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In vitro generation of mature dopamine neurons by decreasing and delaying the expression of exogenous Nurr1

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SUMMARY

Neural stem/progenitor cell (NSC/NPC) cultures can be a source of dopamine (DA) neurons for experimental and transplantation purposes. Nurr1, a steroid receptor transcription factor, can overcome the limitations associated with differentiation of cultured NPCs into DA neurons. However, forced Nurr1 expression in NPC cultures generates non-neuronal and/or immature DA cells. We show here that the Nurr1 level and period of expression crucially affect the differentiation and maturation of Nurr1-induced DA neurons. Mature DA neurons were generated by manipulating Nurr1 expression patterns to resemble those in the developing midbrain.

KEY WORDS: Parkinson's disease, Dopamine neurons, Nurr1, Neuronal maturation, Doxycycline inducible expression system

INTRODUCTION

In vitro differentiation of stem cells is essential for generating specific cell types for experimental and clinical use, and our understanding of developmental processes has aided manipulation of cell differentiation. Dopamine (DA) neurons in the midbrain are of particular interest because of their purported physiological and clinical significance in Parkinson's disease, schizophrenia and drug addiction. Genes involved in these cells' development have been manipulated in an attempt to induce differentiation of DA neurons from neural progenitor cells (NPCs) in vitro. *Nurr1*, a midbrain developmental gene, is regarded as the most promising candidate. Studies have shown that forced expression of Nurr1 efficiently induces the DA phenotype in differentiated NPCs; however, it yields non-neuronal or immature DA cells (Kim et al., 2003; Sakurada et al., 1999; Park et al., 2006b; Kim et al., 2007; Sonntag et al., 2004). Nurr1 expression in the developing ventral midbrain (VM) is initiated during the DA progenitor stage and is tightly controlled within set physiological limits (Ang, 2006). In previous studies, Nurr1 expression was forced in proliferating NPCs, without considering its physiological expression pattern. By contrast, we developed an efficient doxycycline-inducible retroviral vector system, which enables tight control of the levels and the timing of transgene expression in cultured NPCs. We show here that manipulating exogenous Nurr1 expression patterns to match those of the developing VM generates morphologically and phenotypically mature DA neurons that exhibit presynaptic dopamine functions in vitro.

MATERIALS AND METHODS

Construction of doxycycline-inducible retroviral vectors

A novel doxycycline-inducible retroviral vector, pCURT, was constructed by engineering the following DNA fragments into the retroviral vector pCL (Park et al., 2008): (1) the tetracycline-responsive promoter (pTRE)

upstream of the multiple cloning site (MCS); (2) reverse tetracycline-controlled transactivator 3 (rtTA3) under the human ubiquitin promoter (pUbC); and (3) the red fluorescent protein (mCherry) gene under the LTR promoter. Another vector, pCURT5, was constructed by inserting the 5' untranslated region (5'-UTR) of the mouse CREB gene (−147 to −1) between pTRE and the MCS. A schematic drawing of these GFP-fusion vectors is shown in supplementary material Fig. S3A.

NPC cultures and retroviral transduction

NPCs were cultured as previously described (Park et al., 2008). NPCs derived from the cortex of rat embryos at embryonic day (E) 14 were grown in vitro for 3–4 days using basic fibroblast growth factor (bFGF; 20 ng/ml) and then passaged. After subsequent bFGF-mediated NPC expansion, the cells were transduced with retroviruses expressing Nurr1 (pCL-Nurr1, pCURT5-Nurr1) or GFP (pCURT-GFP, pCURT5-GFP) at multiplicities of infection (MOI) of 5–50, as previously described (Bae et al., 2009). Cell differentiation was induced one day after infection by withdrawal of bFGF for 7–15 days. Conversely, NPC cultures that express Nurr1 endogenously and give rise to DA neurons were derived from rat VM at E12. After in vitro expansion the VM-NPCs were differentiated without passage.

Immunocytochemical analysis

Immunofluorescent staining of cultured cells or embryonic VM tissue sections (25 µm) was performed using primary antibodies, as previously described (Park et al., 2006a) (supplementary material Table S1). Between 30 and 50 anti-Nurr1-stained cells were randomly selected from at least three independent cultures, and their fluorescence intensities were determined by LAS image analysis (Leica). Fiber lengths of tyrosine hydroxylase-positive (TH+) DA cells were determined as described previously (Park et al., 2006b).

Additional experiments

Real-time PCR (RT-PCR), dopamine uptake and release assays, electrophysiological analyses, cell counting and statistical analyses were performed as previously described (Rhee et al., 2011).

RESULTS AND DISCUSSION

Supraphysiological levels of Nurr1 in cultured NPCs generate immature DA cells

E14 cortical neural progenitor cells (NPC) cultures express little or no endogenous Nurr1 and do not differentiate into DA neurons (data not shown). Following transduction with Nurr1-expressing retroviruses (25 MOI), 50–70% of these cells began expressing TH, a marker specific to DA neurons, six days after in vitro differentiation (day 7 post-transduction). However, the TH+ cells

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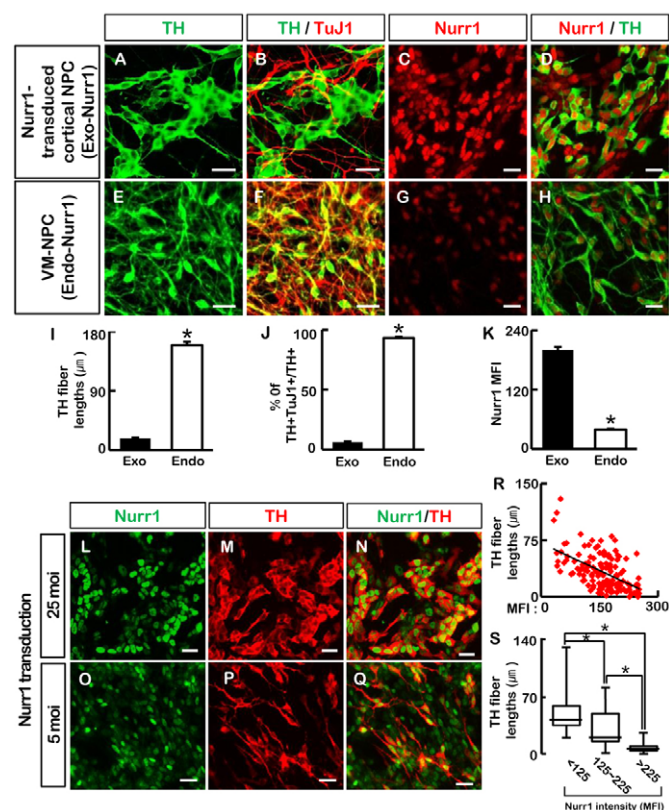


Fig. 1. The level of Nurr1 expression in NPCs determines the neuronal maturity of Nurr1-induced DA cells. (A–K) Differences in neuronal maturity and Nurr1 levels in TH+ cells induced by exogenous and endogenous Nurr1. Cortical NPCs were transduced with Nurr1 retroviruses (pCL-Nurr1, 25 MOI) and differentiated for six days. Neuronal differentiation of TH+ cells induced by exogenous Nurr1 (Exo, A–D) was compared with endogenous Nurr1 in E12 VM-NPC cultures (Endo, E–H) in terms of TH+ fiber lengths (I) and percentage of TH+TuJ1+ cells in total TH+ cells (J). Exogenous and endogenous Nurr1 levels were quantified by the mean fluorescence intensities (MFI) of individual anti-Nurr1-stained cells (K). (L–S) Negative correlation between Nurr1 expression and morphological maturity of Nurr1+TH+ cells. Cortical NPCs were transduced with higher (25 MOI) and lower (5 MOI) Nurr1 virus titers. Seven days after differentiation, cells were stained for Nurr1 and TH (L–Q). Each dot in R represents the Nurr1 intensity (MFI) and TH+ fiber length (μm) of a single Nurr1+TH+ cell. * $P < 0.01$. Scale bars: 50 μm.

were either undifferentiated or morphologically immature without extensive neurite outgrowths (Fig. 1A,D,I), and only some expressed the neuron-specific marker TuJ1 (Fig. 1B,J). Formation of immature DA cells had been reported in virtually all previous gain-of-function studies of Nurr1 in primary NPC cultures (Kim et al., 2003; Sakurada et al., 1999; Park et al., 2006b; Kim et al., 2007; Sonntag et al., 2004). By contrast, we obtained populations enriched for TH+ cells (5–15% of all cells) from NPCs derived at E12 from naive VM tissue, where DA neurons are generated in vivo (Fig. 1E,H) (Studer et al., 1998; Lee et al., 2003). These TH+ cells, derived without Nurr1 transduction, were morphologically mature with multiple and extensive outgrowths (Fig. 1E,H,I), and virtually all expressed TuJ1 (Fig. 1F,J). Within the differentiated E12 VM-NPC cultures, endogenous expression of Nurr1 was localized to TH+ DA neurons (Fig. 1G,H). Combined with the known role of Nurr1 as a transcription factor for the DA phenotype

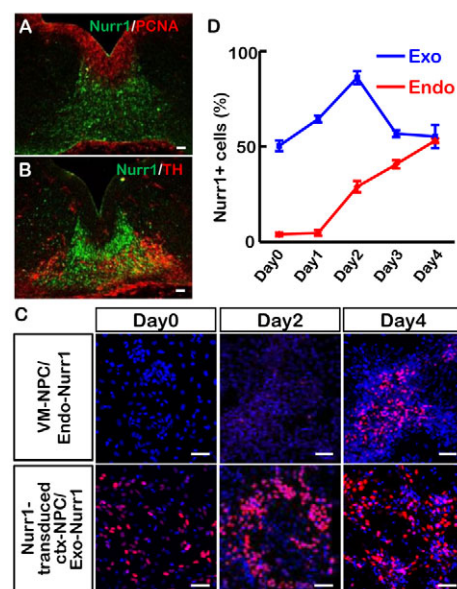


Fig. 2. Nurr1 expression in the developing VM and in vitro VM-NPC cultures. (A,B) Nurr1 expression localized in the intermediate and mantle zones of rat VM (E14). E14 VM slices were immunostained for Nurr1 and PCNA (A) or Nurr1 and TH (B). PCNA, proliferating cell-specific nuclear antigen. (C) Nurr1 expression in E12 VM-NPC cultures (Endo; upper panels) and Nurr1-transduced E14 cortical NPC cultures (Exo; lower panels) on in vitro differentiation day 0 (last day of proliferation) to day 4. (D) Percentage of Nurr1+ cells in total cells. Error bars represent s.e.m. Scale bars: 100 μm.

in the developing VM (Sakurada et al., 1999), these findings suggest that the in vitro generation of DA neurons from VM-NPCs is induced by endogenous Nurr1. When we compared Nurr1 levels in individual cells by immunofluorescence, the mean fluorescence intensities (MFI) were much greater in the Nurr1-transduced cultures (25 MOI) than in the endogenous Nurr1 cells (Fig. 1C,G,K). To determine whether Nurr1 levels affect neuronal differentiation of TH+ cells, we transduced cortical NPCs with a reduced Nurr1 viral titer (5 MOI) and found that the MFI of the Nurr1-transduced cells was comparable to that of the endogenous Nurr1 cells (30.95 ± 1.32 and 39.02 ± 1.49 MFI, respectively). Greater neurite outgrowth was observed in the TH+ cells in cultures transduced with the lower rather than the higher Nurr1 titers (Fig. 1L–S). In individual Nurr1+TH+ cells, a negative correlation was observed between Nurr1 intensity and TH+ neurite length (Fig. 1R,S). Impaired neuronal differentiation of the cells expressing high levels of Nurr1 was also confirmed by the absence of TuJ1 (Bae et al., 2009) (supplementary material Fig. S1A–C). This Nurr1-dependent effect was not specific to cortical NPCs, as it was also observed in NPCs from other brain regions (supplementary material Fig. S2). These findings suggest that Nurr1 expression is crucial for differentiation and maturation of TH+ DA cells, and that the immaturity of Nurr1-induced DA neurons can be overcome by reducing Nurr1 levels.

Endogenous Nurr1 expression in late VM precursor cells

In the developing VM, Nurr1 expression was not detected in the proliferating ventricular zone, but in the intermediate to mantle zones (Fig. 2A,B) (Perlmann and Wallen-Mackenzie, 2004),

suggesting that Nurr1 expression begins during the late precursor cell stage. A similar Nurr1 expression pattern was observed in the E12 VM-NPCs cultures in vitro: Nurr1 was not detected during the proliferation period but began to appear two days after differentiation (Fig. 2C, upper panels, and 2D). Approximately 0.5–1 day after Nurr1 expression began, TH appeared in a subpopulation of Nurr1-positive (Nurr1+) cells in the VM-NPC cultures (data not shown). This sequential expression pattern resembles that of the mouse embryonic VM, where Nurr1 expression starts at E10 and TH expression begins at E11 (Saucedo-Cardenas et al., 1998). By contrast, in our and other gain-of-function studies of Nurr1 in vitro, Nurr1 expression was initiated in the highly proliferating NPCs, in which high levels of Nurr1 were detectable from the early differentiation period (Fig. 2C, lower panels, and 2D). Therefore, using an inducible vector system, we sought to determine whether Nurr1 expression during the early period is associated with immature neuronal differentiation of Nurr1-induced DA cells.

Development of an efficient doxycycline-inducible expression system

We developed pCURT, a novel doxycycline-inducible retroviral vector. Expression of the reporter (GFP) engineered into pCURT (pCURT-GFP) was tightly controlled by doxycycline (supplementary material Fig. S3). However, the doxycycline-induced GFP levels were still weak and restricted to a small proportion of NPCs (supplementary material Fig. S3B,C). One function of the 5'-UTR region of mRNA is to increase protein translation (Falcone and Andrews, 1991). When the 5'-UTR sequence from CREB was inserted directly after the pTRE promoter (pCURT5; supplementary material Fig. S3A), exogenous expression of GFP increased markedly over a range of doxycycline concentrations (supplementary material Fig. S3B,C). Moreover, withdrawal of doxycycline efficiently extinguished exogenous GFP expression in both vector systems (supplementary material Fig. S3D). Based on these findings, we used the pCURT5 vector system in subsequent Nurr1 experiments.

Generation of mature DA neurons by delaying and lowering Nurr1 expression in vitro

Proliferating cortical NPCs were transduced with the Nurr1-expressing pCURT5 (pCURT5-Nurr1; Fig. 3A), and cell differentiation was induced one day after transduction. Exogenous Nurr1 expression began within 24 hours of the doxycycline treatment (1 $\mu\text{g}/\text{ml}$; Fig. 3B, right) and TH+ DA cells were readily generated by the doxycycline-induced Nurr1 expression (supplementary material Fig. S4). Nurr1 expression continues in adult midbrain DA neurons and is thought to play a role in their maintenance (Kadkhodaei et al., 2009). The TH+ cells disappeared within five days of the end of doxycycline treatment (supplementary material Fig. S4C,D), which supports the role of Nurr1 in maintaining DA phenotypes. Nurr1 levels were readily controlled by the selected doses of doxycycline (Fig. 3C). Using this inducible system we confirmed the negative correlation between Nurr1 levels and the length of TH+ fibers again in cultures treated with different doses of doxycycline (supplementary material Fig. S5). In addition, following cell differentiation, TuJ1 was present in a lower proportion of transduced (mCherry+) cells in cultures that had been treated with a higher dose of doxycycline (2 $\mu\text{g}/\text{ml}$ versus 0.5 $\mu\text{g}/\text{ml}$) (supplementary material Fig. S6). Interestingly, the impaired TuJ1 expression recovered when

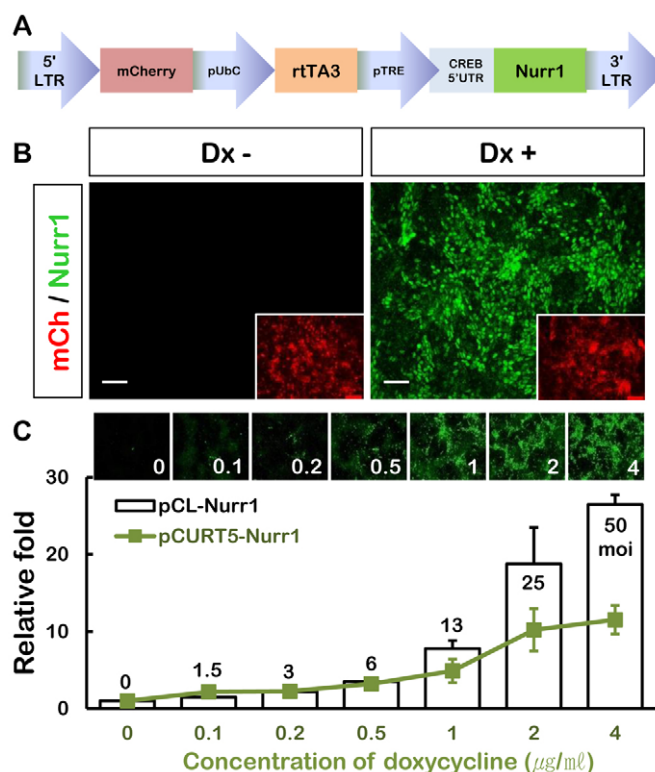


Fig. 3. Manipulation of exogenous Nurr1 expression with the doxycycline-inducible retroviral vector pCURT5. (A) Schematic of the pCURT5 vector expressing Nurr1 (pCURT5-Nurr1). The mCherry gene used for determination of MOI is driven by the LTR promoter. (B) Efficient induction of the Nurr1 transgene by doxycycline treatment. E14 cortical NPCs were transduced with pCURT5-Nurr1, cultured in the absence (left) or presence (right) of doxycycline (1 $\mu\text{g}/\text{ml}$) for three days, then stained for Nurr1. Inset: red fluorescence showing mCherry expression in the transduced cells. Scale bars: 100 μm . (C) Doxycycline dose-dependent expression of transgenic Nurr1. Nurr1 mRNA expression in the cultures treated with 0–4 $\mu\text{g}/\text{ml}$ doxycycline was quantitatively determined using RT-PCR (green). Upper panels show representative images of anti-Nurr1-stained cells at various doxycycline concentrations. Bars in C represent Nurr1 mRNA expression levels induced by the original retroviral vector system (pCL-Nurr1), with the titers indicated. Error bars represent s.e.m.

doxycycline was withdrawn (supplementary material Fig. S7), indicating that the Nurr1-mediated inhibitory effect on neuronal differentiation is reversible.

Next, we examined the effect of Nurr1 expression timing on the neuronal maturity of Nurr1-TH+ cells using two doxycycline treatment schedules: (a) 1 $\mu\text{g}/\text{ml}$ throughout the differentiation period (+7), and (b) 1 $\mu\text{g}/\text{ml}$ starting two days after differentiation (–2/+5). Delaying Nurr1 expression was intended to reflect the physiological patterns of Nurr1 expression observed in the developing VM and in VM-NPC cultures (Fig. 2). Nurr1 expression on the final day of differentiation was not significantly different in the two doxycycline treatments (data not shown). However, the morphological maturity of TH+ cells, estimated by measuring fiber lengths on differentiation day 7, was considerably greater in the cultures in which Nurr1 expression was delayed (–2/+5) than in those that expressed Nurr1 throughout (+7) (total fiber length in a TH+ cell = $60.03 \pm 4.77 \mu\text{m}$ vs $24.58 \pm 2.95 \mu\text{m}$,

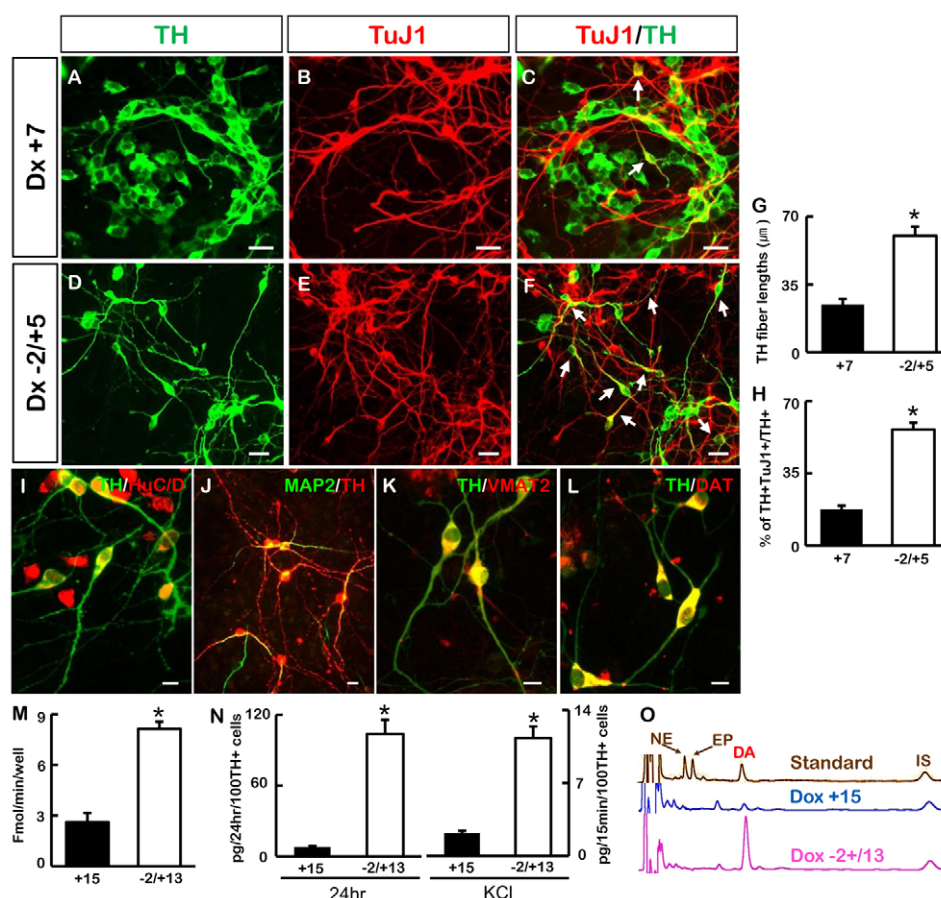


Fig. 4. Generation of mature DA neurons by delaying Nurr1 expression. E14 cortical NPCs transduced with pCURT5-Nurr1 were differentiated using two different doxycycline treatments: 1 μ g/ml during the entire seven- or fifteen-day differentiation period (+7 or +15) and 1 μ g/ml doxycycline starting two days after differentiation (–2/+5 or –2/+13). (A–H) After staining with TH (A,D) and TuJ1 (B,E), the neuronal maturity of the Nurr1-induced TH+ cells was estimated from TH+ fiber lengths (G) and TH+TuJ1+ cells in total TH+ cells (H). Arrows indicate TH/TuJ1 co-expressing cells. (I–L) Mature DA neuronal phenotypes of the TH+ cells generated by the delayed doxycycline treatment (Nurr1 expression) were assessed further by colocalization of mature neuronal (I,J) and DA neuronal (K,L) markers. Presynaptic DA neuronal function was assessed by DA uptake (M) and DA release assays (N,O). For data shown in M, DAT-mediated specific DA uptake calculated by subtracting nonspecific uptake (with nomifensine) from total uptake. For data shown in N, DA levels estimated in the medium conditioned for 24 hours (left) and induced by 56 mM KCl for 30 minutes (right). O shows typical HPLC chromatograms for DA released for 24 hours (lower and middle traces) along with that of standard mixture (upper trace). EP, epinephrine; NE, norepinephrine. An internal standard (3,4-dihydroxybenzylamine) was used for quantification of DA concentrations. * $P < 0.01$, $n = 3$ (M) and $n = 4$ (N) independent cultures. Error bars represent s.e.m. Scale bars: 50 μ m (A–F); 20 μ m (I–L).

respectively, $n = 40$ cells per group, $P < 0.01$; Fig. 4A,D,G). Furthermore, a greater proportion of TH+ cells expressed TuJ1 in the cultures in which doxycycline treatment was delayed (percentage of TH+TuJ1+ cells in total TH+ cells = $56.22 \pm 3.33\%$ vs $17.59 \pm 1.77\%$, respectively, $n = 3$ independent cultures, $P < 0.01$; Fig. 4A–F,H). These findings indicate that the Nurr1 expression period is another crucial factor in DA neuron maturation. In our tests, the delayed doxycycline schedule (1 μ g/ml, –2/+5) yielded the best results. Under these conditions, the TH+ cells co-expressed mature neuronal markers (HuC/D, MAP2) and mature dopamine neuronal markers (VMAT2, DAT) (Fig. 4I–L). In addition, presynaptic DA neuron function was much greater in cultures with delayed Nurr1 expression, as measured by DAT-mediated dopamine uptake (Fig. 4M), DA release (Fig. 4N,O) and electrophysiological single-cell recording (supplementary material Fig. S8). These findings collectively suggest that mature functional presynaptic DA neurons are generated when the level and timing of recombinant Nurr1 expression are controlled.

In our study, high levels of Nurr1 expression in the early-stage NPCs yielded DA cells lacking neuronal shapes and phenotypes, suggesting that Nurr1 inhibits neuronal differentiation in a cell-autonomous manner. Given the role of Nurr1 as an epigenetic repressor (Saijo et al., 2009), and the crucial effects of pro-neuronal genes (such as the bHLH family) on neuron development (Ross et al., 2003), strong Nurr1 expression probably represses pro-neuronal factors and/or their binding to the promoters of neuronal phenotype genes via an epigenetic control mechanism. In addition to its intrinsic inhibitory role, Nurr1 can extrinsically promote neuronal differentiation of NPCs by inducing secretion of multiple neurotrophic factors (Bae et al., 2009; Volpicelli et al., 2007). As these two opposite actions of Nurr1 co-exist in Nurr1-transduced cultures, their neuron yields are varied, depending on the virus titer (supplementary material Fig. S1D–J) (Bae et al., 2009).

Decreased and delayed Nurr1 expression was crucial for neuronal differentiation and maturation of the Nurr1-TH+ cells; however, it also decreased the DA neuron yield (supplementary

material Fig. S9). In addition, exogenous Nurr1 failed to induce markers specific to the midbrain (Lee et al., 2010). These issues could be addressed by co-expressing Foxa2, known to induce the expression of numerous midbrain-specific genes and facilitate Nurr1-induced DA neuron differentiation (Lee et al., 2010). As Foxa2 has a different expression pattern from Nurr1 during midbrain development, this strategy requires the development of another efficient inducible system, in which the expression of multiple genes could be controlled simultaneously but independently, based on their individual developmental patterns. Despite these remaining concerns, our study is of value as the first work demonstrating complete development of NPCs into fully mature DA neurons with presynaptic function, by manipulating the levels and timing of expression of a single Nurr1 gene to match the physiological conditions observed in the developing brain. This concept could be applied more widely to generate other biologically relevant cells in vitro.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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