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Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y

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Summary Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder in industrialized countries. Present cell culture models for PD rely on either primary cells or immortal cell lines, neither of which allow for long-term experiments on a constant population, a crucial requisite for a realistic model of slowly progressing neurodegenerative diseases.

We differentiated SH-SY5Y human dopaminergic neuroblastoma cells to a neuronal-like state in a perfusion culture system using a combination of retinoic acid and mitotic inhibitors. The cells could be cultivated for two months without the need for passage. We show, by various means, that the differentiated cells exhibit, at the molecular level, many neuronal properties not characteristic to the starting line.

This approach opens the possibility to develop chronic models, in which the effect of perturbations and putative counteracting strategies can be monitored over long periods of time in a quasi-stable cell population.

Keywords: Dopaminergic neurons, mitotic inhibitors, neuronal differentiation, neuronal markers, perfusion culture, retinoic acid

Abbreviations

araC	cytosine β-D-arabinofuranoside
BDNF	brain derived neurotrophic factor
BrdU	bromodeoxyuridine
DA	dopamine
DAT	dopamine transporter
DRD2	dopamine receptors type 2
FBS	fetal bovine serum
FdUr	5-fluoro-2′-deoxyuridine
HMBS	hydroxymethylbilane synthase
HRP	horseradish peroxidase
MAP-2	microtubule-associated protein 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NeuN	neuronal nuclei
NeuroD1	neurogenic differentiation 1
PD	Parkinson's disease
PDL	poly-D-lysine
RA	retinoic acid
SN	*substantia nigra*
TH	tyrosine hydroxylase
Ur	uridine

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Introduction

PD is a slowly progressive degenerative neurological disorder resulting from a degeneration of dopamine-producing neurons in the *substantia nigra* (SN) (Dauer and Przedborski, 2003). Various *in vivo* and *in vitro* models exist for PD. The most prevalent *in vivo* models rely on rodents and primates. However, such models are inherently expensive, there is an interspecies variability and also animal-to-animal variation in sensitivity to specific neurotoxins and drugs used (Bove et al., 2005).

The present *in vitro* (cell culture) models use primary cells or immortal cell lines. Neither cell type, however, represents a suitable model for a chronic, progressive disease such as PD. Primary cells cannot be cultured for a sufficiently long period due to the onset of replicative senescence (Blander et al., 2003), while immortal cells replicate too quickly for long-term effects on a cell to be determined. In the latter case, the cells are typically differentiated for 2–3 days, until then they sprout neurite-like processes. Regardless of the source, cells are treated with neurotoxins for a short period of time, on the order of 3–5 days. This is far from optimal if one wants to establish a chronic model.

Usage of rodent cells (be it primary or immortalized lines, such as PC12) faces the added problem of slight but relevant metabolic differences between rodents and humans (Herman, 2002). Human dopaminergic neuroblastoma cell lines are better suited for developing PD models

because they have biochemical properties of human neurons *in vivo* (Sherer et al., 2001). Moreover, since they are tumor derived cell lines, they continuously divide and can provide the required quantity of cells for different experiments, without exhibiting a large variability (Biedler et al., 1973). However, these cell lines do not have all the characteristics of adult neurons in the brain, and, due to immortality, still have the disadvantage of a short doubling time (Biedler et al., 1973). One way to circumvent these shortcomings is differentiation of these cells to dopaminergic, neuron-like, cells.

Neuronal differentiation can be induced *in vitro* by exposure to different agents such as: tetradecanoylphorbol acetate, brain derived neurotrophic factor (BDNF), norepinephrine, retinoic acid (RA) etc. (Encinas et al., 2000; Laifenfeld et al., 2002; Presgraves et al., 2004). In the case of RA-induced differentiation, one can observe the formation of neurites whose length increases with time of exposure. Moreover, there is an increased synthesis of neurospecific enzymes (such as acetylcolinesterase), neurotransmitters (catecholamines like dopamine, DA), changes in the cytoskeleton markers (neurofilaments) and electrophysiologic modifications as seen in normal neurons (Melino et al., 1997). All these effects are due to RA induction of numerous gene products, including transcription factors, structural proteins, neurotransmitters, neuropeptide hormones, growth factors, enzymes and cell surface receptors (Maden and Hind, 2003). After treatment with RA, cells arrest in the G1-phase of the cell cycle, DNA synthesis is inhibited and growth inhibition can be detected already at 48 h after treatment (Melino et al., 1997).

Most differentiation protocols for the SH-SY5Y cell line involve usage of RA as sole differentiation factor, with differentiation performed over a few days. After this differentiation period, cells were considered to be differentiated based primarily on their morphology, without much additional characterization. In several studies, SH-SY5Y cells were treated 48 h with 10 μM RA and the differentiation was assessed by measuring the neurite length, i.e. the neurites had to be longer than 50 μm (Nicolini et al., 1998). Due to the short differentiation protocol (which is insufficient for a terminal differentiation), the follow-up experiments with neurotoxins had to be performed over 24 h, which necessitated high doses of neurotoxins, not physiologically relevant. Similarly, Maruyama et al. (1997) differentiated SH-SY5Y cells for 3 days with 10 μM RA, but differentiation was appreciated purely on the basis of morphological changes and arrest of proliferation. It is unclear whether cells differentiated this way accurately exhibit neuronal characteristics without a detailed molecular analysis.

During *in vivo* neurodifferentiation various proteins experience changes in their expression levels as a consequence of cellular specialization. In order to compare undifferentiated with differentiated cells, the following neuronal markers were interesting for us. Tyrosine hydroxylase (TH) catalyzes the rate-limiting step in the synthesis of DA and other catecholamines, namely the conversion of tyrosine to dihydroxyphenylalanine. This makes TH the marker of choice for dopaminergic neurons (Gates et al., 2006). At the subcellular level, TH is found in small, punctate structures (Hashemi et al., 2003). Synaptophysin, which is an integral membrane glycoprotein, is a marker for synaptic vesicles that store and release classical neurotransmitters. Thus, its presence indicates secretory activity typical for neurons and neuroendocrine cells (Gaardsvoll et al., 1988). Dopamine receptors type 2 (DRD2) are expressed in neurons of the midbrain, caudate and limbic system (Nestler and Aghajanian, 1997). Dopamine transporter (DAT) is a sodium-dependent DA reuptake carrier expressed only in dopaminergic neurons and has higher levels of expression in SN *pars compacta* (Storch et al., 2004). Microtubule-associated protein 2 (MAP-2) is an abundant neuronal cytoskeletal phosphoprotein that binds to tubulin and stabilizes microtubules, essential for the development and maintenance of neuronal morphology, cytoskeleton dynamics and organelle trafficking (Binder et al., 1985). Tau is a heterogeneous group of microtubule stabilizing proteins associated with several diseases. In the normal brain, Tau is localized in the axons of neurons (Wood et al., 1986). βIII-tubulin is a neuron-specific class of tubulin. During development, the relative abundance of this protein increases with the rate of neuronal differentiation (Lee et al., 1990). Nestin is a member of the family of intermediate filaments and is expressed mainly in neuroepithelial stem cells/precursors. Nestin is not expressed in mature cells and terminal neuronal cell differentiation is associated with down-regulation of this protein (Duggal and Hammond, 2002). Laminin is a major glycoprotein component of basement membrane involved in neuronal survival, differentiation, growth cone guidance and neurite growth (Timpl and Brown, 1994). Neuronal nuclei (NeuN) is a vertebrate neuron-specific nuclear antigen with unknown function. Developmentally, NeuN immunoreactivity is observed after the neurons become postmitotic and no reactivity has been observed in the proliferative zones (Mullen et al., 1992). Neurogenin is a transcription factor that induces neurogenesis and inhibits the differentiation of neural stem cells into astrocytes (Ma et al., 1996). Neurogenic differentiation 1 (NeuroD1) is a member of the basic helix-loop-helix transcription factors family implicated in

growth and differentiation of neurons and is expressed in postmitotic cells (Lee et al., 1995). A suitable model for PD should use cells that exhibit as many of these markers as possible.

Encinas et al. (2000) established a differentiation protocol for SH-SY5Y cells using RA and BDNF. They obtained homogenous populations of fully differentiated neuronal cells and thoroughly analyzed the differentiated cells by different methods. This is one of the few studies (Rebhan et al., 1994; Encinas et al., 2000; Edsjo et al., 2003) in which the cells were differentiated up to 12 days. Also, it is one of the rare examples where differentiated cells were extensively characterized by analyzing different neuronal markers. However, the system they developed would not have been suited for the long-term, perfusion, culture system we aimed to develop. A perfusion culture system is characterized by constant, slow addition of fresh media and removal, at the same rate, of the used media. This procedure has the advantage that, especially for long-term culture, the cells are kept in a quasi-constant environment, avoiding both sudden changes in the concentrations of nutrients and accumulation of toxic metabolites (Minuth et al., 1999). Since we planned to cultivate the cells for weeks, under constant renewal of medium, the cost of BDNF to be added to the culture medium would have been very high. This required the establishment of a different protocol for differentiation, which would not rely on expensive growth factors.

The cell culture presented in this paper yields differentiated cells that are very close to primary dopaminergic neurons. These differentiated cells present many neuronal markers at both mRNA and protein levels. Furthermore, we show that, as a consequence of differentiation, these cells exhibit a decrease of the mitotic active, proliferating population. Thus, such a culture is best suited for a long-term chronic intoxication and treatment strategy as would be the case for a PD model.

Materials and methods

Cell culture

SH-SY5Y cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were grown to confluence in T-25 flasks (Nunc) in Dulbecco's Modified Eagles Medium (DMEM) supplemented with L-glutamine, sodium pyruvate, 1000 mg/l D-glucose and aminoacids (Gibco/Invitrogen #31885) to which were added 20% heat inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) and Hepes (10 mM), in a 5% CO_2 humidified incubator at 37°C. Cultures were split twice a week and cells were seeded at 2.5×10^4 cells/cm^2.

Cells were plated at 2×10^5 cells/coverslip in 1 ml medium on 12 mm glass coverslips precoated with poly-D-lysine (PDL) (Beckton-Dickison). Plated coverslips were maintained in 4-well dishes (Nunc), in DMEM supplemented with 20% FBS, in a 5% CO_2 incubator for two days at 37°C in order to allow the cells to better adhere to coverslips and multiply them to the necessary density. Primary rodent cultures were kindly provided by G. Gille's group and were prepared according to Gille et al. (2002). After two days in these conditions, the coverslips were transferred into the perfusion culture system (Minucells and Minutissue Vertriebs GmbH, Bad Abbach, Germany). The system was connected to a peristaltic pump (Ismatec), which was set to 1 ml/h, equivalent to a total medium exchange within 3.5 h for the 6 coverslips perfusion container. Differentiation was started in L-15 medium (Invitrogen) supplemented with 10% FBS and all-*trans* retinoic acid (RA, 10 µM final concentration) for 14 days. After this, RA was removed from media and mitotic inhibitors (10 µM FdUr, 10 µM Ur and 1 µM araC) were added for the following 10 days. After these 10 days, the medium was supplemented only with FdUr and Ur for the rest of the time in culture. The protocol was modeled after Pleasure et al. (1992). Treatment with these mitotic inhibitors was typically performed for a total of 16 days.

Table 1. *List of antibodies used in the present work*

Antibody (reported specificity)	Protein accession number for the human counterpart	Supplier	Fold dilution	
			WB	IF
Tau (rabbit)	P27348	Chemicon	1000	100
TH (mouse)	P07101	Chemicon	1000	1000
MAP2 (rabbit)	P11137	Chemicon – Boehringer Mannheim	2000	1000
βIII tubulin (mouse)	Q13509	Sigma	1000	1000
Nestin (mouse), human specific	P04179	Chemicon	1000	1000
DAT (rat)	Q01959	Advanced Targeting Systems, San Diego, CA	500	NT
Synaptophysin (mouse)	P08247	Chemicon	500	100
NeuN (mouse)	Antigen identity unknown	Chemicon	1000	500
α tubulin (DM1α mouse)	NA	Sigma	NA	500
BrdU (rat) BU1/75	NA	abcam, Cambridge, UK	NA	200
Laminin B2 chain (rat)	P55268	Chemicon	1000	1000
Donkey anti mouse, donkey anti rabbit, TexasRed coupled	NA	BioRad	NA	100
Alexa Fluor 594 donkey anti rat	NA	Molecular Probes	NA	500
Anti actin (mouse), monoclonal AC-40	P68032	MPI-CBG Dresden, Antibody Facility	2500	NA
HRP-coupled secondary	NA	BioRad	3000	NA

NA not applicable, *NT* not tried, *WB* Western blotting, *IF* immunofluorescence.

Table 2. *List of primers used in the present work*

Gene symbol/name	Primer sequence 5′–3′ forward/reverse	Product length (bp)
DAT	Fw 5′-GAC TTT CTC CTG TCC GTC ATT GGC T-3′	278
	Rv 5′-GAG AAG AGA TAG TGC AGC GCC CAG-3′	
Tau	Fw 5′-GCG GCA GTG TGC AAA TAG TCT ACA A-3′	203
	Rv 5′-GGA AGG TCA GCT TGT GGG TTT CAA T-3′	
MAP2	Fw 5′-CAT GGG TCA CAG GGC ACC TAT TC-3′	209
	Rv 5′-GGT GGA GAA GGA GGC AGA TTA GCT G-3′	
DRD2	Fw 5′-TGC AGA CCA CCA CCA ACT ACC TGA T-3′	224
	Rv 5′-GAG CTG TAG CGC GTA TTG TAC AGC AT-3′	
Synaptophysin	Fw 5′-ATT GTG CCA ACA AGA CCG AGA GT-3′	195
	Rv 5′-CAG GAA GAT GTA GGT GGC CAG AG-3′	
Laminin	Fw 5′-GTT TAA CGA TCC CAA AGT TCT CAA GTC C-3′	208
	Rv 5′-GCA GGC ATT CAC TGG CAC TTT CC-3′	
HMBS	Fw 5′-TCG GGG AAA CCT CAA CAC C-3′	155
	Rv 5′-CCT GGC CCA CAG CAT ACA T-3′	
Nestin	Fw 5′-TGG CTC AGA GGA AGA GTC TGA-3′	148
	Rv 5′-TCC CCC ATT TAC ATG CTG TGA-3′	
βIII tubulin	Fw 5′-GGC CTC TTC TCA CAA GTA CG-3′	317
	Rv 5′-CCA CTC TGA CCA AAG ATG AAA-3′	
Neurogenin1	Fw 5′-GCC TAC AAC TAC ATC TGG GCT CTG-3′	173
	Rv 5′-GGC TGG GCT ACT GGG GTC A-3′	
NeuroD1	Fw 5′-CCG TCC GCC GAG TTT G-3′	118
	Rv 5′-GCG GTG CCT GAG AAG ATT G-3′	
TH	Fw 5′-GCC CTAC CAA GAC CAG ACG TA -3′	90
	Rv 5′-CGT GAG GCA TAG CTC CTG A-3′	

Quantitative real-time RT-PCR analysis

Total cellular RNA was extracted from undifferentiated and differentiated cells using the RNeasy total RNA purification mini kit (Qiagen) followed by treatment with RNAse-free DNAse. The reverse transcription was performed with SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen) and the obtained cDNA was used for the real time PCR reaction at 1 μg DNA/reaction. The DNA was amplified in a MX3000P thermocycler (Stratagene) using Brillant SYBR Green QPCR Master Mix (Stratagene) with primers at 1 μM final concentration using an annealing temperature of 60°C. Primer sequences (forward, reverse) and expected lengths of the amplified products are listed in the Table 2. Results are expressed relative to the housekeeping gene hydroxymethylbilane synthase (HMBS) that is considered to be the unity.

Western blotting analysis

Differentiated cells were removed from coverslips and undifferentiated cells were removed from flasks with trypsin/EDTA, washed with PBS and incubated with hot Laemmli sample buffer supplemented with Complete protease inhibitors (Roche) for 10 min. The protein concentration was determined using the BCA protein assay kit (Pierce). Ten micrograms of protein were loaded per minigel lane and separated on a 4–20% SDS-polyacrylamide gradient gel (Invitrogen), then electroblotted onto the nitrocellulose membrane (Schleicher and Schuell, 0.22 μm). Blocking was performed with a PBS/5% skimmed milk/0.5% Tween-20 solution. Membrane strips were incubated with the primary antibody (see Table 1) at 1 μg/mL, washed and incubated with the secondary, horseradish peroxidase (HRP)-coupled antibodies. Protein bands were revealed with the Enhanced chemiluminescence kit (Amersham) and recorded on Amersham Hyperfilm. Gels were scanned and lane densitometry analysis was performed using the ImageJ software (Rasband, 2006). Molecular weight was estimated using MagicMark (Invitrogen) and Prestained Protein Marker, Broad Range (New England Biolabs).

Immunofluorescence characterization of differentiated cells

Undifferentiated and differentiated SH-SY5Y cells and rodent primary dopaminergic neurons, all cultivated on glass coverslips precoated with PDL, were fixed in 4% paraformaldehyde, then permeabilized with 0.1% Triton X-100 in PBS. Cells were incubated in blocking buffer (2% BSA in PBS) for 20 min and then incubated with various primary antibodies for 30 min. Subsequently, the cells were incubated with the secondary, Texas Red coupled antibody, and, after a brief wash, with the FITC-coupled anti-tubulin antibody DM1α, in order to counterstain for the cytoskeleton. Finally, the coverslips were mounted on microscope slides in mounting medium containing *p*-phenylenediamine as antifade and DAPI for DNA staining. Fluorescence images were acquired using two microscopes: a Leica DMIRE2 inverted microscope equipped with a Leica DC350FX camera and a Zeiss Axioplan 2 equipped with a Hamamatsu C4742-95 camera.

BrdU incorporation

Undifferentiated and differentiated SH-SY5Y cells were incubated for 72 h in media supplemented with 10 μM BrdU (differentiated cells in the absence of mitotic inhibitors). For immunofluorescence, cells were treated as above, with the exception that the permeabilization step was followed by a DNA denaturation using 4N HCl at room temperature for 15 min in order to make the DNA accessible to the antibody.

Statistics

Results were obtained, in general, from at least three independent experiments (six for the RT-PCR analysis). Results are presented as mean values and error bars represent SEM. For assessing difference, two-tailed Student's *t*-test or Mann–Whitney test for unpaired samples were performed using the program InStat, with p values <0.05 considered significant (*).

Results

Differentiation of the SH-SY5Y cell line

The differentiation process was performed with RA in a perfusion system that requires the cells to be grown on coverslips. We used PDL precoated coverslips, since cells adhered poorly on plain glass and plastic coverslips were not suitable for fluorescence microscopy.

Morphological changes were seen for most cells after just 3–5 days, consistent with other reports (Encinas et al., 2000). Many cells elongated and extended neuritic processes (Fig. 1B). However, the original SH-SY5Y culture is comprised of two types of cell populations, which can actively interconvert: the substrate-adherent, differentiation resistant 'S' subtype and the neuronal-like, RA-sensitive, 'N' subtype (Ross et al., 1983). Due to the incapability of RA to induce differentiation (and thus growth arrest) of the 'S' subtype, this population would have overtaken the 'N' population. In order to filter out the undifferentiated cells, we used mitotic inhibitors (araC, Ur and FdUr), in the absence of RA for another 16 days. After this step, cells formed clusters connected via long processes that resembled axons. In order to make a valid comparison, the differentiated cells were compared not only with undiffer-

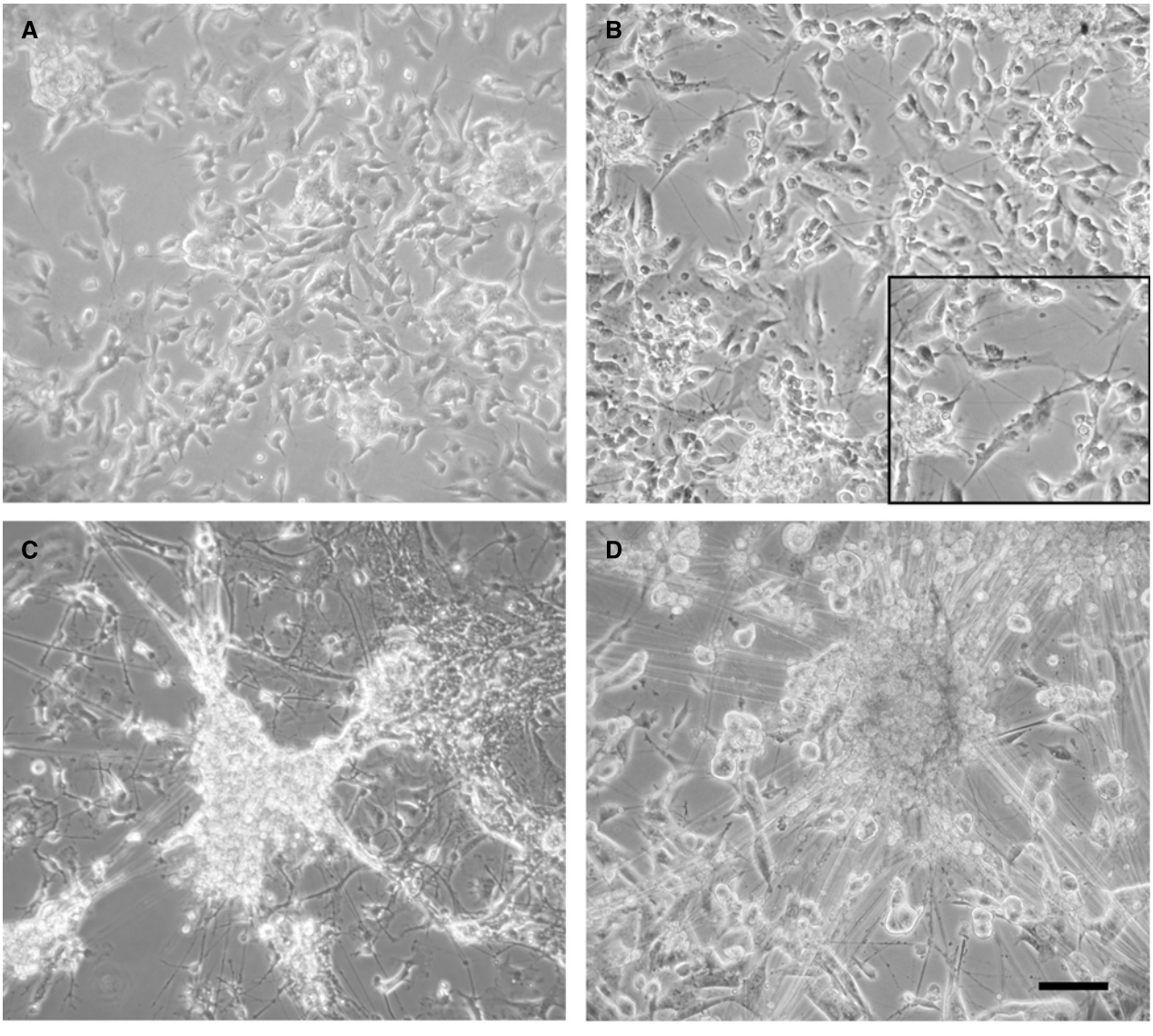

Fig. 1. Morphological comparison between undifferentiated and differentiated SH-SY5Y cells and primary dopaminergic neurons. **A** SH-SY5Y undifferentiated. **B** Eight days RA treatment. **C** Primary rodent dopaminergic neurons cultivated on the same type of coverslips. **D** 14 days RA and 15 days mitotic inhibitors treatment. Inset in **B** emphasizes neurite-like process formation; magnification twice as in the other panels. Scale bar 100 µm

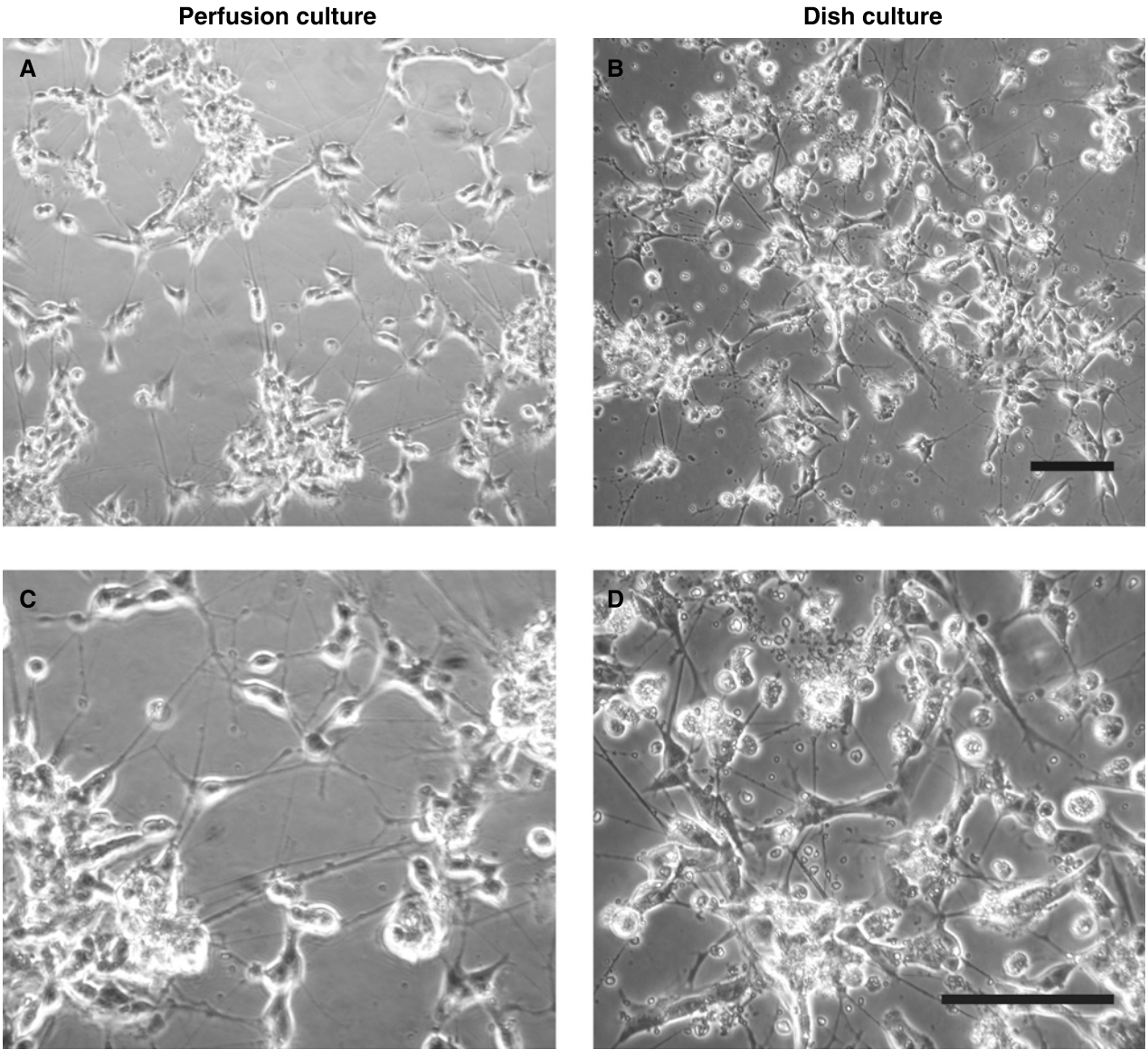

Fig. 2. Comparison between perfusion and plate cultivation during differentiation of cells (14 days RA and seven days mitotic inhibitors). **A** and **C** Perfusion. **B** and **D** dish. Note in **B** and **D** many apoptotic cells (round, bright floating cells) and many cells with a fibroblast-like morphology. Scale bar 100 µm. Same magnification for **A** and **B**, respectively **C** and **D**

entiated SH-SY5Y cells (cultivated in DMEM-20% FBS medium) but also with rodent primary dopaminergic neurons cultivated on identical coverslips (Fig. 1C and D). The differentiated cells had a morphology similar to rodent primary dopaminergic neurons. The differentiation process seemed more successful in the perfusion system compared with the classic cell culture method. As it can be seen in Fig. 2, in the dish culture there are more apoptotic, round cells, compared to perfusion culture (panels B and A, respectively). Moreover, in panel D (dish culture), many more fibroblast-like, undifferentiated cells can be observed compared to panel C.

BrdU staining for proliferation control

It is widely accepted that most of the neuronal cells in the adult brain cease dividing (Cajal, 1928; Gage, 2002). There is evidence for new neurons in the adult mammalian brain. However, proliferation is confined to the olfactory bulb and dentate gyrus (Rakic, 2002). Since the cells seemed to develop a neuronal morphology, and in order to test the efficiency of the mitotic inhibitors treatment, cell duplication was assessed by BrdU incorporation into cellular DNA.

Both types of cells (undifferentiated and differentiated, the latter in the absence of mitotic inhibitors) were incubat-

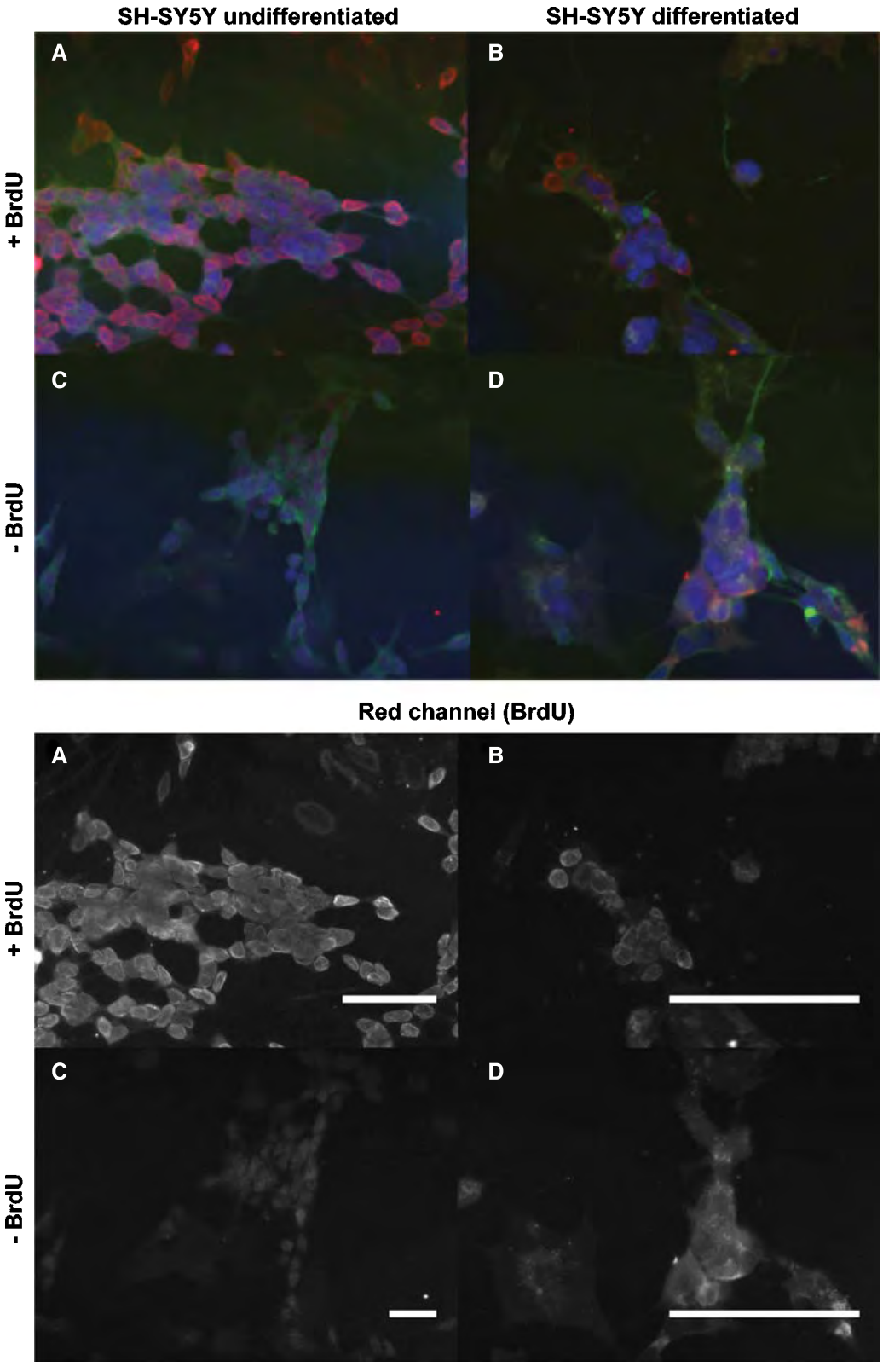

Fig. 3. BrdU incorporation as a control for cell cycle arrest. Top panel: **A** undifferentiated SH-SY5Y cells + BrdU. **B** Differentiated SH-SY5Y cells + BrdU. **C** and **D** No BrdU added (negative control). **C** Undifferentiated SH-SY5Y cells. **D** Differentiated SH-SY5Y cells. Lower panel: the BrdU channel from the top panel (cells have autofluorescence that increases with differentiation). Red: BrdU, Blue: DAPI, Green: tubulin. Scale bar 100 µm

ed with medium containing BrdU for 72 h and subsequently stained for BrdU incorporation. The negative control (undifferentiated and differentiated cells not treated with BrdU, but stained as the other ones) showed that the cells exhibit autofluorescence, which increases after differentiation (Fig. 3, the red channel). The BrdU signal in the differentiated cells is very close to the background (compare panels B and D), whereas the undifferentiated cells incorporated BrdU and led to a strong signal (in panel A) compared to their corresponding control (panel C).

Thus, we concluded that the cell divisions markedly slowed down after the mitotic inhibitors treatment and the differentiated cells are closer to "real" (slow dividing) neurons.

RT-PCR results confirm the differentiation of the cells

To confirm differentiation, we examined several neuronal markers. Mature neurons express specific markers that identify their specialized role in the nervous system. From various known neuronal markers, the twelve presented in the introduction were chosen for this study with the rationale that an increase in their expression (with the exception of nestin) would indicate that the cells are progressing towards a more neuronal phenotype.

As expected, RT-PCR results showed that the mRNA of many neuronal markers increased after differentiation (Fig. 4). For example, a significant change ($p < 0.05$) was observed for Neurogenin, tau, laminin and DRD2, while the message for other proteins (such as MAP2 and DAT) was increased, even if not at a statistically significant level.

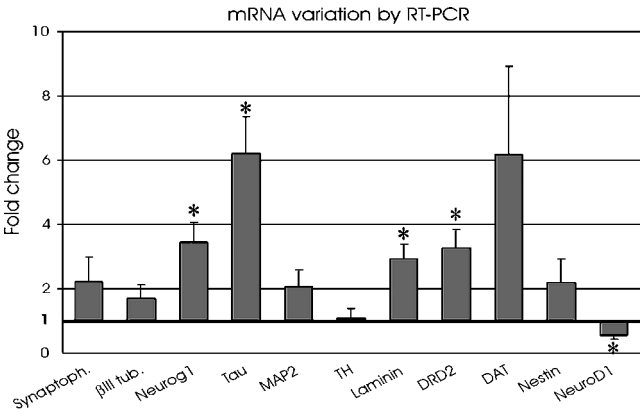

Fig. 4. Variation of neuronal markers after differentiation. Marker mRNA level quantification by QPCR, normalized to undifferentiated cells and HMBS. Reference level is one (mRNA level of marker in undifferentiated cells). The mRNA level decreases after differentiation for NeuroD1 and increases for all the other markers analysed. Asterisk mark statistically significant changes, i.e. $p < 0.05$

Thus, the RT-PCR results suggest that the treatment with RA and mitotic inhibitors led to an increase of the message for many neuronal markers.

Western blotting analysis

To confirm that changes in mRNA level resulted in changes in protein levels, we examined candidate markers by Western blotting. The bands corresponding to the proteins of interest (Fig. 5) were quantified using ImageJ and the β-actin band as a reference (Fig. 6).

Since not all proteins have a commercial antibody available, some antibodies are better than others and several large proteins are difficult to transfer, only a subset of the

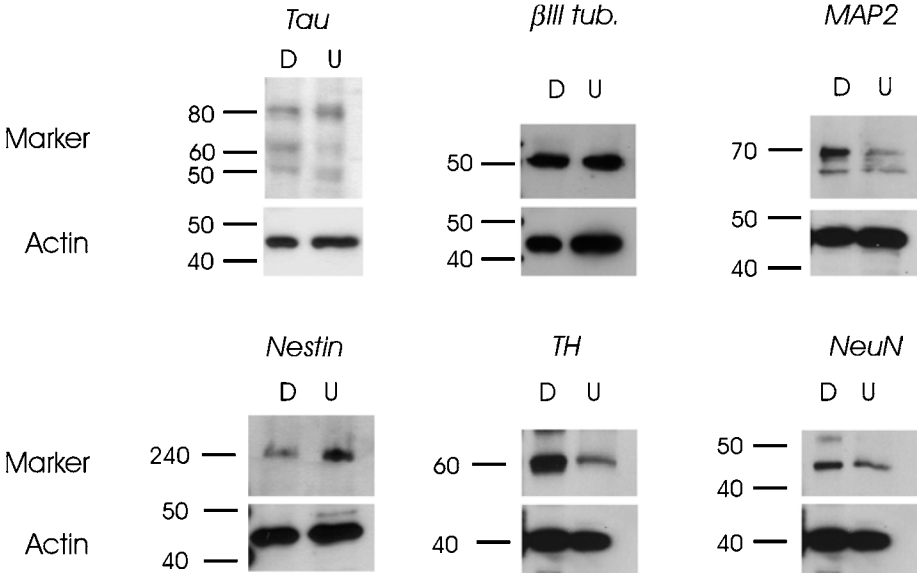

Fig. 5. Western blot analysis of marker proteins. D means differentiated SH-SY5Y cells. U means undifferentiated SH-SY5Y cells. Actin was used as loading control. Numbers on the left represent the molecular weight in kDa

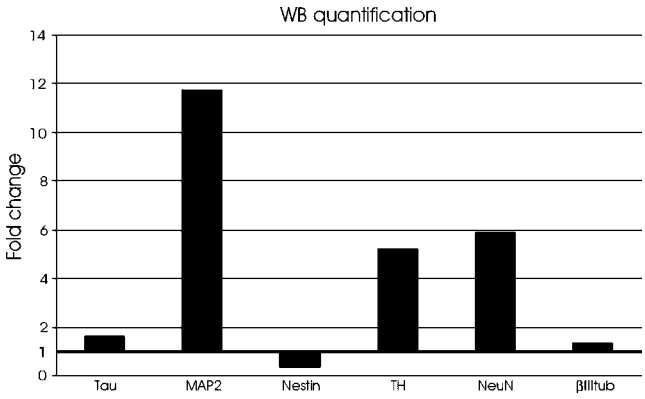

Fig. 6. Quantification of Western blot results for marker proteins from Fig. 5 and two other independent experiments. Comparison between undifferentiated and differentiated SH-SY5Y cells. A level of one means marker protein level unchanged with respect to undifferentiated cells (reference level). Nestin protein levels decrease, as is the case in neurons. Other markers show an increase in protein amount after differentiation

markers tested by RT-PCR could be assessed by Western blotting. Based on the results of Western blotting, MAP2, TH and NeuN increased following the differentiation protocol. Moreover, nestin, a marker for neuronal progenitor cells that decreases during differentiation, was decreased in SH-SY5Y differentiated cells (see Figs. 5 and 6), compared to undifferentiated SH-SY5Y. We concluded that the mRNA of the upregulated genes was indeed translated into increased protein amounts in the cell.

Immunostaining of the cells

We wanted to investigate whether the marker proteins are not only expressed differently in undifferentiated and differentiated cells, but also whether these proteins are localized where they are normally found in neurons. Thus, we have performed immunolabeling for eight different neuronal markers. To have a better comparison, we stained in parallel primary cell cultures of mouse dopaminergic neurons, cultivated on the same type of coverslips (glass, PDL precoated). However, the attachment of the differentiated cells to the glass coverslips was poor; during differentiation, the cells form a network that is very fragile and prone to detaching and, therefore, the number of cells recovered after the staining procedure was usually low. Nevertheless, the staining results were reproducible and consistent.

As it can be seen in Fig. 7, the red signal for the various markers is stronger in the differentiated cells than in the undifferentiated ones. TH, synaptophysin, βIII tubulin (to a lesser extent), MAP2 and laminin showed an increase upon differentiation, consistent with the RT-PCR and Western blotting results. The staining pattern of differentiated cells is close to that of rodent primary dopaminergic neurons and different from undifferentiated SH-SY5Y cells. Thus, the localization of the proteins agreed with our expectations and previous reports (Hashemi et al., 2003).

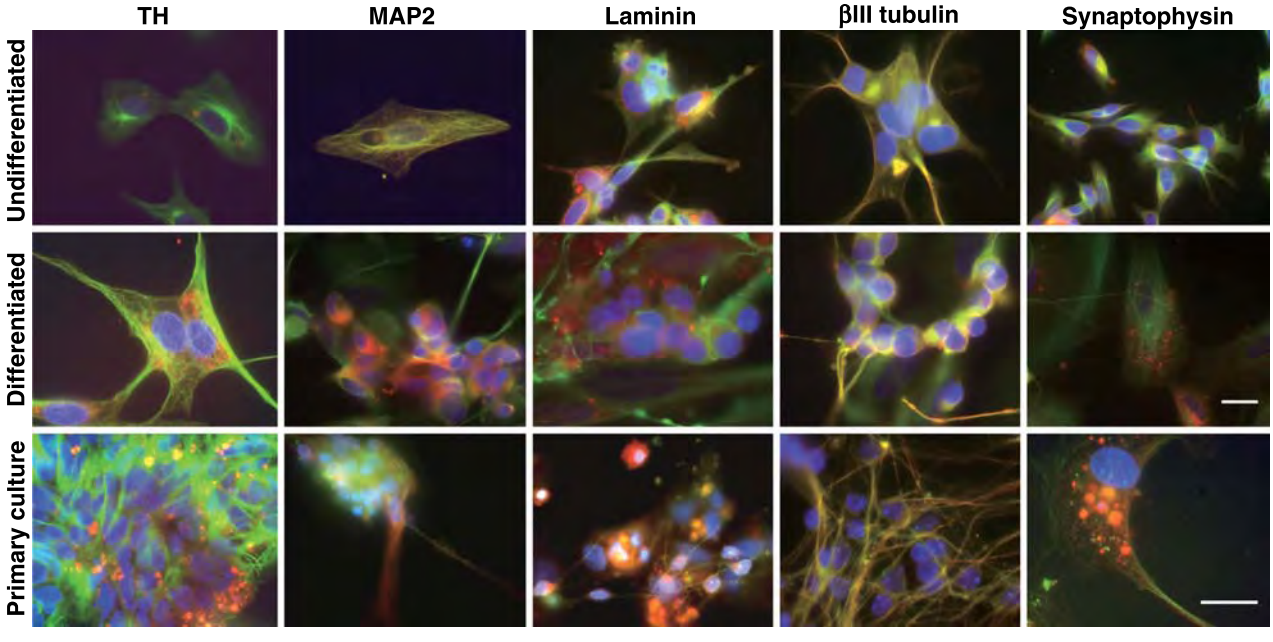

Fig. 7. Immunofluorescence staining for neuronal markers. The comparison was made between undifferentiated, differentiated SH-SY5Y cells and primary dopaminergic neurons derived from mouse embryos. Blue: DAPI, Red: antibody against the respective neuronal marker (Texas Red coupled), Green: DM1α (anti-tubulin) antibody coupled with FITC, Yellow: colocalization of red and green. Scale bar 20 μm for all images. The bottom-right panel is at a different magnification than the remainder of pictures

Taken together, these results suggest that the neuronal markers are expressed and localized as in neuronal cells.

Discussion

In the present work we show that the human dopaminergic neuroblastoma cell line SH-SY5Y can be differentiated to dopaminergic neurons using a specific protocol and a perfusion culture system. The results presented here show that these cells can be differentiated further than has been reported up to now (Nicolini et al., 1998; Maruyama et al., 1997). We have also performed a thorough characterization of the differentiated cells and have shown that many neuronal characteristics can be attained using this protocol.

While animal models probably mimic more accurately aspects of a disease, there are several distinct disadvantages, most obviously, time and cost. In a live animal, many variables can perturb the study of different mechanisms. Cell culture models present the advantage that they are more easily to perform and repeat, whilst being time- and cost-saving. This makes them a good candidate for preliminary studies on the efficiency of various substances, especially when a more controlled setting is required.

In order to have the basic cellular system for developing new oxidative stress models of PD, a human derived cell line was used, which is easier to cultivate than primary neurons, relatively homogenous in composition and closely resembling the cells affected in PD. For this purpose, the human dopaminergic neuroblastoma cell line SH-SY5Y was chosen as a starting point.

The SH-SY5Y cells are often used in cell culture models of PD because they possess many of the qualities of human neurons (Sherer et al., 2001). These cells have neuronal origin, express TH and dopamine-β-hydroxylase, which are specific to catecholaminergic neurons (Ross et al., 1983) and express receptors and transporters for DA and acetylcholine (Biedler et al., 1978; Willets et al., 1995). These cells also express genes associated with neuronal differentiation, including neurofilament proteins and neuron specific enolase among others. Despite expressing all these markers, they are considered immature neuroblasts at different stages of neuronal differentiation (Biedler et al., 1973) and have been shown to maintain the stem cell characteristics and to proliferate in culture for a long time with no contamination (Ross et al., 1983). This is important in the neuroscience and neurotoxicology fields, where the contaminating presence of glial cells, astrocytes and other types of cells can lead to unwanted effects. The SH-SY5Y cell line presents also the advantage that it can be grown and differentiated in the absence of growth factors (Nicolini et al., 1998). The effects of neurotrophic factors used in differentiation are confusing, especially if the cells are further used to study drug-induced neurotoxicity (for example antineoplastic drugs) and the effect of similar trophic factors (Nicolini et al., 1998).

Despite these advantages, there are several differences with respect to neurons, most notably a different expression level of neuronal cell markers (Farooqui, 1994) and confirmed cell proliferation (Pahlman et al., 1995). In particular, undifferentiated SH-SY5Y cells are not an ideal model for dopaminergic neurons as they have a low expression of DAT (Presgraves et al., 2004). Toxicity by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, a neurotoxin widely used in PD pharmacological models) requires the presence of DAT to enter the cells and to be converted to the toxic ion MPP+ (Presgraves et al., 2004). This implies that undifferentiated SH-SY5Y cells are more resistant to MPTP than normal dopaminergic neurons, and are thus not a good starting point for an MPTP-based model of PD (Presgraves et al., 2004). Similarly, the relatively high oxidative stress imposed by DA synthesis makes dopaminergic neurons more susceptible to intoxication by Complex I inhibitors compared to other cells (Barzilai et al., 2001). This was our main reason to generate differentiated cells in order to be further used for a chronic PD model. Another reason to use differentiated cells is to have a constant, non-dividing cell population in order to establish a chronic intoxication model. This would avoid problems stemming from variations in cell numbers and the constant renewal of the cell population. In this respect, primary cells have the disadvantage that they cannot be maintained in culture for very long time whereas immortalized cell lines multiply too much.

We cultivated the cells plated on PDL precoated glass coverslips in a long-term perfusion culture system. This perfusion system is more convenient to use than a normal cell culture dish and the cells can be cultivated for a longer time and under better conditions (Minuth et al., 1999). The perfusion system is characterized by the continuous addition of fresh medium with nutrients and the concomitant withdrawal of the used medium with toxic metabolites. In this way, it is possible to cultivate the cells/tissues in conditions closer to the *in vivo* situation (Minuth et al., 1999, 2000).

The differentiation protocol started with the treatment of cells with RA for 14 days. After 8 days of treatment, cells elongated and exhibited branching similar to neurons (Fig. 1B), as described by several other authors for a shorter treatment (Nicolini et al., 1998; Maruyama et al., 1997). After about two weeks of treatment with RA and another two with mitotic inhibitors to eliminate the proliferating subpopulation, the cells resembled morphologically

the primary rodent dopaminergic neurons cultivated on the same type of coverslips. A BrdU incorporation assay showed that the cells, indeed, stopped proliferating, while RT-PCR, Western blotting and immunofluorescence were used to show that several neuronal markers were upregulated as a consequence of the differentiation protocol.

Quantification of immunofluorescence pictures is prone to many pitfalls; in this particular case, where cells aggregate, it is impossible to do a proper quantification over the entire volume, so the results are only qualitative. Even if the results from RT-PCR and Western blotting were not always in perfect agreement at the quantitative level, both methods, as well as the immunofluorescence suggested that most of the markers tested increased following the differentiation protocol. The immunofluorescence results also show that the proteins localized as expected for a neuronal cell. However, one has to keep in mind that SH-SY5Y cells have neuronal origin, so it is not surprising that, even before differentiation, they already express – albeit at lower levels – proteins that are considered markers for a neuronal cell. Still, there is an obvious signal increase for the above-mentioned markers (Fig. 7). An overview of the neuronal markers variation after differentiation is presented in Table 3.

Patch-clamp would be the ultimate way to prove that the cells are differentiated. However, the differentiated cells are fragile and entangled in a complicated network. Moreover, many cells are packed in large clusters which means patch-clamp would be very difficult (if not impossible) to perform.

Table 3. *Summary of the neuronal markers variation after differentiation*

Neuronal marker	Variation after differentiation			Expected from literature
	RT-PCR	WB	IF	
TH	±	+	+	+ (Hashemi et al., 2003)
MAP2	+	+	+	+ (Binder et al., 1985)
βIII tubulin	+	±	±	+ (Lee et al., 1990)
Tau	+	+	NO	+ (Wood et al., 1986)
Nestin	+	−	NO	− (Duggal and Hammond, 2002)
Laminin	+	NO	+	+ (Timpl and Brown, 1994)
NeuN	NA	+	NO	+ (Mullen et al., 1992)
Synaptophysin	+	NO	+	+ (Gaardsvoll et al., 1988)
Neurogenin1	+	NT	NT	+ (Ma et al., 1996)
DRD2	+	NT	NT	+ (Nestler and Aghajanian, 1997)
DAT	+	NO	NT	+ (Storch et al., 2004)
NeuroD1	−	NT	NT	+ (Lee et al., 1995)

+ = increase, − = decrease.
± no or very small variation, *NO* means no optimal result (problems with the antibody or the protocol, e.g. the transfer on the nitrocellulose membrane in Western blotting).
NT not tried (did not find a working antibody), *NA* not applicable (there is no possibility to design primers for NeuN, as the antigen is not known). *WB* Western blotting, *IF* immunofluorescence.

In conclusion, in the present work we have developed a new cell culture system using human neuroblastoma cells differentiated in perfusion, which allows to better control vital parameters and to maintain the culture for longer time (i.e., weeks instead of just days) (Minuth et al., 2000).

The differentiation protocol presented here has several advantages. Much more time is allowed for the cells to differentiate and ''filter out'' many of the cells that do not undergo differentiation. Other cell culture models utilized short-term (a few days) treatment with RA with/without neurotrophins, tetradecanoylphorbol acetate or norepinephrine (Singh et al., 2003; Laifenfeld et al., 2002). In the present work, the differentiated cells were thoroughly characterized at both the morphological and molecular levels. The results presented suggest that the differentiation protocol was successful and the differentiated cells have a good similarity with primary neurons.

The low division rate of the cells, taken together with our own observations during cell handling, suggests that the population is relatively constant for a long time. A classical culture using cell lines would require splitting the culture every few days, which would skew the results of any viability testing. This new model gives the opportunity to try various neurotoxins in low dose and long time in culture. This way, the differentiated cells can be further used to model PD and other neurodegenerative disorders affecting the dopaminergic system of the brain. Moreover, in these models new potential therapies can be tested for their long-term effect. We are presently developing such a chronic model.

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