatively lower than the 90% achieved with control cells, although these represent sufficiently high values that allow further studies with these cultures. On the other hand, cell recovery is very low, which may be caused by the high percentage of transfected cells (~35%). By the third day, cell viability was evened out in both transfected and control cells (approximately 80%). Cells transfected with BDNF gene-containing plasmid seem to have recovered significantly better than GFP reporter-gene containing plasmids. BDNF is known for its pro-survival and proliferative effects on neural stem cells[62], which may be the cause for this increase in cell recovery, although more data would be required in order to confirm this result, since the opposite was observed on CGR8 cells (see 3.3.3), which greatly prevents any solid conclusion to be drawn from the specific impact of BDNF on cell recovery. Moreover, the fact that the human BDNF gene is being used on mouse derived NSC might have affected his biological expression and function despite the differences in only 11 amino acids (corresponding to a 95.6% homology according to sequences available at *ncbi.nlm.nih.gov*).

Around 35% of the population expresses GFP on the first day. A relatively low amount of literature reporting the lipofection of neural stem cells does not allow for a more solid comparison on transfection efficiencies. Nonetheless, the results obtained report slightly higher values than the ones reported on other occasions. Falk and colleagues achieved around 4% of GFP^+ cells with LF[126], while around 20% transgene-expressing adult rat hippocampal cells were reported by Tinsley *et al*[89]. Plasmids exist within cells as episomes and are, therefore, lost upon division, leading to a decrease in GFP-expressing cells, consisting in 15% of the total cell population by day 3.

A wide variety of new sophisticated commercial formulations are available nowadays, and several other transfection reagents have been used on NSC. Tinsley *et al* tested 6 different reagents, where FuGene and ExGen500 rendered 11% and 16% transgene-expressing adult hippocampal progenitors. The later is a commercial cationic polymer while the former is a non-lipossomal transfection reagent. Cationic polymers are relatively easily manipulated, being flexible in its structure[122], allowing for protonation of the molecules making it easier to interact with other macromolecules such as nucleic acids. Non-liposomal reagents, may they be lipids or polymers, present an alternative to liposomes. They are capable of forming micelles or droplets, containing lipophilic surfaces that interact with cell membranes, forming an encapsulated complex that encloses the genetic material to be delivered. Electroporation has also been widely used on NSC. Richard *et al*, were able to successfully transfect 70% neural precursor cells[101]. In this method, the application of an electrical current momentaneously disrupts the charge gradient across the membrane. While there is not a consensus about the actual mechanism through which DNA enters the cell (either through pore or non-pore formation), the

relatively higher efficiency of this method may be due to the fact that it is relatively independent on the stage of the cell cycle.

However, viral methods still remain the most effective transfection agents. This is logically highly dependent on the type of virus. Retroviruses have provided a landmark in viral gene therapy when in 1990, hematopoietic stem cells were retrovirally transduced *in vitro*, in order to provide a fundamental enzyme, usually lacking in cases of X-linked severe combined immunodeficiency (SCID-X1)[127]. Re-infusion of modified HSC into patients suffering from the disease provided full and sustained correction of the enzyme function. However, retroviruses are incapable of infecting non-replicating cells. Still, they have been used on adult NSC, achieving around 60% of transfection efficiency. Lentivirus is a type of retrovirus, which has been exploited for its ability to infect non-mitotic cells, offering long-term expression of the transgene with relatively low toxicity[4]. NSC have been transduced with lentiviral vectors, preserving differentiation potential and proliferation *in vitro*, as well as their migratory capacity with the CNS[77]. Adenoviruses are non-enveloped icosahedral particles capable of infecting both dividing and non-dividing cells[4]. Adenovirus-mediated transfection has achieved up to 68% efficiently modified mouse NSC *in vitro*[126].

3.4.2 b. Assessment of differentiation potential after transfection

Neural stem cells have the ability to migrate to certain regions in the brain when transplanted, being able to express transgenes[128]. It is important to evaluate the differentiation capacity of transgene-expressing NSC, which would be the basis of a NSC-based therapy taking advantage of these cells as gene vehicles capable of expressing genes coding for clinically meaningful molecules. In order to evaluate the capacity of transfected cells to differentiate into either neurons or glial cells, ReN cells were lipofected as described before (section 2.6.3) and plated at 75000 cells/cm^2 on laminin-coated wells. Differentiation was induced on the next day, following the manufacturer´s protocol, which consists in culturing cells in a 1:1 mix of DMEM/F12 and Neurobasal® Medium, supplemented with B27, without any growth factors. Differentiation was carried out for 12 days, adding fresh medium every 3 days. The results are summarized on figure 3.4.3:

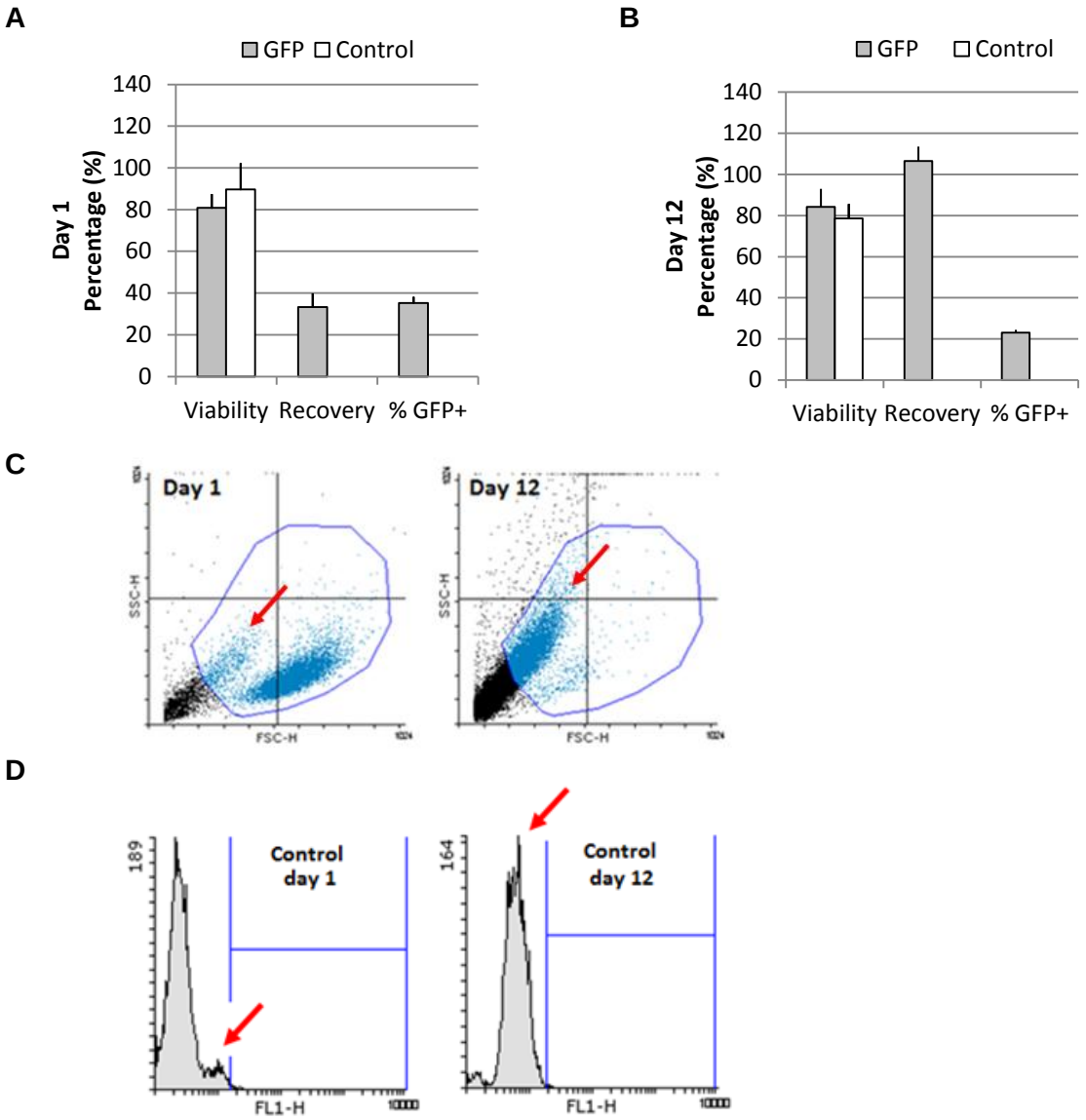


Figure 3.4.3 - ReN cells transfected with Lipofectamine® 2000. One day after transfection, differentiation of the culture was induced. Cell viability, recovery and percentage of GFP+ cells on day 1 (A) and day 12 (B) of differentiation. Dot-plots of flow cytometry analysis of ReN cells transfected with GFP, acquired on day 1 and 12 of differentiation, using a wide gate (C). Histograms based on the previously presented dot-plots (D). Cell viability and recovery was calculated based on the mean value ± SD of 3 independent countings from 3 replicates each. GFP+ cells is presented as the mean ± SD of 2 replicates.

One day after transfection, around 35% of cells express GFP which is consistent with the previous results (see figure 3.4.2a). By the 12th day of differentiation, a shift in the population morphology occurs (figure 3.4.3c). The x-axis is represented by the forward-scattered light (FCS), which is directly related to cell size, while the y-axis represents the side-scattered light (SSC), directly related to cell granularity or internal complexity[129]. Comparing the overall sizes and granularity of the populations along the differentiation process, it is possible to observe a shift into a population presumably consisting of differentiated cells, which present a decreased size as observed on the microscope, as well as cell debris and other particles of reduced size

and complexity, such as organelles. By the 12th day, considering the gate showed in figure 3.4.3c, approximately 23% of the cell population is expressing GFP. However, the real value of GFP expression is probably masked by the presence of cell debris showing autofluorescence. Since there is strong evidence that, during the process of differentiation cells appear to show a reduced size, a wide gate was considered in this case, in order to include both differentiated and differentiating cells (see figure 3.4.3c). By the 12th day, the more evident peak on the histograms (identified by the red arrow) (figure 3.4.3d) probably corresponds to what was initially considered cell debris, and at this point, by differentiated cells as well. However, plasmids are known to be diluted away in the culture upon division, and considering the previous results, where only 15% of cells were expressing the transgene at day 3, it is expected that by the 12th day of culture, a negligible number of cells still expresses GFP. For this reason, another analysis of the data was performed using a smaller gate, comprising the population regarding presumably the initial neural stem cell population at the first day of culture (figure 3.4.4):

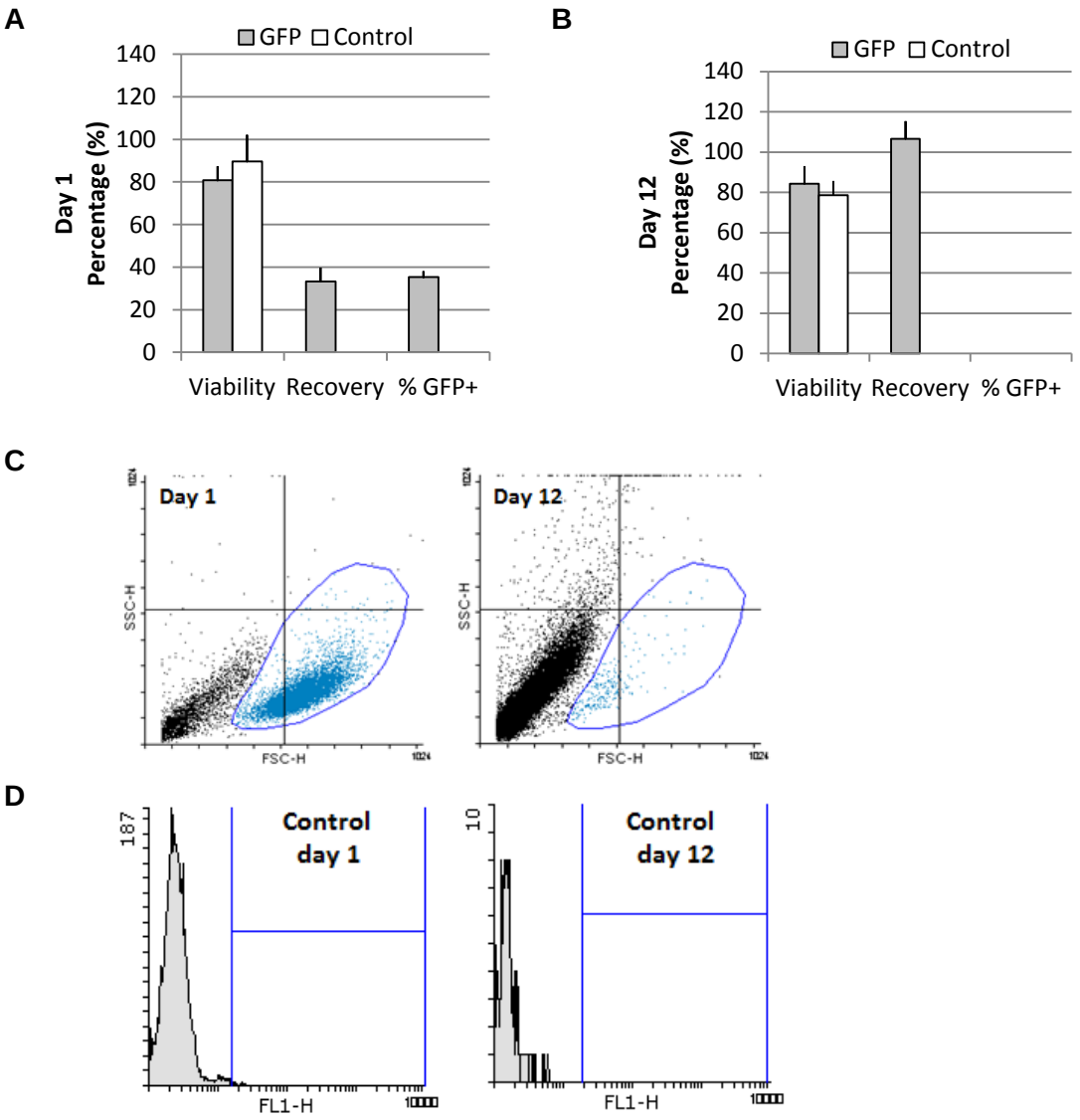


Figure 3.4.4 - ReN cells transfected with Lipofectamine® 2000. One day after transfection, differentiation of the culture was induced. Cell viability, recovery and percentage of GFP+ cells on day 1 (A) and day 12 (B) of differentiation. Dot-plots of flow cytometry analysis of ReN cells transfected with GFP, acquired on day 1 and 12 of differentiation, using a small gate (C). Histograms based on the previously presented dot-plots (D). Cell viability and recovery was calculated based on the mean value ± SD of 3 independent countings from 3 replicates each. GFP+ cells is presented as the mean ± SD of 2 replicates.

On the day post-transfection, around 35% of the population is expressing GFP, according to this analysis, which is in agreement with the previous analysis (see figure 3.4.2). The use of a smaller gate does not change the value of GFP-expressing cells on day 1, since the composition of the gated population (3.4.4c) is virtually the same as the one considered before, taking into account not enough time has passed for the generation of differentiated cells. However, at the end of the differentiation protocol, not enough cells are gated, preventing a statistically significant analysis (less than 1000 gated cells).

The smaller gate is neglecting important data, excluding differentiated cells from the analysis at the end of the differentiation protocol, while the wider gate is including too many events, most of which probably regard autofluorescent cell debris. It is believed the real number of GFP-expressing cells may lie between the two analyses, showed on figures 3.4.3c and 3.4.4c. To confirm this, staining of dead cells with propidium iodide could be carried out in order to distinguish viable GFP-expressing cells from non-viable cells and debris, elucidating about the actual composition of the population highlighted by the red arrow (figure 3.4.3d).

In a parallel assay, ReN cells were plated at 75000 cells/cm^2 on laminin-coated wells, and differentiated for 4 days. Afterwards, the cells were lipofected as described before, in order to evaluate the potential of differentiating cells to take up complexes of cationic lipids and DNA (figure 3.4.5).

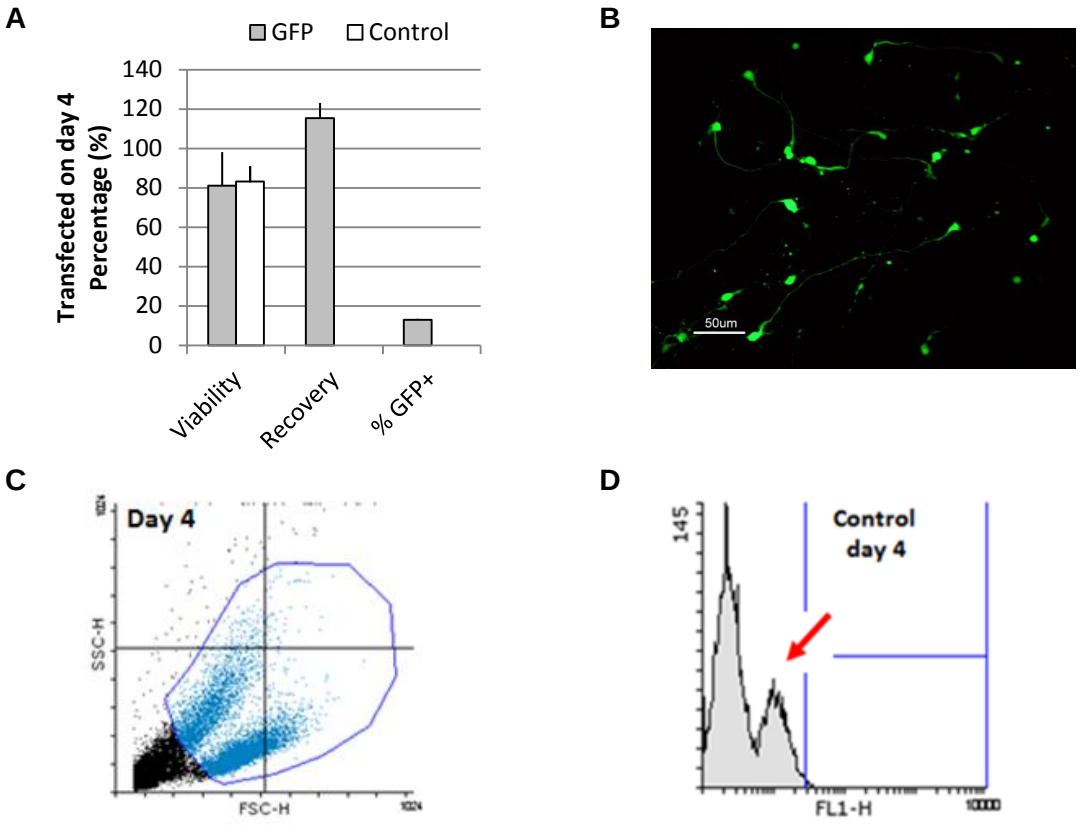


Figure 3.4.5 - ReN cells within 4th of differentiation were transfected with Lipofectamine 2000. Cell viability, recovery and GFP+ cells (A). Cell viability and recovery was calculated based on the mean value ± SD of 3 independent countings from 3 replicates each. GFP+ cells is presented as the mean ± SD of 2 replicates. Fluorescence microscopy picture of differentiating transfected cells (B). Dot-plots of flow cytometry analysis of differentiating ReN cells transfected with GFP, acquired day 4 of differentiation, using a wide gate (C). Histograms based on the previously presented dot-plots (D).

It is known that exogenous transgene expression steadily decreases for 1-2 weeks[130]. In order to attempt a still active and stronger gene expression at the end of the differentiation process, ReN cells were transfected during the differentiation induction. Fluorescence microscopy images showed GFP-expressing cells possessing long neurites (figure 3.4.5b), typical of neuronal committed cells, as opposed to a smaller star-like morphology observed on non-differentiated cells, which hints at the differentiation ability of these cells. Around 13% of the population was able to take up the plasmid and express the transgene, according to flow cytometry results. Nevertheless, this value may as well be masked by the presence of auto-fluorescent cell debris as described before. Performing the analysis with the smaller gate yields only about 3% of GFP-expressing cells (figure 3.4.6). Once more, the real value may lie between those two.

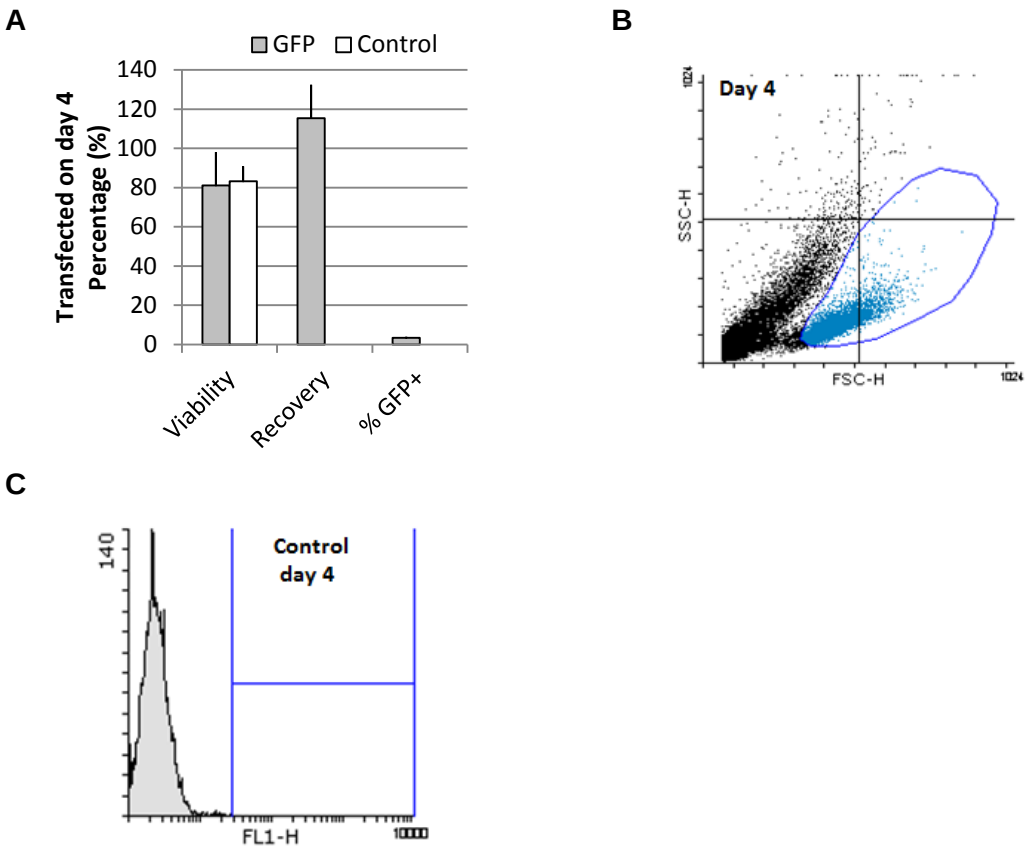


Figure 3.4.6 - ReN cells within 4th of differentiation were transfected with Lipofectamine 2000. Cell viability, recovery and GFP+ cells (A). Cell viability and recovery was calculated based on the mean value ± SD of 3 independent countings from 3 replicates each. GFP+ cells is presented as the mean ± SD of 2 replicates. Fluorescence microscopy picture of differentiating transfected cells (B). Dot-plots of flow cytometry analysis of differentiating ReN cells transfected with GFP, acquired day 4 of differentiation, using a small gate (C). Histograms based on the previously presented dot-plots (D).

While the actual mechanism of lipoplex-mediated transfection is not entirely understood, some authors support that besides the well accepted theory of lipoplex fusion with cells membranes, this might occur during membrane reorganization events that undergo during mitosis. In this stage, the cellular membrane is highly fragile, and so the freshly released DNA from the endosomes has a more direct access to the nuclei of newly formed cells[131]. During differentiation, proliferative events are highly restricted, preventing macromolecules such as lipoplexes to penetrate cells[131], and in fact, lipid-based transfection usually shows poor efficiency in post-mitotic neurons[132], which could explain these results. Although these cells have not reached their final stage of differentiation, the majoritiy of the population is probably in a post-mitotic phase, preventing the lipoplexes from reaching their destination. Neurons should, nonetheless, be able to take up lipoplexes through fusion with the cell membrane, even though they are post-mitotic. However, this seems not to occur naturally. This is presumably due to their high sensitiveness to changes in culture conditions such as pH, osmotic pressure and temperature[132]. Also, the nature of their *in vivo* environment is highly restrictive in the uptake of exogenous molecules as a protective mechanism against external aggressions. Neurons may,

in this way, present themselves cell membranes that engage poorly in interactions with external molecules, especially with the size of lipoplexes. This may be the reason why the most effective transfection techniques on neurons so far, have been more invasive methods such as nucleofection, electroporation and biolistic gene transfer[132,133].

3.5. BDNF effect on the differentiation of ReN cells

Non-transfected ReN cells were cultured during several passages using the conditions described in the section 2.5.2. In parallel, some non-transfected and transfected ReN cells (with LF/pcDNA3.1-BDNF lipoplexes) were subjected to differentiation. A positive control for BDNF effect on the process was created by adding 50 ng/mL of exogenous BDNF to selected cultures. Immunostaining analysis of the cultures was performed as described in section 2.8. The neural stem/progenitor marker Nestin was used to evaluate the multipotency of the culture. Astrocytic marker GFAP and neuronal marker Tuj1 were also used to detect the presence of these cell linages. The nuclear counter-stain DAPI was used as well to identify nuclei (Figures 3.5.1 and 3.5.2).

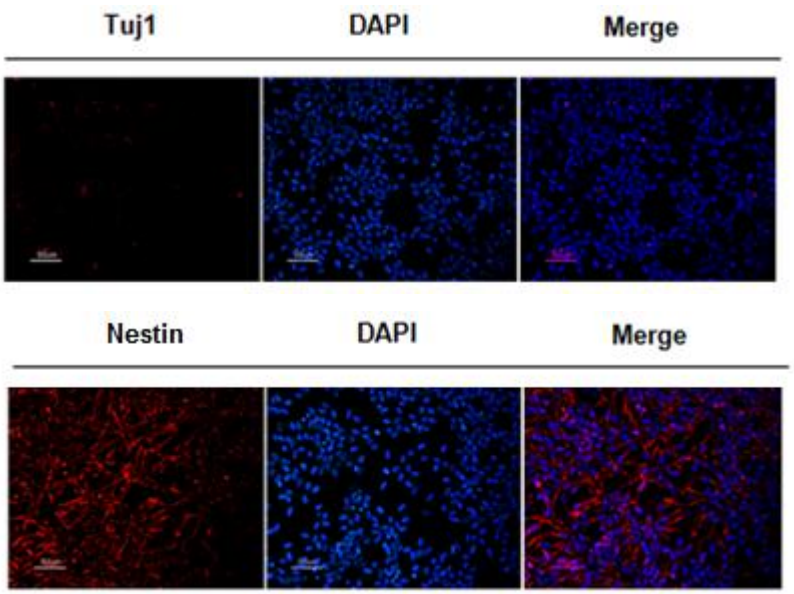


Figure 3.5.1 - Immunostaining of non-differentiated ReN cells. Neuronal cell marker Tuj1 and neural stem/progenitor cells marker Nestin are shown in red. The nuclear counter-stain DAPI in blue. Merged images are shown at the right. Scale bar is 50μm.

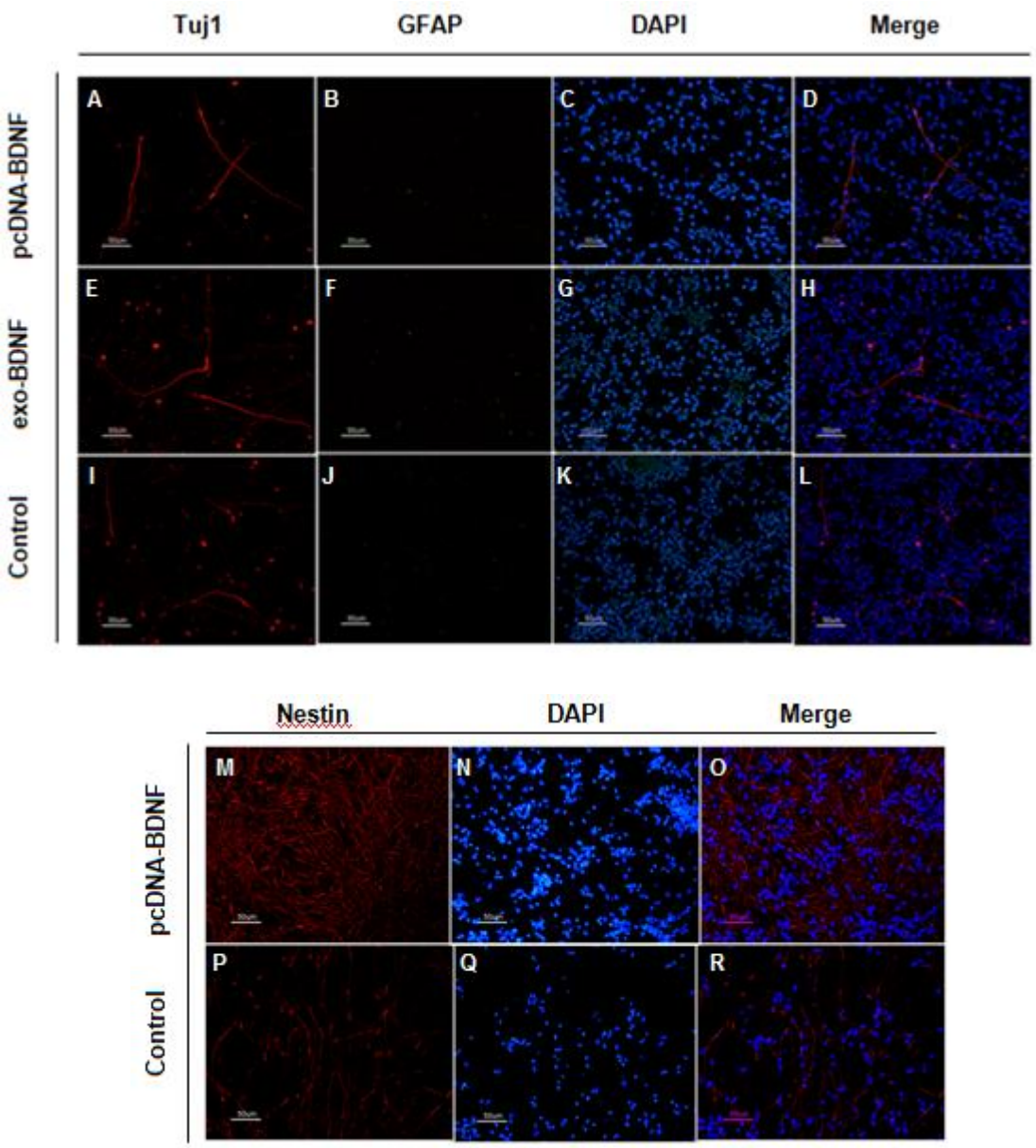


Figure 3.5.2 - Immunostaining of ReN cells transfected with BDNF and differentiated under standard conditions (A-D, M-O); non-transfected ReN cells differentiated with supplemented exogenous BDNF (E-H) and non-transfected ReN cells differentiated under standard conditions (I-L, P-R). The markers Tuj1 and Nestin are shown in red, GFAP in green and the nuclear counter-stain DAPI in blue. Scale bar is 50μm.

Tuj1 is an antibody against the neuron-specific class III β-tubulin marker. It is present in all differentiation conditions, while being absent in non-differentiated cells (Figure 3.5.1) indicating that whichever method was used, neuronal phenotype cells were developed. While this marker does not allow for identification of the specific neuron type produced, the manufacturer´s protocol suggests ReN cells are readily able to differentiate into dopaminergic neurons[116]. One way to address this would be to use antibodies against specific proteins present in this class of

neurons, such as the transcription factor Nurr1, a receptor involved in the development, maturation and survival of DA neurons[134]. GFAP is mainly expressed in astrocytes. It is not possible to observe any kind of positive staining of this marker, although on some occasions, green staining could be observed (data not shown). This does not mean that differentiation into astrocytes was not achieved. It probably suggests that the immunostaining protocol used for astrocytes was not optimal, not allowing for drawing of any solid conclusions. Nestin is a neural progenitor cell marker, that is widely expressed both in transfected and non-transfected cells, showing a similar profile to the expression of this marker on non-differentiated cells, suggesting once more that the differentiation efficiency was very low, as the vast majority of the population still presents multipotency at the end of differentiation. Still, the lipofection process seems not to interfere with the differentiation process, rendering similar immunostaining profiles as the other conditions, rendering Tuj1-positive neurons.

3.6. Qualitative analysis of neurites by ImageJ® software

BDNF promotes neurite outgrowth and number of neurons, which has been proven on several occasions[84,86]. To evaluate this, a library of fluorescence microscopy pictures were analyzed using ImageJ® software, and through the plugin NeuronJ, total neurite and respective lengths were measured in each condition (see 2.10): transfected cells with BDNF; cells exposed to exogenous BDNF; control cells. The results are summarized on figure 3.6.1.

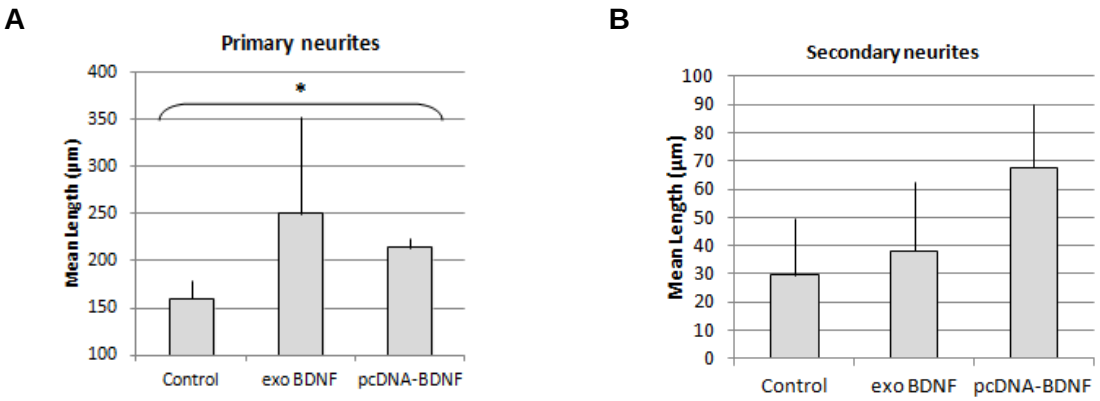


Figure 3.6.1 - Differentiated ReN cells cultured in either normal differentiation conditions (control), with supplemented BDNF (exo-BDNF) or transfected ReN cells with BDNF (pcDNA-BDNF) were immunostained against neuronal-marker Tuj1. Fluorescence microscopy images were analyzed and mean length of primary neurites (A) and secondary neurites (B) were measured in each condition. Results are

displayed as the mean ± SD of 19 independent measurements (n=19). Statistical differences are indicated with * for $p < 0.05$.

Transfected cells seemed to have rendered slightly longer neurites than control cells (215µm vs 160µm, respectively), but shorter than those exposed to the exogenous factor (215µm vs 251µm respectively). It is important to keep in mind that the differentiation process requires the addition of fresh medium every 3 days, which in the case of exo-BDNF samples means that those cells were exposed to a fresh dose of this factor every 3 days. On the other hand, transfected cells were only provided with fresh differentiation medium without any other exogenous factors, which means the outcome observed was derived solely on the BDNF secreted by these cells throughout the entire lifetime of the culture. While it is not possible to know for sure what caused a slight decrease in neurite outgrowth from transfected cells in relation to exo-BDNF exposed cells, one can hypothesize that (1) either the amount of BDNF released by transfected cells was not high enough to provide a more evident increase in neurite outgrowth, or (2) a depletion of the factor has occurred due to its uptake by the cells or by loss of function of the neurotrophin due to the high amount of time it has been present in culture, considering the first 2 days represent the peak of expression of the transgene.
On the other hand, transfected cells seem to have rendered longer secondary neurites than either control or exo-BDNF cells. This suggests that BDNF may promote ramification of cells, which could translate in the generation of more connections with the surrounding cells, creating more communication routes within the neuronal network. One possible explanation to the generation of longer secondary neurites in transfected cells when compared to exogenous BDNF-exposed cells could be related to a more localized effect of the neurotrophin. BDNF is known to present both autocrine and paracrine signaling[135]. When the exogenous factor is added, widespread mixing of the medium allows all the cells in the culture to have access to the neurotrophin. In the transfection process, the presence of the factor in the medium results only from its secretion by transfected cells. In this case, immediate neighbor cells may benefit from the release of this neurotrophin through paracrine signalling, as well as the transfected cells themselves through autocrine signaling, leaving further localized cells unaltered by the lack of access to the neurotrophin. This may have altered the transcriptome of the positively affected cells, leading to the formation of more and longer secondary neurites.

Therefore, these results suggest that BDNF, in fact, promotes neuronal outgrowth, and that even transient expression of this factor enhances the total neurite (both primary and secondary) length, compared to non-treated cells, being the differences significant and noted as early as 12 days in culture.

This is especially relevant since many neurological disorders results in neuronal impairment and severe alterations in cell morphology. Neural stem cells, able to release stable amounts of

neurotrophins such as BDNF, could promote the survival of damaged cells and enhance neurite outgrowth, leading to functional recovery of those cells.

3.7. Glutamate toxicity evaluation by the Alamar blue assay

Glutamate is the main neurotransmitter in the CNS. Nevertheless, when present in high concentrations, it is toxic to neurons[73], and has been shown to reduce cell viability in neuronal cultures[136]. In order to evaluate glutamate toxicity, ReN cells were exposed to three solutions of different glutamate concentrations, and then, cell viability was assessed by the alamar blue assay (Figure 3.7.1). A concentration of 4mM glutamate was chosen for the next experiment. ReN cells within 12 days of differentiation induction were exposed to 4mM of glutamate for 2 hours. After that, the glutamate-containing medium was replaced with conditioned medium and the cells were incubated for 24h in normal conditions. Conditioned medium is herein termed as 3-day old differentiation medium containing BDNF secreted from transfected cells (with LF/pcDNA3.1-BDNF) and supplemented with fresh B27® supplement. In these assays, the respective test-mediums were diluted 1:1 with fresh differentiation medium, after 3h.

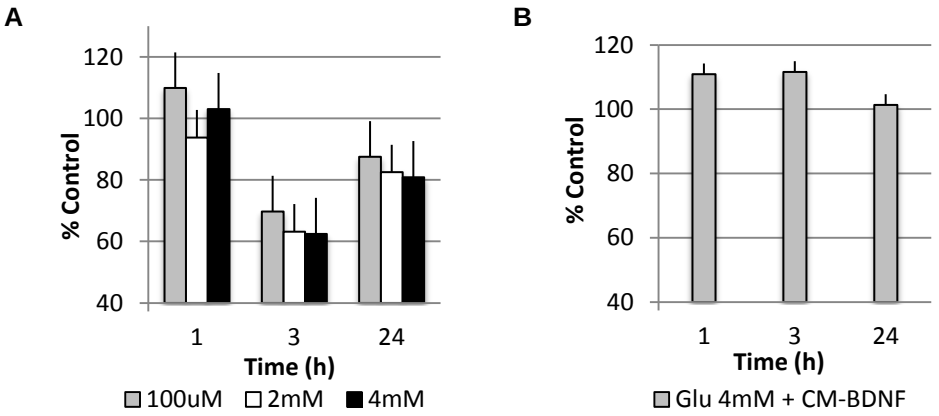


Figure 3.7.1 - Relative fluorescence intensity at different time-points of differentiated ReN cell cultures exposed to different glutamate concentrations (A), and to 4mM glutamate and 3-day old conditioned medium (B). Values are the mean ± SD of 2 replicates.

At 3h and 24h after glutamate exposure, cell viability seems to decrease in a concentration dependent manner, with higher glutamate concentrations affecting more negatively the population, which hints at the presence of glutamate-induced neuronal death. At 24h there is an increase in the fluorescence intensity of all cultures which may be explained by the fact that the 3-day old medium was diluted 1:1 with fresh differentiation medium, which provided fresh nutrients to the culture, improving its cellular activity.

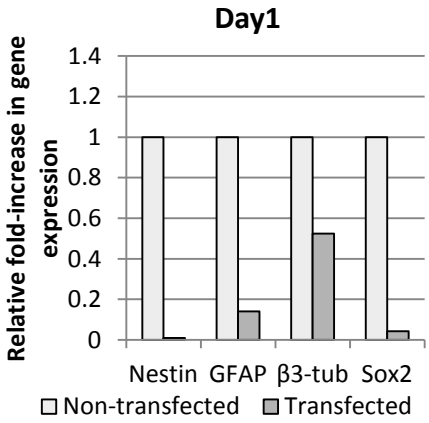
ReN cells were then exposed to 4mM of glutamate for 2 h. After that, cells were incubated with conditioned media. The control was subjected to 3 day-old medium from non-transfected cells.

A 10% increase in fluorescence intensity from BDNF-conditioned media-exposed cells is observed in the first 3 hours. After 24h, the fluorescence intensity is similar to the control. The amount of BDNF present in the medium may have not been sufficient to provide a longer protection from the eventual glutamate-induced neurotoxicity. Also, the concentration of glutamate used may have been too high for BDNF to exert a relevant action. Nonetheless, the increase in fluorescence intensity from conditioned medium-exposed cells occurred in all three time-points, which suggests consistency in the data, which ultimately may indicate that the existing BDNF in the medium has protected the cells, especially neurons, from the harmful effects of this concentration of glutamate. To our best knowledge, this is the first time that it is provided evidence on the effect of BDNF on a human NSC culture after glutamate exposure.

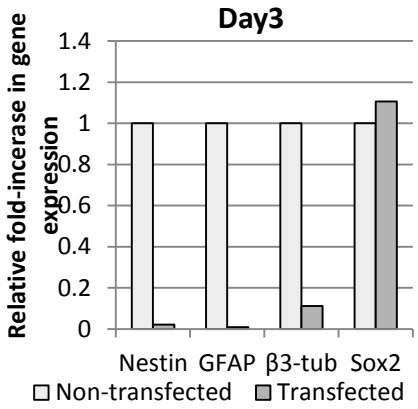
3.8. mRNA quantification by RT-PCR

ReN cells were lipofected with BDNF as described earlier and harvested on the next day ("day 1"). One day post-transfection, differentiation was induced on other cultures, and two days after that (3 days post-transfection) these cells were harvested ("day 3"). The expression of six genes were evaluated by RT-PCR: BDNF, IL-6, GFAP, β3-tubulin, Nestin and Sox2. The results are displayed below and the fold increase was calculated in relation to the expression of the housekeeping gene GAPDH (figure 3.8.1):

A



B



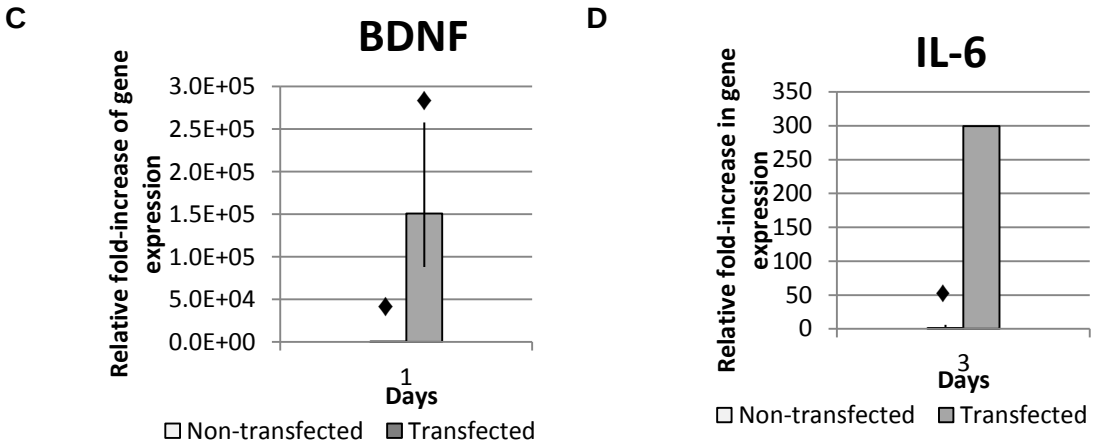
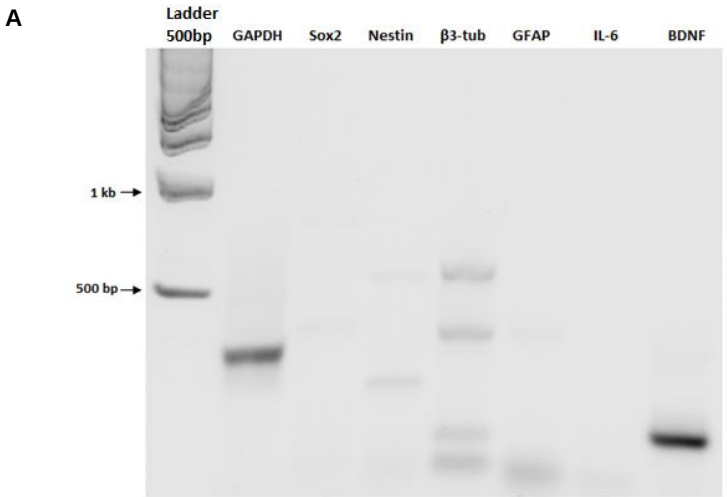


Figure 3.8.1 - Relative fold-increase in gene expression of several genes in BDNF-transfected cells (compared to non-transfected cells), on days 1 and 3. Results are displayed for cell markers Nestin, GFAP, β3-tubulin and Sox2 on day 1 (A) and day 3 (B), for BDNF (C) and IL-6 (D). Values are the mean relative gene expression normalized with respect to endogenous GAPDH. Duplicates were run on ♦ marked samples.

From figure 3.8.2 multiple amplification products for βIII-tubulin are observed, reason why the relative value of gene expression this gene is masked by other non-specific amplification products, so the conclusions drawn from this must be carefully taken. In order to minimize the occurrence of non-specific products of RT-PCR, the protocol was modified (program 2, see section 2.11), and by day 3 all genes presented a unique band, with the fragments appearing with the correct length.



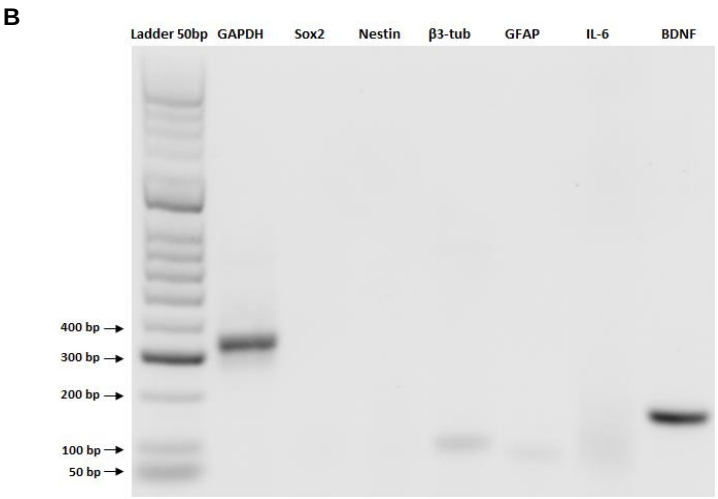


Figure 3.8.2 - RT-PCR reaction products on days 1 (A) and 3 (B). The DNA ladders used were the 500bp ladder (Lonza®) and the HyperLadder 50bp (Bioline®), respectively.

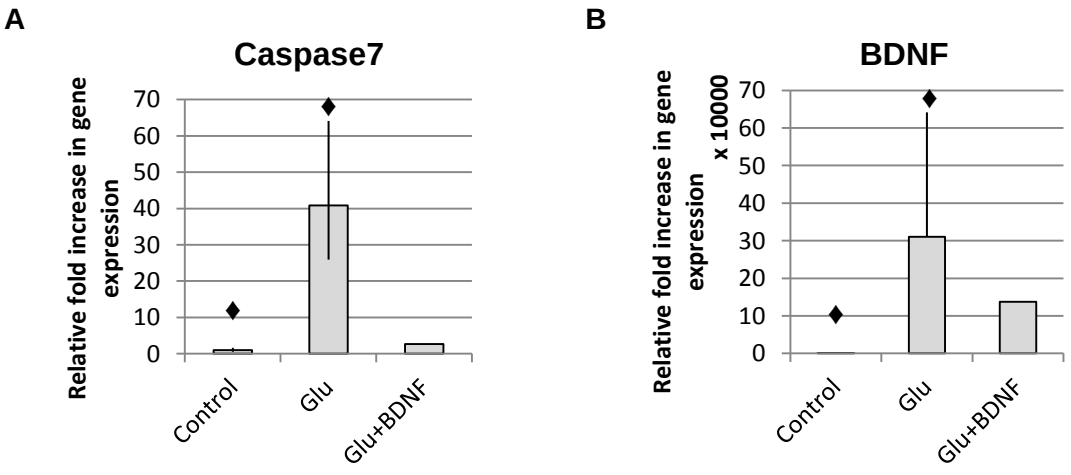
Both neural stem/progenitor and differentiated cell markers are down-regulated in transfected cells (figure 3.8.1). One day after transfection, cells are still in a recovering state, and are maintained in proliferative conditions, reason why Nestin and Sox2 should be extensively expressed. One day post-transfection, cells were exposed to differentiation conditions. Neural stem/progenitor cell markers should be then down-regulated, and an increase in lineage-specific markers should be detected. The opposite seem to have occurred though. Neuronal progenitors have been shown to proliferate for several days after withdrawal of both EGF and FGF-2 mithogens[137]. This was related to the possible existence of a latent period through which cells must undergo before they actually exit the cell cycle and initiate the molecular mechanisms that lead to a committed-fate[137], which could account for the low levels of Nestin and Sox2 expression.

Meanwhile, BDNF shows a 1,5 x 10^5 fold-increase in its expression by day 1. This confirms the successful expression of the transgene on transfected cells. Such a high value of gene expression can be explained by the regulation of its activity by the strong CMV promoter. This promoter has been widely used on neural cell types, enhancing strong transcription of the transgene[4,94]. By day 3, the correct value of BDNF expression could not be evaluated since the difference in the Ct values returned differed on 5 units (23.18 and 18.85) which would strongly undermine the validity of the outcome. The selection of only one of the values would mean that either its expression was increased to 3x10^5 fold or decreased to 7x10^4 fold, when compared to day 1. Although it is not possible to draw any conclusions from this, it is known that the transgene expression starts to decay with time[92]. In particular, transgenes under the control of the CMV promoter, as used in this work, have their peak of expression 1 to 2 days after administration, and then steadily decay during 1 to 2 weeks[130], reason why this value should in theory, be lower than one day post-transfection. The vector used is a regular plasmid containing

unmethylated CpG motifs, which are known to trigger immune responses in the host, through the activation of the TLR9 receptor[95,138]. This potentially led to the increased expression of the pro-inflammatory cytokine IL6, whose expression is potentiated by the presence of the referred motifs[139]. While also the correct value of expression of this cytokine is not known at day 1 (since the Ct values returned differed on 5 units), by day 3 around a 300 fold-increase in its expression compared to non-transfected cells is observed. The degradation of the plasmid and its overall dilution due to proliferation[92] may suggest that by the first day post-transfection, its expression should be highly increased. In fact, using one of the Ct values results in a fold-increase IL-6 expression of approximately 5746. For a more accurate assessment of the actual value of gene expression, the experiments returning significantly different Ct values should be repeated.

Glutamate shows neurotoxicity, mediated by the induction of a calcium influx via NMDA (*N*-methyl-D-aspartate) receptors. This triggers an appropriate response that comes along with glutamate-induced neuronal death[136]. In order to provide some insight about the effect of glutamate and BDNF on a differentiated ReN cell culture, differentiation was induced on ReN cells for 12 days. By the end of the differentiation protocol, long neuronal processes extended throughout the entire culture. A 2 hour-long exposure to 4mM of glutamate was performed by adding the appropriate amount of glutamate to the differentiation medium. After that, fresh B27® supplement was added to 3-day old differentiation medium and the mixture was added to the cells (herein termed “Glu” samples) for 24 hours. To test the effect of BDNF on glutamate-induced neurotoxicity, after the exposure to glutamate, cells were incubated with 3-day old conditioned medium supplement with fresh B27®. This conditioned medium contains BDNF secreted by lipofected cultures with pcDNA3.1-BDNF.

The expression of three genes were evaluated - caspase7, a gene activated upon cell death and an inducer of apoptosis; BDNF and β3-tubulin. The results are displayed on figure 3.8.3:



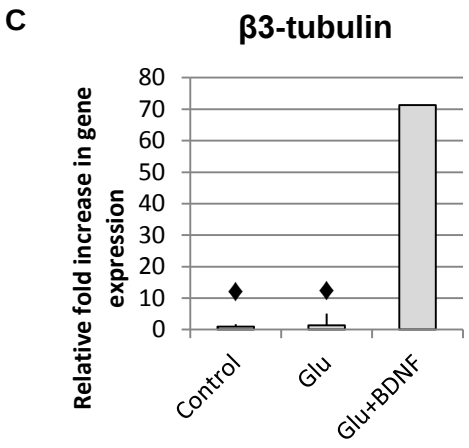


Figure 3.8.3 - Relative fold-increase in gene expression of several genes in glutamate-exposed cells (Glu) and cells incubated with conditioned-media after glutamate exposure (Glu+BDNF) (compared to non-exposed control cells), at the 12th day of differentiation. Results are displayed for Caspase7 (A), BDNF (B) and β3-tubulin (C). Values are the mean relative gene expression normalized with respect to endogenous GAPDH. Duplicates were run on ♦ marked samples.

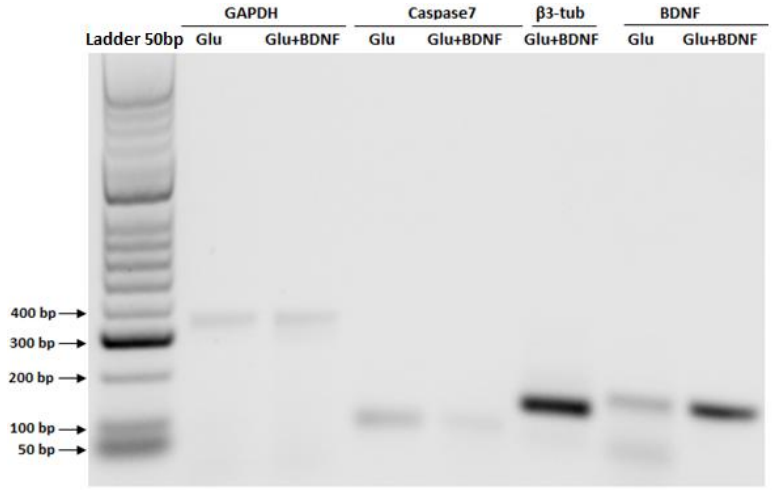


Figure 3.8.4 - Agarose gel of the RT-PCR reaction products. The DNA ladder used was the HyperLadder 50 bp (Bioline®).

This exposure to glutamate led to a major fold-increase in caspase7 activity (~40 fold), confirming extensive apoptotic cell death. On the other hand, cells that were later incubated in BDNF secreted by transfected cells seem to have been spared from the harmful effects of glutamate, reducing the expression of this pro-apoptotic protein to near-control levels. There is extensive evidence of the protective activity of BDNF from glutamate-induced neurotoxicity in cortical neurons[140,141]. However, the mentioned studies have only addressed this question when cells are pre-treated with the neurotrophin. Here, some evidence indicates that even after the aggression has occurred, BDNF can actually rescue or revert this toxicity in some way.

The mechanism of glutamate-induced neurotoxicity has been proposed to occur through the activation of the NMDA receptor by glutamate, which leads to a calcium influx into neurons, which in turn leads to formation of free radicals triggering neurotoxicity[141]. BDNF is known to promote calcium homeostasis, through the activation of genes that regulate calcium and free radicals metabolism[141,142] as well as increasing the expression of the anti-apoptotic protein Bcl-2[143]. So, glutamate-induced neurotoxicity may actually occur, even in the presence of BDNF, although, this neurotrophin may be able to protect neurons from further aggression by the activation of those protective genes within 24 h. On the other hand, BDNF also regulates the degree of phosphorylation and expression of the NMDA receptor units[144], which is in theory, a quicker way to prevent calcium-influx due to NMDA activation by glutamate, which would explain the difference in the results observed within only 24 h.

The BDNF gene is itself up-regulated when cells are exposed to glutamate alone. Despite the real value of gene expression being masked by non-specific products of amplification (figure 3.8.4), BDNF seems to have been, to some degree, up-regulated in this situation. One explanation resides in the fact that glutamate, being the main excitatory neurotransmitter in the CNS, is ultimately closely related to the direct release of neurotrophins. In fact, BDNF and glutamate are closely inter-related and co-regulate one another. During pre-synaptic activity, BDNF enhances the release of glutamate, while the latter increase the transcription and secretion of the neurotrophin[144]. A direct relationship between glutamate exposure and consequent BDNF release has been reported[145]. Its expression decreases when conditioned-medium is added to cells. Possible explanations include (1) the assumption that BDNF indeed prevented glutamate-induced toxicity, and in this sense, this agent did not trigger the release of higher amounts of the neurotrophin, although still enhancing its expression in some degree, or (2) BDNF, enhancing the release of glutamate, which in turn promotes the expression and secretion of the neurotrophin, partially regulates its own expression on a indirect way. These explanations are not mutually exclusive and may have taken place simultaneously.

The exposure to a BDNF-containing medium increases β3-tubulin expression (~70 fold), which confirms that neuronal differentiation is promoted by this neurotrophin, which has been proven on several occasions[84,86,143]. As stated before, the differentiation efficiency was very low throughout this work. The addition of BDNF to the differentiation medium might help to improve its efficiency and in effect, may induce neuronal commitment.

This experiment pretended to simulate an inflammatory environment that is common in neurological disorders such as ischemic brain injury[146], ALS[147] and AD[148], where high concentrations of glutamate have been reported. In AD, damaged cells release high amounts of glutamate as they die, which overstimulates surrounding healthy cells, leading to a phenomenon called excitotoxicity. This eventually leads to widespread cell damage[148]. Neural

stem cells have been proven efficient vehicles of genes, with homing capacity and correct migration *in vivo*[35]. The results presented here may suggest that not only transfected cells with BDNF would benefit from its highly protective properties, but also other cells within that micro-environment, which could be rescued from glutamate-induced neurotoxicity, even preventing healthy cells from this aggression. BDNF alongside many other neurotrophins such as NGF and NT-3 have had their highly beneficial effects proven[142]. However, the delivery of the functional proteins in an attempt to treat neurological disorders is greatly prevented by the BBB[83,149]. *Ex-vivo* modification of neural stem cells to overexpress neurotrophins and other molecules of proven therapeutical interest could be a promising alternative.

Besides gene silencing and plasmid degradation, which have been suggested to occur in this work, the lifespan of transplanted cells is also an important factor to keep in mind. These can range from 1 week for intestinal epithelial cells to lifelong established nerve cells[94], such as CNS cells. In this sense, even though considering the abovementioned barriers to gene therapy, the high amount of transgene expression that has been shown to occur hints that this approach could be helpful in the recovery of milder cases of neurological disorders that are caused by the knockdown of fundamental genes or loss of function of specific proteins.

It is important to highlight that glutamate is the major excitatory neurotransmitter in the CNS, and BDNF, being able to modulate its activity into a positive outcome through the cooperative interactions described, it might also provoke undesired responses such as blocking important synaptic pathways. Therefore, one should be careful in the analysis of these results, and in its hypothetic translation into clinical solutions. In gene therapy, for regenerative medicine applications lower and more stable levels of transgene expression are preferred. In this sense, a range of concentration/levels of expression of this neurotrophin should be determined, within which its activity would result in a desired outcome.

A fair comparison between the three transfection methods used throughout this work is not possible since not all of them were efficiently optimized. Lipofection was the only method whose conditions have been used on both mouse and human neural stem cells, providing significantly different percentages of GFP-expressing cells - 8% and 35%, respectively. The typical doubling time of these cells were investigated, in order to provide some insight about the differences in the results emerging from those two cell lines. ReN cells have an estimated doubling time of 20-30h[116], while CGR8 have around 14 hours[150]. This presumably suggests that the main route of entry of liposomes into cells was not through membrane reorganization events during mitosis. CGR8 cells, presenting higher proliferation rates, should in theory, present higher transfection efficiencies. Liposomes also bind with cell surfaces through electrostatic interactions. These are dependent on the surface components of cell membranes, such as cell matrix components, phospholipids and glycoproteins[92]. In order to better understand why ReN cells seem to be more prone to lipid-based transfection than CGR8 cells, the composition of the cell membranes

of these cell lines should be an interesting parameter to take into account. On the other hand, upon internalization, lipoplexes must release the DNA, and the remaining journey of the plasmid may be quite different in both cell lines. In this sense, cellular uptake may not be the limiting step in the whole process, and it may show similar kinetic profiles on both cell lines. Upon lipoplex destabilization, DNA must diffuse through the cytoplasm, avoiding degradation, reach the nuclear membrane, where it must be transported into the nucleus and finally the transcriptional machinery must be activated for the transgene to be expressed[99,122]. However, live tracking of lipid-DNA complexes would be required for a correct judgement on the actual mechanism of transfection, which is beyond the scope of this work.

4. Conclusion and future trends

Neural stem cells have a tremendous potential as the object of study of neurological disorders, through the establishment of neurological disease models. They can be used from a developmental biology of stem cells standpoint, and in regenerative medicine through gene and cell therapy, either as delivery agents of therapeutic molecules and/or for cell replacement. The use of NSC as therapeutic molecule delivery agents has raised hope for the treatment of certain neurological disorders, considering their capacity to stably express transgenes[89], and their homing capacity into the adult brain[151]. Viral vectors remain the most efficient transfection method for stem cells, and in particular, lentivirus in the case of neural stem cells[151], being most of the clinical investigation based on them. However, serious safety concerns arise from viral-vectors, namely due to immune responses triggered by the host against viral particles and insertional mutagenesis[138].

Non-viral vectors may be an alternative in gene delivery, showing increased safety, with low tumorogenic potential[93] however presenting a decreased efficiency when compared to their viral counter-parts. A successful non-viral gene delivery-based therapy should aim at overcoming the limiting steps of this approach. Clearly understanding the mechanisms of vector uptake by the cells would lead to higher transfection efficiencies. Non-virally transfected neural stem cells have also been showing promising results. GDNF-overexpressing neural stem cells were transduced through calcium phosphate technique. These cells integrated into the host striatum, expressing the transgene for at least 4 months, resulting in protection of dopaminergic neurons from degeneration[152]. The *in vivo* administration of therapeutic molecules faces major issues such as non-specific targeting, loss of function through protein destabilization and decreased bioavailability as a result of poor interaction with the desired cells or tissues. *Ex-vivo* modification of cells, on the other hand, gives researchers a greater control of the system, allowing for the manipulation of the desired cells only. Furthermore, the timing and amount of transgene expression may be susceptible of tight control through the use of adequate systems[115].

The aim of this work was the overexpression of the brain-derived neurotrophic factor in neural stem cells, through non-viral gene delivery strategies. Human neural stem cells were successfully microporated (~20% GFP-expressing cells), while maintaining 70% cell viability. On the other hand, both mouse and human neural stem cells were effectively transfected with Lipofectamine® 2000 (8% and 35% GFP-expressing cells, respectively), while maintaining 70-80% cell viability, which constitute promising results in comparison to the available literature. Furthermore, transfected cells were able to differentiate properly, while however, the efficiency

of the differentiation remained relatively poor. BDNF-transfected human neural stem cells were able to provide neurons with longer primary and secondary neurites, while also promoting neuron ramification, when compared to non-transfected cells.

Neurotoxicity was induced in a differentiated human neural stem cell culture by the addition of glutamate. A slight, but consistent decrease in cellular activity was caused by the exposure to this agent. Secreted BDNF from transfected cells was able to prevent a decrease in cellular activity upon glutamate exposure for the first 3 hours. mRNA quantification by real-time PCR allowed for the observations that secreted BDNF was indeed, able to reduce drastically apoptotic cell death events on glutamate-exposed cells, as well as promoting neuronal differentiation through the up-regulation of neuronal cell-marker Tuj1.

Interesting experiments to be performed would include the exposure of transfected cells to glutamate. This would provide insight about the behavior of these cells faced with glutamate-induced toxicity, in comparison to non-transfected cells exposed to the neurotrophic factor upon the aggression. The evaluation of the paracrine effect of BDNF could be performed through NSC culture on plastic inserts containing permeable membranes. This would allow non-transfected cells to co-exist in the same environment (i.e, media) as transfected cells, while not directly interacting with each other. Finally, important measurements would include protein quantity assessment in the media. This could be performed through ELISA kits using a specific antibody against the human-BDNF protein, or through western blotting.

The conversion of neural stem cell potential into an effective cell-therapy reality must overcome fundamental issues. For one, the determination of the signaling pathways and the major molecules in the process that regulate proliferation, differentiation and migration of these cells is of the utmost importance. In order for the transplanted cells to reproduce known differentiation profiles and kinetics *in vivo*, the adequate chemical cues behind this mechanism should be known. Another problem relates to the immune rejection upon transplant. Overcoming this issue would require autologous transplants to be a viable option. However, the isolation of NSC from the adult brain and achieving meaningful cell numbers through *in vitro* culture still remains challenging. In addition to that, the use of genetically engineered stem cells is still in its infancy, mainly due to concerns about their safety.

Regardless of the abovementioned imposed problems, there is no doubt that a tremendous progression in the mechanisms of neural developmental biology have been made. A quite vast repertoire of evidence has provided meaningful insight into the nature of NSC as clinically valuable products for cell and gene therapy, resulting in very promising outcomes in a wide variety of severe neurological disorders models. Neural stem cell biology can be considered nowadays a solid research field, which integrated within the gene delivery and regenerative medicine fields certainly withholds a promising future.

5. References

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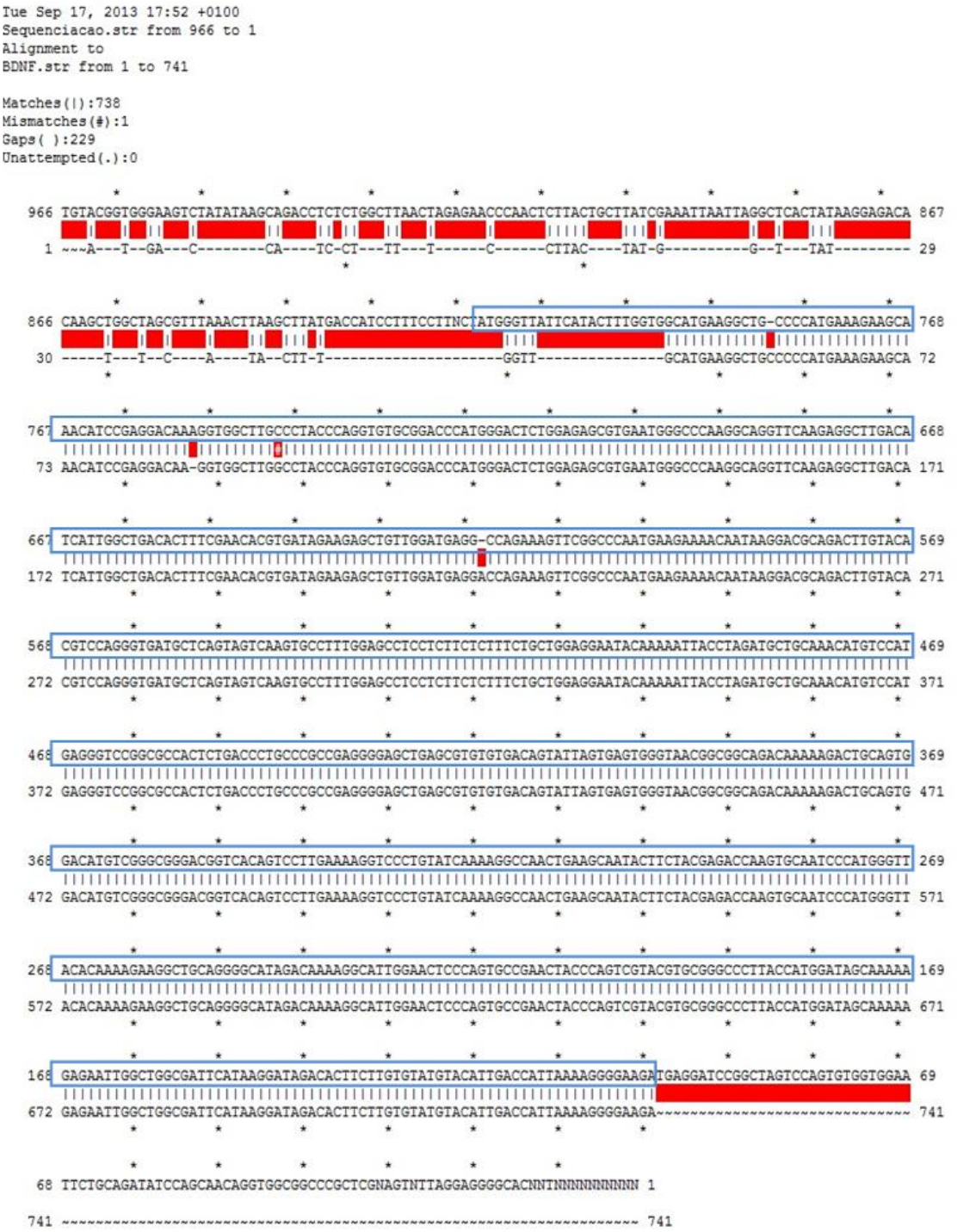
Appendix



Supplementary figure S1 - Sequencing results, showing 738 matching nucleotides, confirming correct cloning of the desired gene into the pcDNA3.1 plasmid. BDNF nucleotide sequence is highlighted in blue. 23 nucleotides are missing in the sequencing results due to incomplete sequencing. By using a reverse primer with a sequence downstream of BDNF we were able to confirm the correct type of the initial base pairs. Both primers were designed by the company StabVida® according to the type

of plasmid in use (pcDNA3.1). The obtained sequence perfectly matched with the sequence showed below that was provided by the supplier company (Origene®).

BDNF nucleotide sequence

5´ATGACCATCCTTTTCCTTACTATGGTTATTTCATACTTTGGTTGCATGAAGGCTGCCCCCATGAAAGA
AGCAAACATCCGAGGACAAGGTGGCTTGGCCTACCCAGGTGTGCGGACCCATGGGACTCTGGAGAG
CGTGAATGGGCCCAAGGCAGGTTCAAGAGGCTTGACATCATTGGCTGACACTTTCGAACACGTGATA
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ATACTTCTACGAGACCAAGTGCAATCCCATGGGTTACACAAAAGAAGGCTGCAGGGGCATAGACAAA
AGGCATTGGAACTCCCAGTGCCGAACTACCCAGTCGTACGTGCGGGCCCTTACCATGGATAGCAAAA
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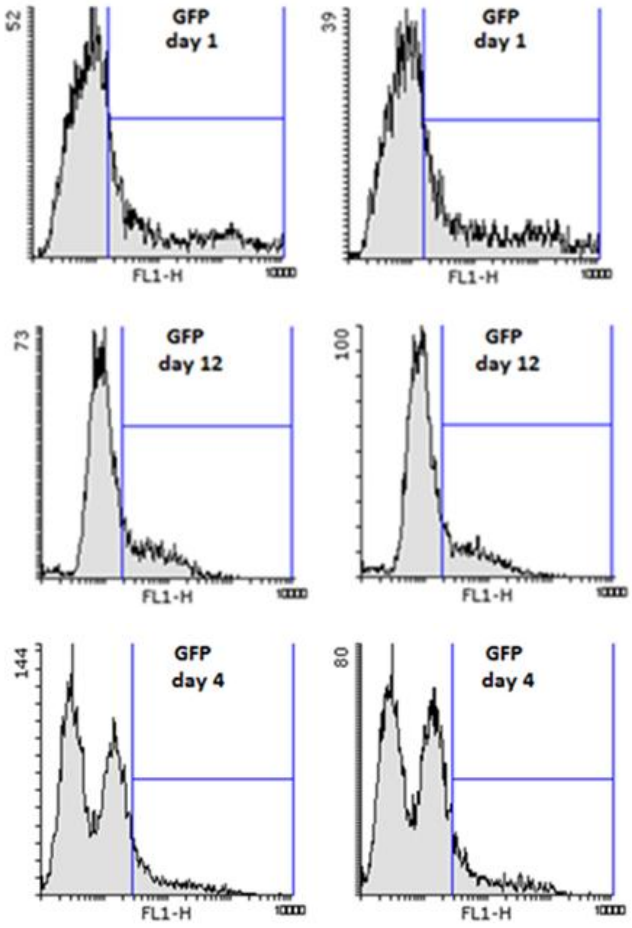
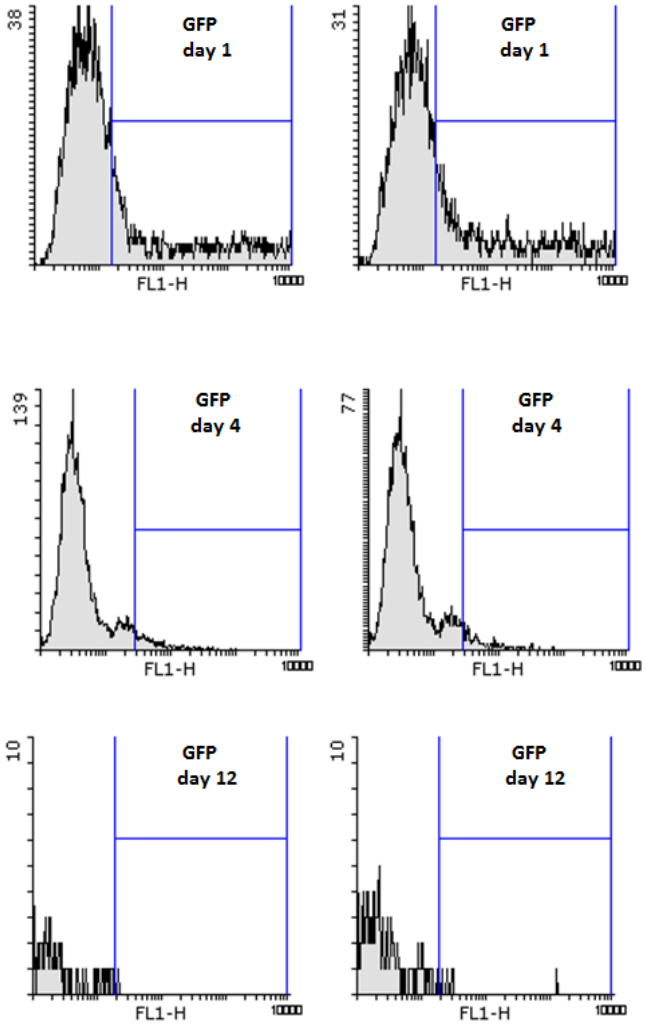
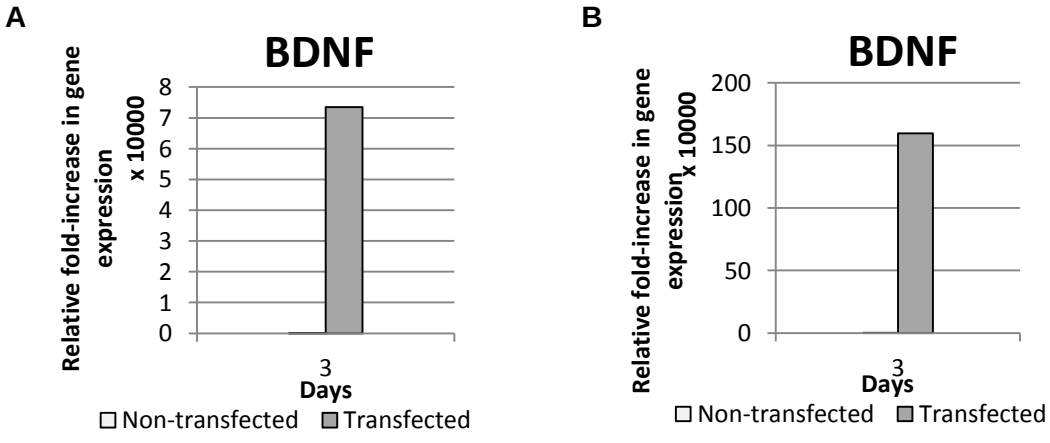
AGA 3´



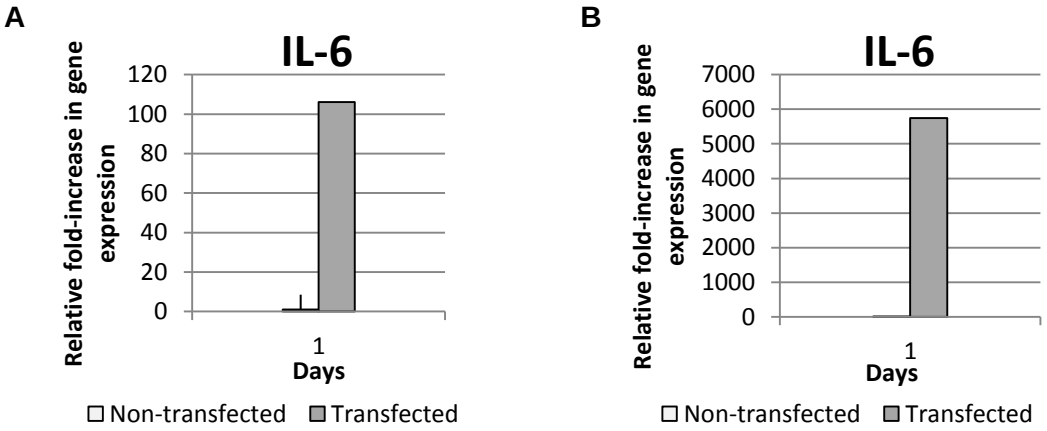
Supplementary figure S2 - Flow cytometry profiles of the ReN cells transfected with GFP at different time points, using the wider gate.



Supplementary figure S3 - Flow cytometry profiles of the ReN cells transfected with GFP at different time points, using the smaller gate.



Supplementary figure S4 - Fold-increase in BDNF expression at the 3rd day of culture, using a Ct of 23.3 (A) or a Ct of 18.8 (B). Both Ct values were returned in the same experiment within one sample, although none were used to prevent an inaccurate analysis.



Supplementary figure S5 - Fold-increase in IL-6 expression at the 1st day of culture, using a Ct of 38.3 (A) or a Ct of 32.6 (B). Both Ct values were returned in the same experiment within one sample, although none were used to prevent an inaccurate analysis.