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Nurr1 Blocks the Mitogenic Effect of FGF-2 and EGF, Inducing Olfactory Bulb Neural Stem Cells to Adopt Dopaminergic and Dopaminergic-GABAergic Neuronal Phenotypes

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Abstract

The transcription factor Nurr1 is expressed in the mouse olfactory bulb (OB), although it remains unknown whether it influences the generation of dopaminergic neurons (DA) (DA neurons) in cells isolated from this brain region. We found that expressing Nurr1 in proliferating olfactory bulb stem cells (OBSCs) produces a marked inhibition of cell proliferation and the generation of immature neurons immunoreactive for tyrosine hydroxylase (TH) concomitant with marked upregulations of *Th*, *Dat*, *Gad*, and *Fgfr2* transcripts. In long-term cultures, these cells develop neurochemical and synaptic markers of mature-like mesencephalic DA neurons, expressing GIRK2, VMAT2, DAT, calretinin, calbindin, synapsin-I, and SV2. Concurring with the increase in both *Th* and *Gad* expression, a subpopulation of induced cells was both TH- and GAD-immunoreactive indicating that they are dopaminergic- GABAergic neurons. Indeed, these cells could mature to express VGAT, suggesting they can uptake and store GABA in vesicles. Remarkably, the dopamine D1 receptor agonist SKF-38393 induced c-Fos in TH⁺ cells and dopamine release was detected in these cultures under basal and KCl-evoked conditions. By contrast, cotransducing the Neurogenin2 and Nurr1 transcription factors produced a significant decrease in the number of TH- positive neurons. Our results indicate that Nurr1 overexpression in OBSCs induces the formation of two populations of mature dopaminergic neurons with features of the ventral mesencephalon or of the OB, capable of responding to functional dopaminergic stimuli and of releasing dopamine. They also suggest that the accumulation of *Fgfr2* by Nurr1 in OBSCs may be involved in the generation of DA neurons.

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Keywords

transcription factors; neural stem cells; dopaminergic neurons; GABAergic neurons; *Fgfr2*

INTRODUCTION

A variety of stem cells are being explored as sources of dopaminergic neurons (DA neurons) that might be used in cell therapy for Parkinson's disease including neural stem cells (NSCs), embryonic stem cells and induced pluripotent stem cells (Parish and Arenas, 2007; Cooper et al., 2012; Lindvall, 2012; Seiz et al., 2012; Studer, 2012). One important way to achieve this goal is to increase the levels of relevant transcription factors through gene transduction into stem cells. This experimental approach is based on the identification of transcription factors that are critical determinants of midbrain DA neurons during development, e.g., Nurr1, Neurogenin2 (Ngn2), Pitx3, Lmx1a, Foxa2 (Zetterstrom et al., 1997; Smits et al., 2003; Andersson et al., 2006; Kele et al., 2006; Kittappa et al., 2007; Jacobs et al., 2009; Ganat et al., 2012; Volpicelli et al., 2012).

Nurr1 is a member of the orphan nuclear receptor family of transcription factors required for the differentiation of mesencephalic DA neurons. This factor induces the expression of dopaminergic genes, such as tyrosine hydroxylase (*Th*), the vesicular monoamine transporter2 (*Vmat2*), or the dopamine transporter (*Dat*) (Saucedo-Cardenas et al., 1998; Sakurada et al., 1999; Kim et al., 2003; Smits et al., 2003; Kadkhodaei et al., 2009; Baron et al., 2012), as well as drives the formation of midbrain-like DA neurons from stem cells (Wagner et al., 1999; Chung et al., 2002; Kim et al., 2002; Martinat et al., 2006; Park et al., 2006a; Theka et al., 2013). The capacity of Nurr1 transduction to generate these neurons from NSCs varies, and it appears to depend on age and species, as well as on the brain region from which the cells are isolated (O'Keeffe et al., 2008; Park et al., 2008). Moreover, sustained Nurr1 expression in NSCs often gives rise to immature DA neurons rather than to functional, mature neurons. In attempts to resolve this problem, Nurr1 has been coexpressed with other transcription factors, such as Pitx3, Ngn2, Mash1/Ascl1, Foxa2, and Lmx1a (Martinat et al., 2006; Park et al., 2006a; Park et al., 2006b; O'Keeffe et al., 2008; Jo et al., 2009; Lee et al., 2010) or the expression of exogenous Nurr1 has been decreased or delayed (Park et al., 2012).

Nurr1 is also expressed in periglomerular (PG) TH⁺-neurons of the olfactory bulb (OB; A16 subtype DA neurons) (Backman et al., 1999; Saino-Saito et al., Developmental Neurobiology 2004), although its influence on the generation of DA neurons from olfactory bulb stem cells (OBSCs) remains unclear (Vergaño-Vera et al., 2006; DíazGuerra et al., 2013). In this study, we transduced Nurr1 into proliferating OBSCs and found that this rapidly inhibited the mitogenic effects of fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) and provokes the induction of an immature neural phenotype associated with TH immunoreactivity and the expression of *Th*, *Dat*, and glutamic acid decarboxylase (*Gad*) genes. The Nurr1-induced neurons differentiated to acquire neurochemical and synaptic markers typical of mature mesencephalic A9/A10 DA neurons or of OB A16 DA-GABAergic neurons, responded to dopaminergic agonists and released dopamine.

Furthermore, our results suggest that regulation of fibroblast growth factor receptor2 (*Fgfr2*) expression by *Nurr1* is part of the mechanism involved in the generation of DA neurons from OBSCs.

METHODS

NSC Cultures

NSCs were prepared from the OB of embryonic day 13.5 (E13.5) CD1 mice and from E13.5 C57Bl/6 mice in some experiments. All animal care and handling was carried out in accordance with European Union guidelines (directive 2010/63/EU) and was approved by the Comisión de Bioética (Ethical Committee) of the CSIC. All efforts were made to ameliorate the suffering of the animals. To obtain brain tissue, pregnant mice were sacrificed by cervical dislocation and the embryos were then decapitated. After dissociating the tissue, the cells were plated and expanded as neurospheres in Dulbecco's modified Eagle medium (DMEM)/nutrient mixture F12 (F12), supplemented with insulin, apotransferrin, putrescine, progesterone, and sodium selenite (N2: DMEM/F12/N2) to which 20 ng/mL FGF-2 (PeproTech) and 20 ng/mL EGF (PeproTech) were added daily. Most experiments were performed with cells between passages 3–10, when the majority of cells exhibit a stable karyotype (Vergano-Vera et al., 2009).

Cloning, Sub-Cloning, Virus Production and Infection of OBSCs with Retroviral Vectors

Viral infection was achieved using a Moloney murine leukemia virus-based retroviral vector (Kim et al., 2003; Park et al., 2008). The retroviral construct pRV-LacZ-IRES-EGFP (referred to simply as the LacZ-EGFP vector) was used as a control in infection experiments. The mouse *Nurr1* and *Ngn2* open reading frames (ORFs) were cloned into the LacZ site resulting in the pRV-Nurr1-IRES-EGFP and pRV-Ngn2-IRES-EGFP vectors (simply referred to as the Nurr1-EGFP and Ngn2-EGFP vectors). These vectors were then used to generate viral particles by transfecting them into human embryonic kidney (HEK) 293T cells, obtaining titers in the range of $3\text{--}5 \times 10^6$ infection units (IU)/mL when measured by flow cytometry of NIH 3T3 cells infected with increasing volumes of supernatant. When the supernatant containing the viral particles was concentrated by ultracentrifugation, the resulting titers were in the range of 10^8 IU/mL. Retroviral particles were also obtained by transfecting the retroviral plasmids into 1F8 cells, a monoclonal cell line derived from a stock of 293GPG (which expresses the vesicular stomatitis virus G (VSV-G) protein and the Gag-Pol (GP) polyprotein) cells (Heinrich et al., 2011). The supernatant containing the viral particles was concentrated by ultracentrifugation and the resulting titers were in the range of $10^9\text{--}10^{11}$ colony forming units (cfu)/mL.

To infect proliferating OBSCs, 3.5×10^6 cells were dissociated and resuspended in culture medium in the presence of 6 $\mu\text{g/mL}$ polybrene, the viral supernatants at multiplicity of infection (MOI) of 75–100 (LacZ-EGFP and Nurr1-EGFP vectors) and of 1000 (Ngn2-EGFP vector), and FGF-2 and EGF (20 ng/mL each). The cells were centrifuged for 1 h and then collected, seeded at a density of 15,000–20,000 cells/cm² and maintained in the presence of FGF-2 and EGF. To calculate the efficiency of infection, proliferating OBSCs were harvested and analyzed by flow cytometry to determine the proportion of cells expressing

enhanced green fluorescent protein (EGFP) (Méndez-Gómez et al., 2011). Two to three days after infection, OBSC cultures contained an average of 16.5% and 18.1% GFP⁺ cells when infected with the Nurr1-EGFP vector and the Nurr1+Ngn2-EGFP vectors, respectively, whereas OBSCs infected with the EGFP vector contained 32.1% green fluorescent protein (GFP⁺) cells. In the experiments, the MOI and the volumes of the viral particles were adjusted to obtain similar percentages of infected cells. As the results obtained with cultures that had different infection efficiencies were indistinguishable, they were combined in the analysis.

Two to three days after infection, OBSCs were passaged and plated for an additional 3–4 days on polyornithine-coated coverslips under conditions that promote proliferation (5000–10,000 cells/cm² with FGF-2 and EGF). Proliferating cells were incubated for 4 h with 5 μ M 5'-bromo-2'-deoxyuridine (BrdU; Roche Diagnostics), a dose previously shown to have no toxic effects on NSC proliferation (Vergaño-Vera et al., 2009), and they were then fixed with 4% paraformaldehyde (PFA).

For cell differentiation studies, neurospheres were disaggregated and plated at a density of 100,000 cells/cm² in DMEM/F12/N2 plus 2% fetal bovine serum (FBS) for 4–20 days. Alternatively, cells were plated at a density of 200,000 cells/cm² in DMEM/F12/B27 plus 2% FBS supplemented every 3 days with 20 ng/mL brain-derived neurotrophic factor (BDNF; Peprotech), 20 ng/mL glial cell-derived neurotrophic factor (GDNF; Peprotech), and 0.5 mM dibutyryl-cAMP (cAMP; Sigma) for a total of 21–22 days. Medium changes were done either with DMEM/N2 plus 2%FBS or with DMEM/B27 plus 2%FBS and all the cells were fixed with 4% PFA.

To induce the expression of the immediate early gene cFos, Nurr1-EGFP cultures were exposed for 2 h to the dopamine D1 receptor (D1R) agonist (*R*)-(+)-SKF-38393 ((*R*)-(+)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride; 20 μ M, Sigma) alone or in combination with the D2 receptor (D2R) agonist (–)-Quinpirole (20 μ M, Sigma) (Darmopil et al., 2008; Suarez et al., 2014), while as controls Nurr1-EGFP cultures were treated with the vehicle alone (sterile H₂O). The cells were finally fixed with 4% PFA and immunostained with antibodies against TH, c-Fos and GFP (Espadas et al., 2012). Both the vehicle and the agonists induced c-Fos in TH negative cells (data not shown). Notably, only the agonists induced c-Fos in TH positive cells, and these cells were counted to find the percentage $\pm$ s.e.m. of TH⁺ and GFP⁺ cells in which c-Fos was induced ($n = 8$ and 4 cultures for SKF and SKF + Quinpirole, respectively).

Immunostaining of Cultured Cells

Cells were incubated overnight at 4°C or room temperature with primary antibodies raised against: GFP (mouse, rabbit and rat antibodies, 1:1000–1:2000: Molecular Probes/Invitrogen, Eugene, OR, Cat. no. A-11120 and A-6455, and Nacalai Tesque, Kyoto, Japan, Cat. no. 04404–84); Nurr1 (rabbit antibody, 1:750–1000: Millipore, Billerica, MA, Cat. no. AB5778), Ngn2 (mouse antibody, 1:10: kindly provided by Dr. D. Anderson, Caltech, Pasadena, CA); TH (mouse and rabbit antibodies, 1:200 and 1:500: Immunostar, Hudson WI, Cat. no. 22941 and Millipore, Cat. no. AB152); MAP2ab (mouse, 1:250: Sigma, St. Louis, MO, Cat. no. M1406); glial fibrillary acidic protein (GFAP) (rabbit, 1:1000: Dako,

Glostrup, Denmark, Cat. no. Z0334); GAD (mouse, 1:75: The Developmental Studies Hybridoma Bank); GABA (rabbit, 1:2000: Sigma, Cat. no. A2052); Calbindin (mouse, 1:5000: Swant, Bellinzona, Cat. no. 300); Calretinin (rabbit, 1:1500: Swant, Cat. no. 7699/4); VMAT2 (rabbit, 1:500: Millipore, Cat. no. AB1598P); GIRK2 (rabbit, 1:100: Alomone Labs, Jerusalem, Cat. no. APC-006); DAT (rat, 1:500: Millipore, Cat. no. MAB369); Synapsin-I (rabbit, 1:1000: Millipore, Cat. no. AB1543); SV2 (mouse antibody, 1:50: Hybridoma Bank, kindly provided by Dr. K.M. Buckley from University of Iowa, IA); vesicular GABA transporter (VGAT; mouse, 1:1000: Synaptic Systems, Cat. no. 131011); c-Fos (rabbit 1:5000: Calbiochem/Merck, Cat. no. PC38); BrdU (mouse antibodies, 1:1000 and 1:100: clone G3G4, the Developmental Studies Hybridoma Bank, University of Iowa Dept. Biological Sciences, Iowa City, IA, and Becton Dickinson, San Jose, CA, Cat. no. 7580). The cells were then incubated with Alexa fluor 405, 488, 568, 594, Texas red, 633, or 647 conjugated secondary antibodies (1:300–1000: Invitrogen) and finally, with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA), before they were mounted in Mowiol solution (Calbiochem, San Diego, CA). Controls were performed to confirm the specificity of the primary and secondary antibodies.

Morphological Analysis

The morphology of TH⁺ neurons was analyzed following transduction with Nurr1-EGFP and Nurr1-EGFP + Ngn2-EGFP (30 TH⁺-neurons per condition). From cell traces obtained using ImageJ software (NIH, Bethesda, MD), the number of primary neurites (any neurite extending directly from the cell body) and neurite branches were counted, and the total neurite length and the cell body perimeter were measured.

Flow Cytometry Analyses and Cell Sorting

OBSCs were plated at a density of 20,000–25,000 cells/cm² and, after 2 days in culture, neurospheres were disaggregated and analyzed by flow cytometry gating of propidium iodide-negative cells to determine the proportion of OBSCs expressing GFP. A FACSAria cytometer (BD Biosciences, San Diego, CA) equipped with a double Argon (488 nm) and Helium-Neon laser (633 nm) was used. Data were collected using a linear digital signal process. The emission filters used were BP 530/30 for GFP (FL1) and BP 616/23 for Propidium Iodide (FL3).

Cells expressing GFP were separated and purified by fluorescent activated cell sorting (FACS) using 20 psi pressure and a 100 μ m nozzle aperture. GFP⁺ cells were collected directly in culture medium in autoclaved 5 mL polystyrene tubes. The cells were immediately counted and incubated in Trizol reagent to extract RNA or seeded in cell culture (see below). The purity of the isolated cells was confirmed by flow cytometry. Debris, dead cells and duplets were always excluded from the analysis.

Data were analyzed with FACSDiva data analysis software (BD Biosciences) and displayed using biexponential scaling.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from the sorted GFP population of OBSCs using Trizol reagent (Invitrogen) and purified with Qiagen RNeasy Mini Kit separation columns (Qiagen). The reverse transcription reaction was carried out with SuperScript III (Invitrogen) to synthesize cDNA from the mRNA.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

The sequences of primer pairs for the RT-qPCR were designed with Lasergene software and are listed in Table 1. All RT-qPCR cDNA products obtained were sequenced and correspond to the expected fragments. The dynamic range of amplification for each primer set was determined using serial dilutions of cDNA. Finally, an optimal cDNA amount, which was within the dynamic range of the primer sets, was selected for the RT-qPCR reactions. A real time qPCR analysis was performed in triplicate with the appropriate controls using Power SYBR Green (Applied Biosystems). The Ct value obtained for each target gene was normalized to the Ct value for *Gapdh* using the comparative comparative threshold cycle (CT) method. Then, gene expression changes in the Nurr1-EGFP condition was compared to the levels of gene expression obtained in the LacZ-EGFP condition, using the CT method (Schmittgen and Livak, 2008) and were expressed as fold changes in log2 scale.

Dopamine Release Assay

Cells expressing GFP were purified by FACS, collected directly in culture medium, plated at a density of 200,000 cells/cm² in DMEM/F12/B27 plus 2% FBS and maintained for 21–24 days in the presence of BDNF, GDNF and cAMP as mentioned above. The culture supernatants were collected to analyze the basal dopamine release. The evoked dopamine release was determined in cells treated with 56 mM KCl in DMEM/B27 for 20 min at 37°C and the supernatants were then collected in the presence of ethylenediaminetetraacetic acid (EDTA) (1 mM) and sodium metabisulfite (4 mM) to prevent dopamine degradation. Dopamine release was measured with Dopamine Research Elisa™ assay kit, according to the manufacturer's instructions (LDN Labor Diagnostika Nord GmbH & Co. KG). The reaction was monitored at 450 nm and the samples were quantified by comparing their absorbance with a reference curve prepared from known concentrations of dopamine. The calculations were done following a nonlinear regression for curve fitting using GraphPad Prism 4 software.

Cell Counts and Statistical Analysis

To determine the number of cultured cells expressing a specific antigen, 10 random fields per coverslip were directly counted using a 40× objective and fluorescence filters (Leica microscope). Alternatively, cells were counted in confocal images (Leica) of optical planes from 10 fields per coverslip or from the whole coverslip. The proportion of cells expressing specific markers and colabeled with GFP (as determined by immunostaining) was calculated with respect to the total number of GFP-expressing cells, and the results were expressed as the mean ± s.e.m. of 4–8 cultures from 3 to 4 experiments. Statistical analyses were performed using a two-tailed Student's *t*-test, applying Welch's correction when the *F*-test indicated that the variances of both groups differed significantly. The differences between

the mean values were considered to be significant when $*p < 0.05$ (GraphPad Prism 4 software).

RESULTS

Forced Nurr1 Expression in OBSCs Blocks Proliferation while Inducing *Th*, *Dat*, *Gad*, and *Fgfr2* Expression and Neuronal Differentiation

Nurr1 is expressed abundantly by the PG TH⁺ neurons of the OB (Backman et al., 1999; Saino-Saito et al., 2004). However, it remains unclear whether Nurr1 influences the proliferation of OBSCs and the generation of DA neurons from these cells (Díaz-Guerra et al., 2013). To address these questions, embryonic OBSCs (Vergaño-Vera et al., 2009) were transduced with a pRV-Nurr1-EGFP or a pRV-LacZ-EGFP vector as a control. These cells proliferated as neurospheres in the presence of the mitogenic factors, EGF and FGF-2 [Fig. 1(A–D)]. To analyze them in more detail they were plated on an adherent substrate in the presence of these mitogens during 4 days, fixed and immunostained with specific antibodies. The large majority ($79 \pm 8\%$) of the transduced GFP⁺ cells expressed Nurr1 in the Nurr1-EGFP infected cultures, whereas this percentage only reached $21.0 \pm 1.1\%$ in the control cultures [$p < 0.001$; Fig. 1 (E,F,K)]. Notably, Nurr1 overexpression was associated with fewer cells incorporating BrdU [$5.0 \pm 0.5\%$ BrdU⁺ cells; $p < 0.001$; Fig. 1(H,L)] compared to $80 \pm 9\%$ in control cultures [Fig. 1(G,L)]. By contrast, Nurr1 induced marked TH expression in the GFP⁺ cells [$69 \pm 8\%$; Fig. 1(J,M)], whereas TH⁺ cells were absent from the control cultures [Fig. 1(I,M)]. The morphology of these TH⁺ cells resembled that of neurons in an early stage of differentiation characterized by the presence of short/small and barely articulated neurites [Fig. 1(J)] (see below). These results indicate that augmenting the levels of Nurr1 inhibits OBSC proliferation and induces their differentiation to a TH neuronal fate.

Then, OBSCs infected with the Nurr1-EGFP or with the LacZ-EGFP vectors were collected 2 days after the infection and purified by FACS [Fig. 2(A)]. More than 90% of the cells (92% and 95% in the Developmental Neurobiology Nurr1-EGFP and LacZ-EGFP conditions, respectively) expressed GFP after sorting [Fig. 2(B,C)]. Gene expression analysis was performed on these cells by RT-qPCR. The level of *Nurr1* mRNA was 7.5-fold higher in the Nurr1-EGFP infected cultures than in the controls whereas the expression of *Egfp* was similar in both conditions [Fig. 2(D)]. Nurr1 overexpression provoked a marked 5–10 fold upregulation in the levels of *Th*, *Dat*, and *Gad* transcripts [Fig. 2(D)] demonstrating that Nurr1 induces both dopaminergic and GABAergic cell fates in proliferating OBSCs. In contrast, Nurr1 did not change *Ng2* expression. To explore the molecular mechanisms modulated by Nurr1, the expression levels of cellcycle related genes (*cyclins*, *p21*, *p27*, and *p53*) (Malumbres, 2011), *Fgfr1* and *Fgfr2* (Nieto-Estevez et al., 2013), and apoptosis related genes (Alavian et al., 2014) were measured. This analysis revealed a sharp upregulation of *Fgfr2* and a slight downregulation of *cyclin D1* by Nurr1 [Fig. 2(E)]. No changes were observed in the expression of *Bcl2* and *Bax* indicating that the effects of Nurr1 were not dependent on the regulation of apoptosis. These results suggest that the increased levels of *Fgfr2* may be involved in the differentiation effects induced by Nurr1 in OBSCs.

Next, we tested the effect of Nurr1 in differentiation conditions, plating OBSCs on an adherent substrate in the absence of the mitogens. In such conditions, the majority of GFP⁺ cells expressed Nurr1 in the Nurr1-EGFP infected cultures, both four ($70.9 \pm 8.5\%$; $p < 0.001$) and 7 days ($84.2 \pm 10.2\%$; $p < 0.001$) after the initiation of differentiation [Fig. 3(B,D,E)], as opposed to only $8.0 \pm 1.5\%$ and $4.6 \pm 0.5\%$ of control cells, respectively [Fig. 3(A,C,E)]. Relative to the number of DAPI⁺ cells, $11.5 \pm 1.4\%$ of the cells were Nurr1⁺ in Nurr1-EGFP infected cultures in contrast to only $3.0 \pm 0.9\%$ in the control cultures [Fig. 3(F)]. This percentage of DAPI⁺ cells expressing Nurr1 (11.5%) was similar to the average percentage of OBSCs expressing GFP (13.2%; see Methods) further supporting a high coexpression of GFP and Nurr1 in the Nurr1-EGFP infected cultures. Endogenous Nurr1 protein could be detected in GFP⁻ cells in these cultures, suggesting that the Nurr1⁺ cells observed in the control cultures were not due to the infection of OBSCs with the control EGFP vector. Nonetheless, the endogenous Nurr1 expression was unable to induce detectable TH [see Fig. 4(A)].

These data indicate that sustained Nurr1 overexpression is maintained during OBSC differentiation and, thus, we performed a time-course analysis of the abundance of TH⁺ cells growing under differentiation conditions on day 4, 7, 15 and 20 [Fig. 4(A–J)]. No TH⁺ cells were found at these time points in LacZ-EGFP infected cultures [Fig. 4(A,K) and data not shown], whereas the proportion of GFP⁺ cells infected with Nurr1-EGFP that were TH⁺ remained between 55% and 75% at all these time points [Fig. 4(C–K)]. In the Nurr1-EGFP cultures, the proportion of cells expressing TH relative to the total number of DAPI⁺ cells was $10.2 \pm 2.7\%$ and $8.8 \pm 2\%$ at 4 and 7 days, respectively [Fig. 4(L)], similar to the percentage of Nurr1⁺ cells relative to DAPI⁺ cells [Fig. 3(F)]. The majority of TH⁺ cells possessed relatively small cell bodies with fine neurites [Fig. 4(E,G,I); arrows]. Together, these findings suggest that increasing Nurr1 in OBSCs induces both TH expression and neuronal differentiation in the large majority of transduced cells. However, some neurons maintained an immature morphology characterized by the presence of relatively short/small neurites [Fig. 4(E,G,I); arrowheads] (see below) suggesting that Nurr1 alone may not be sufficient for all the infected cells to develop a fully mature morphology.

The Effects of Nurr1 and Ngn2 Cotransduction on the Morphology and Presynaptic Protein Expression of OBSC-Derived TH⁺ Neurons

Given the fact that expressing Nurr1 in OBSCs did not appear to promote the full morphological maturation of all the neurons generated, we examined whether coexpressing Nurr1 and Ngn2 in OBSCs promoted neuronal maturation and/or increased the number of TH⁺ cells. We chose to coexpress Ngn2 as this transcription factor is necessary for the development of midbrain DA neurons *in vivo* (Andersson et al., 2006; Kele et al., 2006) and when overexpressed, it increases the production of DA neurons from NSCs (Park et al., 2008). Accordingly, OBSCs were cotransduced with pRV-Nurr1-EGFP and pRV-Ngn2-EGFP vectors, or with the pRV-Nurr1-EGFP vector alone as a control. Transduction of Ngn2 alone did not induce TH expression in the OBSCs [Fig. 5(A,B)] and, thus, we focused our study on how Ngn2 and Nurr1 coinfection affected the differentiation of OBSCs to DA neurons.

Coinfected cultures grew as neurospheres in the presence of EGF and FGF-2 [Fig. 5(C,D)], as when infected with Nurr1-EGFP alone [Fig. 1(B,D)]. When these neurospheres were disaggregated and the cells were plated under differentiation conditions during 4 days, the majority of GFP⁺ cells ($78.2 \pm 1.3\%$) expressed Ngn2, and $23.2 \pm 2.4\%$ of these Ngn2⁺-GFP⁺ cells also expressed Nurr1 [Fig. 5(E-I)]. Hence, cotransduction of Ngn2 and Nurr1 was achieved in at least 18% (78.2×0.23) of the infected GFP⁺ cells. We tested the effect of forced expression of these two transcription factors on the generation of TH⁺ cells after 7 days of differentiation. The cotransduction of Nurr1 and Ngn2 provoked a significant reduction in the percentage of TH⁺ cells ($18.9 \pm 2.3\%$; $p < 0.05$) with respect to those detected in Nurr1-EGFP infected cultures [$63.8 \pm 4.6\%$; Fig. 6(A,B,K)]. However, the percentage of TH⁺ cells that expressed MAP2ab, GFAP and calbindin was similar in both conditions [96–100%, 0% and 51%, respectively; Fig. 6(C–H,K)]. In contrast, only small percentages of TH⁺ cells expressed GAD (a GABAergic marker): 4.1% in the coinfecting cultures and 5.4% in the Nurr1-EGFP infected cultures [Fig. 6(I–K)]. These results suggest that transducing OBSCs with Nurr1 alone induces the generation of TH⁺ neurons with characteristics A10 ventral mesencephalic dopaminergic neurons (calbindin expression) (Mendez et al., 2005; Bjorklund and Dunnett, 2007) and a subpopulation of A16 OB dopaminergic neurons (GAD expression) (Parrish-Aungst et al., 2007; Hurtado-Chong et al., 2009; Kiyokage et al., 2010). They also show that forced Ngn2 coexpression reduces the number of TH⁺ cells and appears to promote a neuronal morphology distinct to that provoked by Nurr1-EGFP alone [compare panels in Fig. 6(H,J) versus (G,I)].

We further studied these two Ngn2-dependent effects by counting the TH immunoreactive cells that have a GABAergic phenotype in the total Nurr1 or Ngn2 populations after 7 days of differentiation (Fig. 7). To identify GABAergic cells we used a mouse GAD antibody and a rabbit GABA antibody in combination with a rabbit Nurr1 and a mouse Ngn2 antibodies, respectively. Similar numbers of cells per sample were detected with GAD (44.2 ± 0.2 cells) and GABA antibodies (41.1 ± 7.2). As shown in Fig. 7, of the total number of Nurr1⁺ cells detected in the coinfection experiments, $60.9 \pm 2.1\%$ expressed TH and $7.7 \pm 1.4\%$ expressed GAD. In contrast, of the total number of Ngn2⁺ cells, $9.8 \pm 1.4\%$ were TH⁺ and $40.6 \pm 7.2\%$ were also GABA⁺. In the Nurr1-EGFP single infection, of the total number of Nurr1⁺ cells $58.8 \pm 12.5\%$ expressed TH and $9.2 \pm 3.3\%$ expressed GAD. These results show a significant higher correlation between Nurr1 expression and the acquisition of a TH phenotype than between Ngn2 expression and TH ($p < 0.001$). The opposite was found when referring to the GABAergic population ($p < 0.01$).

We also analyzed the morphology of the TH⁺ neurons (Fig. 8) present in Nurr1 and Nurr1/Ngn2 (Nurr1 plus Ngn2) infected cultures after 7 days of differentiation. According to previous studies, the morphology of a neuron changes during the differentiation process progressing from an immature neuron that has short neurites to a more mature neuron that possesses longer neurites (Dotti et al., 1988; Vicario-Abejón et al., 1995; Vicario-Abejón et al., 2000; Jan and Jan, 2010; Yang et al., 2011).

In our experiments, we assessed the number of neurites, the total neurite length and the perimeter of the cell body (Fig. 8). TH⁺ neurons growing in coinfecting cultures had significant ($p < 0.01$) fewer primary neurites (3.1 ± 0.2), neurite branches (5.0 ± 0.5) and

total number of neurites (8.1 ± 0.5) than those growing in cultures infected with Nurr1 alone [7.1 ± 0.5 , 15.7 ± 1.7 and 22.7 ± 2.0 , respectively: Fig. 8(A–C)]. By contrast, the total neurite length of TH⁺ neurons from cotransduced cultures was significantly higher ($1089.6 \pm 72.9 \mu\text{m}$; $p < 0.01$) than that in Nurr1- infected cultures ($567.6 \pm 61.4 \mu\text{m}$; Fig. 8D). Furthermore, the average perimeter of TH⁺ neurons from coinfecting cultures was smaller ($56.7 \pm 1.7 \mu\text{m}$; $p < 0.001$) than in Nurr1-infected cultures ($77.8 \pm 4.2 \mu\text{m}$).

The degree of maturation reached by the Nurr1- and Nurr1/Ngn2-induced dopaminergic neurons was further studied by immunostaining cells grown for 22 days with antibodies against the presynaptic proteins synapsin-I and synaptic vesicle2 (SV2). As shown in Figure 8(E–T) both proteins were expressed in TH⁺ cells and their immunofluorescence signal clustered in puncta. These are features of mature neurons with the capacity to form synapses (Vicario-Abejón et al., 1998). A similar pattern of presynaptic protein expression was found in TH⁺ cells both from the Nurr1 and the Nurr1/Ngn2 coinfecting cultures (Fig. 8, compare panels E–L vs M–T). This fact together with the reduced production of TH⁺ neurons in the coinfection condition [Fig. 6(K)] prompted us to explore the characteristics Nurr1-induced TH⁺ neurons in more detail.

TH⁺ Neurons Develop a Mature Dopaminergic Phenotype in Long-Term Cultures Under Conditions of Differentiation

We thought it was important to determine whether the TH⁺ cells generated in the Nurr1 infected cultures acquired characteristics of mature dopaminergic neurons. When OBSCs transduced with viral particles expressing Nurr1-EGFP were plated under differentiation conditions for 18 to 21 days (Fig. 9), we found that a large percentage of TH⁺ neurons expressed VMAT2 [$76.6 \pm 2.6\%$: Fig. 9(A–D,U)], a monoamine transporter present in mature mesencephalic dopaminergic neurons (Thompson et al., 2005; Ares-Santos et al., 2014). Moreover, more than half of the TH⁺ neurons expressed GIRK2 [$55.2 \pm 4.9\%$: Fig. 9(E–H,U)], a potassium channel characteristic of mature A9 mesencephalic neurons (Mendez et al., 2005; Thompson et al., 2005). Furthermore, the dopamine transporter DAT (Zhou et al., 2009) was detected in $77 \pm 7\%$ of the TH⁺ neurons [Fig. 9(I–L,U)] indicating that these cells acquired a relatively high degree of dopaminergic maturation. DAT was also found in $44.0 \pm 2.3\%$ ($n = 2$) of the TH⁺ neurons growing in Nurr1/Ngn2 infected cultures. The total number of TH⁺ -DAT⁺ cells in these coinfecting cultures was 7.6-fold lower than in the Nurr1-EGFP infected cultures (data not shown). In long-term Nurr1-differentiated cultures we also observed TH⁺ cells labeled with an anticalretinin antibody [$26.4 \pm 6.7\%$: Fig. 9(M–P,U)]. Calretinin marks a subpopulation of mesencephalic dopaminergic neurons (Gonzalez-Hernandez and Rodriguez, 2000) and it is absent from OB TH cells (Parrish-Aungst et al., 2007; Hurtado-Chong et al., 2009). Consistent with the presence of a subpopulation of dopaminergic-GABAergic neurons, VGAT was found to be expressed in $18.2 \pm 0.6\%$ of the TH neurons [Fig. 9(Q–T,U)] most prominently in their soma and dendrites suggesting that the generated cells could be able to uptake and storage GABA in vesicles, according to previous works (Panzanelli et al., 2007; Borisovska et al., 2013).

Next, we explored whether Nurr1-induced TH⁺ neurons responded to functional stimuli by activation of the D1R and D2R (Levey et al., 1993; Darmopil et al., 2008; Darmopil et al.,

2009; Suarez et al., 2014). Moreover, we tested whether these cells could release dopamine. In fact, application of SKF-38393 alone or together with Quinpirole to the culture medium induced c-Fos in $4.6 \pm 1.1\%$ and $4.2 \pm 3.4\%$, respectively, of TH⁺ cells [Fig. 10(A–H,N)] whereas treatment with the vehicle did not induce c-Fos in these cells [Fig. 10(I–L,N)]. Dopamine release was detected in both basal and KCl-dependent depolarization conditions in the range of pg/mL [Fig. 10(M,O)] similar to previously reported values (Lee et al., 2010).

DISCUSSION

The results we report here show a strong effect of Nurr1 on NSCs isolated from the OB (termed OBSCs) to generate DA neurons and a subpopulation of DA-GABAergic neurons. Indeed, we demonstrate that sustaining the levels of Nurr1 in OBSCs induces cell cycle exit, the expression of *Th* and *Dat* genes and the formation of immature TH immunoreactive neurons that develop to express typical markers of mature mesencephalic DA neurons, including GIRK2, DAT, VMAT2, calbindin, and calretinin. A small proportion of TH⁺ neurons also expressed GAD and VGAT demonstrating their GABAergic phenotype. Furthermore, these neurons have the potential to respond to stimuli acting on dopamine receptors and to release dopamine indicating they can develop functional and mature characteristics. These findings show that Nurr1 transduction drives OBSCs to generate dopaminergic neurons with features of ventral mesencephalic A9/A10 DA neurons and of dopaminergic-GABAergic neurons with characteristics of OB A16 DA neurons.

The Role of Nurr1 in Regulating Cell Cycle Exit and TH Neuronal Differentiation of OBSCs

The strong inhibition exerted by Nurr1 transduction on OBSC proliferation in the presence of mitogens concurs with the induction of TH⁺ cell generation. These results indicate that above a certain threshold level, Nurr1 blocks the mitogenic effects of FGF-2 and EGF, and induces the expression of *Th*, *Dat*, and *Gad* genes. Given that all TH-immunoreactive cells were MAP2ab⁺ neurons, and that the morphology of these cells resembles that of immature neurons (Vicario-Abejón et al., 2000), Nurr1 overexpression appears to induce both cell cycle exit and OBSC differentiation to neuronal TH dopaminergic and dopaminergic-GABAergic lineages. No such effect on NSC proliferation has been reported previously (Sakurada et al., 1999; Wagner et al., 1999; Kim et al., 2002; Park et al., 2006a; Park et al., 2006b; Bae et al., 2009), although an earlier study described the downregulation of cell cycle-related genes by Nurr1 overexpression in NSC lines derived from the cerebellum, including *cyclin B2*, *cyclin D1* and *p21* (Sousa et al., 2007). However, Nurr1 overexpression in OBSCs upregulated the *Fgfr2* and caused a minor downregulation of *cyclin D1* suggesting that the actions of Nurr1 in OB cells are mediated by the increase of *Fgfr2*. This effect is in line with recent works reporting that the nuclear accumulation of Fgfr1 and its interaction with Nurr1 induces DA neuron generation from mesencephalic progenitors (Baron et al., 2012; Narla et al., 2013). Thus, a potential interaction between Nurr1 and Fgfr2 might be involved in the inhibition of proliferation and induction of TH differentiation in OBSCs.

Additional experimental support for a role of Nurr1 in proliferation comes from its inhibitory influence on hematopoietic stem cells (HSCs). However, in contrast to OBSCs,

HSCs expressing Nurr1 did not differentiate but rather, they became quiescent (Sirin et al., 2010), indicating that the actions of Nurr1 are dependent on the specific type of stem cell.

Sustained Nurr1 Levels Promote the Functional Maturation of DA Neurons Derived from OBSCs

As aforementioned, our data indicate that increasing Nurr1 levels in OBSCs induces them to adopt a neuronal phenotype similar to that of ventral mesencephalic A9/A10 neurons (GIRK2, VMAT2, calbindin and calretinin expression). It could be argued that GIRK2 and VMAT2 are not exclusive markers for A9/A10 DA neurons as the potassium channel is also expressed in dendrites of PG cells in the OB (Schein et al., 1998), and a recent study also detected *Vmat* mRNA in PG cells (Borisovska et al., 2013), although two previous studies concluded that VMAT could not be detected in OB DA neurons by immunohistochemistry (Peter et al., 1995; Weihe et al., 2006). However, we detected both GIRK2 and VMAT2 proteins in the cell bodies of Nurr1-induced TH⁺ neurons together with the expression of calbindin and calretinin (both of which are absent from OB DA neurons) (Parrish-Aungst et al., 2007; Hurtado-Chong et al., 2009) indicating that Nurr1 induces a mesencephalic DA neuron-like phenotype in OBSCs.

Our results also show that a subpopulation of TH⁺ dopaminergic cells have a GABAergic phenotype as they express GAD and VGAT. OB dopaminergic- GABAergic neurons express VGAT in the soma and dendrites (Borisovska et al., 2013). In fact, TH⁺ PG neurons can establish dendro-dendritic synapses in the OB (Pinching and Powell, 1971; Kiyokage et al., 2010; Kosaka and Kosaka, 2011; Liberia et al., 2012). Accordingly, colocalization of TH and VGAT in Nurr1-induced dopaminergic-GABAergic neurons is consistent with the idea that these cells can uptake and store GABA in vesicles within the soma and dendrites.

As both mesencephalic and OB endogenous neurons produce dopamine (Bjorklund and Dunnett, 2007; Borisovska et al., 2013) it was fundamental to determine dopamine release from the Nurr1-induced DA neurons. Indeed, basal and KCl-evoked dopamine release was detected in the cultures at similar values to those previously reported for Nurr1-transduced stem cells (Lee et al., 2010) although in this study the cotransduction of Nurr1 and Foxa2 resulted in a more mature DA neuron phenotype than in the Nurr1 infection alone. Together with the production of dopamine, the induction of c-Fos after activation of dopamine receptors indicates that OBSC-derived dopaminergic cells can develop features of functional neurons. The fact that c-Fos expression was only detected in a 4.6% of TH⁺ cells in response to SKF is consistent with the small number of DA neurons that express the D1R in the substantia nigra and OB (Levey et al., 1993; Coronas et al., 1997; Gutierrez-Mecinas et al., 2005). Although the D2R is more abundant in these regions than the D1R, the addition of Quinpirole (a D2R agonist) along with SKF did not significantly change the levels of c-Fos induction compared to when the cultures were only treated with SKF. These facts are probably reflecting that *in vivo* most D1R and D2R are concentrated in the postsynaptic neurons that receive dopaminergic innervation rather than in the DA neurons themselves where they function as autoreceptors (Coronas et al., 1997; Gutierrez-Mecinas et al., 2005; Darmopil et al., 2009; Suarez et al., 2014). We also tested the activity of sulpiride, a D2R antagonist (Billings and Marshall, 2003; Suarez et al., 2014) but its effect was

indistinguishable from that of vehicle alone (data not shown). Nonetheless, our findings show that a group of Nurr1-induced TH⁺ cells are capable of responding to functional dopaminergic stimulus.

In contrast to the effects of Nurr1 overexpression, increasing the levels of Ngn2 alone did not induce TH in OBSCs and when cotransduced with Nurr1 it caused a marked reduction in the proportion of TH⁺ cells. In fact, a significant lower correlation between Ngn2⁺ cells and TH⁺ cells compared to Nurr1⁺ cells and TH⁺ cells was found in our cultures. Further more, Nurr1 transduction did not induce *Ngn2* expression suggesting that the effects of Nurr1 in OBSCs are not mediated by Ngn2. By contrast, Ngn2 over-expression increased the production of DA neurons in NSCs isolated from the mouse embryonic cerebral cortex, lateral ganglionic eminence (LGE) and ventral mesencephalon (Andersson et al., 2006; Kele et al., 2006; Park et al., 2008) indicating that the activity of Ngn2 is dependent on the cell-context. In a previous study morphological maturation was provoked by cotransduction of Ngn2 with Nurr1 into NSCs isolated from the ventral mesencephalon (Andersson et al., 2007). These authors reported that Nurr1 overexpression induced the generation of immature TH⁺ neurons, whereas Ngn2 was absolutely necessary for these TH⁺ cells to progress further to neurons expressing mature dopaminergic markers, such as VMAT2. In our study the similar pattern of synapsin-I and SV2 expression found in Nurr1 and Nurr1/Ngn2 infections suggest that the morphological changes of TH⁺ neurons caused by Ngn2 at 7 DIV do not have a correlate in presynaptic protein expression at 22 DIV. Here, we show that high levels of Nurr1 alone directs OBSCs to differentiate into TH⁺ neurons which express molecular markers of mature DA neurons, synaptic proteins, respond to dopaminergic stimulation, and release dopamine supporting the idea that at least a proportion of them have acquired functional properties.

In conclusion, we report novel findings indicating that Nurr1 induces in OBSCs the upregulation of *Fgfr2* mRNA, cell cycle exit, and the generation of a TH⁺ neuronal population that presents typical markers of mature and functional mesencephalic neurons, concurring with the generation of a subpopulation of dopaminergic-GABAergic neurons.

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Figure 1.

Nurr1 transduction of OBSCs inhibits proliferation and induces the generation of immature neurons that express tyrosine hydroxylase. (A–D) Cells isolated from the OB of E13.5 mice were cultured and passaged as floating neurospheres in the presence of FGF-2 and EGF, and they were then infected with viral particles expressing LacZ-EGFP (left panels) or Nurr1-EGFP (right panels). (E–J) Infected floating neurospheres were disaggregated, the cells were seeded on polyornithine and fibronectin coated coverslips, and they were maintained in the presence of FGF-2 and EGF for 4 days. The cells were immunostained with antibodies against Nurr1 and GFP (E, F), BrdU and GFP (G, H) or TH and GFP (I, J). Nurr1 overexpression (F, K) provoked a strong decrease in the percentage of BrdU⁺ cells (H, L) and a sharp increase in the percentage of TH⁺ cells that reached 69% (J, M). The arrows in E, F, G, H indicate double-labeled cells, the arrow-heads in H indicate single labeled GFP⁺ cells. The graphs represent the mean percentage $\pm$ s.e.m. ($n = 6$ cultures per condition) of cells expressing the markers indicated. *** $p < 0.001$ (Student's t -test). Scale bar (shown in B), A–D, 262.4 μ m; E–J, 81.9 μ m.

Figure 2.

Nurr1 transduction of proliferating OBSCs induces the expression of *Th*, *Dat*, *Gad*, and *Fgfr2* genes. OBSCs were cultured and passaged as floating neurospheres and infected with viral particles expressing LacZ-EGFP or Nurr1-EGFP. Two days later, infected floating neurospheres were disaggregated, the GFP⁺ population was purified by FACS and their RNA extracted and transcribed to cDNA (A-C). The expression levels of genes involved in neuronal differentiation, cell cycle, proliferation, and apoptosis were quantified by RT-qPCR. The graphs show that Nurr1 overexpression provoked a strong upregulation of *Th*, *Dat*, *Gad*, and *Fgfr2* mRNA transcripts. Results are the mean $\pm$ SD of three experiments performed in triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 3.

Nurr1 overexpression is maintained during the differentiation of OBSCs. (A–D) OBSCs were cultured and passaged as floating neurospheres and infected with viral particles expressing LacZ-EGFP or Nurr1-EGFP. The neurospheres were then disaggregated, the cells were plated on polyornithine and fibronectin coated coverslips, and they were maintained in the absence of FGF-2 and EGF under conditions of differentiation for 4 and 7 days (A–D). The cells were immunostained with antibodies against Nurr1 and GFP. The arrow in D points to the double-labeled cells shown in the insets at high magnification. In the Nurr1-EGFP infected cultures, the proportion of GFP⁺ cells expressing Nurr1 relative to the total number of GFP⁺ cells was 8.8 and 18.2 fold higher at 4 and 7 days, respectively, than in the LacZ-EGFP infected cultures (E). The percentage of Nurr1⁺-GFP⁺ cells relative to the total number of DAPI⁺ cells was 3.8 times higher in Nurr1-EGFP infected cultures than in the controls (F). The graphs represent the mean percentage $\pm$ s.e.m. ($n = 5$ cultures per condition) of cells expressing the indicated markers. *** $p < 0.001$ (Student's t -test). Scale bar (shown in B), A–D, 93.5 μ m; Insets, 57.9 μ m.

Figure 4.

Sustained Nurr1 expression enhances the long-term differentiation of TH⁺ neurons. OBSCs transduced with viral particles were plated under differentiation conditions during 4 days (A, B: LacZ-EGFP Infection) or during 4, 7, 15 and 20 days in DMEM/F12/N2 plus 2% FBS (C-J: Nurr1-EGFP Infection). The cells were immunostained with antibodies against TH and GFP, and no TH⁺ cells were found following LacZ-EGFP infection and 4 (A), 7 (K) and 15 days after differentiation (not shown). By contrast, the Nurr1-EGFP infected cultures contained 56.875% GFP⁺ cells expressing TH relative to the total number of GFP⁺ cells (K) and 8.8–10.2% of TH⁺-GFP⁺ cells relative to the total number of DAPI⁺ cells (L). The graphs represent the mean percentage $\pm$ s.e.m. ($n = 14$ cultures per condition) of cells expressing the indicated markers. $**p < 0.01$ (%TH⁺ cells at 20 days vs. 15 days; Student's *t*-test). The majority of TH⁺ cells possessed relatively small cell bodies with fine neurites (arrows). Some TH⁺ neurons maintained an immature morphology (arrow-heads). Scale bar (shown in B), A, B, 62.5 μ m; C–J, 50 μ m.

Figure 5.

The cotransduction of Nurr1 and Ngn2 in OBSCs using retroviral vectors. OBSCs transduced with Ngn2-EGFP alone (A, B) or cotransduced with viral particles expressing Nurr1-EGFP and Ngn2-EGFP (C, D) were plated under differentiation conditions during 4 days, and the cells were then double immunostained with antibodies against TH and GFP (A, B) or triple immunostained with antibodies against GFP (E), Ngn2 (F) and Nurr1 (G). The panel in H shows the image resulting from the combination of the three previous panels. In these cultures, $78.2 \pm 1.3\%$ GFP⁺ cells expressed Ngn2 (F, H, I) and $23.2 \pm 2.4\%$ of Ngn2⁺-GFP⁺ cells also expressed Nurr1 (G-I). The arrows indicate single (E-G) or triple (H) labeled cells. The graph represents the mean percentage $\pm$ s.e.m. ($n = 5$ cultures per condition) of cells expressing the indicated markers. Scale bar (shown in B), A, B, 22.2 μ m; C, D, 216.4 μ m; E-H, 27.7 μ m.

Figure 6.

The effects of Nurr1 and Ngn2 on the generation of TH⁺ neurons from OBSCs. An analysis of cell marker expression. OBSCs were infected with viral particles expressing the Nurr1-EGFP vector (left panels) or coinfecting with viral particles expressing Nurr1-EGFP and Ngn2-EGFP (right panels). The neurospheres were then disaggregated and the cells cultured under conditions of differentiation for 7 days. The cells were dual or triple labeled with antibodies against GFP, TH, MAP2ab, GFAP, Calbindin (Calb), or GAD, as indicated in the figure. The arrows point to triple labeled cells. The graph represents the mean percentage $\pm$ s.e.m. ($n = 6$ cultures per condition) of cells expressing the indicated markers (TH⁺-GFP⁺ cells relative to the total number of GFP⁺ cells or marker1-TH1 cells relative to the total number of TH⁺ cells). As seen, the coinfection of Ngn2 and Nurr1 decreased the proportion of TH⁺ cells compared to the single Nurr1 infection ($*p < 0.05$; Student's *t*-test). In both conditions, nearly all TH⁺ cells expressed MAP2ab and none expressed GFAP, indicative of

their neuronal phenotype. A small proportion of TH⁺ cells were labeled with GAD (a GABAergic marker). Scale bar (shown in B), A, B and E, F, 44 µm; C, D, 11 µm and G-J, 13.2 µm.

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Figure 7.

The effects of Nurr1 and Ngn2 on the expression of dopaminergic and GABAergic cell markers in neurons generated from OBSCs. OBSCs were coinfecting with viral particles expressing Nurr1-EGFP and Ngn2-EGFP or infected with viral particles expressing the Nurr1-EGFP vector. The neurospheres were then disaggregated and the cells cultured under conditions of differentiation for 7 days. The cells were triple labeled with antibodies against GFP, Nurr1, Ngn2, TH, GAD, or GABA, as indicated in the figure. The arrows point to the triple labeled cells. The graphs represent the mean percentage $\pm$ s.e.m. ($n = 4$ cultures per condition) of cells expressing the indicated markers relative to the total number of Nurr1⁺ cells or relative to the total number of Ngn2⁺ cells. ** $p < 0.01$, *** $p < 0.001$ (Student's t -test). Scale bar (shown in X), 97.2 μ m.

Figure 8.

The effects of Nurr1 and Ngn2 on the morphology and expression of synapsin-I and SV2 in TH1 neurons. OBSCs infected with the Nurr1-EGFP particles (A) or coinfecting with particles expressing Nurr1-EGFP and Ngn2-EGFP (B) were plated under differentiation conditions for 7 in vitro (DIV). Cells were double immunostained with antibodies against GFP and TH, and a morphological analysis was then performed on the TH⁺ cells using Image J software. The graphs represent the mean percentage $\pm$ s.e.m. ($n = 30$ cells per condition) of the number of primary neurites, neurite branches, and the total number of neurites per TH⁺ neuron (C), and the total neurite length (D). The coinfection of Ngn2 and Nurr1 decreased the number of neurites, while it increased the total neurite length compared to the single Nurr1 infection. ** $p < 0.01$; Student's t -test. Scale bar (shown in B), 35 μ m. OBSCs transduced with viral particles expressing Nurr1-EGFP (E-L) and Nurr1 plus Ngn2-EGFP (M-T) were plated in DMEM/F12/B27 plus 2% FBS supplemented with 20 ng/mL BDNF, 20 ng/mL GDNF and 0.5 mM dibutyryl-cAMP for 22 DIV. The cells were then triple immunostained with antibodies against TH, GFP and synapsin-I (E-H, M-P) or SV2 (I-L, Q-T). The arrows indicate the presence of synapsin-I⁺ and SV2⁺ puncta in TH⁺ neurons some of which are visualized in the insets at high magnification. Scale bar (shown in H), 32.4 μ m; insets, 13 μ m.

Figure 9.

TH⁺ neurons derived from Nurr1-transduced OBSCs have molecular characteristics of mature dopaminergic and dopaminergic-GABAergic neurons. OBSCs transduced with viral particles expressing Nurr1-EGFP were plated in DMEM/F12/B27 plus 2% FBS for 18–21 days supplemented with BDNF, GDNF, and cAMP. The cells were then triple immunostained with antibodies against TH, GFP, and VMAT2 (A–D), GIRK2 (E–H), DAT (I–L), calretinin (Calr; M–P), or VGAT (Q–T). The graph (U) represents the mean percentage $\pm$ s.e.m. ($n=4$ cultures per condition) of TH⁺ cells expressing the indicated markers relative to the total TH⁺ cell population. Note that the majority (55–77%) of TH⁺ neurons were also positive for VMAT2, Girk2, and DAT, and that 25% of the TH⁺ neurons were also positive for calretinin. An 18% of TH⁺ neurons expressed VGAT. Scale bar (shown in D), 38.1 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 10.

TH⁺ neurons derived from Nurr1-transduced OBSCs develop functional dopamine receptors and release dopamine. OBSCs transduced with viral particles expressing Nurr1-EGFP were plated for 21–24 days. The cultures were treated with 20 μ M (*R*)-(+)-SKF-38393 alone (A–D) or in combination with 20 μ M (–)-Quinpirole (E–H). Control cultures were treated with the vehicle (steril H₂O) (I–L). The cells were immunostained with antibodies against TH, c-Fos and GFP. The agonists induced c-Fos in TH positive cells, and these cells were counted to find the percentages $\pm$ s.e.m. of TH⁺ and GFP⁺ cells in which c-Fos was detected ($n = 8$ and 4 cultures for SKF and SKF 1 Quinpirole, respectively) (N). Scale bar (shown in D), 40.8 μ m. Basal and KCl- evoked dopamine release was measured after sorting the Nurr1-infected OBSCs which were then plated in cell culture during 21–24 days (M), using the Dopamine Research Elisa™ assay kit according to the manufacturer's instructions. The reaction was monitored at 450 nm. Quantification of samples was achieved by comparing their absorbance with a reference curve prepared with known concentrations of dopamine ($n = 17$; O). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1

Primers used for the Gene Profile Analyses of OBSCs by RT-qPCR

Gene	Primer (5'-3')	Length (bp)
<i>Bax</i>	Forward	115
	Reverse	
<i>Bcl2</i>	Forward	256
	Reverse	
<i>Ccnb2</i>	Forward	143
	Reverse	
<i>Ccnd1</i>	Forward	194
	Reverse	
<i>Ccnd2</i>	Forward	230
	Reverse	
<i>Ccnd3</i>	Forward	202
	Reverse	
<i>Cdkn1a (p21)</i>	Forward	199
	Reverse	
<i>Cdkn1b (p27)</i>	Forward	294
	Reverse	
<i>Egfp</i>	Forward	219
	Reverse	
<i>Fgf1</i>	Forward	188
	Reverse	
<i>Fgf2</i>	Forward	107
	Reverse	
<i>Gad</i>	Forward	180
	Reverse	
<i>Gapdh</i>	Forward	233
	Reverse	
<i>Neurog2</i>	Forward	392
	Reverse	

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Gene	Primer (5'-3')	Length (bp)
<i>Nr4a2 (Nurr1)</i>	Forward	180
	Reverse	
<i>p53</i>	Forward	122
	Reverse	
<i>Slc6a3 (Dat1)</i>	Forward	147
	Reverse	
<i>Th</i>	Forward	255
	Reverse	