Chapter 13

# Neural Fate of Mesenchymal Stem Cells and Neural Crest Stem Cells: Which Ways to Get Neurons for Cell Therapy Purpose?

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Additional information is available at the end of the chapter

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## 1. Introduction

The treatment of neurological disorders represents a critical issue in clinical research, since no complete recovery of patients can be achieved with actual therapeutic means, despite symptomatic improvements. Indeed, whereas restricted brain areas still house cells competent to generate newborn neurons in adulthood, those neural stem cells are present in restricted amounts. Moreover, this limited neurogenesis does not seem to be sufficient to enable neuronal regeneration in cases of traumatic, ischemic or degenerative lesions of the central nervous system. Therefore, other sources of neural cells have to be considered in a cell therapy objective.

Stem cells are characterized as cells endowed with continuous self-renewal ability and pluri- or multipotentiality, and could consequently give rise to a wide panel of cell types. Non-germinal stem cells are classified into different categories: (1) Embryonic stem cells (ES) are found in the inner cell mass of blastocyst and are pluripotent stem cells that can generate any mature cell of each of the three germ layers; (2) Induced pluripotent stem cells (iPS) are adult somatic cells that are reprogrammed into pluripotent cells with ES-like abilities; (3) Somatic stem cells are tissue-specific and more restricted than ES cells in terms of differentiation capabilities. They can be isolated from various fetal and adult tissues, which make them an attractive supply of material for cell therapy. Indeed, while neurons have already been successfully generated from ES cells [1] or iPS cells [2, 3], the use of adult somatic stem cells definitely remain of significant interest regarding technical, ethical and immunological issues concerning cell transplantation for brain diseases. In this

regard, mesenchymal stem cells (MSCs) and neural crest-derived stem cells (NCSCs) that can be found in various locations of the adult organism (and even in perinatal tissues) represent an important source of easily-accessible multipotent cells to use in a cell therapy purpose.

In this chapter, we will describe the major features of MSCs and NCSCs isolated from five different tissues, which constitute the main exploited and accessible sources for cell isolation in an objective of cell therapy protocols for neurological disorders (Figure 1). Moreover, we will detail the multiple ways they can generate neuron-like cells *in vitro*. Indeed, numerous culture conditions and differentiation protocols do exist and are demonstrated as efficient, supporting the fact that neural differentiation can occur through different cellular signaling mechanisms. Therefore, we will review the various signaling pathways that could trigger the neural fate adoption of MSCs and NCSCs, and the related cell-based therapy experiments that have been done downstream.

## 2. Different types of mesenchymal stem cells

Mesenchymal stem cells (MSCs) are plastic-adherent, fibroblast-like cells, which are conventionally able to self-renew and differentiate into tissues of the mesodermic lineage, such as bone, adipose tissue and cartilage. Whereas those cells have traditionally been isolated from bone marrow stroma, many reports have now described the presence of MSCs in a variety of fetal, perinatal and adult tissues, including peripheral blood, umbilical cord Wharton's Jelly and blood, fetal liver and lungs, adipose tissue, skeletal muscles, amniotic fluid, synovium and circulatory system, where they work as supportive cells and maintain tissue homeostasis.

### 2.1. Bone marrow mesenchymal stem cells

The initial study of non-hematopoietic bone marrow (BM) cells was performed by Friedenstein et al. in the late 80's [4, 5]. After establishing single cell suspensions of BM, they showed that those cells were able to generate colonies of adherent fibroblast-shaped cells when cultured *in vitro*. They demonstrated that these colony-forming unit - fibroblasts (CFU-F) presented the ability to undergo osteogenic differentiation [5, 6]. These bone marrow mesenchymal stem cells (BM-MSCs) were then demonstrated as multipotent progenitors that were able to self-renew [7] and could differentiate into any cell of the mesodermic lineage, like osteoblasts, chondrocytes, or adipocytes [8, 9]. More interestingly, it has been showed that BM-MSCs were able to "trans-differentiate" into cells with endodermal or ectodermal characteristics [10], and particularly into neuron-like cells [11-15]. These stem cells are therefore raising huge interest, since they represent a promising source of material for cell therapy protocols, such as mesenchymal tissue engineering or neurological disorder treatments as well.

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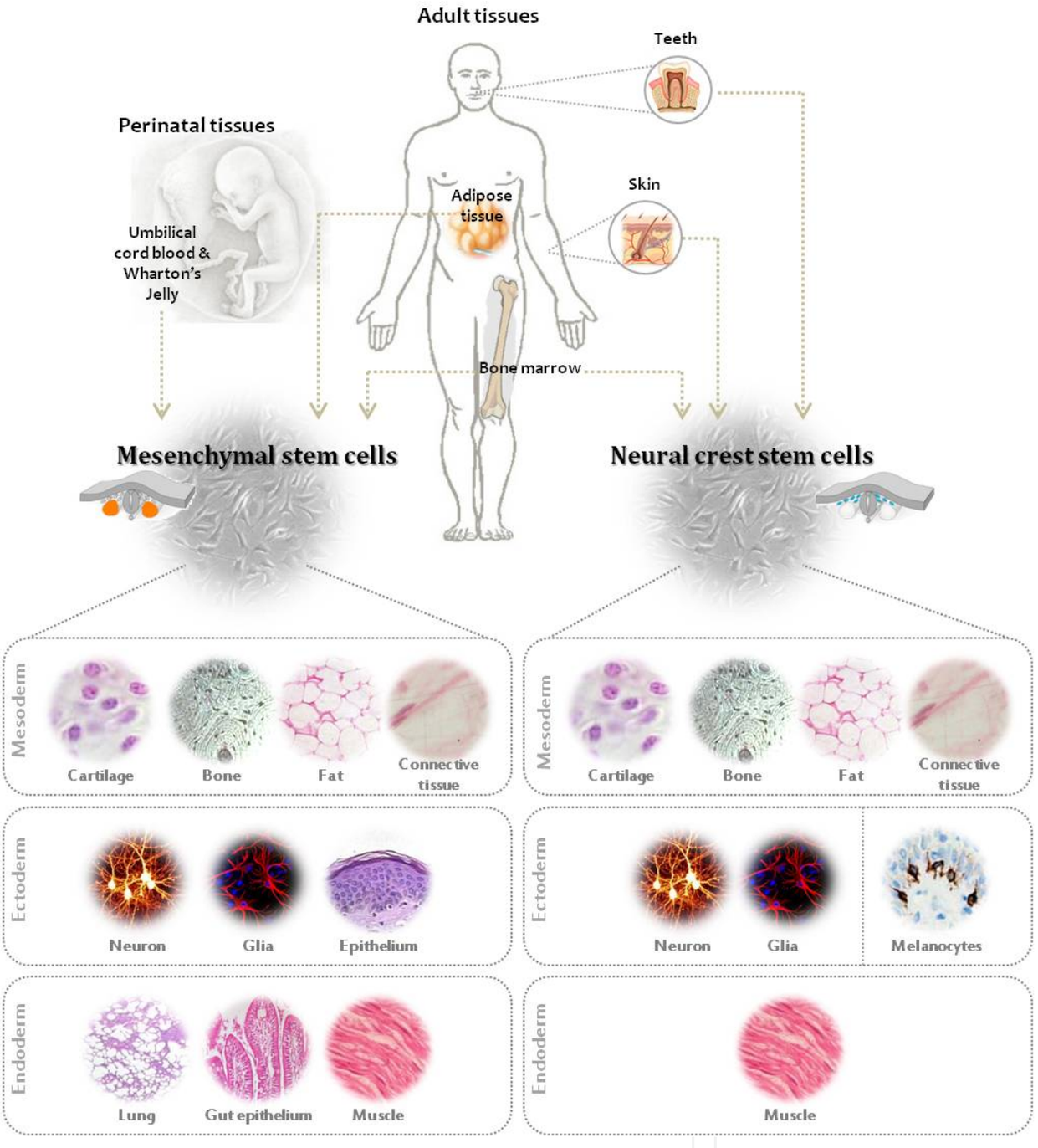


**Figure 1.** Mesenchymal and neural crest stem cells from different perinatal and adult tissues. The upper part describes the presence of mesenchymal and/or neural crest stem cells in various adult tissue. The lower part describes cell fate that have been demonstrated for each type of stem cells regarding the mesodermic, endodermic or ectodermic cell lineage.

The main debate concerning BM-MSCs resides in the lack of exact phenotypic characterization, due to the absence of specific membrane markers and non-standardized culture methods. Consequently, several groups described BM-MSCs with a wide variety of different phenotypes: Verfaillie's group described a rare population of cells in human BM stroma as

mesodermal adult progenitor cells (MAPCs)[12, 16]; D'Ippolito and collaborators cultured cells in low oxygen tension and characterized marrow isolated adult multilineage inducible (MIAMI) cells [17, 18]; whereas a lot of other groups kept the mesenchymal stem cell concept as defined by Pittenger et al. [9].

In addition to the phenotypic differences of BM-MSCs which are inherent to culture settings, it has been demonstrated that BM stroma was a mixed population of cells arising from different embryonic lineages. Although adult BM-MSCs were commonly considered to be of mesodermal origin (bone marrow mesenchymal stem cells) [19], several studies have shown that some adult BM-MSCs derive from the embryonic neural crest [20-24] (see paragraph below). Hence, the different studies that are detailed below often describe BM-MSCs without distinguishing mesenchymal and neural-crest derived cells.

### 2.2. Adipose tissue stem cells

Similarly to the main part of BM stroma, adipose tissue derives from mesodermic lineage and contains stem cells able to differentiate into bone, cartilage, fat and muscle. Likewise, adipose tissue mesenchymal stem cells (AT-MSCs) can adopt a neural-like phenotype [25, 26], which makes them another potential source of cells to use in replacement therapy.

### 2.3. Umbilical cord blood and Wharton's jelly mesenchymal stem cells

Wharton's jelly constitutes the gelatinous matrix of umbilical cord. Mainly composed of collagen fibers, proteoglycans and stromal cells, this tissue has also been described to enclose mesenchymal cells endowed with stem cells properties (WJ-MSCs); They are easily cultured and expanded *in vitro,* and are able to differentiate into a wide range of cell types, including neural cells [27-29].

Whereas adult peripheral blood only contains a tiny number of MSCs, umbilical cord blood is a richer source and allows to culture adherent MSCs more efficiently. These umbilical cord blood MSCs (UCB-MSCs) are considered as a more primitive population, but can be largely expanded and maintained in long term culture [30], and were described to be an osteogenic, adipogenic, chondrogenic and even a neurogenic cell population [31-33]. Altogether, these data confirmed umbilical cord as a new source of cells for cellular therapeutics for stromal, bone, and, potentially, neural repair [34].

## 3. Different types of neural crest stem cells

During embryonic development of vertebrates, neural crest is specified at the border of the neural plate and the non-neural ectoderm after gastrulation. During neurulation, the neural folds both join at the dorsal midline to form the neural tube. Subsequently, neural crest cells (NCC) from the roof plate of the neural tube undergo an epithelial to mesenchymal transition (EMT), delaminating from the neuroectoderm. Those multipotent NCC then migrate towards different locations in the body where they differentiate into various cell types,

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including melanocytes, craniofacial cartilage and bone, smooth muscle, peripheral and enteric neuron, and glia.

In the past few years, multipotent and self renewing neural crest stem cells (NCSCs) have been described to persist in the adult organism. Those post-migratory NCSCs were found in the gut [35], the skin [36-38], the cornea [39], the heart [40], the teeth [41, 42], the dorsal root ganglion and the bone marrow [21, 22, 43]. As the skin, the teeth and the bone marrow constitute the most easily-accessible and available sources of NCSCs to use in therapy protocols, we will describe those three tissues more precisely.

### 3.1. Bone marrow neural crest stem cells

Regarding the striking similarities of bone marrow stromal cells and embryonic NCSCs concerning their neural differentiation potential, the question of the presence of a neural crest-derived cell subpopulation in bone marrow was raised. Indeed, the mesodermal origin of bone marrow stromal cells was definitely queried since a study of Takashima et al. demonstrated that a first wave of mesenchymal stem cells in the embryo derives from the Sox1-positive neuroepithelium through a neural crest stage [20].

Lately, convincing evidence for the subsistence of NCSCs in bone marrow emerged from a study by Nagoshi et al. They isolated neural crest-derived cells from the bone marrow using Wnt1-Cre/FloxedEGFP mice for *in vivo* fate mapping. Those cells could be propagated in sphere cultures for a couple of passages. A bit more than 3% of these isolated $EGFP^+$ cells had the capacity to differentiate into neurons, glia, and smooth muscle cells (this proportion was sustainably increased by collagenase treatment, suggesting their tight contact with bone surface) [21, 22]. The same group used another transgenic mouse (P0-Cre/FloxedEGFP) to isolate NCSCs among the bone marrow stromal cells, and demonstrated their ability to differentiate into neural crest lineages but also into mesenchymal lineages such as adipocytes, chondrocytes and osteocytes [22]. Using a Wnt1-Cre/FloxedLacZ transgenic mouse, the group of Wislet-Gendebien generated neural-crest derived clones of passage 5 bone marrow stromal cells, and compared them with mesenchymal clones. They showed that the two types of populations were surprisingly similar at the transcriptomic level and in terms of differentiation abilities, and that both of them could give rise to neurons [43]. Altogether, the different results about bone marrow NCSCs make those cells as exciting as their MSCs neighbors in a context of therapy. Moreover, their neural crest origin may confer them particular additional properties in a perspective of nervous system repair.

### 3.2. Skin-derived neural crest stem cells

Using the same type of Wnt1-Cre reporter mice, the groups of Sieber-Blum and Toma identified neural crest-derived stem cells in the facial skin of adult mice and humans [36, 37, 44, 45]. Those skin-derived precursors (SKPs) are located in the dermal papillae and in the hair follicle, and are able to differentiate *in vitro* into neurons, smooth muscle cells, Schwann cells and melanocytes.

Other skin-derived NCSCs, termed epidermal NCSCs (EPI-NCSCs) were isolated from the bulge region of whisker follicles. Similarly to SKPs, EPI-NCSCs can give rise to neurons, smooth muscle cells, Schwann cells and melanocytes [37, 44, 46]. As an easy-accessible autologous source of highly multipotent stem cells, the skin and its SKPs and EPI-NCSCs are of significant interest in cell therapy.

### 3.3. Dental neural crest stem cells

The dental pulp is the connective tissue that forms the inner part of the teeth, and contains ondotoblasts which are responsible of dentin formation. Few years ago, a population of stem cells has been identified in dental pulp, and is thought to arise from the embryonic cranial neural crest [47, 48]. These dental pulp stem cells (DP-SCs) are endowed with high proliferative potential, self-renewal ability and multi-lineage differentiation [42, 49], making them an attractive tool for stem cell therapeutic strategies. Whereas the DP-SCs are isolated from the adult teeth, the same type of stem cells can be found in the human exfoliated deciduous teeth (SHED cells), identified as immature DP-SCs.

The properties of self-renewal and multi-lineage differentiation ability of all described stem cells make them truly attractive candidates for cell therapy. Furthermore, they offer the non-negligible advantage of being easily obtained without invasive method. Indeed, whereas umbilical cord is usually intended to trash after birth and can rather be preserved in order to collect cells, bone marrow aspiration, lipo-aspiration, or teeth extraction are non-heavy procedures that are commonly performed in clinical context. Those procedures could even be performed in patients when needed, allowing autologous grafts and avoiding immunological rejects. Additionally, the use of MSCs/NCSCs, from either adult origin or isolated from umbilical cord, get round the ethical problems related to fetal cells use. Moreover, those cells are supposed to be safer than embryonic stem cells or induced pluripotent stem cells in terms of tumorigenicity and genomic modifications [24, 50].

## 4. Signaling pathways involved in neural differentiation of MSCs and NCSCs

As already mentioned, a wide range of culture settings for MSCs/NCSCs neural differentiation were commonly experienced, with some of them giving rise to substantial results. Hence, it can be inferred that MSCs/NCSCs can give rise to neuronal cells through the activation/deactivation of many intracellular signaling pathways. In this chapter, we will focus on the analysis of several differentiating protocols that highlighted specific signaling pathways in neuronal fate decision. We will dissect those pathways from the ligand molecules to the different cellular and molecular effectors that are involved within, up to the gene expression modulation. We will then describe the different downstream effects experimentally observed after activation of those pathways, in the context of neural fate adoption by different type of MSCs/NCSCs and summarized in Figure 2 and Table 1.

### 4.1. Cyclic-adenosine-monophosphate and PKA signaling pathway

Cyclic-adenosine-monophosphate (cAMP) is a well-known intracellular messenger, which is physiologically synthesized from adenosine-triphosphate by a membrane-anchored adenylyl cyclase, when this last is induced by an active G protein-coupled receptor. In the cytoplasm, cAMP essentially activates the protein kinase A (PKA), which then reach the nucleus where it supports the phosphorylation of a transcription factor (cAMP responsive element binding protein, or CREB protein). Once phosphorylated, CREB protein binds CREB-binding protein (CREBB protein or CBP), and with the support of different co-factors, join specific DNA sequences and regulate the expression of different genes (coding for c-fos, brain derived neurotrophic factor (BDNF)[51], or tyrosine hydroxylase (TH)[52]). The destruction of intracellular cAMP is mediated by phosphodiesterases (PDE), which convert cAMP into AMP, then regulating cAMP cytoplasmic concentrations. This cAMP-dependent pathway has been demonstrated to be fundamental in embryonic development, neural cells survival and other processes like long term memory and neuronal plasticity [53-55].

cAMP is frequently used in culture media to induce MSCs/NCSCs into neural lineage, as well as other molecules which raise the intracellular cAMP levels. For example, forskolin activates adenylyl-cyclase, dibutyryl-cAMP (db-cAMP) and 3-isobutyl-1-methylxanthine (IBMX) both act as inhibitors of phosphodiesterases, and 8-bromo-cAMP activates PKA and is long-acting because of its resistance to degradation by phosphodiesterases.

The main cytoplasmic target of cAMP, which is the PKA, has effectively been demonstrated to mediate neural differentiation. Wang et al. studied the impact of PKA activation on neurite outgrowth and on neural markers glial acidic fibrillary protein (GFAP) and neurofilament (NF) expression. They observed that the complete inactivation of this kinase led to a total absence of neural differentiation in UCB-MSCs, while the level of phosphorylated CREB was upregulated in forskolin-treated cells (this effect was inhibited in presence of PKA inhibitor) [56]. The involvement of PKA in the neural differentiation of MSCs was also confirmed by several others studies [57,58].

According to Lepski et al., neuronal differentiation of human BM-MSCs resulted from a specific mechanism dependent upon the PKA pathway. Indeed, they demonstrated that the presence of a PKA inhibitor in the induction medium impaired the differentiation process (induced by IBMX, coupled with brain-derived neurotrophic factor (BDNF), see paragraph 3.4.), and that CREB was phosphorylated in differentiated MSCs [59]. Moreover, MSC-derived cells showed significant voltage-dependent ionic currents ($Na^+$, $K^+$ and $Ca^{2+}$ currents).

Besides, UCB-MSCs were induced to neural outcome with db-cAMP and IBMX treatment, which was demonstrated to be necessary and sufficient for neurite-like outgrowth and for nuclear receptor related 1 protein (Nurr1) expression. Nurr1 is known to play a key role in dopaminergic system maintenance. In addition, those data showed evidence for cAMP-pathway control on differential phosphorylation of TH isoforms [60].

Lin et al. studied the ability of granulocyte-macrophage colony-stimulating-factor (GM-CSF) to promote neural differentiation in BM-MSCs through the phosphorylation of CREB [61]. Indeed, GM-CSF-treated BM-MSCs expressed higher levels of neuron specific enolase (NSE)

over time, whereas transiently increased nestin expression. In parallel, a substantial increase of phosphorylated-CREB level was observed in the GM-CSF-treated BM-MSCs compared with control (CREB levels were not different between the two groups), and that the kinetics of this increase was consistent with the progress in neural differentiation.

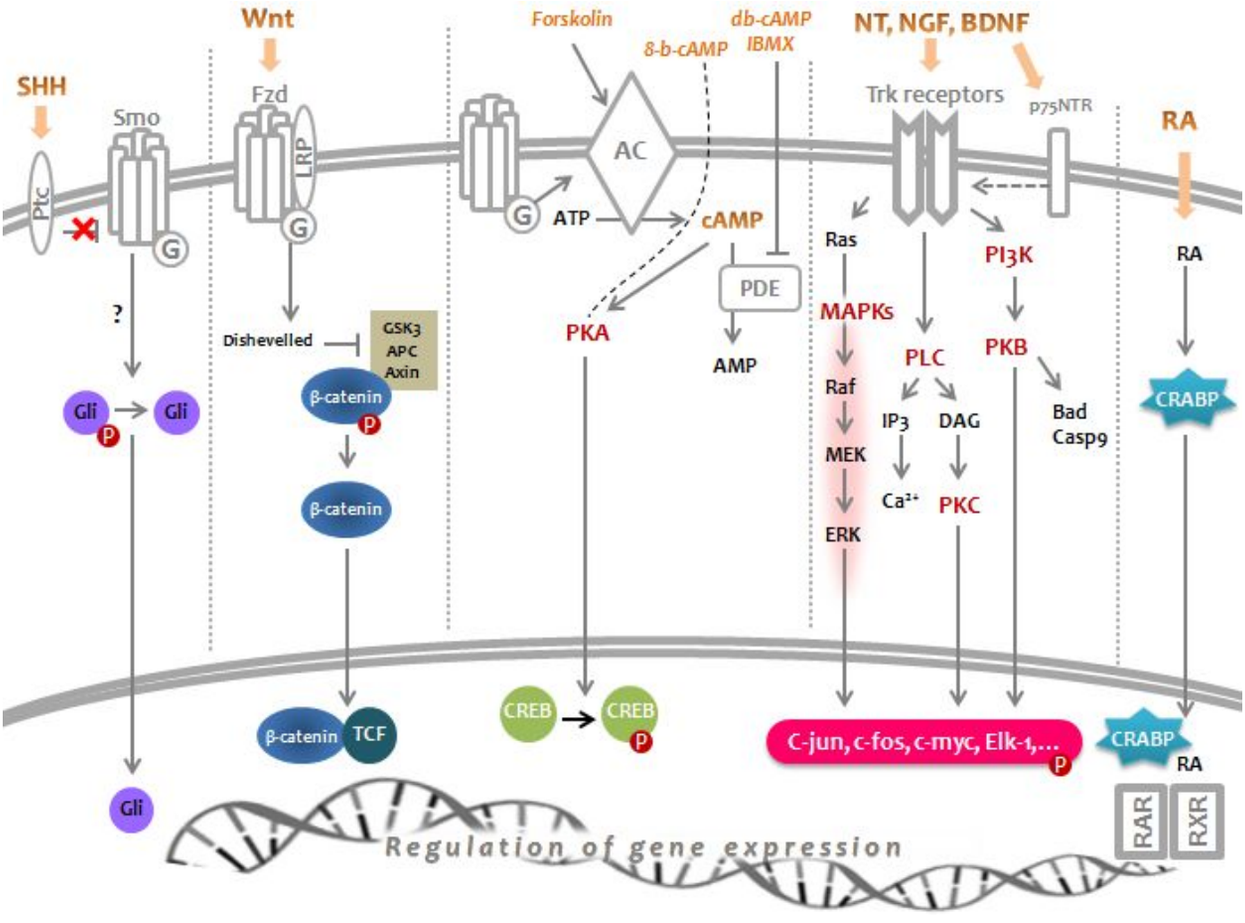


**Figure 2.** Different signaling pathways are involved in neural differentiation of MSCs and NCSCs.Each pathway and modulating substances are described in the text.

While cAMP pathway is supposed to be involved in neural differentiation of MSCs, its precise role in this differentiation process still needs to be defined. Zhang et al. showed evidence for the involvement of cAMP in two differentially-regulated processes, which are early transient neuron-like morphology changes, like cytoskeleton rearrangement, and later neural markers expression associated with neuronal function, but demonstrated that cAMP-treated BM-MSCs did not achieve complete differentiation [62]. Another hypothesis was made by Rooney et al., who examined the effect of intracellular cAMP elevation on BM-MSCs' fate [63]. They demonstrated that forskolin and 8-bromo-cAMP induced a transient increase in βIII-tubulin expression and changes in cell morphology, but no expression of growth associated protein 43 (GAP-43) [64] was seen in the neural-like BM-MSCs, excluding authentic neurite formation. They therefore concluded that this effect was mostly due to a modification of culture conditions rather than in a differentiation process.

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| | *Cell type* | *Passage* | *Pathways* | *Induction protocol* | *Protocol length* | *Neural phenotype* | *Electro-physiological profile* | *Inhibitor* | *Reference* |
|---|---|---|---|---|---|---|---|---|---|
| **1** | **UCB-MSCs** | ns | cAMP-PKA-CREB | Forskolin | 1 to 7 days | NF, GFAP | No | H89, U0126 | [56] |
| **2** | **BM-MSCs** | P12-P24 | cAMP-PKA-CREB, NT | 1) EGF, bFGF. 2) cAMP, IBMX, BDNF | 1) 1 week. 2) 12 hours | NF-200, NFM, NeuroD, MAP2, NeuN, GABA | Inward Na+ currents and outward K+ currents | PKAi fragment 6–22 amide | [59] |
| **3** | **BM-MSCs** | P3-P6 | cAMP-PKA-CREB | GM-CSF | 6 to 96 hours | Nestin, NSE, GFAP | No | No | [61] |
| **4** | **BM-MSCs** | P0? | cAMP-PKA | Forskolin, IBMX | 1 hour to 2 days | βIII-tubulin, GFAP, NSE | No | H89 | [62] |
| **5** | **BM-MSCs** | ns | cAMP | Forskolin / 8-bromo-cAMP | 6 and 24 hours / 1 and 4 days | βIII-tubulin | No | No | [63] |
| **6** | **BM-MSCs** | ns | NT, SHH | SHH, FGF8, bFGF (+ BDNF) | 12 days | NeuN, TH, βIII-tubulin, DAT | Inward Na+ currents and outward K+ currents | No | [65, 66] |
| **7** | **BM-MSCs** | P3-P8 | cAMP, RA, SHH | Forskolin, SHH and/or RA | 2 days | Nestin, Sox2, NSE, GFAP, synapsin, ACh | Neuronal resting potential | No | [67] |
| **8** | **BM-MSCs** | P3-P5 | cAMP, NT, RA, SHH | 1) bFGF, Forskolin. 2) Forskolin, IBMX, RA, SHH, BDNF | 7 days | GATA3, Sox10, GluR4, Irx2, calretinin, MAP2, NeuN, βIII-tubulin | No | No | [68] |
| **9** | **MIAMI cells** | P3-P9 | cAMP, NT, RA, SHH | 1) bFGF. 2) NT-3, SHH, RA, FGF8. 3) Forskolin, NT-3, BDNF, NGF, GDNF. | 1) 24h. 2- 2 days. 3) 3-7 days | NSE, GFAP, βIII-tubulin, NF-L, NF-M, Nurr1, TH | Inward Na+ currents and outward K+ currents | No | [69] |
| **10** | **AT-MSCs** | ns | cAMP, NT, RA, SHH | bFGF, IBMX. 2) SHH,RA. 3) BDNF, GNDF | 6 hours. 2) 1 week. 3) ns | βIII-tubulin, ChAT, Nkx2.2, Pax6, Hb9, Olig2 | No | No | [70] |
| **11** | **UCB-MSCs** | P5-P8 | cAMP-PKA, NT, RA | RA, IBMX, NGF, bFGF | 8 hours to 7 days | GFAP, NF-L, NF-M, NF-H, NSE, Nurr-1, TH, Tau | No | H89 | [60] |
| **12** | **BM-MSCs** | P4 | cAMP, PKC, RA | Forskolin, IBMX, TPA / RA | Up to 48 hours / 7 days | βIII-tubulin, GFAP, NSE, NF-M | No | No | [71] |

| | *Cell type* | *Passage* | *Pathways* | *Induction protocol* | *Protocol length* | *Neural phenotype* | *Electro-physiological profile* | *Inhibitor* | *Reference* |
|---|---|---|---|---|---|---|---|---|---|
| **13** | **BM-MSCs** | P3-P4 | cAMP, RA (RARβ) | 1) RA. 2) Forskolin | 1) 24 hours. 2) ns | Nestin, NSE, MAP2 | Neuronal resting potential | No | [72] |
| **14** | **DP-SCs** | P0? | RA | 1) EGF, bFGF. 2) bFGF. 3) bFGF, RA | 1) 7 days. 2) 7 days. 3) 7 days. | Nestin, βIII-tubulin, NF-M, NFH, PSA-NCAM | Inward Na+ currents | No | [41] |
| **15** | **MIAMI cells** | P5-P9 | cAMP, NT | 1) EGF, bFGF. 2) bFGF. 3) bFGF, NT-3. 4) 3) Forskolin, NT-3, BDNF, NGF. | 1) 10 days. 2) 24 hours. 3) 2 days. 4) 3 days | βIII-tubulin, NF-M, NF-H, NF-L, GalC | No | No | [73] |
| **16** | **MIAMI cells** | ns | NT (TrkC)-Rac1-Mek1/2-Erk1/2 | 1) bFGF. 2) NT-3. | 1) 24 hours. 2) 48 hours | βIII-tubulin, NF-M, NF-H, NF-L, Nestin | No | U0126, K252a, NSC23766 | [74] |
| **17** | **SKPs** | P3-P9 | NT | 1) bFGF, EGF. 2) NT-3, NGF, BDNF | 1) 2-3 weeks. 2) 2-3 weeks. | NF-M, GAP43, βIII-tubulin, MAP2 | No | No | [37] |
| **18** | **BM-NCSCs** | P5 clones | MAPKs | Co-culture with CGN | 5 days | βIII-tubulin | Inward Na+ currents and outward K+ currents (PA for BM-MSCs clones) | PD98059 | [43] |
| **19** | **SKPs** | ns | NT | 1) bFGF, EGF. 2) NGF | ns | NF-M, βIII-tubulin, GFAP | No | No | [75] |
| **20** | **UCB-MSCs** | ns | NT-Raf1-MAPK/ERK, RA | bFGF, RA, BDNF | 7 days | βIII-tubulin, NeuN, GFAP, MBP | No | LY294002, PD98059 | [76] |
| **21** | **UCB-MSCs** | ns | NT (TrkB)-Raf1-MAPK-ERK, B-catenin | BDNF gene transfection | | βIII-tubulin, NeuN, GFAP, MBP | No | K252A | [77] |
| **22** | **WJ-MSCs** | P3 | NT | BDNF / HCNP / rDHE | 14 days | MAP2, AChT | No | No | [78] |

| | *Cell type* | *Passage* | *Pathways* | *Induction protocol* | *Protocol length* | *Neural phenotype* | *Electro-physiological profile* | *Inhibitor* | *Reference* |
|---|---|---|---|---|---|---|---|---|---|
| **23** | **AT-MSCs** | ns | cAMP-NT | 1) bFGF, EGF, BDNF. 2) IBMX | 1) 3 days. 2) 48 hours | βIII-tubulin, GFAP | No | No | [79] |
| **24** | **BM-MSCs & DP-SCs** | P3 | cAMP-NT | bFGF, EGF, BDNF, IBMX | 3-5 days. | GFAP, c-fos, NF, HNK1, enolase-2, βIII-tubulin, MAP2, Sox2, Tenascin-C, Connexin-43 and nestin | No | No | [80] |
| **25** | **DP-SCs** | P1-P4 | cAMP, PKC, RA | 1) bFGF, 5-azacytidine. 2) bFGF, IBMX, TPA, db-cAMP, Forskolin, NT-3, NGF. 3) db-cAMP, NT-3. | 1) 48 hours. 2) 3 days. 3) 3-7 days. | βIII-tubulin, NF-M, GFAP, NeuN, NSE | Inward Na+ currents and outward K+ currents | No | [81] |
| **26** | **SKPs** | P3 | NT, RA | 1) RA. 2) NT-3 | 1) 7 days. 2) 7 days. | βIII-tubulin, GFAP, MAP-2, NeuN | No | K252a, Pep5 | [82] |
| **27** | **SKPs** | ns | cAMP, NT, RA | 1) RA, NT-3, BDNF, NGF, db-cAMP | 2 weeks to 1 month | PGP9.5, NF, FMI-43, NMDAR | No | No | [83] |
| **29** | **BM-MSCs** | P3-P4 | cAMP-MAPK-MEK-ERK-Raf | Forskolin | 48 hours | βIII-tubulin, NF200, NSE | No | PD98059 | [58] |
| **30** | **BM-MSCs** | ns | cAMP-PKA, PKC, MAPK-MEK-ERK | 1) bFGF. 2) Forskolin. | 1) Overnight. 2) Up to 7 days | NF | No | K252a, KT5720, AG879, KN-62, LY294002, PD98059 | [57] |
| **31** | **BM-MSCs** | P0? | cAMP-Wnt | bFGF, IBMX, Forskolin, Wnt1 | 3 to 7 days | Ngn1, Brn3a, NeuroD, P2X3, GluR2, GluR4 | No | No | [84] |

("ns" indicates that the passage or the incubation length are not specified. "No" indicates that no electrophysiological results are described in the study or that no inhibitor has been tested to confirm the pathway). **Hedgehog signaling pathway**

**Table 1.** In vitro protocols for neural differentiation of different types of MSCs/NCSCs and detailed results.

The hedgehog-mediated signalization is involved in the embryonic development, and its main ligand, Sonic Hedgehog (SHH) plays a role of morphogenic factor, defining which fate has to be applied to cells at each place of the embryo: its role in the nervous system organization is crucial and depends on precise concentration gradients which are essential for a correct patterning of the embryo. Indeed, SHH signaling is the chief actor in the definition of the dorsoventral axis of the nervous system. This signalization mostly takes place in cell cilia, and implies two different receptors that, once activated, generate a tricky reorganization of cytoplasmic protein complexes. SHH binds its receptor Patched1 (Ptc), relieving Ptc-mediated inhibition of a second receptor Smoothened (Smo): Ptc leaves the cilium where Smo then accumulates and induces the activation of Gli family of transcription factors (Gli1, Gli2, and Gli3) [85, 86].

SHH was used to induce dopaminergic differentiation of human BM-MSCs, as demonstrated by Trzaska et al. After a 12-days incubation with SHH, coupled with fibroblast growth factor 8 (FGF8) and basic fibroblast growth factor (bFGF), a great number of cells turned into putative dopaminergic neurons, as showed by tyrosine hydroxylase (TH) expression and electrophysiological features. Those cells showed higher expression of neuronal markers, and downregulated genes which are involved in cell cycle regulation, like cyclin-dependent kinase 2 (CDK2) and proliferating cell nuclear antigen (PCNA), indicating that they entered a post-mitotic fate [65, 66].

Qi and al. analyzed the abilities of rhesus monkey BM-MSCs to differentiate into cholinergic neural cells. While SHH alone in the culture medium did not trigger any modification of resting potential, they demonstrated that BM-MSCs exhibited neuronal resting membrane potential when retinoic acid (RA) was present in the culture medium, and under the combination of both SHH and RA. Moreover, cells from the SHH+RA inducing group expressed higher levels of synapsin and acetylcholine (ACh), indicating that the combination of both signals was the best way to obtain cholinergic neurons [67].

Many other studies demonstrated a synergic role of SHH and RA in neural induction of BM-MSCs. Kondo et al. identified those two signals as sensory factors, showing that SHH+RA application leaded to the expression of glutamatergic sensory neuron markers (including GATA3, Sox10 or GluR4) by treated BM-MSCs [68]. On the other hand, the combination of SHH+RA added to FGF8 (before neurotrophin incubation) was showed to promote dopaminergic fate in MIAMI cells [69], which expressed TH and other molecules involved in dopaminergic differenciation, like Nurr1.

Human AT-MSCs were induced to neural differentiation through the action of SHH and RA. After induction, immunochemical labeling showed βIII-tubulin, choline acetyltransferase (ChAT), and NSE expression. The differentiated cells were then characterized by RT-PCR and results showed that those cells were restricted to a ventral spinal fate (Nkx2.2, Pax6, Hb9, and Olig2), suggesting that those cells could be good candidates for motoneurons generation, in the context of spinal cord injuries therapy [70].

### 4.2. Retinoic acid signaling pathway

Retinoic acid (RA) is physiologically metabolized from retinol, thanks to the sequential action of cellular retinol-binding protein (CRBP), retinol dehydrogenase (RoDH) and retinaldehyde dehydrogenases (RALDHs). Once in the cytoplasm, RA is bound by cellular RA-binding protein (CRABP) and enters the nucleus to bind its specific receptors (RARs) and the retinoid X receptors (RXRs), which themselves heterodimerize and bind to DNA sequences known as the RAREs (RA-response elements). This activates transcription of target genes (Hox genes, Oct4,...) [87]. This RA signalization is involved in brain development and more particularly in the definition of the anterio-posterior axis of the nervous system, by regulating the expression of Hox genes in defined localized domains of the embryo [88].

Whereas SHH was often coupled with RA in cell culture differentiation medium, the implication of RA signaling in neuronal differentiation was also studied without a combination with SHH. RA was demonstrated to act on the up-regulation of NF-L expression in UCB-MSCs, while applied with IBMX, db-cAMP, nerve growth factor (NGF) and bFGF [60]. This study also highlighted the role of RA and cAMP/PKA pathways in the differential phosphorylation of TH during differentiation. Indeed, neurally differentiated cells express neuronal markers as Tau or NSE, whereas TH and Nurr-1 expression assessed their dopaminergic profile.

In the study of Scintu et al., two different protocols were used to differentiate BM-MSCs into neuronal cells. The first one was carried out by activating the cAMP and PKC pathway (with forskolin, TPA and IBMX), whereas the second one consisted in RA treatment. Both protocols leaded to NSE, βIII-tubulin, GFAP and NF positive cells [71].

Similarly, pre-treatment with RA before incubation with forskolin leaded BM-MSCs to express higher levels of nestin, NSE, and microtubule associated protein 2 (MAP2) and exhibit neural-like resting membrane potential and increased intracellular calcium concentration [72]. They also demonstrated that only RA specific receptors RARa and RARc were expressed in native BM-MSCs. Conversely, the expression of RARb was significantly increased in differentiated neurons, suggesting its major role in neural differentiation.

Arthur et al. performed RA treatment of human DP-SCs, which subsequently showed neural morphology and expression of βIII-tubulin, NF-M and NF-H, and more interestingly exhibited electrophysiological activity characteristic of sodium voltage-gated channels, assessing for their potential ability to give rise to functional neurons [41].

### 4.3. Neurotrophic factors and downstream signaling pathways

Neurotrophins are secreted growth factors that are involved in the development of neurons in the nervous system, as well as in their survival and functionality. This family of proteins is constituted by the “prototypical” nerve growth factor (NGF), brain-derived growth factor (BDNF), and the neurotrophins (NT-) 3, 4/5 and 6. Those members promote neural cells to survive, grow, differentiate and function through the activation of high-affinity tyrosine-kinase (tropomyosin-related) receptors (TrkA, TrkB and TrkC are respectively bound by NGF/NT-6, BDNF/NT-4, and NT-3), and through the activation of a common low-affinity re-

ceptor, the $p75^{LNR}$, which has no intrinsic kinase property. While $p75^{LNR}$ activation is sufficient to induce events like neurite formation, its role seems to facilitate the binding of neurotrophins to Trk receptors. After trans-phosphorylation, Trk receptors function as activators of three main signaling pathways, respectively mitogen-activated protein kinases (MAPKs), phospholipase C (PLC) and phosphatidyl-inositol-3-kinase (PI3K) [89,90].

Briefly, the MAPKs pathway consists in a set of sequentially-activated kinase proteins grouped in three main connected cascades, involving regulators of alpha-foetoproteins (Raf), extracellular-regulated kinases (ERK), p38 or jun-kinase 1/2/3, resulting in the phosphorylation of transcription factors and then regulating gene expression. MAPKs are abundantly expressed in the central nervous system (CNS), and ERKs are known to be involved in different processes, including neuronal maturation, survival, and synaptic functions.

The PLC signalization pathway mostly induces intracellular calcium mobilization, but furthermore stimulates protein kinase C (PKC) via the production of diacylglycerol (DAG). The PKC can also be directly activated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and is also able to activate MAPKs pathway.

Finally, PI3K controls another downstream kinase called Akt (also named protein kinase B or PKB) that is a crucial player in cell survival through the regulation of apoptosis among other roles.

Several additional neurotrophic factors are regrouped in a second family, the GDNF family of ligands (GFL). Briefly, the glial neurotrophic factor (GDNF) is a protein that has been shown to promote the survival and differentiation of dopaminergic neurons and motoneurons, and constitutes then an important potential actor in the management of neurological diseases such as Parkinson's disease [91, 92], amyotrophic lateral sclerosis or spinal cord injuries. Neurturin, artemin and persephin are similar ligands that also play a role in cell survival, neurite outgrowth, and cell differentiation migration. Those four members act by the activation of a tyrosine kinase receptor RET, which in association with a co-receptor (GFR$\alpha$), triggers auto-transphosphorylation and downstream signaling processes.

A cocktail of the three main neurotrophins (BDNF, NGF and NT-3) was used to direct neural differentiation of MIAMI cells, which began to develop a complex neuritic arborization and to express neuronal markers, e.g. NF-L or NeuN [69]. Moreover, those differentiated cells showed inward $Na^+$ and outward $K^+$ currents. This study also highlighted the importance of NT-3 in the neural commitment and differentiation step, through the demonstration of a dramatic decrease in viability, βIII-tubulin expression, neuron-like morphology and branching of differentiated cells without NT-3 treatment. Those results were then further detailed and showed the enhanced neural specification from MIAMI cells thanks to epidermal growth factor (EGF) and bFGF pre-treatment [73]; the implication of Rho-GTPase Rac1, which was thought to regulate Mek1/2-Erk1/2 phosphorylation, mediating transcription of genes involved in neural differentiation versus proliferation during NT-3-induced neuronal commitment [74].

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The first assessment of the ability of SKPs to generate neural cells was achieved by the group of Toma, who first described that about 9,4% of SKPs were NF-M, GAP43, βIII-tubulin and MAP2 positive after being treated with NGF, BDNF and NT-3 [37].

When co-cultivated with immature cerebellar granules, nestin-positive BM-MSCs are able to differentiate into functional neuronal cells [93], as showed by βIII-tubulin expression and action potentials firings. After characterizing two subpopulations of BM-MSCs which were respectively derived and non-derived from the embryonic neural crest (after generating clonal cultures), Wislet-Gendebien and collaborators demonstrated that the neural differentiation of BM-NCSCs in co-culture conditions was abolished in the presence of a MAPKs inhibitor. Those results confirmed the importance of MAPKs pathway in neural differentiation of adult BM-NCSCs [43].

After adding NGF in the culture medium of human SKPs, Bakthiari et al. obtained NF-M, βIII-tubulin, S100 and GFAP positive cells, and that after different conditions of cryopreservation [75].

Lim et al. demonstrated the important role of BDNF in neural differentiation of human UCB-MSCs, and provided a deeper study of the different involved pathways. Addition of BDNF in the induction medium leaded to the phosphorylation of Raf-1 and ERK, then to the downstream up-regulation of p35 expression, which was not observed when ERK was blocked with a specific inhibitor. p35 is known as an anti-apoptotic factor able to block procaspase maturation and to protect neurons from cell death [94, 95].

They also analyzed the contribution of BDNF to cell viability, and demonstrated an up-regulation of the anti-apoptotic gene Bcl2, which was mediated by the activity of PI3K and Akt phosphorylation [76]. Few years later, after genetically modifying UCB-MSCs through transfection with a BDNF-expressing plasmid, they showed the expression of βIII-tubulin, NeuN, GFAP and myelin basic protein (MBP) in differentiated cells, associated with an up-regulated phosphorylation level of TrkB, Raf-1 and ERKs [77].

BDNF was also used to induce bi-multipolar morphology and MAP2 expression in WJ-MSCs. When used in combination with hippocampal cholinergic neurostimulating peptide (HCNP) and/or denervated hippocampal extract (rDHE), WJ-MSCs turned into Choline-acetyltransferase (ChAT) positive cells [96].

Coupled with IBMX, BDNF was able to induce GFAP and βIII-tubulin expression in AT-MSCs, as demonstrated by Ying et al. [79]. The same combination of IBMX and BDNF was used to differentiate DP-SCs into GFAP, c-fos, NF, HNK1, enolase-2, βIII-tubulin, MAP2, Sox2, Tenascin-C, Connexin-43 and nestin positive cells [80].

Neural induction of DP-SCs was achieved by Kiraly et al. through the activation of both cAMP and PKC signaling pathways. After reprogramming by 5-azacytidine treatment, cells where treated with IBMX, db-cAMP, forskolin, TPA, NGF and NT-3, and showed an increase in neurogenin-2, βIII-tubulin, NSE, NF-M and GFAP expression, while electrophysiological recordings revealed voltage dependent sodium channels activity [81].

The study of Zhang et al. demonstrated that RA induced SKPs to neural differentiation through the up-regulation of the transcription factor NeuroD and the cell-cycle regulatory protein p21 [82]. In the meantime, RA also induced $p75^{NTR}$ up-regulation that leaded to apoptotic cell death. They showed that when treated with NT-3 after RA induction, the survival and neural differentiation of SKPs were improved significantly, and cell apoptosis induced by RA was decreased. These effects were reversible as confirmed by the way of a $p75^{NTR}$ inhibitor Pep5 instead of Trk receptor inhibitor K252a.

The three pathways of RA, cAMP and NT were recruited together to differentiate SKPs into neuronal cells. After adding RA, db-cAMP, NGF, BDNF and NT-3 to the culture medium, Lebonvallet et al. identified NF and PGP9.5 positive cells, which were also able to incorporate FMI-43 staining, indicating the presence of synaptic vesicles. Furthermore, they showed an overexpression of neuron-related genes in differentiated SKPs [83].

Kim et al. studied the involvement of non-neurotrophin-activated MAPKs pathway. They showed that cAMP and PKA (resulting of forskolin treatment) promoted the phosphorylation/activation of B-Raf, MEK and ERK [58]. Confirmation was specified with the use of an inhibitor of MAPKs pathway that induced a significant decrease in neural features of forskolin-treated BM-MSCs. The same observation was carried out by Jori et al., confirming that neural-like BM-MSCs reverted to uncommitted cells when cultured with a MEK-ERK inhibitor [57].

### 4.4. Wnt signaling pathway

The Wnt signaling pathway is constituted by a network of proteins that are involved in the regulation of multiple developmental events during embryogenesis, but also in adulthood, in several physiological processes and tissue homeostasis through cell fate specification, differentiation, or proliferation..

Wnt proteins act on cells by binding Frizzled (Fzd)/low density lipoprotein (LDL) receptor-related protein (LRP) receptor complex. When Wnt signal is inactive, the levels of cytoplasmic transcription factor β-catenin are kept low through continuous proteasome-mediated degradation, which is regulated by a complex including glycogen synthase kinase-3β (GSK-3), Axin, and Adenomatous Polyposis Coli (APC). Once Wnt ligands activate Fzd/LRP, the degradation pathway is inhibited (through the activity of Dishevelled (Dsh)) and β-catenin accumulates in the cytoplasm. After nuclear translocation, it interacts with T-cell specific transcription factors (TCF) among others, which allows transcription regulation [97, 98].

Kondo et al. exposed that BM-MSCs induced to neural differentiation (with forskolin and IBMX) showed significant dose-dependent upregulation of sensory neurons markers Ngn1, NeuroD, Brn3a and P2X3 when the induction medium was supplemented with recombinant Wnt1 (whereas Wnt3a exhibited comparable but slighter effects)[84]. Glutamate receptors GluR2 and GluR4 were also up-regulated in those conditions.

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## 5. Implications in cell therapy

With regards to their accessibility and their multipotentiality, adult and perinatal MSCs and NCSCs constitute ideal stem cells to use in cell therapy. As it has been shown that those cells could give rise to neuron-like cells via multiple ways of induction, we can infer that they could be of valuable interest in the treatment of neurological lesions. In this paragraph, we will collect the results of some studies that focused on cell therapy of Parkinson's disease and spinal cord injuries, using different types of MSCs/NCSCs and different ways to differentiate them into neurons before being transferred in animal models. Those results are summarized in the Table 2.

### 5.1. Dopaminergic neurons and Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, with a prevalence of 0,3% of the population in industrialized countries, reaching 1% after 60 years of age [110]. This pathology is characterized by typical clinical symptoms, like bradykinesia, rigidity, gait troubles and resting tremor, while the main pathological feature is the loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc), associated with ubiquitinated protein aggregates called Lewy bodies in different locations of the brain [111, 112].

In the last 80's, clinical trials have been started, using fetal mesencephalic dopaminergic neurons to transplant in PD patients [113, 114]. Despite the demonstration of several benefits in terms of clinical symptoms and pathology, few problems remain. Fetal tissue heterogeneity, influence of harvesting methods on the graft efficiency, need of too much fetuses for only one patient, altogether coupled with ethical concerns, left no option but finding other ways to proceed. One of the main goals in this field relies in the replacement of lost dopaminergic neurons in the nigrostriatal system, which could be achieved through the use of different types of stem cells. As explained earlier, MSCs/NCSCs are interesting candidates in this objective [115].

In this paragraph, we will review the results of some studies aiming to differentiate diverse types of MSCs and NCSCs in dopaminergic neurons before grafting those cells *in vivo*, using animal models mimicking the symptoms of PD (which are required to study the putative usefulness of stem cells in regenerative therapy).

After *in vitro* differentiation of WJ-MSCs in dopaminergic neural cells using a SHH and FGF8 treatment (in combination with brain-conditioned medium), Fu et al transplanted those differentiated cells inside the striatum of hemiparkinsonian rats, previously treated with 6-hydroxydopamine (6-OHDA). 20 days after transplantation, TH positive cells were found around the implantation site, and those cells were shown to be grafted WJ-MSCs. Moreover, the number of amphetamine-induced rotations (giving idea of motor performances of hemiparkinsonian rats) was decreased, and this decrease was gradual over time, showing an important improvement in the nigrostriatal pathway function [99].

| Cell type | Differentiation protocol | Animal model | Histology | Behavioral aspects | Ref. |
|---|---|---|---|---|---|
| Dopaminergic neurons - Parkinson's Disease | | | | | |
| WJ-MSCs | SHH, FGF8 (+ brain-conditioned medium) | 6-OHDA rat | Survival after 20 days<br>TH positive grafted cells. | Decrease of amphetamine-induced rotations over time. | [99] |
| BM-MSCs | SHH, FGF8, GDNF | 6-OHDA rat | Transient survival<br>No differentiation | No | [100] |
| DP-SCs / SHED | Nothing | 6-OHDA rat | TH-positive grafted cells | Decrease of apomorphine-induced rotations over time. | [101] |
| | Forskolin, SHH, FGF8, GDNF | (in vitro) | In vitro expression of βIII-tubulin, MAP2, and TH | No | |
| BM-MSCs | Forskolin, IBMX, db-cAMP and TPA | 6-OHDA rat | <4 weeks survival<br>Few TH expression<br>Many GABA positive cells | No | [102] |
| Motoneurons - Spinal Cord Injuries | | | | | |
| BM-MSCs | 8-bromo-AMP and Rolipram | Rat with contused SC | A2B5, NCAM and B3T positive cells | Recovery of hind limb stepping and hind limb weight support | [103] |
| BM-MSCs | RA + NT-3 overexpression | Rat with transected SC | MAP2, NF and GFAP positive cells<br>Reduction of cystic cavity size<br>Rescue of surviving neurons | Improvement of hindlim locomotion | [104] |
| BM-MSCs | Hb9 and Olig2 overexpression + RA, forskolin, SHH | Injured organotypic SC slice | Integration in slice<br>ChAT positive cells | No | [105] |
| BM-MSCs | RA, db-cAMP, forskolin, IBMX | Rat with contused SC | In vitro expression of MAP2, NSE, nestin, βIII-tubulin<br>Reduced cystic cavity | Improvement in motor performances | [106] |
| UCB-MSCs | Nothing | Rat with hemisection SC injury | Accumulation near the lesion<br>Reduction of lesion size<br>Enhanced axon regrowth | Enhancement of functional locomotor abilities | [107] |

| Cell type | Differentiation protocol | Animal model | Histology | Behavioral aspects | Ref. |
|---|---|---|---|---|---|
| SKPs | Nothing | Rat with contused SC | Survival rate of<br>Reduced cavity size<br>Myelination of endogenous axons<br>Recruitment of endogenous Schwann cells | Heightened sensory responses | [108] |
| | Forskolin (+ neuregulin-1β) | Rat with contused SC | Survival rate of<br>Reduced cavity size<br>Myelination of endogenous axons<br>Recruitment of endogenous Schwann cells<br>Bypass of the lesion<br>Reduced gliosis<br>Axonal growth | Increased BBB score and Basso locomotor subscore<br>Decease in the number of hind limb errors<br>No impact of sensitivity to sensory stimuli | |
| EPI-NCSCs | Nothing | Rat with contused SC | No migration, no proliferation<br>Close contact with host neuritis<br>βIII-tubulin, GAD67, RIP and MBP positive cells.<br>No GFAP | No | [109] |

("No" indicates that no behavioral testing is described in the study. Protocols describing *in vitro* differentiation in the meantime than *in vivo* studies are also included in the table, as well as some *in vivo* studies using non-differentiated cells).

**Table 2.** Different results obtained in cell-based therapy experiments using MSCs and NCSCs.

On the other hand, Khoo et al showed that neuronal-primed human BM-MSCs (with SHH, FGF8, GDNF and other growth factors) did survive transiently in the brain of 6-OHDA-treated rats, but no further differentiation in functional dopaminergic neurons was observed, even when a co-transplantation with olfactory ensheating cells (OECs) was performed to enhance to graft efficiency [100].

The same cocktail was used by Wang et al. to differentiate SHED cells (DP-SCs) into dopaminergic neurons, this time supplemented with forskolin. Differentiated cells were βIII-tubulin, MAP2 and TH positive *in vitro*. They further characterized naïve SHED cells by transplanting them into the striatum of a hemiparkinsonian rat. While some TH-positive cell

bodies were found in the graft zone, a significant decrease in apomorphine-induced rotations was observed, attesting of a beneficial effect of the cell transplantation [101].

cAMP pathway was recruited to differentiate BM-MSCs (after EGF/bFGF-induced sphere formation) in dopaminergic neurons, using forskolin, IBMX and db-cAMP (coupled with the PKC activator TPA) [102]. Differentiated cells showed *in vitro* expression of βIII-tubulin, neurofilament, Nurr-1, TH, AADC and GIRK2. After transplantation into the striatum of 6-OHDA rats, few cells were βIII-tubulin and TH positive, whereas a higher number of grafted cells became GABA-positive (maybe due to the striatal environment mainly composed of GABA neurons). Unfortunately, no behavioral observation was described by Suon et al.

### 5.2. Motoneurons and spinal cord injuries

Whereas peripheral nerves are able to regenerate when a lesion occurs, the motoneurons and nervous fibers in the spinal cord can't be replaced in case of spinal cord injury (SCI). Indeed, traumatic spinal cord injury results in a wide panel of pathophysiological events counteracting any possibility of neural regeneration, and those events are generally grouped in two phases. The primary injury phase can be due to either contusion or compression, and is characterized by section of axons, necrosis, degeneration, oligodendrocytes apoptosis, gliosis and macrophage infiltration. Altogether, those events lead to secondary lesions like ischemia, inflammation, alteration of ion balance, insult of the blood-brain-barrier, lipid peroxidation and glutamate-induced excitotoxicity. Despite a slight spontaneous recovery, all those events collectively constitute an environment that hampers axonal regeneration [116]. Since the clinical consequences of such lesions are dramatic and rarely reversible (para-, hemi-, tetraplegy, respiratory problems, loss of sphincters control, all leading to important socio-economic issues), it's crucial to find out efficient therapies to improve the recuperation of motor function.

Stem cell grafting has been suggested as a therapeutic strategy for spinal cord repair, hence the obtainment of mature motoneurons is critical.

Human BM-MSCs were induced to differentiate into neural cells through the activation of cAMP signaling pathway, via the addition of 8-bromo-cAMP and Rolipram (inhibitor of phosphodiesterases) in the culture medium. Those neurally-induced BM-MSCs were then transplanted into a segment of the spinal cord of rats, previously wounded by contusion. After confirming neural nature of differentiated cells by immunostaining of A2B5, NCAM, B3T, *in vitro* as well as *in vivo* after transplantation, behavioral testing of rats revealed that the motor recovery (assessed by hind limb stepping and weight support) was significantly different at 2 to 12 weeks post-recovery in the group that was transplanted with neurally-induced BM-MSCs when compared with the control groups that received non differentiated BM-MSCs and saline solution [103].

Another protocol was tested by Zhang et al., who treated BM-MSCs with RA before genetically modifying them to overexpress the gene coding for the neurotrophin 3 (NT-3) [104]. Once they've showed that RA pretreatment enhanced NT-3 expression and secretion by MSCs after genetic engineering, they transplanted cells into the transected spinal

cord of rats. Some transplanted cells were positive for MAP2, NF and GFAP labeling. Moreover, cell transplantation leaded to the reduction of cystic cavity, improvement of local environment, rescue of surviving neurons from retrograde atrophy, and improvement of hind limb locomotion.

Liqing et al. induced AT-MSCs to neural fate through the action of SHH and RA, and showed that those cells expressed the transcription factors Nkx2.2, Pax6, Hb9, and Olig2, suggesting those cells as attractive nominees to become mature motoneurons [70]. While no *in vivo* testing has been performed in this study, another experiment focusing on ventral spinal-specific transcription factors was carried out by Park and al., who genetically modified human BM-MSCs to express Hb9 and Olig2, just before treating them with neural induction medium consisting in sequential incubation with RA, forskolin, SHH and FGF. *In vitro*, those cells were excitable and were able to connect muscle fibers; while after transplantation into an injured organotypic spinal cord slice culture, they survive and integrate the slice, while expressing motoneurons-specific markers, e.g. ChAT [105].

Pedram et al. performed comparative study of the potencies of BM-MSCs to take part in the repair of spinal cord damages, either when neurally-differentiated than when used in their native state [106]. BM-MSCs were cultured following a multi-step protocol, in presence of RA and bFGF, db-cAMP, forskolin and IBMX. After assessing the neural nature of differentiated cells (expression of MAP2, NSE, nestin, and βIII-tubulin), they transplanted cells into the lesion cavity of contused rat spinal cords. Either undifferentiated BM-MSCs or neurally-induced BM-MSCs transplantation leaded to a reduced cavity, but a significant improvement in motor performances was observed in rats that received neurally-differentiated BM-MSCs (compared to control group and native BM-MSCs-transplanted group).

Obtaining myelinating glial cells is another way to manage spinal cord injuries. In that purpose, Biernaskie et al. studied the effect of SKPs on spinal cord lesions, when transplanted in their naïve state or when pre-differentiated into Schwann cells (using forskolin and neuregulin-1β) [108]. They showed that a graft of both the naïve SKPs and SKPs-derived Schwann cells leaded to a reduced cystic cavity size, and that the cells myelinated host axons and recruited host Schwann cells. Still, the SKPs-derived Schwann cells were the only ones to generate a bridge across the lesion and to induce a growth-permissive environment, while a substantial improvement was observed at the behavioral level (Increased BBB score and Basso locomotor subscore and decrease in the number of hind limb errors).

Significant enhancement of functional locomotor abilities was observed by Schira et al. after transplantation of unrestricted UCB-MSCs into the surrounding area of a hemisection injury, accompanied by cell accumulation near the lesion, reduction in its size and enhanced axon regrowth [107]. This study gives an example of what can be observed using naïve stem cells in cell therapy, without any pre-differentiation. In the same way, Sieber-Blum et al. transplanted EPI-NCSCs in the core of a spinal cord lesion, and observed βIII-tubulin, GAD67, RIP and MBP positive cells among the grafted cells. Those cells were tightly close to endogenous neuritis, but did not show any sign of proliferation nor migration in the tissue [109].

## 6. Conclusions

Mesenchymal stem cells (MSCs) and neural crest stem cells (NCSCs) are multipotent cells that are able to generate a wide range of cell types, including neural cells, which makes them incredibly interesting in restorative therapies for patients suffering from neurological diseases. A lot of induction protocols indicate that many signaling pathways may be involved in the neural fate of MSCs and NCSCs. Indeed, the signalization pathways of cAMP, Retinoic acid, Hedgehog, Wnt and the neurotrophins-activated pathways have been implicated into the maturation of adult MSCs/NCSCs into neural-like cells. After an induction process consisting in various activators, lengths and conditions of culture, treated cells adopt a neural morphology express markers (at the transcriptome level as well as at the protein level) that are usually described to characterize neurons at different developmental stages [117, 118] in MSCs as well as in NCSCs. Despite the expression of those specific neural markers, only a tiny number of *in vitro* protocols were able to provide convincing evidence for a neuron-specific electrophysiological signature of the differentiated cells.

During neural development, immature neural cells undergo a differentiation process towards functional neurons through different stages that are accurately defined by specific electrophysiological features. Briefly, the first currents that occur in the cell consist in voltage-dependent outward potassium currents. As maturation proceeds, voltage-dependent inward calcium and sodium currents arise sequentially. The ultimate step is finally characterized by the elicitation of action potential through the activity of several mature voltage-gated sodium channels: an important depolarization triggers intracellular modifications, proteins activation, and vesicular trafficking that are required for proper synaptic chemical and electrical function/transmission [119-121]. As clearly observed in Table 1, even if a few data attest of electrophysiological activity in MSCs/NCSCs-derived neuron-like cells (as showed by sodium and potassium currents), there is no sufficient evidence for action potential firings and for an appropriate neuronal function.

As showed by the diagram on the figure 3, most of the collected studies describe the cAMP signaling pathway to play a key role in the neural differentiation of MSCs/NCSCs. On the other hand, neurotrophins are often used for neural differentiation, whereas MAPKs have been shown to be involved too. RA represents the third most used signaling molecule. We can see that the most part of studies showing significant electrophysiological recordings use RA treatment for differentiation, suggesting its noteworthy role in this process (16,1 % of the total number of studies (5/31) and 62,5 % of the number of studies showing significant electrophysiological recordings (5/8)). Additionally, a major number of protocols were performed in association with cAMP pathway activation. On the other hand, 40% of the differentiation protocols using SHH signalization (2/5 studies) were able to induce changes in electrical activity (see Table 1). That presumably raises the question of a role for RA and SHH in the last stages of maturation of MSCs/NCSCs into neural-like cells.

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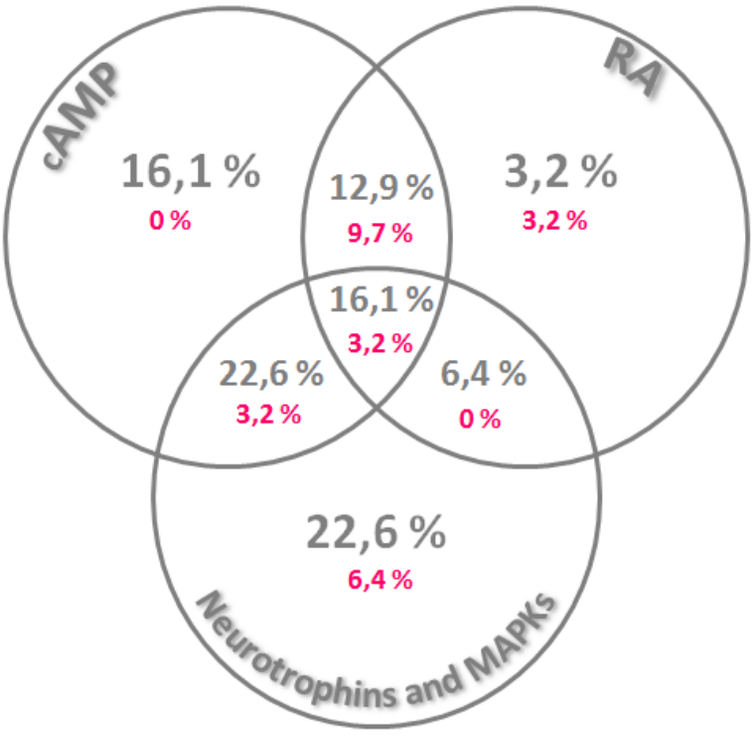


**Figure 3.** Percentages of differentiation protocols involving cAMP, RA or neurotrophins/MAPKs signaling pathways among the 31 detailed studies. Percentages are expressed in regard to the total number of studies. Numbers in red indicate the percentages of studies describing significant electrophysiological recordings.

Overall, we tend to conclude that although the cells express neural-specific proteins and exhibit a preliminary electrical activity [122, 123], MSCs and NCSCs do not seem to be able to fully differentiate and generate functional neurons in order to reach the objective of cell-based therapy in human neurological treatments.

However, several *in vivo* studies based on MSCs/NCSCs-derived neural cells demonstrate a significant improvement of symptoms and lesions in animal models of neurological disorders, such as Parkinson's disease or spinal cord injury [24]. In these studies, the transplantation of differentiated MSCs/NCSCs leads to the limitation of the lesions and the recovering of motor functions. Regarding other successful cell therapy experiments based on the transplantation of non-differentiated MSCs/NCSCs, we can infer that the experimental enhancement is more likely to be due to the intrinsic properties of the grafted cells, and not to a genuine differentiation process nor to an authentic neuronal electrical activity. Indeed, even if their ability to generate neurons is present but quite limited, they still display important immunomodulatory and anti-inflammatory properties, they secrete a lot of growth and neurotrophic factors, they modulate apoptosis processes, and promote endogenous precursors recruitment [124-128]

In conclusion, those observations confirmed the significance of MSCs and NCSCs use in cell therapy procedures to treat several neurological disorders, sustaining their high capacity to protect or restore neural tissue through many proceedings that are probably more owed to intrinsic abilities than to neuronal differentiation.

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