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# The Catecholaminergic RCSN-3 Cell Line: a Model to Study Dopamine Metabolism

IRMGARD PARIS[a], JORGE LOZANO[a], SERGIO CARDENAS[a], CAROLINA PEREZ-PASTENE[a], KATHERINE SAUD[a], PATRICIO FUENTES[a], PABLO CAVIEDES[a], ALEXIES DAGNINO-SUBIABRE[a], RITA RAISMAN-VOZARI[b]; TAKESHI SHIMAHARA[e], JOHN P. KOSTRZEWA[c], DAVID CHI[d], RICHARD M. KOSTRZEWA[c], RAÚL CAVIEDES[a,†] and JUAN SEGURA-AGUILAR[a,*]

[a]*Molecular and Clinical Pharmacology, ICBM, Faculty of Medicine, Casilla 70000, Santiago-7, Chile;* [b]*INSERM U679, Neurology and Experimental Therapeutics, Hôpital de la Salpêtrière, Université Pierre et Marie Curie-Paris, France;* [c]*Departments of Pharmacology and* [d]*Internal Medicine, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA;* [e]*NBCM, CNRS, Gif sur Yvette, France. jsegura@med.uchile.cl*

[†]Please address correspondence regarding use of the RCSN-3 line to Pablo Caviedes (pcaviede@med.uchile.cl)

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**RCSN-3 cells are a cloned cell line derived from the substantia nigra of an adult rat. The cell line grows in monolayer and does not require differentiation to express catecholaminergic traits, such as (i) tyrosine hydroxylase; (ii) dopamine release; (iii) dopamine transport; (iv) norepinephrine transport; (v) monoamine oxidase (MAO)-A expression, but not MAO-B; (vi) formation of neuromelanin; (vii) vesicular monoamine transporter-2 (VMAT-2) expression. In addition, this cell line expresses serotonin transporters, divalent metal transporter, DMT1, dopamine receptor 1 mRNA under proliferating conditions, and dopamine receptor 5 mRNA after incubation with dopamine or dicoumarol. Expression of dopamine receptors $D_2$, $D_3$ and $D_4$ mRNA were not detected in proliferating cells or when the cells were treated with dopamine, $CuSO_4$, dicoumarol or dopamine-copper complex. Angiotensin II receptor mRNA was also found to be expressed, but it underwent down regulation in the presence of aminochrome. Total quinone reductase activity corresponded 94% to DT-diaphorase. The cells also express antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. This cell line is a suitable *in vitro* model for studies of dopamine metabolism, since under proliferating conditions the cells express all the pertinent markers.**

*Keywords:* Cell; Dopamine; Neuromelanin; SH-SY5Y; PC12; VMAT-2; Tyrosine hydroxylase

## INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized pathologically by the selective degeneration of neurons in the substantia nigra pars compacta and locus coeruleus, and the presence of Lewy bodies in the remaining neurons (Jackson-Lewis and Smeyne, 2005), generating a dopaminergic imbalance (Mehler-Wex *et al.*, 2006). Identifying the neurotoxin(s) (Berg *et al.*, 2006; Segura-Aguilar and Kostrzewa, 2006)

*Corresponding author: Tel.: +56 2 978 6057; FAX: 56 2 737 2783; E-mail: jsegura@med.uchile.cl

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responsible for neurodegeneration of melanin containing neurons in the nigro-striatal system is a key step, in order to develop new therapeutic agents in PD. Cell lines such as PC12 and SH-SY5Y have been the most utilized as preclinical *in vitro* experimental models to study this neurodegenerative process in PD via neurotoxins such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or rotenone. In the present work, we present evidence of a new cell line, named RCSN-3, which was established from the substantia nigra of an adult rat. The cell line exhibits characteristics of central dopaminergic neurons, and could represent a valuable tool in study of the pathophysiology of PD and in the evaluation of potential pharmacological agents for treatment.

## MATERIAL AND METHODS

### Establishment of Cell Line (RCSN-3)

The substantia nigra of a 4 month old Fisher 344 rat was carefully dissected, and the tissue was minced and suspended in 3 ml of PBS containing 0.12% (w/v) of trypsin (Sigma, St. Louis, MO) and incubated for 30 min at 37°C. Afterwards, the trypsin reaction was stopped by adding an equal volume of plating medium, consisting of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 nutrient mixture (1:1; Sigma) modified to contain 6 g/l glucose, 10% bovine serum, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma). The suspension was centrifuged and the pellet resuspended in 2 ml of plating medium. The tissue was dissociated by passages through a fire-polished Pasteur pipette, and the cells plated onto a collagen 60-mm culture dish at a density of 40,000 cells/cm$^2$. At the time of seeding, the plating medium was supplemented with 5% (v/v) of medium conditioned by the UCHT1 rat thyroid cell line, which reportedly induces transformation *in vitro* (Caviedes *et al.*, 1993; 1994; 2002). After 24 h, the initial plating medium was replaced by feeding medium consisting of DMEM/Ham's F12 nutrient mixture (1:1) modified to contain 6 g/l glucose, 10% bovine serum, 2.5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma) and 10% UCHT1 conditioned medium. The cultures were kept in an incubator at 37°C with 100% humidity and an atmosphere of 5% $CO_2$ and were monitored routinely for appearance of transformation foci or morphological changes, which became evident after variable periods of time (7 - 8 months) and signaled the establishment of the RCSN cell line.

### Cell Culture Conditions

The RCSN-3 cell line grows in monolayers, with a doubling time of 52 h, a plating efficiency of 21% and a saturation density of 56,000 cells/cm$^2$ when kept in normal growth media composed of DME/HAM-F12 (1:1), 10% bovine serum, 2.5% fetal bovine serum, 40 mg/l gentamicine sulphate (Dagnino-Subiabre *et al.*, 2000; Paris *et al.*, 2001; Martinez-Alvarado *et al.*, 2005). The cultures were kept in an incubator at 37°C with 100% humidity, and the cells did growth very well both at an atmosphere of 5% or 10% $CO_2$.

### Immunofluorescence Analysis with Confocal Microscopy

Coverslips containing control RCSN-3 cells grown to 50% confluence were washed twice with Dulbecco's phosphate buffered saline pH 7.4 (PBS). They were then fixed for 30 min with methanol at -20ºC. The cells were rinsed twice with PBS and blocked with 1.5% bovine serum albumin diluted in PBS for 40 min. The blocking solution was then aspirated and the cells were rinsed with PBS. The coverslips were incubated with the primary antibody (rabbit anti-DAT, Sigma Chemical Co. St Louis, MO, USA; rabbit anti-NET and rabbit anti-5HTT, Chemicon International) at a dilution of 1:1,000; 1:1,000 and 1:500 in PBS, respectively, overnight. The primary antibody was aspirated, and the cells were washed three times with PBS. After washing, the cells were incubated with the secondary antibody (biotinylated anti-rabbit IgG [H+L], Vector Laboratories) diluted 1:250 in PBS for 1 h.

### Dopamine Determination by HPLC

Samples of RCSN-3 cell supernatant fractions were frozen on dry ice and stored at -80°C for less than 2 weeks. All frozen samples were filtered (0.45 μm), then injected directly via a 20 μl loop onto a microdialysis MD-150 x 1 micro bore analytical column using a mobile phase consisting of 1.7 mM 1-octanesulfonic acid, 25 μM EDTA, 10% acetonitrile, and 0.01% triethylamine in 75 mM phosphate buffer at pH 3.0 and a flow rate of 0.6 ml/min.

A guard cell (250 mV) and an analytical cell (-175 mV) were used with the Coulochem data analysis system to integrate peak areas and to so analyze concentrations of DA.

**Transmission Electron Microscopy**

RCSN-3 cells (not treated) were washed three times with PBS, pH 7.4 and fixed in 3% glutaraldehyde for 240 minutes, washed 3 times and post-fixed in 2% osmium tetraoxide for 60 minutes at room temperature. The cells were dehydrated in an ascending ethanol battery ranging from 20 to 100%, and were later placed in 100 % ethanol for 10 minutes and finally embedded in epon-812 resin. Ultra thin sections were made and impregnated with 4% uranyl acetate and Reynold's lead citrate. The sections were visualized in a Zeiss EM-900 transmission electron microscope at 50 kV, photographed, the negatives were scanned at 600 x 600 ppi resolution, and the images obtained were analyzed later in a PC compatible computer using customized software.

**Western Blotting**

Lysates of RCSN-3 cells were separated on SDS-polyacrylamide-gel electrophoresis (10% w/v for α-synuclein and 13% for DT-diaphorase). The separated proteins were then transferred electrophoretically to a 0.2 μm nitrocellulose membrane. After blocking with a solution of 0.5% skim milk containing 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.025% Tween 20 for at least 4 h, the membrane was incubated with polyclonal antibodies against human α/β-synuclein, polyclonal antibodies anti-NQO1 or anti-actin (H-90) (Santa Cruz Biotechnology, USA) overnight at room temperature in the same buffer. The membrane incubated with α-synuclein was washed and incubated with anti-goat alkaline phosphatase-linked antibodies while the membranes incubated with anti-NQO1 or anti actin were incubated with a secondary antibody, goat anti-rabbit IgG HRP conjugated or mouse anti-goat IgG HRP conjugated at dilution 1:10000 (Santa Cruz Biotechnology). The bands of α-synuclein (FIG. 5C) were detected using BCIP/NBT (Zimed laboratories Inc.) where lane 2 contained 4-fold more protein than lane 1. The bands of DT-diaphorase and actin were determined with Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and developed with Kodak film.

**RT-PCR**

The RT-reaction was performed using a Thermoscript RT-PCR system (Life Technologies) with Oligo $(dT)_{20}$ as primers. The amplification of ssDNA of antioxidant enzymes was performed by PCR reaction using the following primers: $D_1$, 5'-CGATGTGTTTGTGTGGTTTGGGTG-3' and 5'-TATGACCGATAAGGCTGGGGACAG-3'; $D_2$, 5'-TGTGTTCATCATCTGCTGGCTGC-3' and 5'-GCGTGTTCCCTGCTTTCCTATGTG-3'; $D_3$ 5'-AGCATCCTGAACCTCTGTGCCATC-3' and 5'-GGATTCAGGGCACTGTTCACATAGC-3'; $D_4$ 5'-CCTGATGTGTTGGACGCCTTTC-3' and 5'-AATCAGACGAACGAAAGCCGCC-3'; $D_5$ 5'-GCTGGGATTACAGAGGCAACTG-3' and 5'-GTGAGAGGTGAGATTTTG-3'; DMT1, 5'-CTGAGCGAAGATACCAGCG-3' and 5'-GGAGCCATCACTTGACCACAC-3'.

## RESULTS AND DISCUSSION

RCSN-3 is a cell line derived from adult rat substantia nigra which grows in mono layer. In proliferating conditions, the cells express enzymes involved in catecholamine function such as tyrosine hydroxylase, dopa decarboxylase and dopamine -hydroxylase (FIG. 1). These results clearly suggest the catecholaminergic lineage of RCSN-3 cells. Nevertheless, this alone does not confirm their capability to synthesize and secrete dopamine. To address this issue, we incubated the cells in fresh medium and collected the supernatant at 15 and 60 min. The presence of dopamine in the supernatant of cells incubated 15 min (65 ± 2 nM) and 60 min (73 ± 1 nM) suggested that RCSN-3 cells not only synthesize dopamine but also release it from the cell. The latter has also been confirmed with single cell amperometry studies using carbon fiber electrodes (data not shown). This feature of RCSN-3 cells is a clear advantage with respect to PC12 or SH-SY5Y cells, since it is expressed with little manipulation of the culture condition.

PC12 is a cell line that was originally derived from a pheochromocytoma (a tumor of the adrenal gland) that developed in an irradiated rat (Greene and Tischler, 1976). Under standard culture conditions, they exhibit properties similar to those of immature rat adrenal chromaffin cells. When grown in the presence of nerve growth factor (NGF), PC12

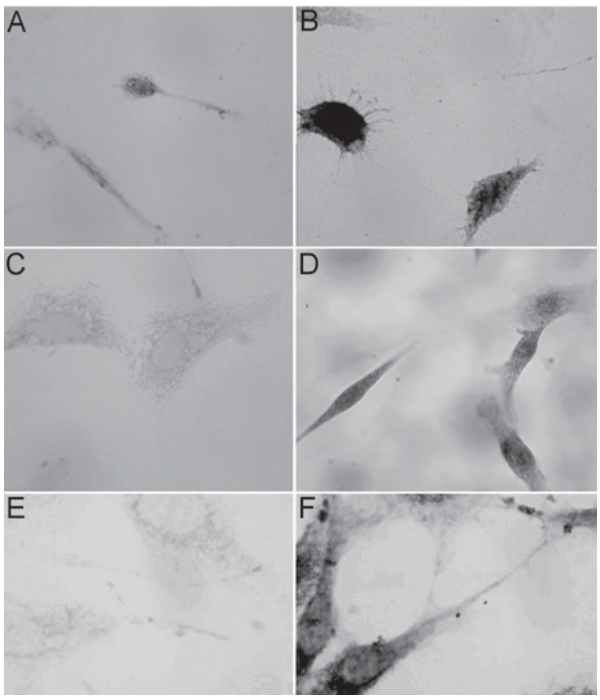


FIGURE 1 Immunostaining against tyrosine hydroxylase, dopa decarboxylase and dopamine-β-hydroxylase. RCSN-3 cells were incubated in the absence of antibodies (**A,C,E**) and in the presence of antibodies against tyrosine hydroxylase, dopa decarboxylase and dopamine-β-hydroxylase, as described under Material and Methods (**B, D** and **F**, respectively).

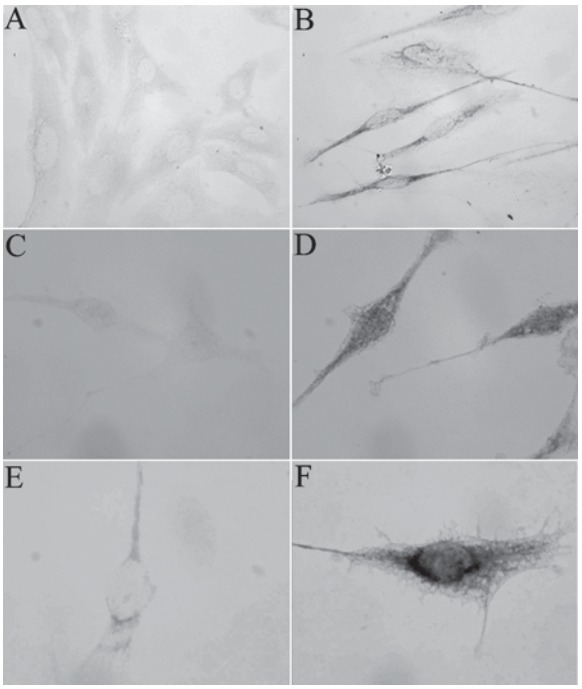


FIGURE 2 Immunostaining against dopamine, norepinephrine and serotonin transporters. RCSN-3 cells were incubated in the absence of antibodies (**A,C,E**) and in the presence of antibodies against dopamine, epinephrine and serotonin, as described under Material and Methods, (**B, D** and **F**, respectively).

cells extend neurites, they become electrically excitable, they respond more efficiently to exogenously applied acetylcholine, and they increase their expression of calcium channels and the biosynthesis of several neurotransmitters (Greene and Tischler, 1982). PC12 cells grown in the presence of NGF resemble sympathetic neurons, and they have been widely used as an experimental model for preclinical studies on neurodegeneration mechanisms in Parkinson's disease.

The other cell line mentioned above, SH-SY5Y, is a third generation neuroblastoma, cloned from SH-SY5. The original cell line was isolated from a metastatic bone tumor in 1970. The cells possess an abnormal chromosome 1, which exhibits an additional copy of the 1q segment, referred to as trisomy 1q. SH-SY5Y cells are known to be dopamine-β-hydroxylase active, cholinergic, glutamatergic and adenosinergic. The dividing cells can form clusters, which reminisce of their cancerous nature, but certain treatments such as retinoic acid and BDNF can force the cells to extend processes and differentiate. Therefore, to have a model cell line which produces and releases dopamine under proliferating conditions would constitute an important advantage, as *in vitro* differentiation protocols can be timely and costly. Both PC12 and SH-SY5Y cells have been used as model cell lines for studies on mechanisms of action of neurotoxins in Parkinson's disease but also in the study of other diseases and to evaluate the effect of neurotoxins (Copeland *et al.*, 2005; Massicotte *et al.*, 2005; Chiasson *et al.*, 2006; Duka and Sidhu, 2006; Langer *et al.*, 2006).

Both PC12 and SH-SY5Y cells exhibit dopamine- and norepinephrine-transport (Seitz *et al.*, 2000; Jiang *et al.*, 2004; Hashimoto *et al.*, 2005) while only PC12 cells have been reported to transport serotonin (King *et al.*, 1992). RCSN-3 cells present immunopositive staining against dopamine, norepinephrine and serotonin transporter (FIG. 2) which is in agreement with results obtained when the

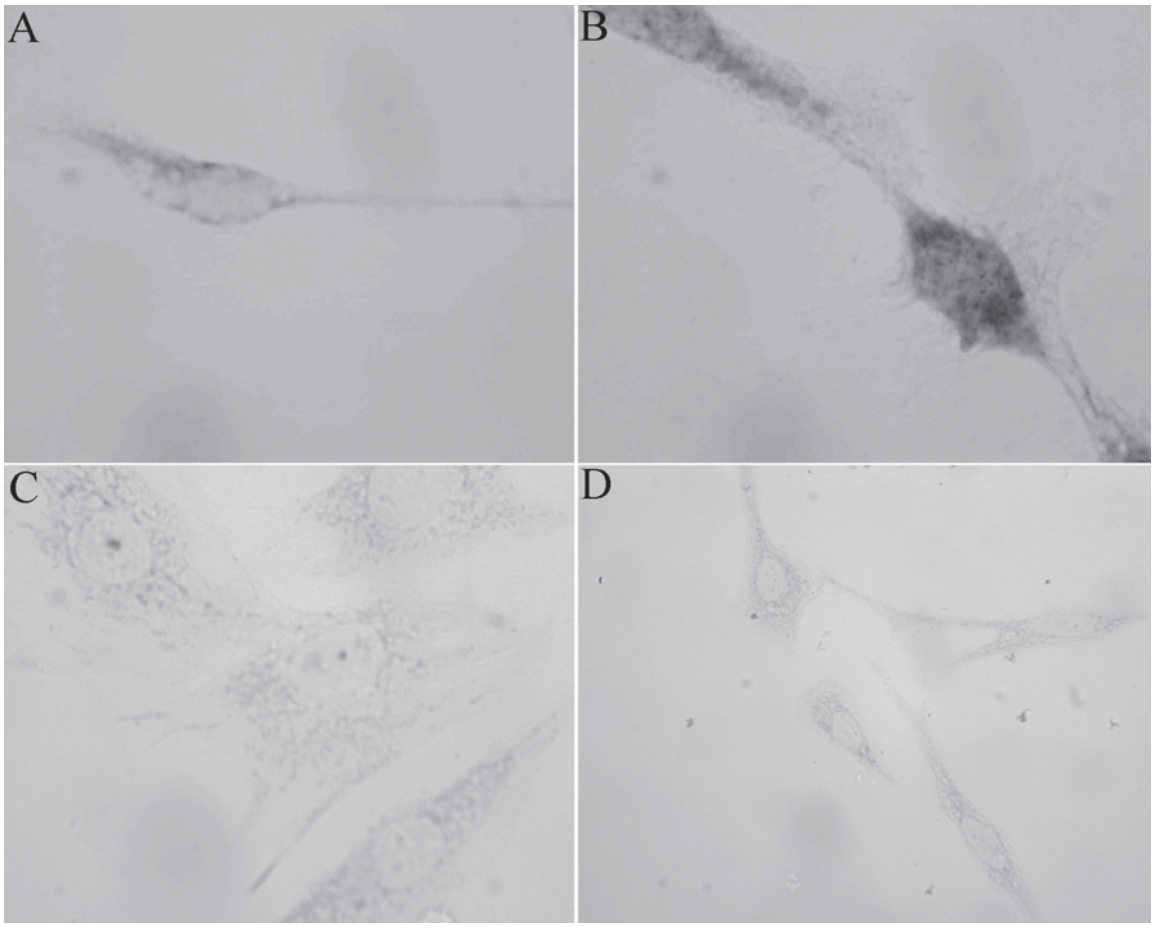


FIGURE 3 Immunostaining against monoamine oxidase-A and -B. RCSN-3 cells were incubated in the absence of antibodies (**A,C**) and in the presence of antibodies against monoamine oxidase A and B, as described under Material and Methods (**B** and **D**, respectively).

uptake of dopamine-FeCl complex was measured in the absence and presence of nomifensine, an inhibitor of dopamine transport; reboxetine, an inhibitor of the epinephrine transporter, and imipramine, an inhibitor of both norepinephrine- and serotonin-transporters (Paris *et al.*, 2005a). Nomifensine was found to also inhibit dopamine uptake (Martinez-Alvarado *et al.*, 2001) and dopamine-Cu complex (Paris *et al.*, 2001). Aminochrome uptake into RCSN-3 cells was inhibited with nomifensine (Arriagada *et al.*, 2004). The existence of dopamine-, norepinephrine- and serotonin- transporters confers an interesting specificity to this cell line, since most neurtoxins are transported into the cell via such membrane-bound proteins (Table I). In this regard, dopamine-Fe complex is toxic in RCSN-3 cells, but its toxicity is dependent on its uptake via monoaminergic transporters, as this toxicity is inhibited by nomifensine and reboxetine (Paris *et al.*, 2005a, 2005c, e, f). This complex was not toxic in CNh cells, derived from the cerebral cortex of a fetal mouse, which lacks monoaminergic transport. However, the complex was also toxic in the RCHT hypothalamus cell line, which only expresses norepinephrine transport (Paris *et al.*, 2005b,d).

RCSN-3 cells have immunopositive staining against monoamine oxidase (MAO)-A but not MAO-B (FIG. 3). These results are in agreement since MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is not toxic in RCSN-3 cells while $MPP^+$ (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium ion)is extremely toxic. The lack of neurotoxic effects of MPTP on RCSN-3 cells relies on the inability of these cells to metabolize MPTP to $MPP^+$, as determined by HPLC (Aguilar-Hernandez *et al.*, 2003). $MPP^+$ is also toxic both in PC12 and SH-SY5Y cells (Xu *et al.*, 2005; Lu *et al.*, 2006).

Formation of neuromelanin has been reported in

PC-12 cells after 14 days of treatment with L-dopa (Sulzer *et al.*, 2000). Overexpression of tyrosinase in SH-SY5Y cells resulted in formation of melanin pigments in cell soma. Interestingly, the expressed tyrosinase protein was initially distributed in the entire cytoplasm, and later accumulated to form catecholamine-positive granular structures 3 days after induction (Hasegawa *et al.*, 2003). However, RCSN-3 cells undergoing proliferation in the absence of extracellular L-dopa or dopamine, form melanin (Fuentes *et al.*, 2005; 2007). Granules with double membranes were observed in the cytoplasm,

Table I Dopamine and aminochrome uptake into RCSN-3 cells

| Conditions | % uptake | Reference |
|---|---|---|
| $^{3}$H-DA | 100 | Martinez-Alvarado *et al.* , 2001 |
| $^{3}$H-DA + nomifensine | 22 | |
| $^{64}CuSO_4$-DA | 100 | Paris *et al.* , 2001 |
| $^{64}CuSO_4$ | 15 | |
| $^{64}CuSO_4$-DA + nomifensine | 23 | |
| $^{64}CuSO_4$-DA + 2 mM DA | 37 | |
| $^{3}$H-AM | 100 | Arriagada *et al.* , 2004 |
| $^{3}$H-AM + nomifensine | 20 | |
| $^{59}FeCl_3$-DA | 100 | Paris *et al.* , 2005a |
| $^{59}FeCl_3$ | 88 | |
| $^{59}FeCl_3$-DA + 2 mM DA | 16 | |
| $^{59}FeCl_3$-DA + nomifensine | 57 | |
| $^{59}FeCl_3$-DA + imipramine | 37 | |
| $^{59}FeCl_3$-DA + reboxetine | 28 | |

Table II The expression of mRNA determined by RT-CPR

| Cell Determinant | Expression | Reference |
|---|---|---|
| CuZn-Superoxide dismutase | + | Paris *et al.* , 2001; Martinez-Alvarado et al., 2001 |
| Mn-Superoxide dismutase | + | |
| Glutathione peroxidase | + | |
| Glyceraldehyde 3-P-dehydrogenase | + | |
| Angiotensin receptor II | + | Dagnino-subiabre et al., 2000 |
| DT-diaphorase | + | Dagnino-subiabre et al., 2000; Hurtado-Guzman C et al., 2002 |
| Actin | + | Arriagada et al., 2004 |
| Divalent metal transporter 1 | + | |
| Dopamine receptor $D_1$ | + | |
| Dopamine receptor $D_2$ | - | |
| Dopamine receptor $D_3$ | - | |
| Dopamine receptor $D_4$ | - | |
| Dopamine receptor $D_5$ | + | |

which were phagocytosed by vacuoles (FIG. 4; Fuentes at al., 2007). Further, ferrous ion capture study, a cytochemical technique that identifies melanin, was positive in proliferating RCSN-3 cells, and the label was enhanced by differentiation (Arriagada *et al.*, 2002).

Other interesting features in RCSN-3 cells are (i) the expression of α-synuclein, determined with Western blot (FIG. 5A); (ii) expression of DT-diaphorase, determined with Western blot (FIG. 5B) and RT-PCR (Table II); (iii) expression of mRNA for gluthatione peroxidase, CuZn-superoxide dismutase, catalase, actin, glyceraldehyde 3-phosphate dehydrogenase, dopamine receptor $D_1$ and $D_5$, and the transporter for divalent metals (DMT1).

Table III The effects of neurotoxins on RCSN-3 cells

| Conditions | % Cell Death | Reference |
|---|---|---|
| Control | 3.7 | Paris *et al.*, 2001 |
| $CuSO_4$ | 6.3 | |
| DA | 1.3 | |
| dicoumarol | 1.9 | |
| $CuSO_4$ + dicoumarol | 34 | |
| DA + dicoumarol | 1.4 | |
| $CuSO_4$-DA | 18 | |
| $CuSO_4$-DA + dicoumarol | 95 | |
| Control | 3.2 | Aguilar Hernandez *et al.*, 2003 |
| MPTP | 3.9 | |
| $MPP^+$ | 6.6 | |
| dicoumarol | 10.5 | |
| MPTP + dicumarol | 9.8 | |
| $MPP^+$ + dicoumarol | 27 | |
| Control | 1.2 | Martinez-Alvarado *et al.*, 2001 |
| Salsolinol | 23 | |
| dicoumarol | 9.3 | |
| Salsolinol + dicoumarol | 56 | |
| Control | 2.6 | Arriagada *et al.*, 2004 |
| DA | 5.8 | |
| $Mn^{3+}$ | 4.4 | |
| dicoumarol | 1.8 | |
| AM | 8.3 | |
| AM + dicoumarol | 56 | |
| Control | 4.1 | Paris *et al.*, 2005 |
| DA | 8.4 | |
| $FeCl_3$ | 7.8 | |
| dicoumarol | 8.3 | |
| $FeCl_3$-DA | 15 | |
| $FeCl_3$-dicoumarol | 44 | |
| $FeCl_3$-dicoumarol + nomifensine | 20.2 | |
| $FeCl_3$-dicoumarol + reboxetine | 16.7 | |
| Control | 4.8 | Fuentes *et al.*, 2007 |
| Reserpine | 9.9 | |
| DA | 14 | |
| dicoumarol | 8.4 | |
| Reserpine + dopamine | 19.8 | |
| Reserpine + dopamine + dicoumarol | 47.5 | |

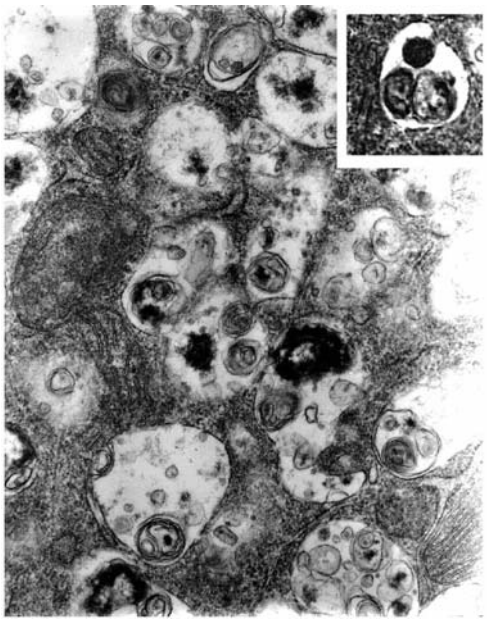

FIGURE 4 Transmission electron microscopy on RCSN-3 cells. Transmission electron microscopy of RCSN-3 cells reveals the presence of melanin. A phagocytic vacuole under endocytosis of a melanin body is shown in the insert (x 30,000).

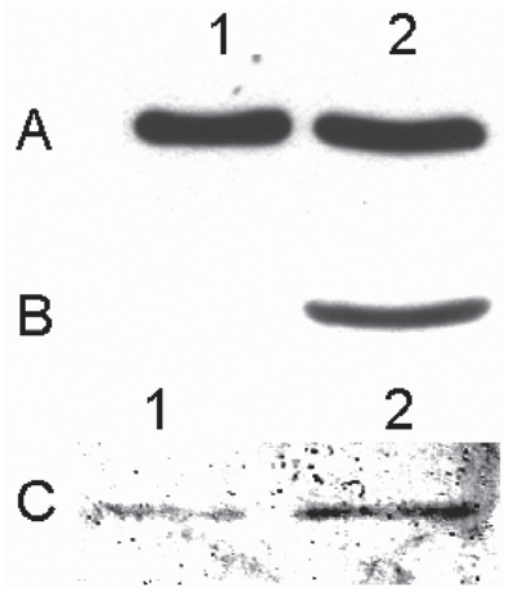


FIGURE 5 Determination of expression of DT-diaphorase and α-synuclein in RCSN-3 cells. The immunostaining against DT-diaphorase (**A**) and actin (**B**) in lane 1 of pure DT-diaphorase and lane 2 protein extract from RCSN-3 cells and the immunostaining against α-synuclein (**C**) lane 1 and 2 protein extract from RCSN-3 were determined as described under Material and Methods.

However, expression of $D_5$ dopamine receptors was only detected when the cells were treated with dicoumarol while $D_2$, $D_3$ and $D_4$ were not detected (Table II).

RCSN-3 cells have been very useful in study of the specific neurotoxic actions of neurotoxins which are dependent on uptake via dopamine, norepinephrine or serotonin transport. Cu-dopamine or Fe-dopamine complex are only cytotoxic in cells expressing monoaminergic transporters (Paris *et al.*, 2001; 2005a,b) (Table III). The neurotoxic effects of $MPP^+$ and aminochrome are also dependent on specific transport mechanisms in RCSN-3 cells (Aguilar-Hernandez *et al.*, 2003; Arriagada *et al.*, 2004). Aminochrome toxicity has been proposed as a pre-clinical experimental model to study cell death in dopaminergic neurons (Paris *et al.*, 2007a,b), and RCNS-3 cells are very suitable for this purpose since (i) aminochrome is transported into the cells; (ii) aminochrome can also be formed inside the cells by inhibiting VMAT-2 with reserpine (Fuentes *et al.*, 2007); (iii) the cells produce neuromelanin under proliferating conditions; (iv) the cells express α-synuclein; and (v) the cells are very easy to grow and do not require costly and time consuming differentiation procedures to express relevant catecholaminergic features, unlike the other aforementioned PC12 and SH-SY5Y cell lines.

## CONCLUSIONS

RCSN-3 cells have features similar to PC12 or SH-SY5Y cells, which makes this cell line a suitable preclinical *in vitro* model to study the neurodegenerative process of neuromelanin containing dopaminergic neurons in Parkinson's disease. The most relevant features of RCSN-3 cells are their ability to express (i) enzymes required for dopamine synthesis and dopamine release; (ii) dopamine-, norepinephrine- and serotonin-transporters; (iii) MAO-A; and (iv) VMAT-2. The cells also (v) form melanin under proliferating conditions and (vi) express DT-diaphorase, which prevents neurotoxic effects of aminochrome during dopamine oxidation (Lozano-Gonzalez *et al.*, 2005). However, the most important advantages of RCSN-3 over PC12 or SH-SY5Y cells are that 1) RCSN-3 cell do not require expensive and time consuming differentiation procedures to induce these dopaminergic features, and 2) that RCSN-3 cells produce neuromelanin under proliferating conditions, which is an important feature, since the dopaminergic neurons which degenerate in PD are melanin-containing neurons.

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