Development/Plasticity/Repair

The Generation of Dopaminergic Neurons by Human Neural Stem Cells Is Enhanced by Bcl-X_L, Both *In Vitro* and *In Vivo*

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Progress in stem cell biology research is enhancing our ability to generate specific neuron types for basic and applied studies and to design new treatments for neurodegenerative diseases. In the case of Parkinson's disease (PD), alternative human dopaminergic (DAergic) neurons other than primary fetal tissue do not yet exist. One possible source could be human neural stem cells (hNSCs), although the yield in DAergic neurons and their survival are very limited.

In this study, we found that Bcl-X_L enhances (one-to-two orders of magnitude) the capacity for spontaneous dopaminergic differentiation of hNSCs, which then exceeds that of cultured human ventral mesencephalic tissue. Bcl-X_L also enhanced total neuron generation by hNSCs, but to a lower extent. Neuronal phenotypes other than DA were not affected by Bcl-X_L, indicating an exquisitely specific effect on DAergic neurons. *In vivo*, grafts of Bcl-X_L-overexpressing hNSCs do generate surviving human TH^+ neurons in the adult rat 6-OH-dopamine lesioned striatum, something never seen when naive hNSCs were transplanted. Most of the data obtained here in terms of the effects of Bcl-X_L are consistent with an enhanced survival type of mechanism and not supportive of induction, specification, or proliferation of DAergic precursors.

From this *in vitro* and *in vivo* evidence, we conclude that enhancing Bcl-X_L expression is important to obtain human DAergic neurons from hNSCs. These findings may facilitate the development of drug-screening and cell-replacement activities to discover new therapeutic strategies for PD.

Key words: **human neural stem cells; tyrosine hydroxylase; dopaminergic; Bcl-X_L; Parkinson's disease; SOD; GDNF; BDNF**

Introduction

The most common form of human dopaminergic (hDAergic) neuron procurement for Parkinson's disease (PD) research is the use of embryonic ventral mesencephalic (VM) tissue. Practical, biological (cell survival and function), clinical, and in some countries, ethical and legal limitations have stimulated a research effort to find alternative sources to generate hDAergic neurons in a predictable manner and in sufficient numbers (Lindvall et al., 2004). hDAergic neurons undergo continuous oxidative stress imposed by their catecholaminergic neurotransmitter phenotype (Haavik and Toska, 1998; Olanow and Tatton, 1999; Stokes et al., 1999; Barzilai et al., 2001; Blum et al., 2001). TH activity, by itself, generates hydrogen peroxide, and this gets exacerbated further by the auto-oxidation of both L-3,4-dihydroxyphenylalanine (L-DOPA) and DA, contributing many other free radicals. Grafts of VM tissue survive to some extent, but do so at a low rate *in vivo* (Lindvall et al., 2004). In addition, VM cells undergo cell death soon after cell-suspension preparation (Emgard et al., 2003). This can be compensated by grafting cells from multiple embryos, but, in any case, the low survival rate of VM cells limits their application to clinical research (Björklund et al., 2003; Isacson, 2003; Lindvall et al., 2004). In alternative settings, the poor survival of TH^+ neurons or teratoma formation precludes the use of neural stem cell (NSC) or embryonic stem (ES) cell derivatives, respectively (Svendsen et al., 1997; Arenas, 2002; Björklund et al., 2003; Isacson, 2003; Lindvall et al., 2004).

The purpose of the present *in vitro* and *in vivo* study has been, first, to discover genes that could help human NSCs (hNSCs) to cope with a dopaminergic phenotype (using for this purpose the transfection of human tyrosine hydroxylase), and, second and more important, to discover ways to increase the efficiency of generation of true hDAergic neurons from hNSCs, using different neuroprotective strategies aimed at enhancing cell survival. Thus, we have studied the capacity of neurotrophic [BDNF and glial cell line-derived neurotrophic factor (GDNF)], antioxidant (Cu plus Zn superoxide dismutase, SOD1cit), and anti-apoptotic (Bcl-X_L) proteins to enhance transgenic TH expression by

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hNSCs. Even when all of these factors enhanced the survival of TH^+ cells, Bcl-X_L was found to be superior in performance. Next, in the absence of transfected TH, Bcl-X_L-overexpressing clones of hNSCs showed a marked increase in their capacity for spontaneous generation of hDAergic neurons. These results were confirmed using nonimmortalized human neurosphere cultures. Furthermore, *in vivo*, Bcl-X_L-overexpressing hNSCs do generate TH^+ human neurons (against a background of non-TH^+ neuron production by control cells).

The subjacent interest of this work was to show that Bcl-X_L can help to ameliorate the limitations of hNSCs for hDAergic neuron generation, and that the use of this genetic manipulation may be helpful to understand how to overcome these hurdles.

Materials and Methods

Cell culture

Human neurospheres. Forebrain neurosphere cells derived from two human embryos of 6 and 9.5 weeks gestational age (strains 6 week hFBr and 9.5 week hFBr) were kindly provided by Dr. Lars Wahlberg (Karolinska Institute, Stockholm, Sweden) and cultured as floating aggregates in the presence of 20 ng/ml epidermal growth factor (EGF), 20 ng/ml fibroblast growth factor (FGF)-2, and 1 ng/ml leukemia inhibitory factor (LIF) (Carpenter et al., 1999). Permission to use these materials for the present research was granted by local ethical committees and the European Union. hNS1 (formerly called HNSC.100, a model cell line of hNSCs) is a human embryonic forebrain-derived, multipotent, clonal cell line of neural stem cells. hNS1 cell culture conditions are based on a chemically defined HSC medium supplemented with 20 ng/ml each of EGF and FGF-2 (Villa et al., 2000, 2004). Human embryonic kidney 293T (HEK293T) cells (American Type Culture Collection, Manassas, VA), were used as a control non-neural cell line of human origin and cultured at 37°C in complete DMEM, 10% fetal bovine serum (FBS; Invitrogen, San Diego, CA), 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. Cells from the rat hippocampal progenitor stem cell line HiB5 were cultured as HEK293T cells, but at their permissive temperature of 33°C (Frederiksen et al., 1988).

All cell cultures were proliferated at 5% CO_2 and normal atmospheric oxygen levels (resulting in 20% O_2). When indicated, some cultures were differentiated at 5% O_2 and 5% CO_2 in a dual control O_2–CO_2 incubator (Forma).

Expression vectors and transfection. Expression vectors used in the present study were all derived from pIRES1hyg (Clontech, Cambridge, UK). p(LacZ)IREShyg:LacZ cDNA was excised as a *Bam*HI–*Not*I insert from pcDNA3.1/Myc-His/lacZ (Invitrogen) and subcloned into *Bam*HI–*Not*I of pIRES1hyg.

p(rhGFP)IREShyg:*Renilla raniformis* green fluorescent protein (GFP) cDNA was excised as an *Eco*RI–*Not*I insert from pFB-rhGFP (Stratagene, La Jolla, CA) and subcloned into pCR2.1 (Invitrogen). From this one, a *Bam*HI–*Not*I fragment was excised and subcloned into pIRES1hyg.

p(hTH)IRES1hyg was generated by subcloning human tyrosine hydroxylase I as a *Bam*HI insert excised from pMLVTH (Lundberg et al., 1996) into the *Bam*HI site of pIRES1hyg.

p(BDNF)IREShyg, *p(GDNF)IREShyg,* and *p(Bcl-X_L)IREShyg* have been described previously (Villa et al., 2000).

p(SOD1)IREShyg was constructed after subcloning the SOD1cit cDNA into the *Bam*HI site of pIRES1hyg. Human cytosolic (Cu plus Zn) superoxide dismutase (SOD) 1 (GenBank accession number X02317) was cloned by reverse transcription (RT)-PCR using human total RNA (Clontech) and the following primers: hSOD1c-sense, GCG TGG CCT AGC GAG TTA T; hSOD1c-antisense, GGG CCT CAG ACT ACA TCC AA. Amplified DNA was cloned into pST1-Blue using the Perfectly Blunt Cloning Kit (Novagen, Madrid, Spain), and sequenced.

Vectors were transfected using Lipofectamine reagents (Invitrogen), following recommendations of the supplier. When used, drug selection of stable transfectants was performed with 50–150 μg/ml hygromycin B (hyg; Calbiochem, La Jolla, CA).

Immunocytochemistry and immunoblotting (Western blot)

At the specified time points, cultures were rinsed with PBS and fixed for 10 min in freshly prepared 2 or 4% PFA in 0.1 M phosphate buffer, for β-galactosidase (β-gal) activity development [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) stain], or stored in cryoprotective solution until used for immunocytochemistry (ICC). For staining, cultures were rinsed and blocked for 1 hr in 5% normal horse serum. Cultures were then incubated overnight at room temperature with monoclonal antibodies (mAbs) against TH (1:1000; clone TH-2; Sigma, St. Louis, MO), β-III-tubulin (β-III-tub) (1:1000; Sigma), or DA (1:400; clone M32192; Fitzgerald, Concord, MA), followed by 1 hr of incubation with biotinylated horse anti-mouse (1:200; BA2001; Vector Laboratories, Burlingame, CA), and developed by incubation with ABC reagent (Vector Laboratories) and DAB reaction. Immunofluorescent stains used FITC (horse anti-mouse; 1:200; Vector Laboratories) or Texas Red (horse anti-mouse; 1:200; Vector Laboratories)-conjugated antibodies. Cell nuclei were counterstained with Hoechst 33258 at 0.2 μg/ml. In some cases, double staining for β-III-tub or DA and TH was performed using anti-TH rabbit polyclonal antibody (pAb) from Chemicon (1:1000) and β-III-tub or DA as indicated above. For Western blot analyses, 30 μg of protein from dividing or differentiated cultures was assayed. Samples were electrophoresed and transferred onto nitrocellulose membranes overnight. Primary antibodies were anti-TH mAb (1:2000; Sigma), rabbit anti-Bcl-X_L pAb (1:1000; Transduction Laboratories), anti-Nurr1 mAb (1:1000; Transduction Laboratories), anti-β-III-tubulin mAb (1:1000; Sigma), anti-human neuron-specific enolase (hNSE) mAb (1:300; Chemicon), anti-β-actin mAb (1:5000; Sigma), and rabbit anti-β-F1-ATPase pAb [1:10,000; a gift from Prof. J. M. Cuezva, Center of Molecular Biology Severo Ochoa (CBMSO)] (Arco et al., 2002). The blots were developed using horse anti-mouse antibodies conjugated to peroxidase (HAM-PO) or goat anti-rabbit antibodies conjugated to peroxidase (GAR-PO) [HAM-PO: 1:5000 (Vector Laboratories); GAR-PO: 1:10,000 (Nordic Immune)] and developed using the ECL system (Amersham Biosciences, Arlington Heights, IL).

For double ICC for β-III-tub and serotonin, choline acetyltransferase (ChAT), GABA, glutamate (Glu), TH, nestin, doublecortin (Dcx), and microtubule-associated protein 2 (Map-2)-cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (for GABA and Glu, 0.1% gluteraldehyde was included in the fixative). Cultures were rinsed, blocked, and incubated overnight at 4°C with anti-ChAT mAbs or pAbs from Chemicon (1:500), rabbit anti-serotonin (1:1000; Sigma) or anti-GABA (1:1000; Sigma), anti-Glu (1:2000; Sigma), anti-TH mAb (1:1000; Sigma), rabbit anti-nestin (1:500; generous gift from Prof. Urban Lendahl, Karolinska Institute), or goat anti-Dcx (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), alone or in combination with mouse anti-β-III-tub (as described above). For double ICC for β-III-tub and Map-2, we used a rabbit anti-β-III-tub (1:1000; a generous gift from Dr. F. J. Díaz-Nido and F. Wandosell, CBMSO) combined with a mouse anti-Map-2 (1:1000; clone HM-2; Sigma). In some cases, double ICC for TH and GABA was performed as described above. For detection of primary antibodies, fluorescence-labeled Texas Red (horse anti-mouse; 1:200; Vector Laboratories) or Alexa 488 (goat anti-rabbit; 1:400; Jackson ImmunoResearch, West Grove, PA) were used according to the specifications of the manufacturer.

Differentiation of hNSCs into DAergic neurons

hNS1 cells consistently generate a small number of DAergic neurons after differentiation under standard conditions in culture, a potential that has been exploited to study the effects of Bcl-X_L. Cells are differentiated on poly-L-lysine (10 μg/ml; Sigma)-coated plastic by removal of growth factors (EGF, FGF-2), and in some cases, by the addition of 0.5% heat-inactivated fetal bovine serum, for 12 d or the times indicated.

Human neurosphere cells were differentiated on poly-L-lysine plus laminin (10 and 2 μg/ml laminin, respectively)-coated plastic by removal of growth factors (EGF, basic FGF, LIF) and in the presence of a dopaminergic-inductive mixture (Stull and Iacovitti, 2001), containing 100 nM phorbol 12-myristate 13-acetate (TPA; Sigma) and 100 ng/ml acidic FGF (Peprotech, Rocky Hill, NJ). Neurosphere cells, and some

hNS1 cultures, when indicated, were differentiated under low oxygen conditions (5%) in a dual control O_2–CO_2 incubator (Forma).

DA determination by reverse-phase HPLC coupled to electrochemical detection

hNS1 cells or Bcl-X_L-overexpressing hNS1 cells (clone 5) were differentiated in the presence of 0.5% of fetal bovine serum for 11 d. Cells were lysed in 200 μl of 0.1N perchloric acid containing 0.8 mg/ml glutathione by freeze-thawing, and the supernatants were collected for analysis of intracellular DA levels. Samples were frozen at −80°C and analyzed by HPLC coupled to electrochemical detection (HPLC-EC). For assays of DA release, parallel cultures were treated with 60 mM KCl at 37°C for 20 min in 300 μl of HBSS containing Ca^{2+} and 0.8 mg/ml glutathione. The extracellular medium was collected, filtered through a 0.2 μm filter, and frozen at −80°C until HPLC-EC analysis (Lotharius et al., 2002). Assays were conducted in quadruplicate, and experiments were repeated three times.

Bcl-X_L overexpression in hNS1 and human neurospheres

hNS1 cells were transfected with *p(Bcl-X_L)IREShyg* and selected with hygromycin (100 μg/ml), to generate a polyclonal Bcl-X_L-overexpressing line. From this line, subclones were isolated by limiting dilution. In the experiment described in Figure 7, human neurospheres were transfected with *p(Bcl-X_L)IREShyg* at day 0, and differentiation was started at day 2. Cells were allowed to differentiate for 5 d under the DA-inductive conditions described above.

Image and data analyses on cell cultures

Analyses and photography of fluorescent or immunostained cultures were done in inverted Zeiss (Oberkochen, Germany) Axiovert 135 or Leica (Nussloch, Germany) DM IRB (equipped with a digital camera Leica DC100) microscopes. In some experiments, digitized images were captured using Leica IM500 software. Image analyses were performed using NIH Image software. Statistical tests were run using Prophet Software (NIH).

In vivo *transplantation*

Adult female (250–300 gm) Wistar rats, housed and treated according to the guidelines of the European Community (86/609/EEC), were anesthetized with a mix of ketamine and dontor and 6-OHDA-lesioned in the right median forebrain bundle (MFB) at the following coordinates (in mm): anteroposterior (AP), −3.7; mediolateral (ML), −1.6; dorsoventral (DV) (skull), −8.8, with the tooth bar set at −3.3. Fourteen days later, the rats were grafted into the denervated striatum with cellular suspensions of proliferating control hNS1 cells ($n = 6$) or proliferating Bcl-X_L clone 5 ($n = 6$) at the following coordinates: AP, +1; lateral, −3; DV (from dura), −4.5, with the incisor bar set at −2.3. The cells were bromodeoxyuridine (BrdU)-labeled *in vitro*, before grafting (1 μM for 3 d), and 400,000 cells were implanted as a single deposit (cell density of 150–200,000 cells/μl). The animals were immunosuppresed with cyclosporine A (Neoral; Novartis, Summit, NJ: 100 μg/ml in drinking water, starting 48 hr before grafting). Four weeks after grafting, rats were intracardially perfused with freshly prepared, buffered 4% paraformaldehyde. Brains were postfixed for 12 hr, dehydrated in 30% sucrose, and sectioned (30 μm, freezing microtome) for free-floating immunohistochemistry analyses. Serial sections were processed for TH using mouse monoclonal antibodies (clone TH-2; 1:2000; Sigma) or rabbit polyclonal anti-TH antibodies (AB152; 1:1000; Chemicon). Monoclonal anti-human nuclei (MAB 1281; 1:500; Chemicon) or anti-BrdU (clone BU33; 1:1000; Sigma) were used to detect all grafted and surviving human cells. Migrating neuroblasts were detected using anti-Dcx antibodies (C-18; 1:1000; Santa Cruz Biotechnology), and human neurons were specifically stained using an anti-hNSE antibody (MAB324; 1:2000; Chemicon). For the unambiguous detection of human TH-expressing neurons, double immunohistochemistry was performed combining TH antibodies with those for human nuclei (h-Nuc) or hNSE. Secondary antibodies were biotinylated horse anti-mouse (1:200; BA2001; Vector Laboratories) [followed by ABC (Vector Laboratories) and Ni-DAB reaction]. For immunofluorescence (IF), secondary antibodies were Texas Red (horse anti-mouse; 1:100; Vector Laboratories) and Alexa 488 (goat anti-rabbit; 1:400; Molecular Probes, Eugene, OR).

Histological analyses

Immunostained sections were viewed and analyzed on a Leica DM IRB inverted fluorescence microscope equipped with an X-Y-Z motorized stage operated by Olympus (Ballerup, Denmark) CAST-GRID software for stereological, unbiased morphometric analyses. Graft survival (on the basis of detection of h-Nuc-stained cells) was analyzed by two methods: (1) anteroposterior extent of grafted cells present in coronal sections, and (2) actual graft volume measurement using stereological methods. TH^+ neurons were all counted in every graft-containing section in every animal, and the total number of TH^+ neuron was estimated on the basis of sectioning protocol. In animals grafted with naive hNS1 cells, no TH^+ neurons were detected.

For double IF analyses, sections were analyzed on a Microradiance Confocal microscope (Bio-Rad, Hercules, CA) in the *z*-axis for unambiguous assignment of stained human nuclei to a TH^+ cytoplasm. Overlapped and single Z-sections are shown in Figure 8*F*. In other cases, colocalization of cytoplasmic stains was performed on 1-μm-thick confocal sections (Fig. 8*G*).

Results

Transgenic human TH overexpression

In a previous study, we have shown that when TH is overexpressed in hNSCs, it induces an upregulation of p53, a decrease in mitotic activity, and profound nuclear damage (Liste et al., 2004) (Fig. 1, see table). As a result, stable and highly expressing TH^+ hNSC lines could not be generated. As an additional extension of these data, a colony-forming assay was conducted in the present study. Cells were transfected with bicistronic vectors (gene of interest: IRES-hyg^R), selected for 1 month with 100 μg/ml hygromycin, and then fixed and stained to determine the frequency of clones expressing the gene of interest (Fig. 1*A*). As shown in Figure 1, *B* and *C*, no TH^+ clones were generated from hNS1 cells (but $LacZ^+$ clones were generated at the expected rate) (Fig. 1*C*,*D*). HiB5 and HEK293T cells yielded a high percentage of TH^+ clones. It is important to note at this point that hNS1 cells express both GTP-cyclohydrolase I (GTP-CH1, responsible for BH_4 synthesis) and aromatic amino acid decarboxylase (AADC; required for the conversion of L-DOPA to DA), thus allowing for TH being fully active and rendering the cells with a full catecholamine synthesis pathway (Haavik and Toska, 1998; Liste et al., 2004).

Analyses of helper genes able to enhance the capacity of established hNSC lines for transgenic TH expression

To identify factors that could be neuroprotective in this context, TH was coexpressed with other candidate neuroprotective factors such as BDNF, GDNF, SOD1cit, or Bcl-X_L (or with an empty vector). Cultures were fixed and immunostained 10 d after cotransfection (Fig. 2*A*). As hypothesized, BDNF, GDNF, and SOD1cit coexpression resulted in an amelioration of a net twofold increase in TH^+ cells. The effects of Bcl-X_L were much more remarkable, however, resulting in a net increase of approximately fourfold in the total number of cells obtained ($n = 12$; see legend to Fig. 2 for statistics). In control experiments, cotransfection of LacZ and Bcl-X_L resulted in no increase in the number of β-gal^+ cells obtained (actually a reduction to 90%) compared with LacZ plus the empty vector ($n = 12$; data not shown), ruling out unspecific Bcl-X_L effects related to the transfection procedure itself.

We therefore became interested in examining whether a pharmacological anti-apoptotic block could also have a similar effect, and we performed parallel experiments using the broad-range, pan-caspase inhibitor z-VAD-fmk (50 μM, or DMSO-only as a control). Z-VAD-fmk treatment resulted in a 24.04 ± 6.55-fold increase in the number of TH^+ cells compared with the DMSO control ($n = 12$), consistent with the presence of a caspase-

TH-induced neurotoxicity in hNSCs.

	LacZ positive	TH positive
Nuclear damage	6 ± 2	27 ± 2 (p<0.001)
P53 expression	4 ± 3	33 ± 8 (p<0.05)
BrdU incorporation	57 ± 6	24 ± 9 (p<0.05)

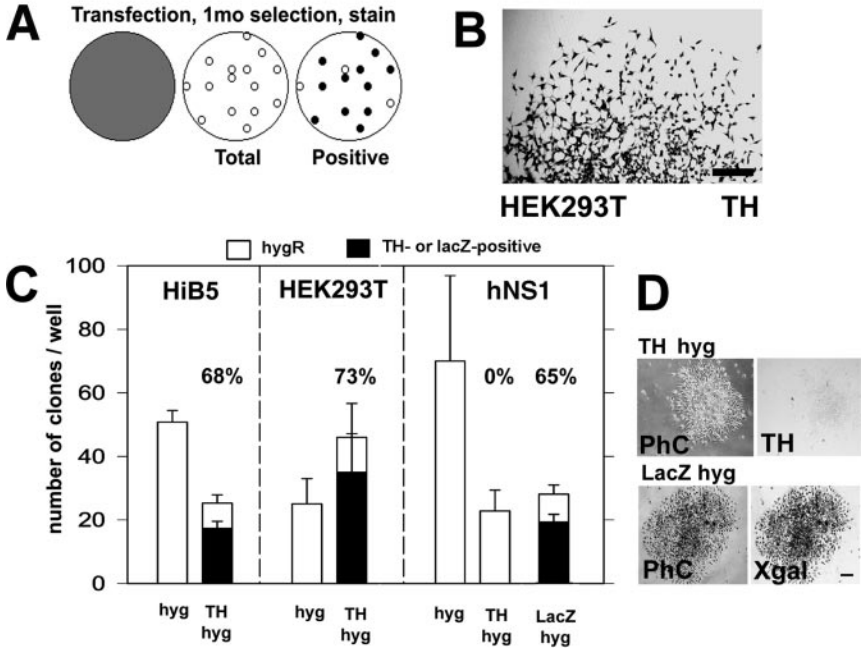

Figure 1. Transgenic TH overexpression: colony formation assays. Top, Summary of the main findings related to cytotoxicity and genotoxicity induced by the overexpression of TH in hNSCs [adapted from Liste et al. (2004)]. *A,* Experimental design: the cells are transfected and subjected to hygromycin selection for 1 month. Afterward, the cultures are fixed and stained for β-galactosidase activity or immunostained for TH. Total and positive clones were subsequently quantified. *B,* An example of an immunostained TH^+ clone derived from HEK293T cells. Scale bar, 100 μm. *C,* Quantifications of the total number of clones (open bars) and those among them positive for β-gal or TH (closed bars, percentages are given above bars), using as substrate the indicated cell lines. Hyg, Empty vector (CMV-IRES-hyg, also coding for hyg^R); TH hyg, CMV-TH-IRES-hyg vector; LacZ hyg, CMV-LacZ-IRES-hyg. *D,* An example of negative (for TH) or positive (for β-gal) clones photographed under phase-contrast (PhC) or bright-field (TH or X-gal panels). Scale bar, 100 μm.

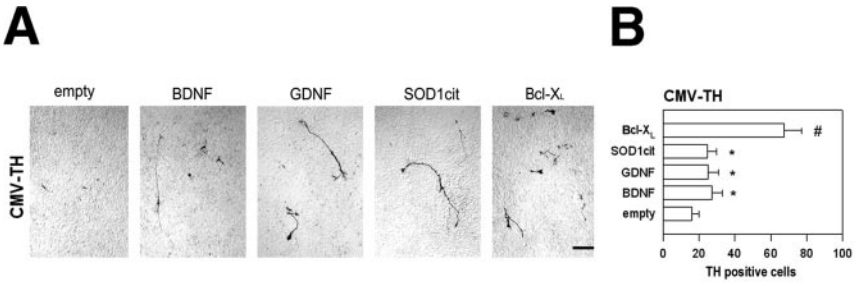

Figure 2. Transgenic TH overexpression: coexpression of TH and helper genes enhances the number of TH^+ hNS1 cells. *A,* Microphotographs illustrating the increased number of TH^+ cells after cotransfection of a TH-coding vector with other vectors coding for BDNF, GDNF, SOD1cit, Bcl-X_L, or the empty vector, as a control. Scale bar, 100 μm. *B,* Quantification of the number of TH^+ cells in the cotransfection experiments shown in *A* ($n = 12$; four independent samples in each of three separate experimental rounds); $^{\#}p < 0.0001$, Bcl-X_L vs all other groups; $^{*}p < 0.005$, different from the empty vector group (Student's *t* test).

mediated cell-death process operating in the present experimental setting.

Finally, we explored a possible interaction between TH and Bcl-X_L coexpression and culture in high (20%) or low (5%) oxygen tension. Low oxygen per se did not increase the rate of TH^+ cells obtained by transfection (96 ± 4.2% of those at high O_2; $n = 6$) or modify Bcl-X_L effects (fold enhancement of TH^+ cell yield: 2.63 ± 0.47 and 3.01 ± 0.53 at high and low oxygen, respectively; $n = 6$; nonsignificant difference).

Given the remarkable effects of Bcl-X_L on TH expression observed in cotransfection experiments, the rest of the present study focused on this particular antiapoptotic protein. To this end, we generated stable cell lines coexpressing both TH and Bcl-X_L. In three independent experimental rounds (each one performed on quadruplicate cultures), vigorously growing hyg^R cell lines were isolated, expressing large amounts of Bcl-X_L, but none of them expressed detectable levels of TH protein, either by ICC or by Western blot analysis (data not shown). These negative data are consistent with the notion that high-level transgenic TH expression is toxic and precludes cell division (Liste et al., 2004). As mentioned above, in the transient (10 d) transfection experiments shown in Figure 2, couples or colonies of TH-positive cells were not observed either after cotransfection of Bcl-X_L or under low-oxygen conditions. Considering that the cell cycle of hNS1 cells is ~40 hr (Villa et al., 2000), finding small clones of positive cells would have been expected for a nontoxic transfected protein.

Bcl-X_L action on spontaneous hDAergic neuron generation by established hNSCs

The capacity for TH^+ neuron generation by hNSCs (hNS1 cells), although of low magnitude (<0.1% of total cells; see control cell data in Figs. 3, 5), provides a baseline over which to test the putative enhancing effects of Bcl-X_L on dopaminergic differentiation. It is worth mentioning that both proliferating and differentiated hNS1 cells express Nurr1 (Figs. 3, 4), indicating that the cells should be able to differentiate along a dopaminergic lineage. hNS1 cells also express GTP-CH1 and AADC (Liste et al., 2004) and are dopamine β-hydroxylase (DBH)-negative (data not shown).

Stably Bcl-X_L-overexpressing hNS1 cells were studied under conditions of high or low oxygen and the presence or absence of FBS. A clear-cut TH signal was only detected in the case of Bcl-X_L-overexpressing cells differentiated in low oxygen and in the presence of 0.5% FBS (Fig. 3*A*,*C*, bottom blot). Quantitative single-cell analysis revealed a more than one order of magnitude increase in the number of TH^+ neurons generated (15-fold) (Fig. 3*D*,*E*). The absence of differences between naive and hyg^R hNS1 cells rules out an effect caused by the coexpression of hyg^R gene or drug selection. Finally, in the absence of FBS, there was no detectable TH expression under any of the

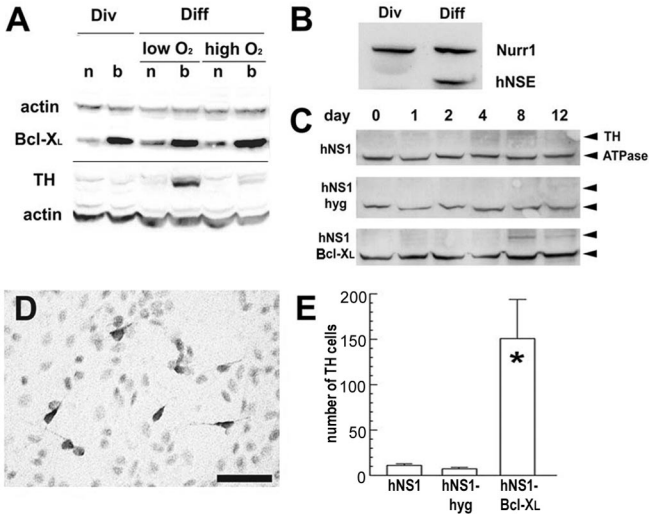

Figure 3. Spontaneous TH neuron generation by stable Bcl-X_L-overexpressing polyclonal hNS1 cells. *A,* Western blots of naive (n) or Bcl-X_L-expressing (b) hNS1 cells under division (Div) or differentiation (Diff) conditions. Cells were differentiated in the presence of 0.5% FBS for 12 d, at low (5%) or high (20%) O_2 tension. Blots were double-stained for Bcl-X_L or TH (or α-actin, as a loading control). When the same experiment was performed in the absence of serum during differentiation, no TH expression could be detected (data not shown). *B,* Nurr1 expression in dividing (Div) and differentiated (Diff) control hNS1 cells. Note that differentiated cells upregulate a mature neuronal marker such as hNSE. *C,* Time course of appearance of TH protein in the indicated cell lines, during differentiation in low O_2 and 0.5% FBS. Blots were probed for TH and β-F1-ATPase as a loading control. TH expression was clearly detectable after 8 d of differentiation, and only in the Bcl-X_L cells. *D,* TH ICC in cultures parallel to those shown in *A*. Differentiated TH$^+$ human neurons are illustrated only in the case of Bcl-X_L-expressing cells, low O_2, and 0.5% FBS. Scale bar, 50 μm. *E,* Quantification of TH$^+$ neuron generation rate. hNS1-hyg cells are cells stably transfected with the IRES-hyg empty vector and hyg selected for 1 month (*$p < 0.01$ Bcl-X_L vs the two other groups; Student's *t* test). The net increase in TH$^+$ neuron generation rate is 15-fold.

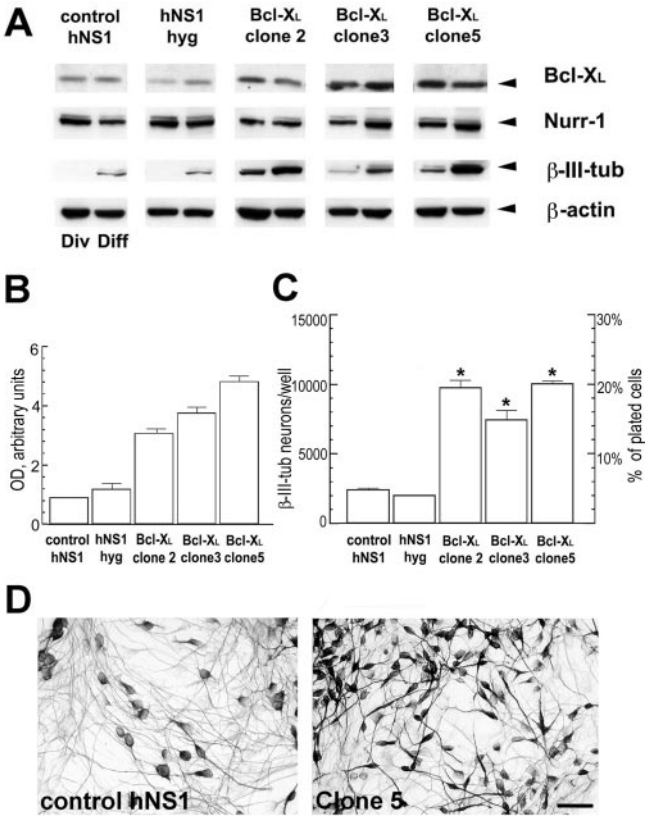

Figure 4. Enhanced spontaneous generation of human neurons by hNS1 cell subclones expressing different levels of Bcl-X_L. *A,* Bcl-X_L, Nurr1, and β-III-tub Western blots of naive hNS1 cells or cells genetically modified for hyg^R or Bcl-X_L overexpression (three representative clones, 2, 3, and 5, are shown). Cells were probed under both division (Div) and differentiation (Diff) conditions (12 d in 0.5% FBS, low O_2). *B,* Quantification of Bcl-X_L overexpression levels in the different cell lines used in these assays (data from four individual Western blots from independent cultures were averaged after normalizing OD values to that of the control cells). Every group was different from all of the other groups (one-way ANOVA, followed by Newman–Keuls; $p < 0.05$). *C,* Quantification of neuron generation rate (β-III-tub$^+$ cells) by the cell lines shown in the Western blot in *A*. The asterisks denote a significant difference between the indicated groups (see Results for details). *D,* Immunocytochemistry of human β-III-tub$^+$ neurons generated after differentiation of the control hNS1 cells and Bcl-X_L-overexpressing hNS1 subclone 5, in the same conditions as in *A*. Scale bar, 50 μm.

tested conditions, either by Western blot or by ICC. In summary, the best conditions for the generation of TH$^+$/DAergic human neurons from hNS1 cells involve (1) a background of increased Bcl-X_L, (2) differentiation in the presence of serum, and (3) low oxygen tension.

In terms of the time course of TH expression (Fig. 3*C*), control cells (both naive and hyg^R) did not express detectable levels of TH at any time point, whereas Bcl-X_L-overexpressing cultures did, starting at day 8. These results do not support the view that the effects of Bcl-X_L could be caused by enhanced survival of TH$^+$ cells generated in the first few days of differentiation, which would have subsequently died in their absence.

To further investigate the action of Bcl-X_L on a more powerful experimental substrate, we isolated subclones from the Bcl-X_L-overexpressing polyclonal cell line, showing various Bcl-X_L levels (Figs. 4*A*,*B*, 5*A*). Importantly, Bcl-X_L overexpression persists after differentiation. β-III-tubulin quantification after differentiation revealed a net threefold to fourfold increase in the number of neurons generated by the Bcl-X_L clones when compared with the naive and hyg^R cell lines ($p < 0.01$; Tukey test; all three Bcl-X_L clones vs both control cell lines) (Fig. 4*A*,*C*,*D*). The total number of neurons generated in the best case (clone 5) represented 20.2% of the total number of cells plated. The increase in total neuron production was very similar in all three clones studied, regardless of Bcl-X_L expression levels (Fig. 4*C*). In contrast, TH expression levels and TH$^+$ neuron generation after differentiation correlated with Bcl-X_L expression levels (Fig. 5). [Note that Nurr1 expression levels were unaffected by Bcl-X_L overexpression or differentiation (Fig. 4*A*)]. Comparison of Bcl-X_L and TH expression levels showed a Bcl-X_L expression threshold, over which TH neuron generation occurred at high rates (Figs. 4*B*, 5*A*,*F*,*G*).

The neuronal and dopaminergic nature of the TH$^+$ cells was confirmed by coexpression of TH and β-III-tub in all TH$^+$ cells and positive stain for DA (Fig. 5*C–E*). Furthermore, the cells synthesized dopamine and released it after high potassium depolarization (Fig. 5*I*), again confirming the neuronal nature of the DAergic cells generated. As expected from Western blot and ICC data, DA production and release were significantly higher in Bcl-X_L-overexpressing cells than in control ones. TH$^+$ neuron generation rate (Fig. 5*F*) by these clones accounted for up to 2.18% of the total cells plated (Bcl-X_L clone 5), in fact greatly exceeding the rate of TH$^+$ neuron generation by serially passaged human VM cultures [~0.3% (Storch et al., 2001)]. The net increase in TH$^+$ neuron generation reached almost two orders of magnitude (91-fold, control hNS1 vs Bcl-X_L clone 5). These hDAergic neurons stained negative for p53 (data not shown), suggesting the absence of neuronal damage.

The effect of Bcl-X_L on DAergic neuron generation was specific for the DAergic neuronal phenotype, as determined by comparing the generation of other neurotransmitter phenotypes by

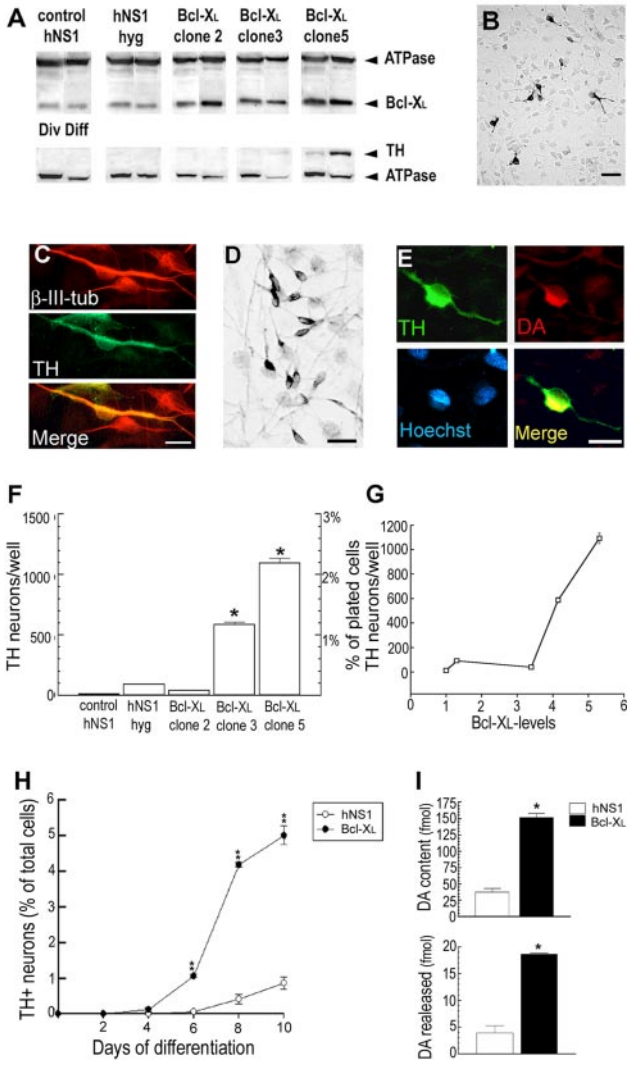

Figure 5. Enhanced spontaneous generation of TH^+/DA^+ neurons by hNS1 subclones expressing different levels of Bcl-X_L. *A,* Bcl-X_L and TH Western blots of naive hNS1 cells or cells genetically modified for hyg^R or Bcl-X_L overexpression (three representative clones, 2, 3, and 5, are shown). Cells were probed under both division (Div) and differentiation (Diff) conditions (12 d in 0.5% FBS, low oxygen). *B,* Immunocytochemistry of human TH^+ neurons generated after differentiation of the Bcl-X_L hNS1 subclone 5 in the same conditions as in *A*. Scale bar, 50 μm. *C,* Double ICC for β-III-tub and TH after differentiation of Bcl-X_L hNS1 subclone 5. Note colocalization of both markers, indicating the neuronal nature of TH^+ cells. Scale bar, 20 μm. *D,* Dopamine ICC in differentiated cells from the Bcl-X_L subclone 5 (DAB development). Scale bar, 50 μm. *E,* TH^+ neurons (green) produce dopamine (red), illustrating that differentiation of Bcl-X_L subclone 5 results in the generation of human dopaminergic neurons. Scale bar, 20 μm. *F,* Quantification of the rate of TH^+ neuron generation by the different cell lines. The asterisks denote a significant difference from all of the other groups ($n = 5$; $p < 0.01$; Tukey test). The rate of TH^+ neuron generation by clone 5 is 2.18% of the total number of plated cells (right axis). *G,* Diagram illustrating the relationship between number of TH^+ neurons and level of Bcl-X_L overexpression, supporting the notion of a threshold- or a dose–response-type of effect for Bcl-X_L after DAergic neuron generation. *H,* Time course of TH^+ neuron generation during the differentiation period in control (hNS1) and Bcl-X_L-overexpressing hNS1 cells (subclone 5). Data are expressed as mean ± SEM ($n = 5$). $^{**}p < 0.01$ (Student's *t* test). *I,* HPLC-EC determinations of DA production and release by control (hNS1) and Bcl-X_L-overexpressing cells (subclone 5) after differentiation. The graphs represent the mean ± SEM of three independent experiments, each performed in quadruplicate. $^{*}p < 0.001$ (Student's *t* test).

control and Bcl-X_L-overexpressing hNSCs (Fig. 6). For these determinations, differentiated cells were costained for β-III-tubulin and GABA, glutamate, TH, ChAT, and serotonin to determine the percentage of neurons expressing each of the phenotypes (Fig. 6*A*). Cholinergic neurons were never found, and serotonergic ones were only occasionally detected in these cultures under the present conditions. No differences in the generation of GABA or Glu neurons were observed (Fig. 6*B*). In contrast, DAergic neuron generation was strikingly enhanced, from close to up to 10% of the total neuron population. Therefore, the effects of Bcl-X_L described above are specific to dopaminergic neurons. The majority of TH^+ neurons do not coexpress GABA (~70%), being thus similar in phenotype to midbrain-DAergic neurons (Isacson et al., 2003; Perrier et al., 2004). There was, however, ~30% of $TH^+/GABA^+$ neurons (31.8 ± 1.7% in control hNS1 cells and 28.5 ± 1.4% in Bcl-X_L-overexpressing cells; $n = 5$; $p > 0.05$, nonstatistically significant difference; Student's *t* test), with a phenotype could resemble that of olfactory bulb DAergic neurons (Gall et al., 1987; Max et al., 1996).

As deduced from data in Figure 6*B*, some of the β-III-tubulin neurons did not express any of the different phenotypes studied here. However, all of the β-III-tubulin cells were negative for nestin and immunopositive for Dcx and Map-2 (>95%) (Fig. 6*C*). As reported recently by Markakis et al. (2004), these β-III-tubulin cells could express several other neurotransmitters and/or neuropeptides different from the ones studied here.

Bcl-X_L effects on TH^+/DAergic neuron generation by nonestablished hNSCs (neurospheres)

To further extend and generalize our observations on the effects of Bcl-X_L, we were interested in determining whether Bcl-X_L could also enhance the dopaminergic differentiation capacity of nonestablished, serially passaged, human neurosphere cell strains (Fig. 7). To this end, the cells (two independent strains, 6 week hFBr pass6 and 9.5 week hFBr pass18) were attached to a poly-L-lysine-coated surface and transfected 48 hr later with GFP or Bcl-X_L coding vectors [or mock-transfected (no DNA)]. After transfection, we differentiated the cells in low oxygen tension for 5 d under the influence of a minimal dopaminergic inductive mixture [TPA and acidic FGF (see Materials and Methods) that results in dopaminergic neuron maturation] (Stull and Iacovitti, 2001). Efficiency of transfection was 12–15% (Fig. 7*C*) (as determined by GFP fluorescence). These two neurosphere strains, similarly to the hNS1 cell lines, also express the transcription factor Nurr1 (detectable by Western blot; data not shown). As shown in Figure 7, *A* and *B*, Bcl-X_L greatly enhanced the number of TH^+-differentiated neurons (5.5- to 7-fold, 9.5 week or 6 week hFBr cells, respectively). The TH^+ neuron generation rate increased from a baseline (mock- or GFP-transfected cells) of 1–1.5% up to 7–8.2% of the total number of cells ($p < 0.01$; Tukey test; $n = 5$, applicable for both neurosphere strains).

Bcl-X_L-overexpressing hNSCs generate TH^+ neurons after grafting to the adult striatum

One month after cell implantation of control or Bcl-X_L (clone 5) cells into complete 6-OHDA-lesioned animals (MFB lesion), three of five rats receiving control cells or four of six animals receiving Bcl-X_L cells had surviving grafts, as determined by the presence of h-Nuc or BrdU positively stained cells (Fig. 8*A*,*B*). No signs of tumor formation were observed in any transplanted animal, according to previous studies (Rubio et al., 2000; Villa et al., 2002). Cell migration from the transplant core into the host parenchyma, as detected by h-Nuc immunoreactivity, reached 86 ± 4 or 283 ± 24 μm (control or Bcl-X_L cells, respectively), surrounding the implantation site (mediolateral extent), being thus more notorious for Bcl-X_L cells than for control cells (Fig. 8*A*). Grafted cells were found to preferentially migrate through striatal gray matter, rather than invading white-matter tracts

(Fig. 8*A*, high-magnification panels, *B*). Overall, graft survival was clearly enhanced in the case of Bcl-X_L cells. AP extension of the grafts was mostly enhanced for transplants of Bcl-X_L cells, which spanned 1.12 ± 0.043 mm rostrocaudally, compared with control cells (0.5 ± 0.1 mm) ($p < 0.05$; Mann–Whitney *U* test). Total graft volume, determined according to stereological procedures, was also increased from 0.02 ± 0.01 to 0.52 ± 0.04 mm^3 (for control or Bcl-X_L cell implants, respectively; $p < 0.01$; *t* test). Total counts of h-Nuc^+ cells could not be reliably determined because of the heavy, dense cellular packing at the core of the transplants (Fig. 8*A*).

hNSCs generate very few neurons *in vivo* when implanted at non-neurogenic regions, such as the striatum, as expected from a neural stem cell (Rubio et al., 2000; Martínez-Serrano et al., 2001; Villa et al., 2002). In agreement with this background, grafted control hNS1 cells generated few Dcx^+ or $hNSE^+$ neurons in the present experiment. In contrast, Bcl-X_L cell implants seem to be richer in Dcx^+ and $hNSE^+$ cells (Fig. 8*C*,*D*). These findings are consistent with the *in vitro* differentiation data previously described in the present work (Fig. 4).

In vivo TH^+ neuron generation capacity was quantified in all stained sections for every surviving graft. Whereas no TH^+ neurons were found in any section from animals grafted with naive cells, an average of 170 ± 16 cells per graft were quantified in Bcl-X_L cell-transplanted animals (DAB ICC) (Fig. 8*E*) (the human nature of all TH^+ neurons was confirmed by double staining for h-Nuc in parallel sections). The magnitude of this difference (which essentially represents an all-or-none type of situation) resembles the previously described data obtained in the *in vitro* experiments (Figs. 3, 5). In addition, these data also help to establish proof-of-principle of the prominent role of Bcl-X_L in human TH^+ neuron generation from hNSCs, in the present case *in vivo*. TH^+ neuron morphology is shown at high magnification in Figure 8, *F* and *G*. TH^+ neurons have been unambiguously identified as being of human origin after examination of h-Nuc plus TH double-IF sections (Fig. 8*F*), and of neuronal nature (TH–hNSE double stains) (Fig. 8*G*).

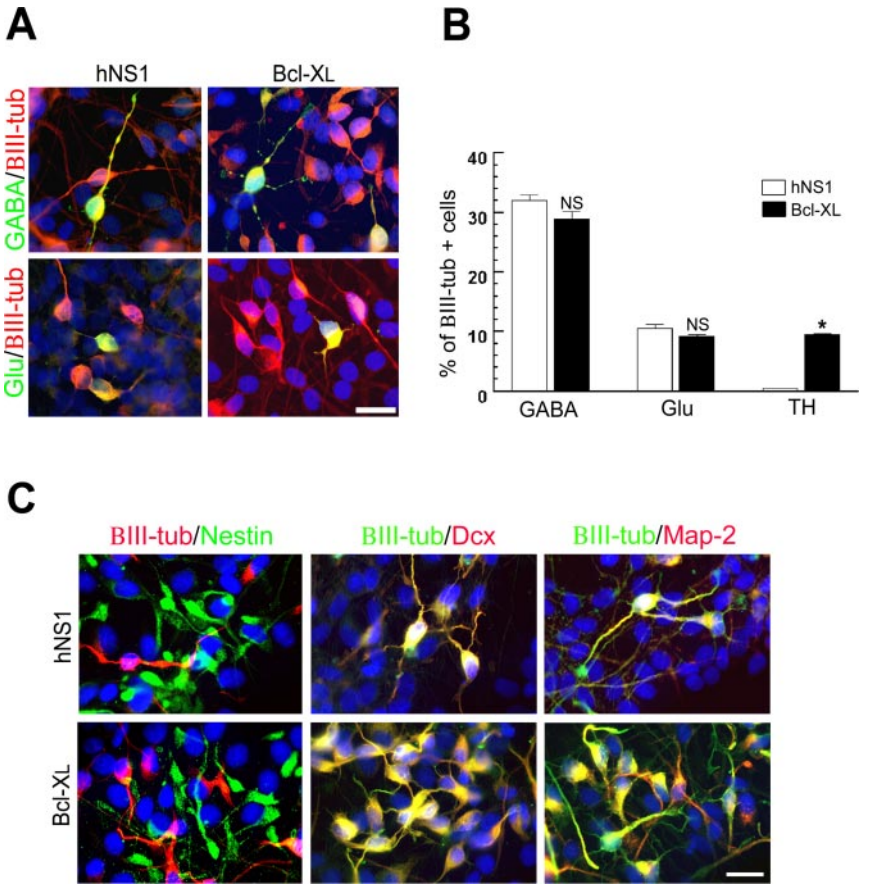

Figure 6. Bcl-X_L effects on multiple neurotransmitter phenotypes. *A,* Double ICC for β-III-tub (red) and GABA or Glu (green) after differentiation of control hNS1 or Bcl-X_L-overexpressing hNS1 cells (subclone 5). Hoechst nuclear staining appears in blue. Scale bar, 20 μm. Costainings for β-III-tub and TH were shown in Figure 5. *B,* Quantification of the percentage of β-III-tub^+ neurons staining positive for GABA, Glu, or TH. Graphs represent the mean ± SEM ($n = 6$); *$p < 0.001$ (Student's *t* test). NS, Nonsignificant difference. *C,* Double IF of differentiated control (hNS1) or clone 5 cells (Bcl-X_L) for β-III-tub and nestin, Dcx, or Map-2. Note the absence of double β-III-tub–nestin-positive cells, and that the majority of β-III-tub^+ neurons costained for Dcx or Map-2. Hoechst 33258 nuclear staining is shown in blue. Scale bar, 20 μm.

Discussion

Discovery and relevance of the effects of Bcl-X_L

Consistent with previous studies (Liste et al., 2004), presently (Fig. 1) we have obtained additional data indicating that the overexpression of TH cannot be achieved in hNSCs, because of cytotoxic and genotoxic effects, coursing with an apoptotic mechanism. After testing several possible neuroprotective genes, Bcl-X_L was found to be much more effective than BDNF, GDNF, and SOD1cit in counteracting TH-induced cell death (Fig. 2). After conducting these TH transfection experiments, we studied a more relevant process: the spontaneous generation of DAergic neurons by hNSCs, in the absence of TH transfection. Both v-myc immortalized hNSCs (Figs. 3–6) and human forebrain neurospheres (Fig. 7) displayed a greatly enhanced capacity for DAergic neuron generation after Bcl-X_L overexpression. Remarkably, the hNS1 cells studied here express Nurr1, GTP-CH1, and AADC, being negative for DBH; v-myc is fully downregulated after differentiation in hNS1 cells (Villa et al., 2000, 2004). Also, TH^+ human neurons generated from hNS1 cells produce and release dopamine (Fig. 5). In the case of neurosphere cells, the DA-inductive protocol used here results in the full expression of the DAergic phenotype and generation of DAergic neurons (Stull and Iacovitti, 2001). These experiments on neurosphere cells are important to rule out the possibility that the reported Bcl-X_L effects on hNS1 cells could be caused by v-myc expression. It is also worth mentioning that transfection efficiency in neurospheres was only 12–15% of all cells in culture, and, if extrapolated, would result in an increase of 30- to 60-fold yield in TH–DAergic neurons.

As shown here (Fig. 8) (see also Rubio et al., 2000), control hNSCs generate very few neurons in the adult lesioned or intact striatum and do not generate TH-expressing neurons at all. Consistent with other studies (Svendsen et al., 1997; Fricker et al., 1999; Englund et al., 2002), neurons were only seen at or close to the implantation site. Present *in vivo* experiments suggest that Bcl-X_L overexpression helps human TH^+ neurons to survive in a

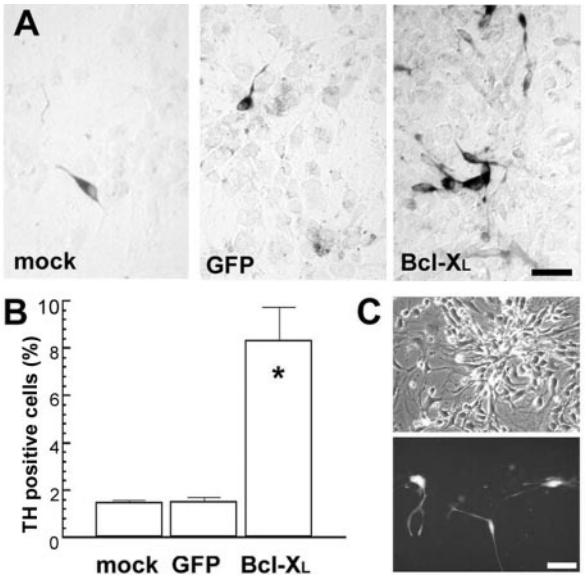

Figure 7. Enhancement of DAergic neuron generation by Bcl-X_L in nonimmortalized neurosphere cultures of human forebrain neural precursor cells. Data in this figure correspond to experiments performed using the 6 week hFBr neurosphere strain (see Materials and Methods for details). *A,* Photomicrographs of TH^+ human neurons after mock transfection or after genetic modification for GFP or Bcl-X_L expression. Two days after transfection, the cells were differentiated for 5 d in the presence of a DA inductive mixture, before fixation. Scale bar, 50 μm. *B,* Quantification of the rate of TH^+ neuron generation by the transfected cultures. Data are expressed as percentage of plated cells (100,000 cells per well; $n = 5$; 4–6 analyzed fields per sample summing up to 800–1200 cells analyzed in total). The asterisk indicates a significant difference between the Bcl-X_L group and the other two groups ($p < 0.01$; Tukey test). *C,* Photomicrographs (phase contrast and fluorescence) of GFP-transfected cells. Transfection efficiency, determined by GFP fluorescence 48 hr after transfection at the time when differentiation period starts, was 15%. Scale bar, 50 μm.

non-neurogenic site such as the adult striatum, in an all-or-none manner. Such clear-cut data serve as a proof-of-principle type of evidence for Bcl-X_L action. Behavioral effects were not studied here, because the design of this grafting experiment aimed to match that of a previous study in which a small number of cells ($\sim 10^5$) were instrastriatally implanted for a short survival time (1 month) (Rubio et al., 2000). Indeed, it is well known that human fetal grafts require several months to mature and start showing any functional improvement (Brundin et al., 1986, 1988). After analyses of the transplants, indeed, the number of TH^+ neurons found in the Bcl-X_L grafts was relatively small (<200 per animal), far distant from the number of cells needed to exert any behavioral effects in the present model (Lindvall et al., 2004). Behavioral effects might be easily attained by transplanting more cells, possibly at multiple locations, to obtain a better reinnervation of the striatum (our ongoing work).

Three grounds of evidence substantiate the relevance of the findings reported here: First, the magnitude of the enhancement of TH–DAergic cell generation by Bcl-X_L is remarkably high (Figs. 3–7), not a few-fold increase over control levels but rather an increase of one to two orders of magnitude. Second, experiments with different designs performed on different cellular systems yielded similar results (Figs. 2–7). Third, Bcl-X_L expression greatly enhances the survival of human TH^+ neurons generated from hNSCs *in vivo*, in an all-or-none manner (Fig. 8). Therefore, the present data support the notion that Bcl-X_L actions can be generalized to multiple human DAergic neuron source cellular systems in both *in vivo* and *in vitro* settings. During the course of the present study, Bcl-X_L has been reported to increase the yield of TH neurons *in vitro* and *in vivo* from mouse ES cells (Shim et al., 2004), which complements our data [first reported in preliminary form by Martínez-Serrano et al. (2003)]. Enhancement of TH neuron generation in mouse ES cells was below a twofold increase for every parameter studied (TH^+ and DAT^+ or $calbindin^+$ cells). DA production and release enhancement was limited to a 25% increase. After transplantation, a minor increase in the number of TH^+ cells and graft volume was observed ($\leq$50% increase). Therefore, the effects of Bcl-X_L seem to be more prominent in hNSCs than in mouse ES cells. For mouse ES cells, the underlying mechanisms explaining the effects of Bcl-X_L were not investigated, and were just assumed to be anti-apoptotic (Shim et al., 2004). Also, supporting our findings, Bcl-X_L has been shown to protect human neuroblastoma SHSY5Y cells from 6-OHDA toxicity (Jordan et al., 2004).

The reasons to choose Bcl-X_L here as the anti-apoptotic factor were that (1) Bcl-X_L is the most potent anti-apoptotic molecule in the Bcl-2 family because of its multiple actions at different levels (for review, see Benn and Woolf 2004). Furthermore, during CNS development, its absence is not complemented by other members of the family (like Bcl-2, Bcl-X_L null mutation is embryonically lethal in mice, with the pups showing remarkable CNS development deterioration). (2) Whereas other members of the Bcl-family, such as Bcl-2, are barely or not expressed during adulthood, Bcl-X_L is highly expressed in dopaminergic neurons in adult rodents (Krajewska et al., 2002). (3) Bcl-2 has been tested on rodent substantia nigra cells, showing no effects on DAergic neuron survival after transplantation in the 6-OHDA-lesioned rat (Anton et al., 1995; Schierle et al., 1999).

The effects of Bcl-X_L discovered here are of higher magnitude than those reported for other neuroprotective strategies used in rodent ES and progenitor cells. Thus, low oxygen (Studer et al., 2000) or antioxidants (*N*-acetylcysteine and dipyridamole) (Rodríguez-Pallarés et al., 2001, 2002) have been shown to exert moderate effects on survival of rat VM DAergic neurons (twofold to threefold enhancement). Low oxygen, in the present study, did not alter the outcome of TH transfection experiments but was crucial for the spontaneous generation of hDAergic neurons by Bcl-X_L-overexpressing hNSCs (Figs. 3–7)

Mechanisms of Bcl-X_L action on hNSCS

DAergic neurons usually die through caspase-dependent apoptosis (Han et al., 2003; Janumyan et al., 2003; Vila and Przedborski, 2003; Milosevic et al., 2004; Tamm et al., 2004). Also, the most widely accepted biological action of Bcl-X_L is to counteract apoptosis preventing translocation of pro-apoptotic molecules to the mitochondria, and thus preserving its integrity, blocking Cit C release and caspase activation (Benn and Woolf 2004). As discussed below, most data generated here point to a Bcl-X_L-mediated survival mechanism.

Survival

The concept that the DAergic phenotype is toxic for neurons has been extensively described in the literature and has been extended here for hNSCs and their derivatives (see also Liste et al., 2004). We show here that: (1) Bcl-X_L is more potent in terms of counteracting TH-induced cell death than other neurotrophic and antioxidant proteins that only partially prevent cell death. (2) The effects of Bcl-X_L are mimicked by the pan-caspase inhibitor z-VAD-fmk [we also determined that caspase 3 gets activated after TH expression in hNSCs (M. Ramos and A. Martínez-Serrano, unpublished observations)]. (3) Time course data (Figs. 3, 5) are consistent with Bcl-X_L enhancing the survival of TH^+ neurons simultaneously with their

generation. (4) *In vivo*, Bcl-X_L-expressing grafts show more cells globally (both BrdU- and h-Nuc-positive) (Fig. 8), indicating a survival action.

Induction and specification

(1) Bcl-X_L does not induce TH expression in dividing cells or differentiation of proliferating cells. (2) Bcl-X_L does not alter Nurr1 expression. (3) *In vivo*, there are more surviving cells in the Bcl-X_L grafts, globally, and not only more TH^+ cells. (4) In the case of an inductive mechanism or of a respecification of neuronal progenitors toward a DAergic phenotype, this should have occurred at the expense of other phenotypes, which is not so, as shown in Figure 6.

Proliferation of DAergic precursors

(1) Such proliferation was not detected in any experiment (Figs. 3*D*, 5*B*, 6*A*, 7*E*). If a Bcl-X_L-induced proliferation of precursors was taking place, one would expect to see small groups of TH^+ neurons, which was never the case. (2) The time course of TH^+ cell generation (Fig. 5*H*) showed that TH^+ neurons appeared earlier in time in Bcl-X_L-overexpressing cells than in control cells (instead of more TH+ cells appearing later). (3) *In vivo*, proliferation was not evident, because the number of $BrdU^+$ and h-Nuc^+ cells was similarly increased in the case of Bcl-X_L and control grafts [if so, one would have expected more h-Nuc^+ cells and less $BrdU^+$ cells (because of label dilution) for Bcl-X_L grafts].

In summary, all the experiments reported here consistently point to a survival mechanism, rather than to other putative actions of Bcl-X_L. From the data reported here, it is clear that hNSCs of forebrain origin are endowed with the potential to generate human DAergic neurons, but this potential is normally masked, by unknown reasons. Further study of Bcl-X_L actions will surely help to understand the relevant cellular processes beyond this observation and to discover the underlying cellular mechanisms regulating human DAergic neuron development and neurodegeneration, which is poorly understood at present (Dauer and Przedborski, 2003; Vila and Przedborski, 2003). Hopefully, further research in this line (and its extension to ventral mesencephalic cells) and refinement of these new biotechnological tools will help to accelerate discoveries aimed at cell replacement in PD.

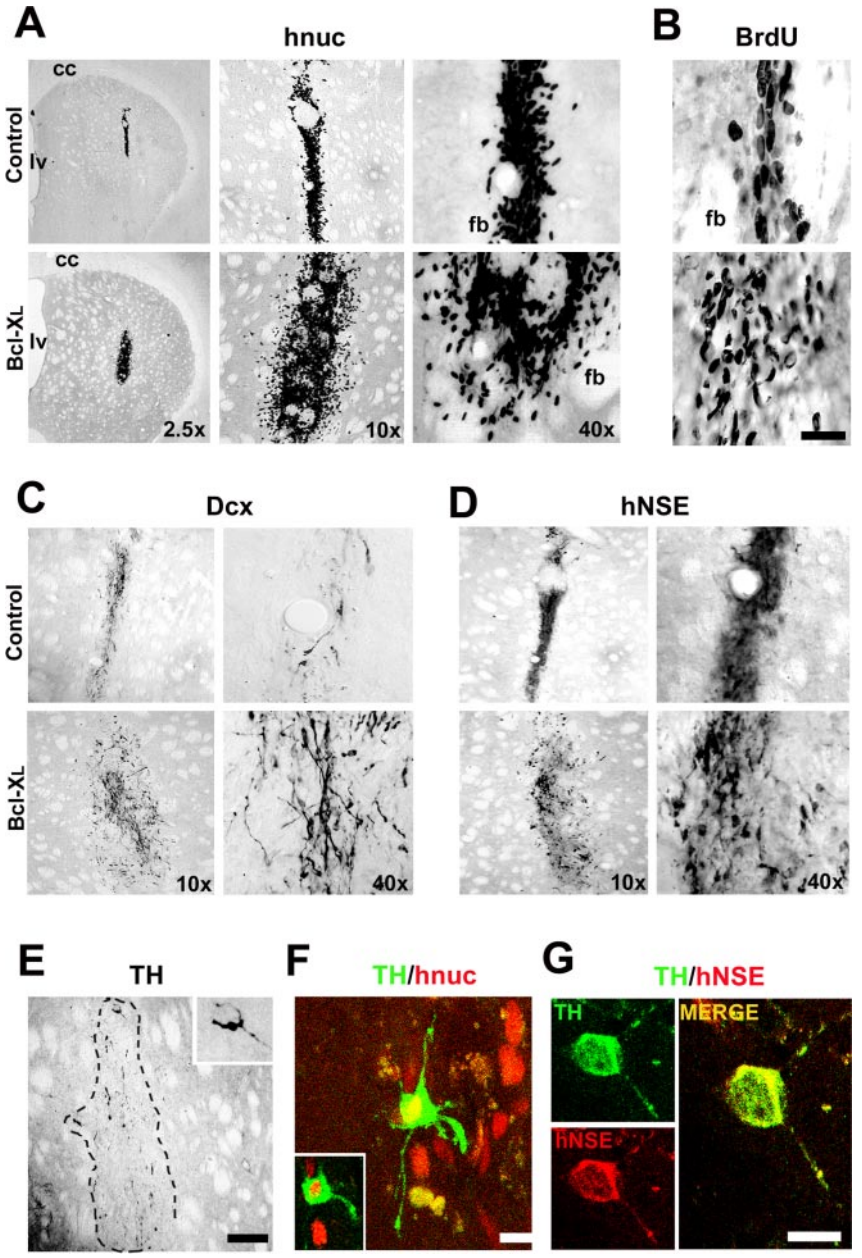

Figure 8. Transplantation of Bcl-X_L-overexpressing hNSCs into the adult rat 6-OHDA-lesioned striatum results in human TH^+ neuron generation and survival. *A*, Human nuclei-stained sections to illustrate survival of transplanted control hNS1 cells (top row) and Bcl-X_L-overexpressing hNS1 cells (bottom row) into the host striatum (1 month survival time). Panels are arranged from left to right to show increasing magnifications. Note the nondisruptive integration of the transplanted cells into the host parenchyma, the increased migration of Bcl-X_L cells, and also the preferential integration and migration through gray matter regions of the neostriatum. cc, Corpus callosum; lv, lateral ventricle; fb, fiber bundles. *B*, Parallel sections stained for BrdU, showing confirmatory evidence to that obtained from h-Nuc-stained sections. Scale bar, 20 μm. Dcx (*C*) or hNSE (*D*) stainings of parallel sections show neurons generated from grafted control or Bcl-X_L-overexpressing hNSCs. *E*, A TH-immunostained (DAB development) parallel section showing a transplant of Bcl-X_L hNSCs. The dashed line represents the graft–host boundary. The inset shows a high magnification of a human TH^+ neuron. Transplants of naive hNSCs cells did not contain any TH^+ neurons (data not shown), despite reasonably good survival, as shown in *A–D*. Scale bar, 100 μm. *F*, *G*, High-magnification views of TH^+ human neurons: TH^+ human nuclei double IF (*F*) illustrates the human origin of TH^+ cells [the larger photograph shows an overlay of a confocal *z*-axis series, out of which a single section (1 μm depth) is shown in the inset]. Scale bar, 10 μm. *G*, TH plus hNSE double IF illustrates the human and neuronal nature of TH^+ cells. Scale bar, 10 μm.

References

Anton R, Kordower JH, Kane DJ, Markham CH, Bredesen DE (1995) Neural transplantation of cells expressing the anti-apoptotic gene bccl-2. Cell Transplant 4:49–54.

Arco A, Morcillo J, Martínez-Morales JR, Galian C, Martos V, Bovolenta P, Satrústegui J (2002) Expression of the aspartate/glutamate mitochondrial carriers aralar1 and citrin during development and in adult rat tissue. Eur J Biochem 269:3313–3320.

Arenas E (2002) Stem cells in the treatment of Parkinson's disease. Brain Res Bull 57:795–808.

Barzilai A, Melamed E, Shirvan A (2001) Is there a rationale for neuroprotection against dopamine toxicity in Parkinson's disease? Cell Mol Neurobiol 21:215–235.

Benn SC, Woolf CJ (2004) Adult neuron survival strategies: slamming on the brakes. Nat Rev Neurosci 5:686–700.

Björklund A, Dunnett SB, Brundin P, Stoessl AJ, Freed CR, Breeze RE, Levivier M, Peschanski M, Studer L, Barker R (2003) Neural transplantation for the treatment of Parkinson's disease. Lancet Neurol 2:437–445.

Blum D, Torch S, Lambeng N, Nissou MF, Benabid AL, Sadoul R, Verna JM (2001) Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. Prog Neurobiol 65:135–172.

Brundin P, Nilsson OG, Strecker RE, Lindvall O, Astedt B, Bjorklund A (1986) Behavioural effects of human fetal dopamine neurons grafted in a rat model of Parkinson's disease. Exp Brain Res 65:235–240.

Brundin P, Strecker RE, Widner H, Clarke DJ, Nilsson OG, Astedt B, Lindvall O, Bjorklund A (1988) Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: immunological aspects, spontaneous and drug-induced behaviour and dopamine release. Exp Brain Res 70:192–208.

Carpenter MK, Cui X, Hu Z-Y, Jackson J, Sherman S, Seiger A, Wahlberg L (1999) In vitro expansion of a multipotent population of human neural progenitor cells. Exp Neurol 158:265–278.

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889–909.

Emgard M, Halin U, Karlsson J, Bahr BA, Brundin P, Blomgren K (2003) Both apoptosis and necrosis occur early after intracerebral grafting of ventral mesencephalic tissue: a role for protease activation. J Neurochem 86:1223–1232.

Englund U, Björklund A, Wictorin K (2002) Migration patterns and phenotypic differentiation of long-term expanded human neural progenitor cells after transplantation into the adult rat brain. Brain Res Dev Brain Res 134:123–141.

Frederiksen K, Jat PS, Valtz N, Levy D, McKay R (1988) Immortalization of precursor cells from the mammalian CNS. Neuron 1:439–448.

Fricker RA, Carpenter MK, Winkler C, Greco C, Gates MA, Björklund A (1999) Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. J Neurosci 19:5990–6005.

Gall CM, Hendry SH, Seroogy KB, Jones EG, Hycock JW (1987) Evidence for coexistence of GABA and dopamine in neurons of the rat olfactory bulb. J Comp Neurol 266:307–318.

Haavik J, Toska K (1998) Tyrosine hydroxylase and Parkinson's disease. Mol Neurobiol 16:285–309.

Han BS, Hng HS, Choi WS, Markelonis GJ, Oh TH, Oh YJ (2003) Caspase-dependent and–independent cell death pathways in primary cultures of mesencephalic dopaminergic neurons after neurotoxin treatment. J Neurosci 23:5069–5078.

Isacson O (2003) The production and use of cells as therapeutic agents in neurodegenerative diseases. Lancet Neurol 2:417–424.

Janumyan YM, Sansam CG, Chattopadhyay A, Cheng N, Soucie EL, Penn LZ, Andrews D, Knudson CM, Yang E (2003) Bcl-XL/Bcl-2 coordinately regulates apoptosis, cell cycle arrest and cell cycle entry. EMBO J 22:5459–5470.

Jordan J, Galindo MF, Toornero D, González-García C, Ceña V (2004) Bcl-XL blocks mitochondrial multiple conductance channel activation and inhibits 6-OHDA-induced death in SH-SY5Y cells. J Neurochem 89:124–133.

Krajewska M, Mai JK, Zapata JM, Ashwell KW, Schendel SL, Reed JC, Krajewski S (2002) Dynamics of expression of apoptosis-regulatory proteins Bid, Bcl-2, Bcl-X, Bax and Bak during development of murine nervous system. Cell Death Differ 9:145–157.

Lindvall O, Kokaia Z, Martínez-Serrano A (2004) Stem cell therapy for neurodegenerative disorders–how to make it work. Nat Med 10:S42–S50.

Liste I, Navarro B, Johansen J, Bueno C, Villa A, Johansen TE, Martínez-Serrano A (2004) Low level tyrosine hydroxylase (TH) expression allows for the generation of stable TH+ cell lines of human neural stem cells. Hum Gene Ther 15:13–20.

Lotharius J, Barg S, Wiekop P, Lundberg C, Raymon HK, Brundin P (2002) Effect of mutant alpha-synuclein on dopamine homeostasis in a new human mesencephalic cell line. J Biol Chem 277:38884–38894.

Lundberg C, Horellou P, Mallet J, Björklund A (1996) Generation of DOPA-producing astrocytes by retroviral transduction of the human tyrosine hydroxylase gene: *in vitro* characterization and *in vivo* effects in the rat Parkinson model. Exp Neurol 139:39–53.

Markakis EA, Palmer TD, Randolph-Moore L, Rakic P, Gage FH (2004) Novel neuronal phenotypes from neural progenitor cells. J Neurosci 24:2886–2897.

Martínez-Serrano A, Rubio FJ, Navarro B, Bueno C, Villa A (2001) Human neural stem and progenitor cells: *in vitro* and *in vivo* properties, and their potential for gene therapy and cell replacement in the CNS. Curr Gene Ther 1:279–299.

Martínez-Serrano A, Navarro B, Bueno C, Villa A, Liste-Noya I (2003) Bcl-X_L mediated blockade of cell death is crucial to enhance the generation of tyrosine hydroxylase expressing human neurons from neural stem cells. Soc Neurosci Abstr 29:672.12.

Max SR, Bossio A, Iacovitti L (1996) Co-expression of tyrosine hydroxylase and glutamic acid decarboxylase in dopamine differentiation factor-treated striatal neurons in culture. Brain Res Dev Brain Res 91:140–142.

Milosevic J, Storch A, Schwarz J (2004) Spontaneous apoptosis in murine free-floating neurospheres. Exp Cell Res 294:9–17.

Olanow CW, Tatton WG (1999) Etiology and pathogenesis of Parkinson's disease. Annu Rev Neurosci 22:123–144.

Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L (2004) Derivation of midbrain dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci USA 101:12543–12548.

Rodríguez-Pallares J, Rey P, Soto-Otero R, Labandería-Garcia JL (2001) *N*-acetylcysteine enhances production of dopaminergic neurons from mesencephalic-derived precursor cells. NeuroReport 12:3935–3938.

Rodríguez-Pallares J, Caruncho HJ, Guerra MJ, Labandería-Garcia JL (2002) Dipyridamole-induced increase in production of rat dopaminergic neurons from mesencephalic precursors. Neurosci Lett 320:65–68.

Rubio FJ, Bueno C, Villa A, Navarro B, Martínez-Serrano A (2000) Genetically perpetuated human neural stem cells engraft and differentiate into the adult mammalian brain. Mol Cell Neurosci 16:1–13.

Schierle GS, List M, Martinou JC, Widner H, Nitera P, Brundin P (1999) Differential effects of Bcl-2 overexpression on fibre outgrowth and survival of embryonic dopaminergic neurons in intracerebral transplants. Eur J Neurosci 11:3073–3081.

Shim JW, Koh HC, Chang MY, Roh E, Choi CY, Oh YJ, Son H, Lee YS, Studer L, Lee SH (2004) Enhanced *in vitro* midbrain dopamine neuron differentiation, dopaminergic function, neurite outgrowth, and 1-methyl-4-phenylpyridinium resistance in muse embryonic stem cells overexpressing Bcl-XL. J Neurosci 24:843–852.

Stokes AH, Hastings TG, Vrana KE (1999) Cytotoxic and genotoxic potential of dopamine. J Neurosci Res 55:659–665.

Storch A, Paul G, Csete M, Boehm BO, Carvey PM, Kupsch A, Schwarz J (2001) Long-term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells. Exp Neurol 170:317–325.

Studer L, Csete M, Lee SH, Kabbani N, Walikonis J, Wold B, McKay R (2000) Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. J Neurosci 20:7377–7383.

Stull ND, Iacovitti L (2001) Sonic hedgehog and FGF8: inadequate signals for the differentiation of a dopamine phenotype in mouse and human neurons in culture. Exp Neurol 169:36–43.

Svendsen CN, Caldwell MA, Shen J, ter Borg MG, Rosser AE, Tyers P, Karmiol S, Dunnett SB (1997) Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. Exp Neurol 148:135–146.

Tamm C, Robertson JD, Sleeper E, Enoksson M, Emgard M, Orrenius S, Ceccatelli S (2004) Differential regulation of the mitochondrial and death receptor pathways in neural stem cells. Eur J Neurosci 19:2613–2621.

Vila M, Przedborski S (2003) Targeting Programmed cell death in neurodegenerative diseases. Nat Rev Neurosci 4:365–375.

Villa A, Snyder EY, Vescovi A, Martínez-Serrano A (2000) Establishment and properties of a growth factor dependent, perpetual neural stem cell line from the human CNS. Exp Neurol 161:67–84.

Villa A, Navarro B, Martínez-Serrano A (2002) Genetic perpetuation of *in vitro* expanded human neural stem cells (HNSCs): cellular properties and therapeutic potential. Brain Res Bull 57:789–794.

Villa A, Navarro-Galve B, Bueno C, Franco S, Blasco MA, Martínez-Serrano A (2004) Long-term molecular and cellular stability of human neural stem cell lines. Exp Cell Res 294:559–570.