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Chapter 5

Neural Stem Cell: Tools to Unravel Pathogenetic Mechanisms and to Test Novel Drugs for CNS Diseases

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1. Introduction

1.1. Neurogenesis, stem cells and cellular models of diseases

In a study published in 1992, Weiss and Reynolds at University of Calgary, demonstrated for the first time that cells isolated from the brain of adult mice have the ability to proliferate in vitro and differentiate into neurons, astrocytes and oligodendrocytes using a specific cocktails of growth factors [1]. In 1999, similar stem/progenitor cells were isolated from the human adult periventricular subependymal zone and expanded in vitro [2].

Neural stem cells (NSC) are self-renewing, multipotent cells residing in the nervous system. NSC during development produce the enormous diversity of neurons, astrocytes and oligodendrocytes within the developing nervous system. However, accumulating evidence has clearly shown that a number of newborn neurons can be generated also from adult NSC, integrates into pre-existing neural circuits and is functional [3]. In the adult brain, neurogenesis is not a diffuse event and occurs in restricted regions, where classical developmental signals and morphogens such as, Bone Morphogenic Proteins (BMPs), ephrins, Noggin, Sonic hedgehog homolog (Shh), and Notch expression are maintained even after differentiation [4]. In particular, the Notch pathway is a highly conserved arbiter of cell fate decisions and is intimately involved in developmental processes [5]. Thus, besides its pivotal role in neural development, it is also involved in the control of neurogenesis, neuritic growth [6], neural stem cell maintenance [7], synaptic plasticity [8] and long term memory [9] both in the developing and adult brain.

In the adult brain the well-established restricted regions of neurogenesis, named niches, are the sub ventricular zone (SVZ) of the lateral ventricle wall and the dentate gyrus subgranular zone (SGZ) of the hippocampus [9]. Several reports describe that neurogenesis may also occur

in other brain areas, including substantia nigra [10], and neocortex [11] even if these studies have not been confirmed by others.

In the currently prevalent view, primary adult SVZ NSCs *in vivo* are slowly dividing, long-term 5-bromo-2'-deoxyuridine (BrdU)-retaining progenitors that exhibit several common features of subventricular radial glia-like astrocytes and ventricular ependymal cells, including morphological characteristics and expression of the glial fibrillary acidic protein (GFAP) and the glycoprotein CD133, also known as Prominin-1.

Adult neurogenesis in either the SVZ or the SGZ is highly sensitive to environmental cues, physiological stimuli and neuronal activity, suggesting that the tailored addition of new neurons might serve specific neuronal functions. In fact, the complex dynamic equilibrium present in healthy adult CNS involves also the participation of functional NSC niches [12, 13].

The occurrence of major events breaking niches homeostasis may overcome their adaptive capacities and contribute or lead to disease. In CNS, various pathogenic events acting by different mechanisms may cause neural cell loss and chronic inflammation. Several agents and mediators sustaining these mechanisms also act on niche homeostasis and it is therefore expected that these two conditions may have a deep impact on NSC biology and niche properties. The neurogenesis within SVZ niche is differentially activated in various neurodegenerative pathologies. An increase in endogenous neurogenesis in the lateral ventricular walls occurs in several diseases including traumatic brain injury, vascular dementia, Huntington's disease, multiple sclerosis or epilepsy and physiological events such as ageing. [14-19]. In these pathophysiological conditions, a common feature leading to neurogenesis is represented by the upregulation of inflammatory cytokines production. In several cases it has been shown that the niche reaction to inflammation leads to generation of new neurons that integrate within the pre-existing circuitry [20].

In pathological conditions, such as stroke, NSC niches can appear also at unexpected ectopic sites as in brain parenchyma. In particular, part of the stroke-induced ectopic cells positive for doublecortin, a widely accepted marker of neurogenesis, originates from the SVZ resident NSCs that have migrated to the injured site where they differentiated into mature neurons [21] However, it is also possible that part of this ectopic pool may originate locally, leading to an injury-induced ectopic NSC niche formation [22]. Niche reaction to injury may thus proceed by amplification of pre-existing NSC niche and migration toward the damaged site or by activation of local and pre-existing quiescent NSC niches or by conversion of a normal tissue into a newly established NSC niche. The mechanisms and signals driving these reactions are many, some of them peculiar to the pathological condition and most of them endogenous to the NSC niche and already acting in physiological condition.

Altogether the findings described above lead to speculate that NSC may be therapeutically useful for CNS self-repair if properly mobilized and can supply cells for neuronal substitution in CNS injury or degeneration, providing potential therapeutic tools in CNS diseases. NSC, on the other hand, can be also useful for studying the mechanisms and molecules involved in neuronal differentiation, regulation of neurotransmitter biosynthesis and also physiopathology of brain diseases. Indeed, to date, deciphering the molecular and cellular basis of CNS

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disorders has been met with success in animal models for neurological disease. However, while the continued importance of animals in translational research is unquestionable, genetic and anatomical variation between rodents and humans have led to imperfect phenotypic correlations between genetic models and the human disease. Moreover, most neurodegenerative diseases are sporadic and depend on the complex interaction of genetic and environmental risk factors. Thus, it may be difficult to fully clarify these conditions in animals. For these reasons, it would be advisable if neurological disease cellular models should be developed and studied in concert with existing animal models. Recent advances in the areas of stem cell and reprogramming biology seem to provide a novel route to the generation of a wide variety of neural cell types for studying neurological diseases.

A critical point of neurological disease modelling using NSCs is the availability of reliable protocols that efficiently direct stem cells differentiation into the specific neural cell types affected in disorders of interest. Knowledge of the pathways that drives neural differentiation has led to rational approaches now routinely used to direct the differentiation of NSCs in vitro. It appears that a variety of neural phenotypes can be achieved, depending on the combination and timing of the inductive signals to which progenitors are exposed. Recently, several disease-related cell types have been generated in vitro by directed differentiation of NSCs such as spinal motor neurons, midbrain dopaminergic neurons, basal forebrain cholinergic neurons, cortical progenitors and oligodendrocytes [23-38]. Moreover, Lee and co-workers also generated sensory neurons and Schwann cells [39]. One of the first differentiation method has been developed for the production of spinal motor neurons. In particular, NSCs can be directed to differentiate into functional spinal motor neurons when challenged with retinoic acid and Sonic Hedgehog. Retinoic acid induces neuralization and caudalization of stem cells, while the induction of Sonic Hedgehog signalling converts spinal progenitors cells into motor neurons [40]. The NSCs derived spinal motor neurons, when transplanted in developing chick embryos, were able to form long axonal projections into skeletal muscle [27]. The ability to direct the differentiation of NSCs into specific motor neurons could have important implications for modelling of diseases such as amyotrophic lateral sclerosis (ASL) and understanding molecular mechanisms of selective vulnerability. Another clinically relevant neural subtype that has been generated in vitro from NSCs is dopaminergic neurons which could be used as cellular model of Parkinson Disease (PD). One of the strategies used to obtain dopaminergic neurons from NSCs rely on the combined activity of Sonic Hedgehog and FGF8, as first shown by Lee et al. [27]. Methodological improvements to enhance dopaminergic differentiation in vitro include co-culture with immortalized fetal astrocytes and dual inhibition of BMP/TGF beta signalling during neural induction. The in vivo functionality of dopaminergic neurons derived from NSCs has been demonstrated by transplantation assays into the striatum of 6-hydroxydopamine-lesioned parkinsonian rats showing partial recovery of motor function [41-46]. A recent study reported the controlled differentiation of NSCs to basal forebrain cholinergic neurons, the neuronal population affected in Azheimer's disease (AD) and associated with cognitive decline [33]. In particular, two methods have been applied to obtain basal forebrain cholinergic neurons from NSCs: the use of the diffusible ligand BMP9 and the transfection of two developmentally relevant transcription factors, Lhx8 and Gbx1, which were further shown to act downstream of BMP9 signalling. Basal forebrain cholinergic neurons

obtained in these ways were also shown to stably engraft into murin hippocampal slice cultures and to generate electrophysiologically functional cholinergic synapses [33]. In addition, NSCs are also able to differentiate into glial cell types of the CNS. However, directed differentiation protocols and functional characterization of astrocytes derived from NSCs have yet to be reported. In contrast, there are already many approaches to obtain oligodendrocytes from NSCs [47-50]. The ability of these differentiated cells to myelinate axons has been demonstrated both in vitro by co-culture with rat hippoampal neurons [51] and in vivo by transplantation into the mouse model of dysmielination [52]. Moreover, studies evaluating NSCs-derived oligodendrocytes transplants into adult rats have reported improvement of locomotor recovery in both thoracic and spinal cord injury models [53].

Neural progenitors generated in vitro can also be directed towards cell types of the peripheral nervous system (PNS).

Disease modeling using pluripotent stem cells might greatly benefit if the genome of these cells could be readily modified. For instance, the generation of transgenic ''reporter'' cell lines using fluorescent reporter genes under the control of cell type-specific promoters could enable the purification, tracking, and functional characterization of disease cells models after directed differentiation. Indeed, most in vitro differentiation strategies result in a heterogenous population of differentiated cells, which can include progenitors and a variety of cellular intermediates. Thus, having the ability to prospectively identify, purify, and easily track the desired cell type by means of reporter-gene expression can facilitate downstream disease-specific assays, which could be hindered by the presence of other cell types. However, in spite of their self-renewal properties, NSCs cells can still be difficult to genetically manipulate. Various techniques for stem cell genetic modification have been reported, and these can result in random (i.e., transgenic) or targeted integration of the DNA construct [54]. Transgenic approaches include the use of plasmid transfection, lentiviral transduction, transposases, and bacterial artificial chromosomes (BAC) as DNA delivery systems [55-59]. In particular, Placantonakis and colleagues used BAC transgenesis of different reporter constructs to generate motor neuron lineages [59]. Some of the limitations associated with transgenic approaches include the possibility of insertional mutagenesis, transgene silencing or ectopic expression of the transgene due to position effects, and lack of faithful expression of a reporter transgene due to absence of regulatory elements in the promoter fragment driving its expression [56].

Development of methods to accurately correlate how a stem cell-derived neuron in culture correlates ontologically with the in vivo equivalent could be useful. In fact, neuronal dysfunction and degeneration as a result of the neurodegenerative process probably occurs much earlier than the initial neurological manifestations that characterize the disease. For example, prior to the onset of the motor component of the Parkinson disease (PD), a significant number of dopaminergic neurons have already been lost. Moreover, non-motor manifestations can predate motor manifestations by years. In PD, one of the earliest symptoms of disease may be olfactory and autonomic dysfunction and initial alpha-synuclein positive Lewy body pathology may occur in the dorsal motor nucleus of the glosso-pharyngeal and vagal nerves and anterior olfactory nucleus [60]. It would seem reasonable, in addition to studying midbrain dopaminergic neurons, to dissect the molecular causes that lead to degeneration of the motor

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component of PD, to obtain the cell types affected earliest in the disease. Methods to accelerate the time of the pathology in vitro will probably be important in adult-onset disease. Cellular stressors such as oxidative stress, growth factor withdrawal, starvation, selective neurotoxins, and heat shock may reveal differences in NSCs models. Dopaminergic neurons obtained from a neural stem cell line with a known mutation in LRRK2, typical of an early-onset PD, demonstrated increased proportions of caspase-3 activation suggesting a selective vulnerability when exposed to a variety of cellular stressors including hydrogen peroxide, proteosome inhibition, and 6-OHDA exposure [60].

In addition, with better methods to direct differentiation of pluripotent stem cells to non-neuronal cells, it may be possible to recapitulate the relevant microenvironments that are important in several neurodegenerative diseases [61]. In this regard, NSC cell-based co-culture models have provided informative models of the effects of glia in SOD-1-related Amyotrophic lateral sclerosis (ALS). For example, NSC-derived spinal motor neurons cultured in the presence of glial cells, either derived from SOD-1 transgenic mice or primary astrocytes genetically modified to express mutant SOD-1, are selectively vulnerable to the toxic effects [62, 63]. Furthermore, these culture models allowed subsequent searches for candidate mechanisms by which mutant glia exerted its effects and testing of drugs to rescue motor neuron death. Thus, future NSC-based models composed of titrated populations of neurons, glia, and skeletal muscle to create a functional motor unit in vitro would be informative for studies on several neurologic diseases.

2. Isolation and establishment of pluripotent neural cell line endowed with stemness properties

Here we describe briefly, the establishment of a novel neural cell line obtained from mesencephalic primary cultures generated from 11-day-old mouse embryos and showing staminal properties when appropriately cultured.

In particular, these cells were infected with a replication-defective retrovirus bearing c-*myc* and neomycin resistance genes. Neomycin-resistant clones were isolated and a stable cell line was further characterized and named mes-c-myc A1 (A1). The proto-oncogene c-*myc* was chosen because of its efficiency in immortalizing cells including neuroepithelial cells without neoplastic transformation. A1 cells deprived of serum and treated with cAMP show arrest of cell division, which is considered a prerequisite for neural cells to enter a differentiation program. Under both conditions, A1 cells express nestin and vimentin, markers of neural precursors, and the glial fibrillary acidic protein (GFAP), a marker of glial cells. A1 cells present numerous neuronal features, such as peripherin, neuron specific enolase (NSE), microtubule-associated protein 1 (MAP1), N-CAM. In addition, morphologically differentiated A1 cells show functional voltage-gated channels, a neuronal hallmark. Finally, these cells synthesize and accumulate GABA, the principal inhibitory neurotransmitter in CNS. A1 cells present features of neural progenitors and have a broad potential because they may represent a new tool in CNS developmental studies and could be useful in therapeutic applications.

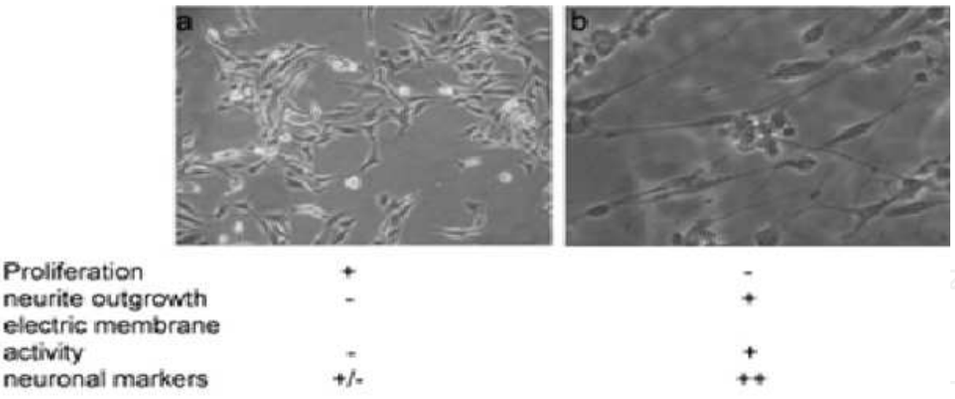

Figure 1. a) Proliferating/undifferentiated A1-mes-c-myc cells and (b) unproliferating/differentiated A1 mes-cmyc cells with their phenotypic profiles. [64].

When cultivated in suspension in a serum free medium with the addition of epidermal growth factor (EGF) with or without basic fibroblast growth factor (bFGF), these cells are able to form neurosphere.

NSCs express EGF and bFGF receptors. The stimulation of these EGF and FGF cell-surface receptors promotes the activation of specific signal transduction pathways, such as the mitogen activated protein kinase (MAPK) and the phospholipase C (PLC) signal pathways [65]. Thus, A1 cells similarly to NSC derived from brain respond to such growth factors by generating neurospheres. Moreover, neurosphere are cellular clones derived from a single neural stem cell which divides to produce other NSC and/or other progenitors cells. Neurospheres can form not only from the clonal progeny of a single NSC, but also by the aggregation of cells into neurospheres [66]. For these reasons, the neurosphere assay (NSA) is one of the most frequently used methods to isolate, expand and also calculate the frequency of neural stem cells (NSCs). Furthermore, this serum-free culture system has also been employed to expand stem cells and determine their frequency from a variety of tumors and normal tissues. In our system, cells were counted and cultured in suspension ($2{,}5\times10^5$/ml) with neurosphere medium [MEM/F12, N_2, bFGF and EGF] in 25 cm^2 flasks with no substrate pre-treatment. Primary neurospheres formed after 4–5 days in vitro once every 7 days they were gently spun down [75 g), mechanically dissociated, and the medium was changed. To evaluate neural differentiation potential, neurospheres were dissociated and plated on poly-D-lysine precoated plates in neurosphere medium. After 6 days in culture, cells were kept for additional 3 days without b-FGF and EGF, and neuronal, astroglial and oligodendroglial differentiation was assessed by immunocytochemical analysis. Immunostaining for markers of neural (β-III-tubulin), astrocyte (GFAP) and oligodendrocyte (O4) cells clearly showed the presence of all three CNS cell types among the neurosphere derived cells. Further characterization of neuronal, astroglial, oligodendroglial and stem cells markers has been performed by real-time PCR. In particular, our analysis identified several stem cell genes and pluripotency-associated gene in neurosphere cells, including nestin, Nanog, and Sox2. By means of real-time PCR we also confirmed the presence of genes of neural cells such as β-III-tubulin and astrocyte such as GFAP. Finally, to definitely confirm the NSCs features of A1 mes-c-myc cells, we determined the self-renewal ability of secondary neurospheres. In particular, primary

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neurospheres were mechanically dissociated into single cells, counted and cultured in neurosphere medium in suspension in 25 cm^2 flasks again. As expected from NSCs, cells dissociated from primary neurosphere were able to generate secondary neurosphere.

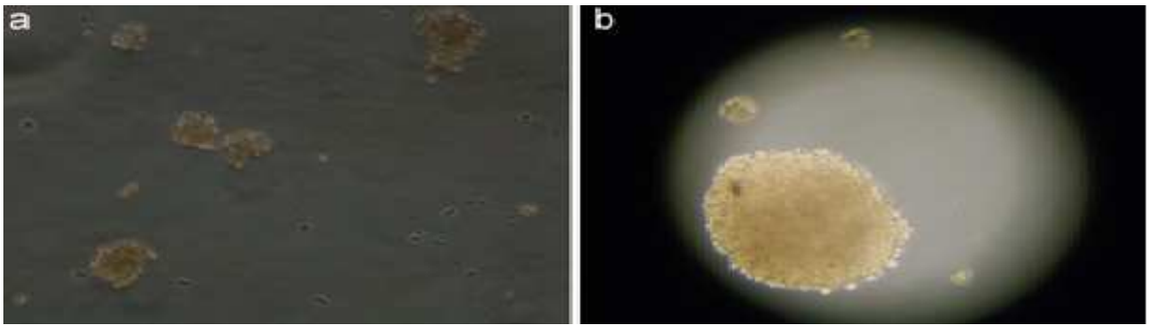

Figure 2. Neurospheres originated from A1mes-c-myc cells 2 days (a) and 10 days (b) after cultured in neurosphere medium.

It cannot be excluded that the contemporary maintainance of precursors and differentiated markers could be attributed to the persistent expression of the exogenous c-myc. However, in our model system the exogenous expression of c-myc does not impair block of proliferation elicited by serum deprivation and/or cAMP treatment. Moreover, it is generally accepted that c-myc is not oncogenic by itself likely because of its dual action as a promoter of cell division and as an apoptotic mediator in absence of growth factors. In addition, it has been reported that in genetically manipulated NSC clones there is a spontaneous and significant down-regulation of c-myc in concomitance with mitogen removal and the onset of differentiation [67, 68]. Moreover, it is worth noting that c-myc is one of the genes used to generate iPS cells. Indeed, viral vectors have been used to transfer transcription factors, such as Oct4, Sox2, c-myc, Klf4, and nanog, to induce reprogramming of mouse fibroblasts, neural stem cells, neural progenitor cells, keratinocytes, B lymphocytes and meningeal membrane cells towards pluripotency [69-71]. Immortalization using the myc transcription factor has proven highly effective at extending the normal life span of human NSCs *in vitro* and maintaining a stable genotype and phenotype. Long term cell expansion with associated karyotype stability is a feature of myc immortalization. Traditionally thought of as a proto-oncogene, it has been recently reported that myc may be a 'stemness' gene driving rapid proliferation yet maintain multipotentiality in stem cells. Indeed, two NSC lines were recently immortalized from human progenitors by means of v-myc and c-myc [72], although useful to test drugs on molecular targets, the human origin of these cells may hamper the efficacy of transplants into mice due to species barrier.

3. NSC lines as model to test new therapeutic compounds

Given the potential of neural stem cells and their differentiated cell types to model keys aspects of neurological diseases, an obvious extension of this platform is represented by its use for drug discovery and predictive toxicology. Nearly 90% of new drugs tested in humans fail to

ultimately come to clinical approval, with central nervous system disorders as a therapeutic area, among those with the highest rate of attrition. Arguably, these failures have resulted from a reliance on imperfect models used during preclinical development.

Neural stem cells (NSCs) are a good model to screen effective drugs that increase neurogenesis. Several classes of drugs have been reported to interfere with NSC homeostasis. Most of the interest has been focused on psychiatric drugs since the early discovery of links between neurogenesis and stress-related disorders. Several extensive reviews have already been published on this topic in the recent years. In particular, association between neurogenesis and depression has been the object of debate since the report of Malberg *et al*. showing, in 2000 by BrdU assay, that chronic antidepressant treatment significantly increased neurogenesis in the dentate gyrus of the hippocampus, and that the new cells became neurons [73-75]. These effects were in agreement with previous data showing that serotoninergic depletion decreased neurogenesis in the dentate gyrus and the SVZ [76]. The effects on neurogenesis were consistent with the time course for the therapeutic action of antidepressants. Chronic treatment with the classical antidepressant fluoxetine accelerated the maturation of immature neurons and enhanced a specific form of long term potentiation in the dentate gyrus; neurogenesis was also linked to the behavioral effect of fluoxetine measured by the novelty-suppressed feeding test [77, 78]. We have recently demonstrated, by means of western blotting and real time PCR, that A1 immortalized cell line is able to express serotonergic markers before and after differentiation. In particular, we demonstrated that A1 cells express the transcripts of the two rate-limiting enzymes necessary for the serotonin synthesis TPH1 and TPH2 and that the two enzymes were differently expressed in proliferating and differentiated cells. We also found that TPHs were modulated by fluoxetine and citalopram, two SSRI drugs widely used in therapy [79, 80].

In addition to synthetic chemicals, recent results show that some natural products also affect cell fate of NSCs [81]. Until recently, neuroprotective effects of natural products have been intensely studied. In particular, methanol extracts of Jeju native plants protected apoptosis induced by hydrogen peroxides. Visnagin, an active component extracted from the fruits of *Ammi visnaga*, which has been used as treatment for low blood-pressure, showed protective effects on kainic acid-induced mouse hippocampal cell death by reducing inflammation. BF-7 extracted from a sericultural product has significant protective effects on amyloid β peptide induced apoptosis through reduction of ROS generation and diminished caspase activity [82-85]. On this issue, also in our lab, we are in progress to test the biological effects of a natural compound on NSCs. In fact, in CNS, aberrant proliferation causes cancer whereas impaired survival of differentiated neurons induces neurodegenerative disorders. In order to find novel therapeutic targets able to inhibit aberrant cell proliferation and/or enhance differentiated cells survival, we analyzed properties of the aqueous extract of *Ruta graveolens* (*Ruta g*. a.e) on differentiated, non-proliferating and undifferentiated, proliferating neural cells. *Ruta g.* is currently used for its diuretic, sedative, and analgesic effects and recent studies described antiproliferative effects on different cancer cells.

In A1 cell system, *Ruta g.* a.e. induces increase of ERK 1/2 (ERKs) phosphorylation and death of A1 proliferating cells. In presence of the ERKs pathway inhibitor, *Ruta g.* a.e.-induced cell

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death decreases, indicating that ERKs is involved in the *Ruta g*. effect on A1 proliferating cells. Moreover, when *Ruta g*. a.e. is added, the number of differentiated A1 cells appears significantly higher as compared to control conditions and the analysis of the cell cycle showed an increased number of cells in G2/M phase in differentiated cells treated with *Ruta g*. a.e.

Thus, our data suggest that A1 cells could represent a model system of neural stem cell line able to allow a deep insight into the mechanisms of regulation neural gene expression and to identify novel therapeutic targets in the development of more useful drugs for the management of disorders of the CNS

4. Conclusion and future perspectives

Research in the area of stem cell biology and regenerative medicine, along with developmental and molecular neuroscience, will further our understanding of drug-induced effects (i.e. death, survival, neurotransmission) on neurons during their development. Moreover, in vitro models of stem cell-derived neural cell lines allow investigators, under control conditions and during intense neuronal growth, to delve deep into molecular mechanisms underlying the actions of various drugs and pathophysiological conditions at various developmental stages. In addition, since NSCs lines are capable to differentiate into non proliferating neuronal phenotypes, they represent a powerful tool to screen drugs exerting different effects according to the cell cycle.

In conclusion, the use of this models will likely lead to fewer pharmacological risks and/or identification of new compounds exerting biological effects on healthy and diseased neurons.

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