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Orphan nuclear receptor Nurr1 induces neuron differentiation from embryonic cortical precursor cells via an extrinsic paracrine mechanism

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ABSTRACT

Nurr1 is an orphan nuclear receptor-type transcription factor (TF) that plays critical roles in midbrain dopamine neuron development. This study demonstrated a novel role for Nurr1 in neuronal/astrocytic differentiation of neural precursor (NP) cells isolated from rat embryonic cortices: overexpression of this TF promoted NP cell differentiation towards neurons at the expense of astrocytic differentiation. Single cell-based lineage analyses and experiments using co-cultures revealed that Nurr1 elicited its neurogenic role in an extrinsic paracrine manner. We defined diffusible factors and downstream neurogenic TFs responsible for the Nurr1-mediated neuronal differentiation.

1. Introduction

Nurr1 (NR4A2) is a transcription factor (TF) in the orphan nuclear receptor family [1]. Analyses of Nurr1-null mice have revealed the roles of this TF in the generation [2,3], maturation, migration, striatal target innervation, and survival [3,4] of dopamine (DA) neurons in the developing midbrain. However, Nurr1 expression is not confined to the developing midbrain, but is also detected in various embryonic brain regions along the neuroaxis, indicating that this TF has roles outside of midbrain DA neuron development [5]. Gain-of-function studies in cell cultures in vitro have revealed Nurr1 roles in cell cycle arrest and morphological maturation of neuronal cells [6,7]. Furthermore, transcriptosome analyses revealed that Nurr1 targets genes involved in axonal outgrowths and guidance, general cell survival and neuron differentiation [8–12], suggesting a broad spectrum of Nurr1 roles in brain development. The purpose of this study was to gain insights into Nurr1-induced differentiation effects in the CNS. We demonstrated that Nurr1 enhances neuron formation from neural precursor (NP) cells isolated from rat fetal cortices, at the expense of astroglial differentiation. The Nurr1-induced effect on precursor cell differentiation occurred by means of a paracrine mechanism via secreting diffusible factors. Furthermore, we showed Nurr1-specific induction of diffusible neurogenic factor expression, with a concomitant decrease in astrogenic cytokines, which may underlie a mechanism for the Nurr1 function.

2. Materials and methods

2.1. Primary CNS precursor culture

NP cells were cultured as described previously [13–15]. Briefly, brain tissue dissected from rat cortices on embryonic day 14 (E14) was mechanically triturated and plated on 10-cm culture dishes pre-coated with 15 μg/ml poly-*L*-ornithine (PLO; Sigma, St. Louis, MO)/1 μg/ml fibronectin (FN; Sigma). NP cells were allowed to proliferate by the mitogenic action of basic fibroblast growth factor (bFGF, 20 ng/ml, R&D Systems, Minneapolis, MN) in serum free N2 medium up to 60–80% cell confluency. To obtain a uniform population of NP cells, clusters of cells were passaged by dissociating them into single cells and plating them onto freshly prepared

Abbreviations: BDNF, brain-derived neurotrophic factor; CM, conditioned medium; CNTF, ciliary neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; LIF, leukemia inhibitory factor; Ngn2, neurogenin2; NP, neural precursor; NT3, neurotrophin 3; NT4/5, neurotrophin 4/5; PFA, paraformaldehyde; TF, transcription factor; TuJ1, tubulin βIII

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PLO/FN-coated coverslips (12 mm diameter; Marienfeld GmbH & Co., KG, Lauda-Konigshofen, Germany) or 6-cm dishes. Upon reaching 50–60% cell confluency by an additional bFGF-mediated proliferation (usually for 1–2 days), cells were subjected to retroviral transductions as described below. On the day following transduction, cell differentiation was induced by withdrawing bFGF from the medium. In some experiments, cells were treated with brain-derived neurotrophic factor (BDNF, 200 ng/ml, R&D systems), glial cell line-derived neurotrophic factor (GDNF, 200 ng/ml, R&D Systems), neurotrophin 3 (NT3, 200 ng/ml; ProSpec-Tany TechnoGene, Rehovot, Israel), or K-252a, an inhibitor of protein kinases activated by Trk-mediated signals (100 nM; Calbiochem, Darmstadt, Germany). Cultures were maintained at 37 °C in a 5% CO_2 incubator, and the media were changed every other day with or without daily supplementation with bFGF.

2.2. Retroviral transduction

NP cells were transduced with retroviruses expressing Nurr1 (or LacZ: control) at viral titers of 6.25–25 multiplicity of infection (MOI). Details of retroviral production and transduction are described in Supplementary data.

2.3. Co-cultures and conditional medium treatments

Untransduced NP cells were co-cultured with Nurr1- or LacZ (control)-transduced NP cells using a co-culture unit depicted in Fig. 3a. Media were collected from E14 cortical NP cell cultures transduced with Nurr1- and LacZ during 3 days of differentiation without medium changes. The conditioned medium (CM) was filtered at 0.42 μm and kept at −70 °C until use.

2.4. Clonal assay

For clonal-density cultures, cells were plated at 3000 cells per 10-cm dish. Isolated single cells were marked on the bottom of the plates with a 3-mm circle and were induced to proliferate with bFGF for 5 days. Only the clusters of cells that grew within the marked circles were referred to as clones and included in analyses. Differentiation phenotypes of clones were determined after 5 days of differentiation.

2.5. Cell counting and statistical analysis

Cell counting was performed in 10–40 randomly chosen microscopic fields for each coverslip, using an eyepiece grid at a final magnification of 200 or 400. One to three cultured coverslips were analyzed in each experiment and 2–4 independent experiments were carried out. Statistical analyses were made by one-way ANOVA (SPSS12.0; SPSS Inc., Chicago, IL).

Methods for immunochemical, immunoblot, RT-PCR and morphometric analysis for neurons are described in Supplementary data.

3. Results and discussion

3.1. Nurr1 promotes neuronal differentiation at the expense of astrocyte differentiation in vitro

Previous studies have suggested that Nurr1 may function in precursor cell differentiation during brain development. Findings have included Nurr1 effects in cell cycle arrest [6], neuron formation [7] and neurite outgrowths [6]. These effects have not been

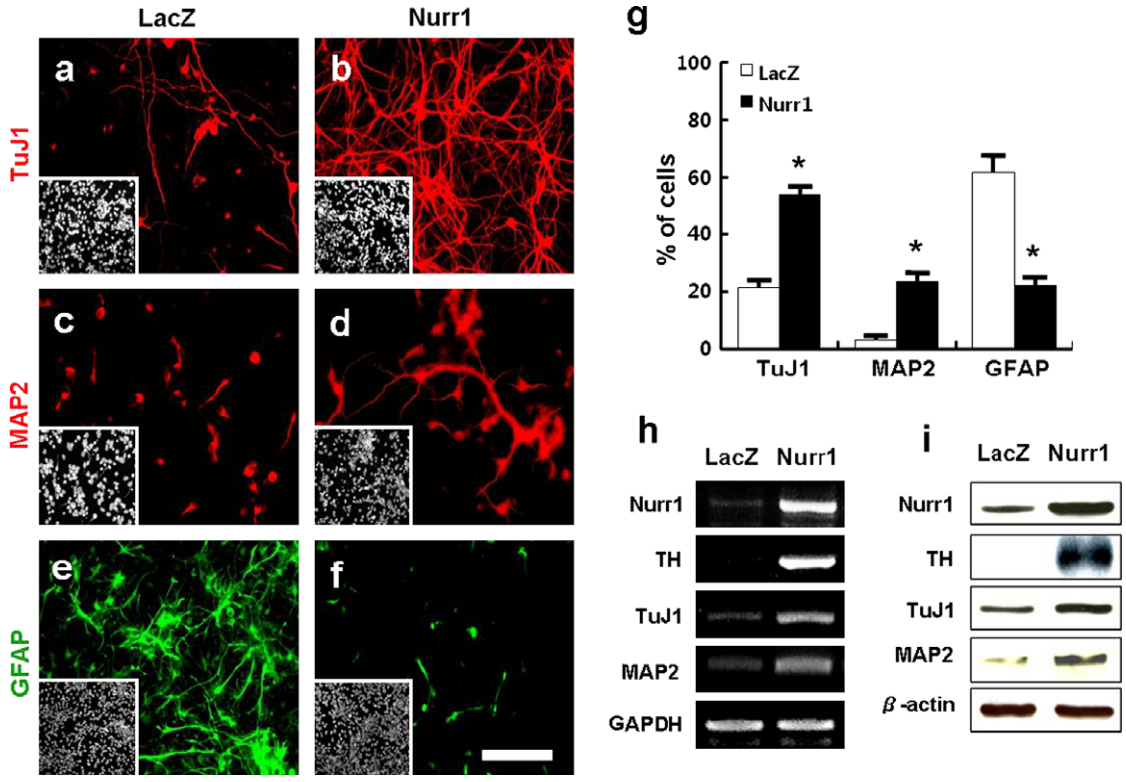

Fig. 1. Nurr1-induced neurogenesis from NP cells in vitro. NP cells were cultured from embryonic rat cortices at E14, and transduced with Nurr1 or LacZ (control). Differentiation phenotypes of the precursor cells were determined at 3 days of in vitro differentiation by immunocytochemical (a–g), RT-PCR (h), and immunoblot (i) analyses for the markers specific to neuron (TuJ1, MAP2) and astrocyte (GFAP). Shown in (a–f) are representative images for cells immunoreactive to TuJ1 (a, b), MAP2 (c, d) and GFAP (e, f). Insets, DAPI nuclear staining of the same field. Scale bar, 40 μm. Graph (g) depicts % immunoreactive cells out of total cells. *Significantly different from LacZ-transduced controls at $P < 0.001$, for each value, $n = 30–40$ microscopic fields from 2 independent experiments. Induction of Nurr1 expression promoted cortical precursors to differentiate towards neurons at the expense of astrocytic differentiation.

systematically investigated. To gain insights into Nurr1-induced differentiation effects in the CNS, we performed gain-of-function experiments for Nurr1 in NP cells derived from rat embryonic cortices. More than 95% of cells in the cultures were nestin+/Ki67+ proliferating neural precursor cells, and <3% were positive for the differentiated neuron-specific marker, tubulin βIII (TuJ1); none of the cells were positive for the astrocytic marker, glial fibrillary acidic protein (GFAP) [16,17]. In the cortical NP cultures, 3.7 ± 0.5% of cells endogenously expressed Nurr1, and the Nurr1-expressing cell population was increased to 80–90% of total cells by retroviral infection with viruses expressing Nurr1 (Fig. 2k–n). Three days after differentiation, 21.1 ± 2.1% of total cells were positive for the neuronal marker TuJ1 in the control (lacZ-transduced) cultures derived from rat embryonic cortices. Induction of Nurr1 expression in the cortical precursors enhanced TuJ1+ neuron yield by 2–3-fold (Fig. 1a, b, and g). A similar increase in the numbers of cells positive for microtubule-associated protein 2 (MAP2), a protein specific to mature neurons, occurred when Nurr1 was overexpressed (3.1 ± 0.7% in the control versus 23.5 ± 2.4% in Nurr1-transduced cultures; Fig. 1c, d, and g). The Nurr1-induced neuron differentiation was further confirmed by enhanced mRNA and protein levels of those neuronal markers (Fig. 1h and i). We also measured decreases in GFAP+ astrocyte numbers in the Nurr1-transduced cultures (GFAP+ cells: 21.4 ± 3.2% in Nurr1-transduction versus 64.2 ± 4.6% in LacZ-transduced cultures; Fig. 1e–g), indicating that Nurr1 promotes cortical precursor cell differentiation toward a neuronal lineage at the expense of astroglial differentiation. The numbers of total viable cells and cells with apoptotic nuclei were not significantly altered by Nurr1 transduction under the culture conditions used (e.g., 2.3 ± 0.6% apoptotic nuclei in LacZ- versus 2.2 ± 0.3% in Nurr1-transduced cultures), indicating that cell survival make a negligible contribution to the Nurr1 effect.

Consistent with a previous study [6], morphological maturation as estimated by neurite outgrowths of TuJ1+ neuronal cells was greater in the cultures transduced with Nurr1 (Supplementary Fig. S1): total TuJ1+ fiber lengths per cell were 540.8 ± 17.8 μm in Nurr1- and 120.1 ± 6 .07 μm in control cultures (n = 150 cells for each value, significantly different at $P < 0.001$).

3.2. Nurr1 exerts its neurogenic effect in a dose dependent manner

As previously demonstrated [13,14], Nurr1 overexpression efficiently induced expression of the DA neuron marker tyrosine

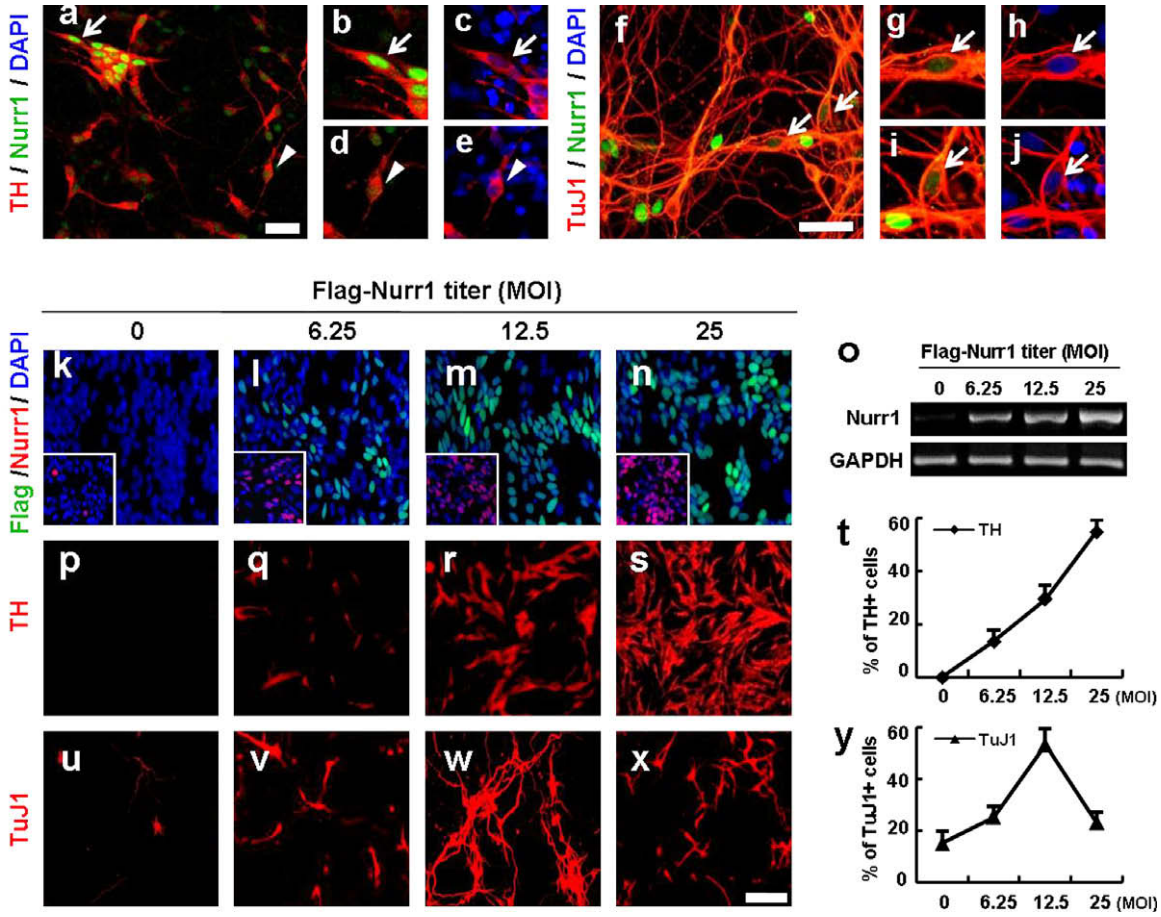

Fig. 2. Differences in Nurr1-induced generation of TuJ1+ neurons versus TH+ cell generation. (a–j) Difference in Nurr1 expression pattern in the cells expressing TH (a–e) and TuJ1 (f–j). The cells indicated by arrow and arrowhead in (a) are further illustrated in panels (b, c), and (d, e), respectively. Panels (c) and (e) are TH/DAPI-stained images in fields identical to (b) and (d), respectively. (f–j) Images from TuJ1/Nurr1 (f, g, i) and TuJ1/DAPI (h, j)-staining. The cells indicated with arrows in (f) are representative TuJ1+/Nurr1+ cells, in which intensities of Nurr1 immunoreactivity were relatively weak. None of the cells with strong Nurr1 immunoreactivity in image (f) was positive for TuJ1. Scale bar, 20 μm. (k–y) Distinct Nurr1-dose effects in the generations of the cells expressing TH (p–t) and TuJ1 (u–y). Cortical precursor cells were transduced with over the Nurr1 (Flag-tagged) virus at titers of (0–25 MOI), and subjected to immunostaining against TH and TuJ1 3 days after differentiation. Exogenous Nurr1 protein levels were determined by immunostaining for Flag and Nurr1 (insets) in panels (k–n). Protein (k–n) and mRNA (o) levels of exogenous Nurr1 were determined. Panels (k–n) are representative images for Flag+ or Nurr1+ (insets) cells at different viral titers. %Flag+ cells of total DAPI+ cells were 49%, 73%, and 87% at 6.25, 12.5, and 25 MOI, respectively. Shown in (p–s) and (u–x) are representative images of TH+ cells and TuJ1+ cells, respectively, at the viral titers indicated. Scale bar, 40 μm. The yields of TH+ and TuJ1+ cells over the Nurr1 viral titers are depicted in (t) and (y).

hydroxylase (TH) in cortical precursor cell cultures (Figs. 1h & i and 2a, p–t). Nurr1 immunoreactivity was localized in virtually all the TH+ cells generated (Fig 2a), indicating an intrinsic, cell-autonomous mechanism in Nurr1-induced TH expression. By contrast, only subpopulations of TuJ1+ cells were positive for Nurr1 in Nurr1-transduced cultures, and intensities of Nurr1 immunoreactivity in the Nurr1+/TuJ1+ neurons were relatively weaker (Fig. 2f–j). These findings suggest dose dependency in Nurr1-induced neuron formation, in which a high dose of Nurr1 inhibits neuron formation in vitro. These results prompted us to examine the yields of TuJ1+ cell in comparison to TH+ cells at various Nurr1 viral titers. TH+ cell yields were always greater at higher Nurr1 titers, at least in the range of titers tested (0–25 MOI) (Fig. 2p–t). TuJ1+ cell yields were enhanced by increasing Nurr1 titers up to 12.5 MOI (Fig. 2u–y). However, the neuronal yield greatly decreased at the highest Nurr1 dose tested (25 MOI). In addition, TH+ cells with strong Nurr1 immunoreactivity were mostly flat and immature or non-neuronal types of cells, while TH+ cells with weak Nurr1 staining intensities had a neuron-like shape with neurite outgrowths (Fig. 2a–e). These findings collectively suggest that low or moderate levels of Nurr1 expression increase neuron formation, but high doses of Nurr1 can intrinsically inhibit neuron differentiation from cortical precursor cells. All the following experiments were performed using a Nurr1 viral titer of 12.5 MOI, unless otherwise specified.

3.3. Extrinsic paracrine factor-mediated mechanism in Nurr1-induced neuronal differentiation

There are two potential explanations for why only minor populations of TuJ1+ cells showed Nurr1 immunoreactivity. First, TuJ1+ neurons may have been intrinsically generated from the precursors expressing low levels of Nurr1, but the exogene expression was too low to be detected with the sensitivity of current Nurr1 immunostaining. Another possibility is that Nurr1-mediated neuron formation occurs in an extrinsic manner, in which Nurr1-expressing cells facilitate neuronal differentiation of neighboring precursors via

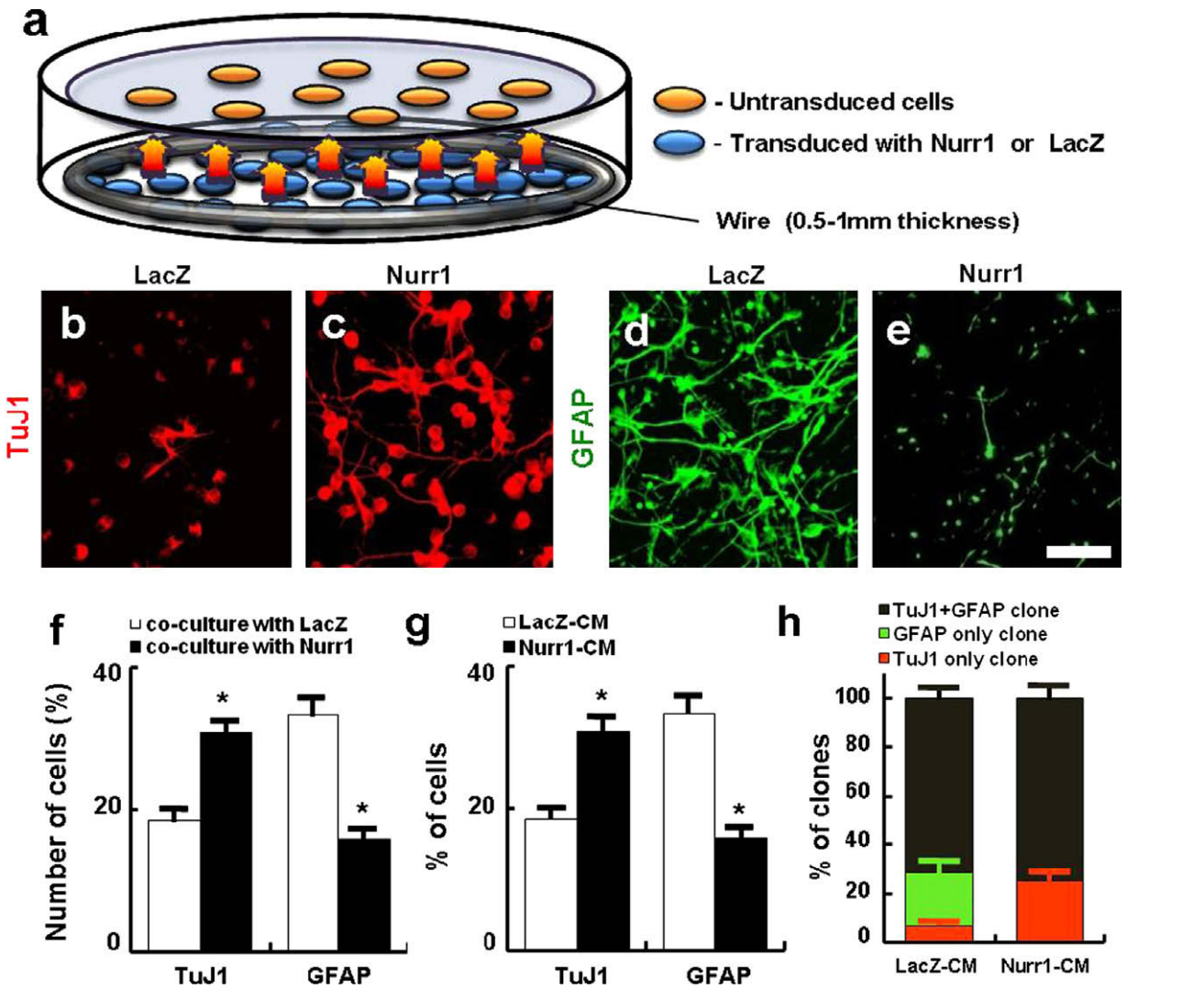

Fig. 3. A paracrine mechanism underlies Nurr1-induced precursor differentiation. (a–f) Co-culture experiments. (a) Schematic drawing of the co-culture system. Untransduced precursor cells (orange in (a)) were co-cultured with the precursors transduced with Nurr1 or LacZ (control) (blue in (a)). Neuron/astrocyte yields of the untransduced cells were determined 3 days after differentiation. Shown in (b–d) are representative images of TuJ1+ (b, c) and GFAP+ (d, e) cells derived from the untransduced precursors co-cultured with LacZ (b, d)- and Nurr1 (c, e)-transduced precursor cells. Scale bar, 20 μm. (g, h) Experiments with CM treatments. The media were conditioned in the cultures transduced with Nurr1 (Nurr1-CM) or LacZ (LacZ-CM, control) during precursor differentiation as described in 'Section 2'. Cortical precursor cells plated at a normal (30,000 cells/cm^2; (g)) and clonal (3000 cells/10 cm dish; (h)) densities were differentiated in the medium supplemented with Nurr1-CM or LacZ-CM (1:1, v:v). Differentiation phenotypes were determined 3 days after differentiation based on % TuJ1+ or GFAP+ cells of total DAPI+ cells (g) and the numbers of clones containing TuJ1+ neuron only, GFAP+ astrocyte only, and mixed neurons and astrocytes (h). *Significantly different from untreated control at $P < 0.001$.

cell-cell contact-mediated signals or secretion of paracrine factors. To investigate the mode of the Nurr1-induced neuron formation, we performed single cell-based lineage selection analyses in cultures plated at clonal density. In the clonal assays, single cells transduced with Nurr1 or LacZ as control generated clones containing neuron-only (0% versus 0%), astrocyte-only (18.2% versus 16.4%), and mixed neuron-astrocyte (81.8% versus 83.6%), in proportions which did not differ significantly (for each value, 56 clones from 3 independent cultures) [13], suggesting Nurr1-induced neuron differentiation does not occur via an intrinsic cell-autonomous mechanism. In order to test whether Nurr1-induced neurogenesis occurred via a paracrine mode, in which diffusible factors released from Nurr1-transduced cells promote neighboring precursors to differentiate into neurons, we adopted a co-culture system. In this system, untransduced cells faced the cells transduced with Nurr1 (or LacZ as the control) without direct contacts (spaced with a gap of 0.5–1 mm; see Fig. 3a). Compared to precursor cells co-cultured with LacZ-transduced cells, cells co-cultured with Nurr1-transduced cells yielded significantly more TuJ1+ neurons at the expense of GFAP+ astrocyte formation (Fig. 3b–f). More TuJ1+ cells were differentiated from precursors co-cultured with cells transduced with higher Nurr1 viral titers (Supplementary Fig. S2a–e). This finding is in a contrast to decreased neuron numbers from the precursors transduced with the highest Nurr1 titer tested (Fig. 2t). Thus, it is likely that high Nurr1 expression in cortical precursor cells intrinsically inhibits neurogenesis in the expressing cells, but exerts more abundant paracrine factor release to promote neuronal differentiation of neighboring precursors. Furthermore, treatment with medium conditioned in Nurr1-transduced cultures yielded the same cortical precursor cell differentiation results. In these experiments, TuJ1+ neurons were 30.8 ± 2% in the cultures supplemented with Nurr1-CM and 18.4 ± 1.4% in those supplemented with LacZ-CM, while GFAP+ astrocytes were 15.8 ± 1.2% (Nurr1-CM) versus 33.4 ± 2.3% (LacZ-CM) (Fig. 3g). In addition, numbers of neuron-only clones were greatly increased in clonal-density cultures treated with Nurr1-CM at the expense of astrocyte-only clones; % neuron-only clones was 25.0% (Nurr1-CM) versus 7.1% (LacZ-CM); % astrocyte-only clones were 0% (Nurr1-CM) versus 21.4% (LacZ-CM) (250 clones from five independent cultures were analyzed, Fig. 3h). The possibility of cross-infection can be excluded because all the results were obtained by using replication-incompetent viruses in the co-culture and conditioned-medium experiments (see Supplementary data). Thus, these findings strongly support the role of an extrinsic paracrine mechanism in Nurr1-promoted neuronal differentiation at the expense of astrocytic differentiation.

3.4. Factors responsible for the Nurr1 effects in enhanced neuron formation and decreased astrocyte formation

We therefore investigated the identity of paracrine factors responsible for Nurr1-induced neuron formation. Notably, mRNA

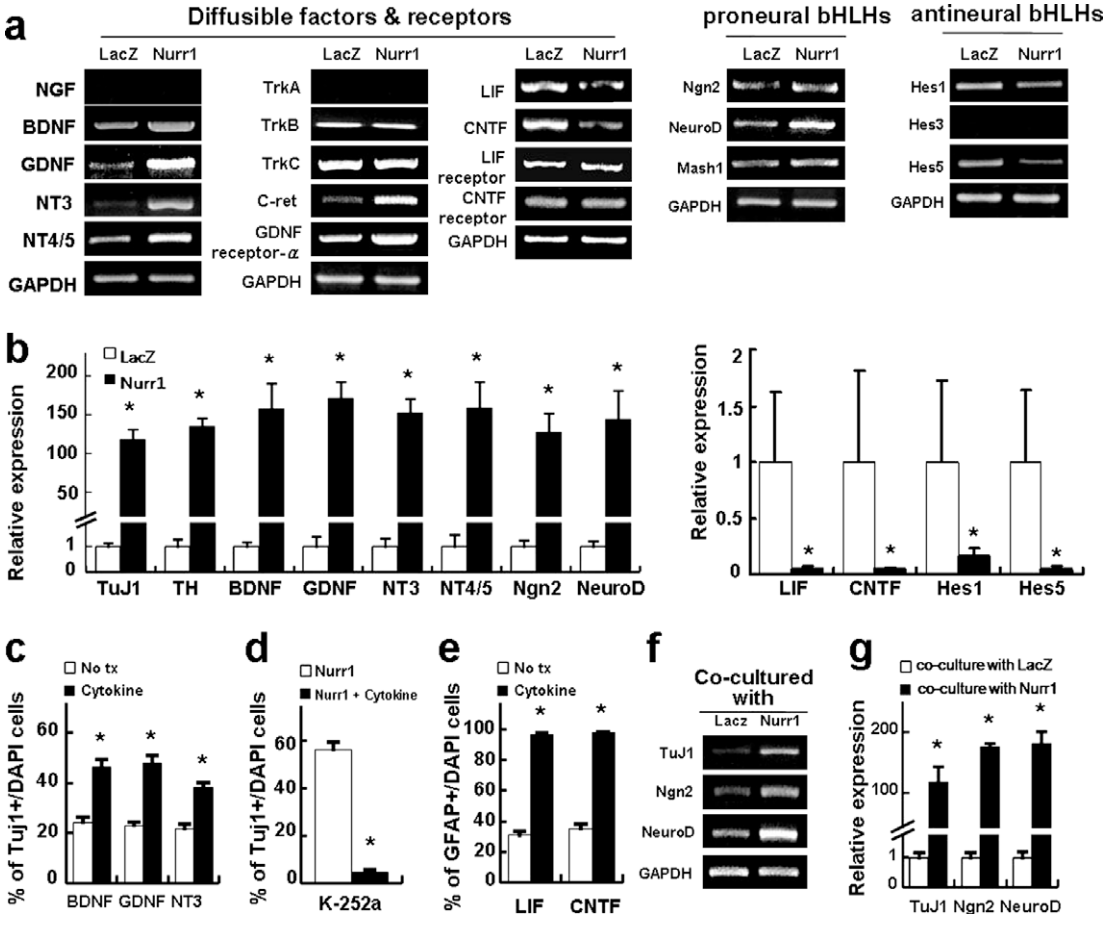

Fig. 4. Molecules responsible for Nurr1-induced neuronal differentiation at the expense of astrocytic differentiation. Semi-quantitative (a) and quantitative (real-time; b) PCR analyses to determine mRNA levels specific to neurogenic/astrogenic neurotrophic factors, receptors, and pro-/anti-neural bHLHs in Nurr1- and LacZ-transduced cultures. The PCR analyses for proneural bHLHs, Ngn2 and NeuroD, were also performed in untransduced cells co-cultured with Nurr1- and LacZ (control)-transduced cells (f and g). In the real-time PCR analyses, mRNA expression was normalized to that of GAPDH and expressed relative to the respective control value (for each value, $n = 6$). (c–e) Effects of neurotrophic factor treatments in neuron/astrocyte yields from NP cells. Untransduced (c, e) and Nurr1-transduced (d) NP cells were treated with the factor indicated during 3 days of differentiation. *Significant at $P < 0.01$.

levels of the neurotrophic factors BDNF, GDNF, NT3 and neurotrophin 4/5 (NT4/5) increased significantly in Nurr1-transduced cells (Fig. 4a and b), increases that were more prominent in cultures transduced with higher Nurr1 viral titers (Supplementary Fig. S2f). Furthermore, mRNA expression of astrocyte differentiation factors leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) was down-regulated in Nurr1-transduced cells. Treatment with the cytokines of the neurotrophic factors BDNF, NT3 and GDNF facilitated neuron formation and decreased astrocytic yield, similar to the effects of Nurr1 transduction, while the opposite cell fate switch was seen following LIF and CNTF treatment (Fig. 4c and e). In addition, blockage of the neurotrophic factor-mediated signals with a specific inhibitor, K-252a, completely abolished Nurr1-induced increase of TuJ1+ cells (Fig. 4d). Together, these findings suggest that Nurr1-induced effects on precursor cell differentiation are at least in part elicited by these diffusible cytokines. Interestingly, the proneural basic-helix-loop-helix (bHLH) transcription factors neurogenin2 (Ngn2) and neuroD mRNAs were greatly enhanced in cultures transduced with Nurr1, while the anti-neural bHLHs Hes-1 and -5 levels were decreased (Fig. 4a and b). Proneural bHLH gene expressions could be induced intrinsically by Nurr1 in the same Nurr1-transduced cells. However, this intrinsic mode of Nurr1-mediated bHLH gene expressions conflicts with our data that support a paracrine mechanism in Nurr1-induced neurogenic effects, as proneural bHLHs elicit their neurogenic roles via directly promoting transcriptions of neuron phenotype genes, in a cell-autonomous mechanism. Thus it is more plausible that the bHLH genes were up-regulated in the neighboring cells by paracrine factors released from Nurr1-transduced cells. Indeed, Ngn2 and NeuroD mRNA levels were clearly up-regulated in untransduced cells co-cultured with Nurr1-transduced cells, in comparison to the controls co-cultured with LacZ-transduced cells (Fig. 4f and g). Furthermore, treatment with BDNF, expression of which was induced by Nurr1, resulted in an enhancement of proneural bHLH gene expressions [15].

In summary, our study provides evidence that Nurr1 creates a milieu that promotes neuron differentiation and inhibits astrocyte formation from embryonic cortical precursor cells. The mechanism underlying the Nurr1 effect is an extrinsic paracrine one, in which Nurr1 upregulates the expressions of the neurogenic neurotrophins BDNF, GDNF, NT3, and NT4/5, and downregulates those of the gliogenic factors LIF and CNTF. In turn, the diffusible neurogenic factors stimulate neighboring precursor cells to differentiate towards neurons via upregulation of Ngn2 and NeuroD TF and downregulation of Hes-1 and 5. Nurr1 expression has been detected in the ventricular zone of the developing cortex from an early embryonic age, and in post-mitotic neurons in the cortical plate of later embryos (Allen Brain Atlas Developing Mouse Brain – http://www.brain-map.org). Our results, therefore, provide important insights into Nurr1 roles in cortical brain development. Further in vitro and in vivo experiments that allow for fine temporal control of Nurr1 expression will be required to confirm the proposed function of Nurr1. Ultimately, the observed effects of Nurr1 in NP cells may potentially be exploited in regenerative medicine, using engineered expression of Nurr1 in damaged brain tissue to promote the release of factors that stimulate endogenous neural stem cells to generate into neurons and thus facilitate functional recovery.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.04.004.

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