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Proneural bHLH neurogenin 2 differentially regulates Nurr1-induced dopamine neuron differentiation in rat and mouse neural precursor cells in vitro

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Abstract **Roles of Nurr1 and neurogenin 2 (Ngn2) have been shown in midbrain dopamine (DA) neuron development. We present here rat and mouse species-dependent differences of Nurr1 and Ngn2 actions in DA neuron differentiation. Nurr1 exogene expression caused an efficient generation of tyrosine hydroxylase (TH)-positive DA cells from rat neural precursor cells (NPCs). Nurr1-induced TH+ cell yields were low and highly variable depending on the origins of NPCs in mouse cultures. Coexpression of Ngn2 repressed Nurr1-induced generation of TH+ cells in rat cultures. In clear contrast, a robust enhancement in Nurr1-induced DA cell yields was observed in mouse NPCs by Ngn2. These findings imply that DA neurons may develop differently in the midbrains of these two species.**

Keywords: Dopaminergic differentiation; Neural precursor cells; Neurogenin 2; Species-dependent differences; Nurr1

1. Introduction

Midbrain dopamine (DA) neurons play principal roles in the regulation of motor behaviors, emotion, and cognition. Dysfunction of these cells is related to Parkinson's disease, schizophrenia, and drug addiction. Development of the midbrain dopaminergic system has been extensively studied because of its physiological and clinical significance. In vivo functional analyses in mice mainly contribute to identifying the critical factors of midbrain DA neuron development. However, the mechanisms of generating midbrain DA neurons are still largely unknown. Neural stem/precursor cells cultured in vitro are regarded as an appropriate tool for studies that aim to gain a deeper understanding of the mechanisms and signals that regulate the differentiation of stem/precursor cells into specific neuronal populations. In addition, research has been focused on the promise of stem cell-based replacement therapies for neuro-degenerative disorders.

Nurr1 is a transcription factor of the orphan nuclear receptor superfamily that is highly expressed in the developing midbrain. Mice lacking Nurr1 exhibit a specific loss of midbrain DA neurons [1–3], suggesting that Nurr1 is essential for midbrain DA neuron development. With respect to the production of midbrain DA neurons for experimental and transplantable purposes in regenerative medicine, interest in Nurr1 has been renewed because engineered Nurr1expression in vitro sufficiently induces the acquisition of DA neuron phenotypes in naïve non-dopaminergic neural precursor cells (NPCs) [4]. However, Nurr1-induced DA cells were either undifferentiated non-neuronal cells [5,6] or morphologically and functionally immature DA neurons [4]. In the search for factors regulating neuronal differentiation of Nurr1-DA cells, neurogenin (Ngn) 2 and Mash1 proneural basic helix-loop-helix (bHLH) factors are the first candidates because of their well-acknowledged pan-neurogenic nature for review, see [7] and proposed roles in midbrain DA neuron development [8–10].

We have recently demonstrated that coexpression of Mash1, a proneural transcription factor homologous with drosophila achaete-scute bHLH, resulted in the morphological and functional differentiation of Nurr1-expressing NPC toward mature DA neurons exhibiting biochemical and electrophysiological functionalities as presynaptic DA neurons [10,11]. Interestingly and surprisingly, however, another class of the atonal-related bHLHs including Ngn1, Ngn2, and neuroD remarkably repressed Nurr1-induced expression of DA neuronal markers in the cultures for NPCs derived from rat embryonic brains [10,11]. These findings are in contrast to the loss-of-function studies in mice that demonstrate a reduced number of DA neurons in developing midbrains by Ngn2 deletion, which suggests that Ngn2 is required in mouse midbrain DA neuron development [8,9]. The inconsistency of the in vivo and in vitro findings may be attributable to the differences between the two experimental systems with a possibility that the in vitro NPC cultures may not reflect physiologic developmental events in certain cases. Alternatively, the contrasting regulatory roles of Ngn2 could represent a real species-dependent difference in midbrain DA neuron development. To help answer this question, we examined Ngn2 roles in Nurr1-induced DA

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neuron differentiation in the cultures of NPCs derived from rat and mouse brains.

2. Materials and methods

2.1. Cultures for NPCs

Cultures of NPCs derived from rodent embryonic brains were performed as described previously [4] with some modifications. Briefly, embryonic brain tissues were dissected from the cortex (Ctx), lateral ganglionic eminence (LGE, anlage of striatum), and ventral midbrain (VM) of rats (Sprague–Dawley (SD), KOATECK, Seoul, Korea) at embryonic day 12 (E12)–E14 or from the identical brain regions of mouse embryos (ICR, KOATECK, E10.5–E12.5). After mechanical trituration, cells were plated at 20,000 cells/cm^2 on culture dishes pre-coated with poly-L-ornithine (PO)/fibronectin (FN) in N2B medium (DMEM/F12, 4.4 μM insulin, 100 mg/l transferrin, 30 nM selenite, 0.6 μM putrescine, 20 nM progesterone, 0.2 mM ascorbic acid, 2 mM L-glutamine, 8.6 mM D(+) glucose, 20 mM $NaHCO_3$, B27 (Invitrogen, Carlsbad, CA)) supplemented with basic fibroblast growth factor (bFGF; 20 ng/ml, R&D Systems, Minneapolis, MN), and epidermal growth factor (EGF, 20 ng/ml, R&D) and were cultured for 4–6 days as a monolayer on the adherent surface. To obtain a uniform population of NPCs, clusters of cells, which were formed by the proliferation of NPCs with the mitogens (bFGF and EGF), were passaged by dissociating them into single cells and plating them onto freshly PO/FN-coated coverslips (12-mm diameter; Marienfeld GmbH & Co. KG, Lauda-Knlgshofen, Germany) [12]. After subsequent precursor expansion up to 50–60% cell confluence, cells were retrovirally transduced as described below. On the day following infection, cell differentiation was induced in N2B medium in the absence of the mitogens for 3–10 days. All the data in this study were obtained in the passaged cultures grown on the adherent surface, unless otherwise noted. Cultures were maintained at 37 °C in a 5% CO_2 incubator. Medium was changed every other day and mitogens were added daily.

"Neurosphere" culture, another widely used culture method for NPCs, was carried out under identical experimental conditions to those described above, except cells were seeded in low adhesive culture dishes (No. 3262 Ultra Low Attachment; Corning, NY) without the PO/FN-coating procedure to prevent cells from attaching to the culture dish surface. Four to six days after cell expansion, floating cell aggregates (neurospheres) were mechanically dissociated into small pieces and subjected to retroviral infection in a cell suspension. Cell differentiation was induced by plating the fragmented neurospheres on PO/FN-coated surfaces and culturing for 3–10 days in N2B.

NPCs were also isolated and cultured from the subventricular zone (SVZ) of adult rat or mouse brains [13]. For details, see the supplementary methods.

2.2. Retroviral construction and transduction

Mouse Nurr1 (mNurr1, Genebank number: GI 7305324), mNgn2 (GI 34328159), rat Mash1 (rMash1, GI 11693147), and human Nurr1 (hNurr1, GI 27894347) and hNgn2 (GI 22477416) cDNAs were engineered into the retroviral vector pCL as previously described [11]. Details for retroviral construction and transduction are given in the supplementary methods.

2.3. Cell counting and statistical analysis

Cell counting was performed in uniform microscopic fields that were randomly chosen across the culture area, using an eyepiece grid at a final magnification of 200× or 400×. Statistical comparisons were performed using SPSS software (version 11.0; SPSS Inc., Chicago, IL). One-way or two-way ANOVA, followed by Tukey's post hoc comparison was applied where appropriate.

Methods for immunochemical, immunoblot, RT-PCR and DA release analyses are described in the supplementary methods.

3. Results and discussion

In order to compare the roles of Ngn2 in Nurr1-induced dopaminergic differentiation in rats and mice, NPCs were isolated and cultured from rat and mouse embryonic brains. To exclude any potential experimental bias, the comparative studies were carried out under various experimental conditions, which included the gathering of precursor cells from various brain regions at variable developmental stages and different culture methods, such as adherent monolayer and floating neurosphere cultures. In particular, we paid special attention to creating identical experimental settings in the rat and mouse cultures.

As was described previously [4], exogenous expression of Nurr1 in rat NPCs efficiently yielded cells expressing TH, a key enzyme of DA biosynthesis, regardless of embryonic age (E12, E14) or the brain region (Ctx, LGE, VM) from which the precursors were isolated (Fig. 1A–F). Of the cells transduced with Nurr1 (Nurr1-IRES-eGFP), as visualized by GFP expression, 49–66% were positive for TH at 2 days after transduction (Fig. 1M). Comparisons of the TH+ cell yields induced by Nurr1 transduction were made between the rat and mouse NPC cultures derived from identical regions at corresponding embryonic days (rat E12 versus mouse E10.5 and rat E14 versus mouse E12.5). Viral transductions were efficient (>90% GFP+/total DAPI+ cells) and indistinguishable in both rat and mouse cultures. Nurr1-induced TH+ cell yields from the mouse NPCs were extremely low, compared with those of the corresponding rat cultures. For instance, the percentages of TH+ cells out of the GFP+ infected cells in rat E14 versus mouse E12.5 cultures were 55.3 ± 4.1% versus 2.5 ± 0.2% (cortex), 59.1 ± 8.2% versus 3.4 ± 0.1% (LGE), and 49.1 ± 3.6% versus 7.9 ± 0.2% (VM) ($n = 300$ microscopic fields for each value from three independent experiments). Unlike the invariably high embryonic age- and region-independent TH+ cell yields in the rat cultures, Nurr1-induced TH+ cell yields were highly variable (2–15% of GFP+ cells), depending on the mouse brain regions and embryonic ages of the precursors. The higher TH+ cell yields were attained in the mouse NPCs derived from younger embryos, and the VM showed the greatest Nurr1 effect in yielding TH+ cells among the brain regions tested. Thus, the most efficient TH+ cell yield among the mouse cultures tested was observed in the NPCs derived from the VM at E10.5. These findings are consistent with previous studies showing that Nurr1-induced TH expression in mouse precursor cells requires factors derived from astrocytes of embryonic midbrain-origin [14,15] and that cells from early embryonic midbrains secrete autocrine/paracrine factors for in vitro precursor differentiation of DA neurons [16].

As previously reported [10,11], coexpression of Ngn2 in rat NPCs resulted in a striking repression of Nurr1-induced derivation of TH+ cells. The inhibitory role of Ngn2 was observed in all rat cultures derived from different developmental ages and brain regions (Fig. 2A, B, E and data not shown). To our surprise, however, the opposite effect of Ngn2 was observed in the mouse cultures; forced expression of Ngn2 resulted in a remarkable (5.3–8.6-fold) enhancement of Nurr1-induced TH+ cell yields in all the mouse cultures tested (Fig. 2C, D and F). For instance, the percentages of TH+ cells among all cells were 38.1 ± 1.7% and 7.3 ± 0.5% ($n = 300$ from three independent experiments) in E12.5 VM cultures transduced with Nurr1 + Ngn2 and Nurr1 alone, respectively. Immunoblots for TH protein levels further confirmed the contrasting roles of Ngn2 in mouse and rat NPC cultures (Fig. 2G).

Neuron numbers were also greatly increased in the Ngn2 co-transduced VM cultures (TuJ1+ cells out of all cells were

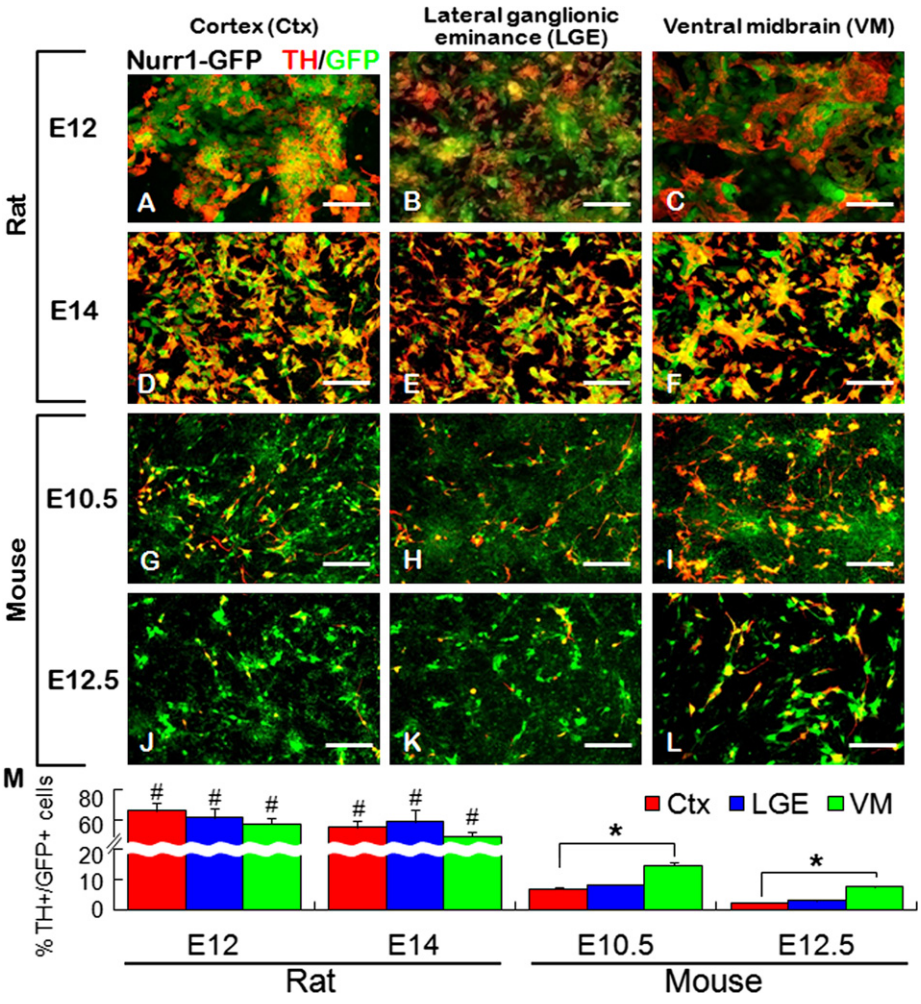

Fig. 1. Expression of TH-induced by exogenous expression of Nurr1 in rat and mouse embryonic NPCs. To compare Nurr1 action to induce DA phenotype expression in rats and mice, NPCs were isolated from identical regions of rat and mouse embryonic brains (Ctx, LGE, and VM) at corresponding developmental ages (rat E12 versus mouse E10.5, rat E14 versus mouse E12.5). The bicitronic pNurr1-IRES-GFP retroviruses were transduced and Nurr1-infected cells were detected by GFP expression. Scale bar, 50 μm. Graph M depicts the percentage of TH+/GFP+ cells out of the total GFP-positive cells 2 days after transduction. Note that TH+ cell yields of the rat cultures are much greater than those of the mouse cultures. [#]Significantly different from the respective mouse cultures at $P < 0.001$. [*]Significance at $P < 0.01$ compared with the mouse cortical cultures at same embryonic days.

4.1 ± 0.1%, 20.0 ± 1.1% and 19.4 ± 1.3% ($n = 300$ from three independent experiments) in E12.5 VM cultures transduced with pCL, mNurr1 and mNurr1 + mNgn2, respectively, Suppl. Fig. S2). Similarly, in Ngn2-deleted mouse embryonic VM, decreased TH+ cell number is accompanied by a decrease in the neuron population [8,9]. Furthermore, we have previously demonstrated a strong enhancement of Nurr1-induced TH+ cell yields by various neurogenic factor treatments in the precursors derived from adult rodent brains [13]. These findings suggest that the enhanced yield of Nurr1-TH+ cells by Ngn2 is closely associated with the neurogenic role of the bHLH. This speculation could be confirmed by determining if Nurr1-mediated DA phenotype induction preferentially occurs in neuronal progeny over other populations such as glial progeny or uncommitted NPCs. On the other hand, previous studies have demonstrated that proneural bHLHs can directly activate the expressions of neuronal subtype-specific genes by cooperation with other subtype determination factors for review, see [17]. Interestingly, En1/2, Lmx1b, Pax2, and Pax5 mRNA expressions related to the midbrain DA neuron development were greater in Nurr1 + Ngn2-transduced VM cultures than those of Nurr1 alone (Suppl. Fig. S3).

A recent study [18] demonstrated an effect of Ngn2 co-expression on enhanced neuronal differentiation of Nurr1-TH+ cells without a significant change of Nurr1-induced TH+ cell yield in the multi-passaged VM precursors of mouse origin. Properties of neural precursors derived from rodent embryos are altered after in vitro cultures with multiple cell passages [19,20]. Indeed, none of the midbrain markers such as Pitx3, En1/2 are detected in the multi-passaged VM cultures in the study of Andersson et al. [18]. By contrast, those markers are invariably expressed as in the embryonic VM tissues in our mouse VM cultures after only short-term NPCs expansion in vitro (Suppl. Fig. S3 and [21]). Therefore, data obtained in our culture conditions are much more likely to reflect the developmental physiology. Another clear difference between their studies and ours is the transduction efficiencies (% Nurr1-transduced cells of total cells; 10–12% in Andersson

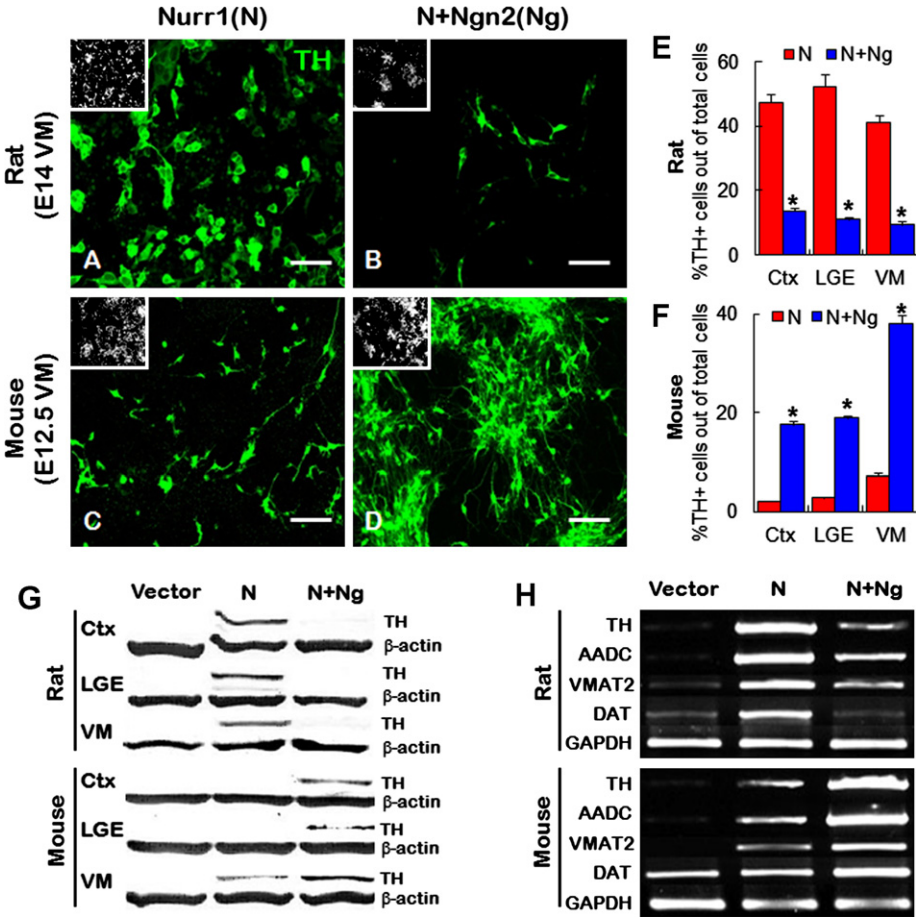

Fig. 2. Contrasting roles of Ngn2 in Nurr1-induced DA neuron differentiation in rat and mouse NPCs in vitro. NPCs derived from rat (E14) and mouse (E12.5) brain regions (Ctx, LGE, VM) were transduced with Nurr1 and Ngn2 (N + Ng) or Nurr1 and control empty vector (N) and then subjected to the immunocytochemical (A–F) and immunoblot (G) analyses for TH and semi-quantitative RT-PCR analyses (H) for the other DA neuron markers (AADC, VMAT2, DAT) after 3 days of differentiation (4 days of post-transduction). In A–D representative images of the TH+ cells in the rat and mouse VM cultures are shown, clearly demonstrating the contrasting roles (inhibitory in rat but inductive in mouse cultures) of Ngn2 in Nurr1-induced TH+ cell yield from rat and mouse NPCs. Insets, DAPI nuclear staining of the same field. Scale bar, 20 μm. *Significantly different from the respective N controls at $P < 0.01$.

et al. [18] versus >90% in this study). Such a low transduction efficiency in the study of Andersson et al. [18] could produce only rare cells in which both Nurr1 and Ngn2 exogenes are introduced together in a cell by co-transduction with a mixture of those viruses, thereby hindering an appropriate determination of the cell autonomous effects of Ngn2 in Nurr1-induced DA neuron differentiation.

The contrasting effects of Ngn2 in rat and mouse DA neuron differentiation were further shown in the mRNA expression of other DA phenotype genes. Compared with the respective Nurr1-transduced controls, the mRNA levels of AADC, VMAT2, and DAT in the Nurr1 + Ngn2 transduced cells were decreased in the rat cultures, but increased in the mouse cultures (Fig. 2H).

TH+ cells derived from Nurr1 + Ngn2-transduced mouse NPCs were morphologically more mature than those from Nurr1-transduced cells as determined by the estimation of TH+ fiber length per cell (157.0 ± 25.4 μm versus 27.4 ± 11.3 μm, $n = 50$, Fig. 3A–C).

Dopamine neuron identity of the TH+ cells generated by Nurr1 + Ngn2 transduction was further confirmed by the co-localization of neuronal (HuC/D, Fig. 3D; TuJ1, Fig. 3E; MAP2, Fig. 3F) and DA neuronal markers (AADC, Fig. 3G; VMAT2, Fig. 3H). Higher proportions of TH+ cells in Nurr1 + Ngn2-cotranduced VM cultures expressed the neuronal and DA neuronal markers than those in the cultures transduced with Nurr1 alone (data not shown). The Ngn2 actions in morphologic and phenotypic maturation of Nurr1-induced DA cells are consistent with the study of Andersson et al. [18]. HPLC analysis revealed an increase in DA release in the cultures transduced with Nurr1 + Ngn2 relative to those of Nurr1-cultures (KCl evoked DA levels were: Nurr1 + Ngn2 10.46 ± 1.03 μg/ml; Nurr1: 3.88 ± 1.18 μg/ml, Fig. 3I). Altogether, these results strongly indicate contributory roles of Ngn2 in the generation of morphologically, phenotypically, and functionally mature DA neurons from mouse NPCs.

Forced expression of Mash1, along with Nurr1, generated morphologically mature TH+ cells without altering Nurr1-induced TH+ cell yield in rat cultures [10,11] (and Suppl. Fig. S4). In contrast, Mash1 in mouse precursor cell cultures not only induced morphological differentiation of Nurr1-TH+ cells but also increased TH+ cell yield (Suppl. Fig. S4), indicating that Mash1 has a role similar to Ngn2 in Nurr1-induced DA neuron differentiation from mouse NPCs. These

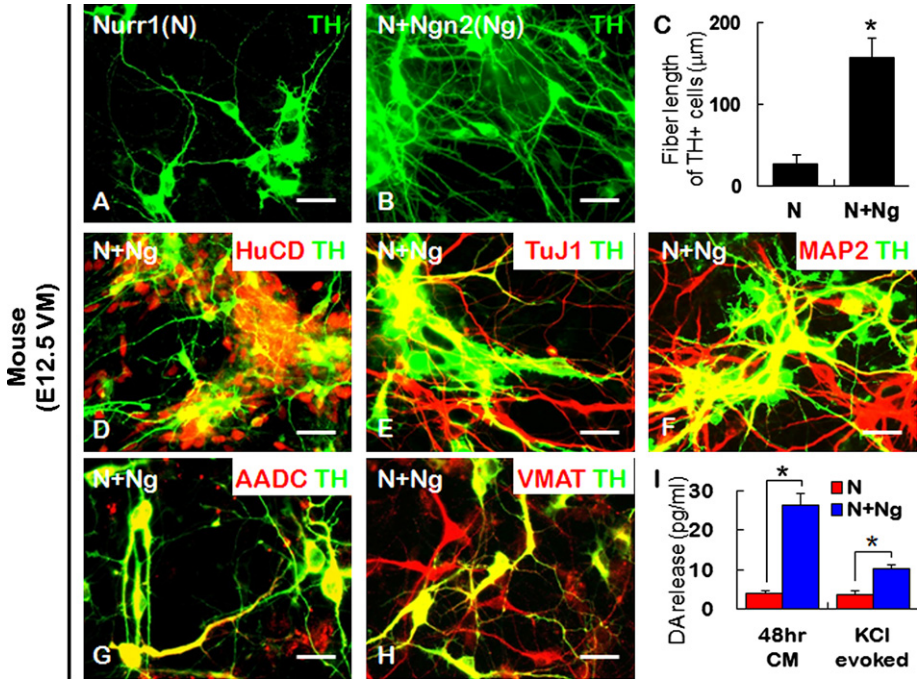

Fig. 3. Ngn2-induced neuronal differentiation of Nurr1-induced TH+ DA cells. NPCs derived from the mouse VM at E12.5 were transduced with Nurr1 + Ngn2 (N + Ng) or Nurr1 (N); this was followed by immunocytochemical, morphometric, and functional analyses at day 6 of the differentiation phase. (A)–(C) Morphometric measurement of TH+ fiber lengths. Shown in A and B are the representative images of TH+ cells in the N- and N + Ng-transduced NPC cultures, respectively. Graph C depicts total TH+ fiber lengths per cell in the N- and N + Ng-cultures. $n = 50$ for each value. (D)–(H) Co-localization of neuronal (HuC/D in D, TuJ1 in E, and MAP2 in F) and DA neuronal (AADC in G, VMAT2 in H) markers in TH+ cells derived from the N + Ng-transduced VM NPCs. Scale bar, 10 μm. (I) HPLC quantification of DA release. The graph shows DA levels in the medium conditioned for 48 h (48 h CM) and released by KCl-evoked depolarization in NB medium with 56 mM KCl for 15 min. (KCl evoked), $n = 3$. *Significantly different from the respective N at $P < 0.01$.

findings are consistent with previous data showing redundancy in the roles of Mash1 and Ngn2 in DA neuron development in the embryonic mouse midbrain [8].

There is a possibility that the differences between rat and mouse NPCs, which are described above, are observed only in specific experimental conditions. In particular, cell density and cell–cell contacts can affect the differentiation properties of precursor cells [16]. However, the similar species-dependent differences were shown in the cultures plated at various cell densities and in unpassaged cultures (data not shown). Furthermore, the species-dependent differences in the efficiency of Nurr1-TH yield and the roles of Ngn2 and Mash1 in Nurr1-TH+ cell generation were also obvious in neurosphere cultures, another method for culturing NP cells in the form of floating cell aggregates (Suppl. Fig. S5). We recently reported that Nurr1 also acts as an efficient inducer of DA neuron differentiation in the NPCs derived from the subventricular zone (SVZ) of the lateral ventricles in adult rats [13]. As shown in the NPCs of rat embryos, coexpression of Ngn2 drastically reduced Nurr1-TH+ cell production from SVZ cells (Nurr1: 4.2 ± 0.9%; Nurr1 + Ngn2: 0.8 ± 0.2%, Suppl. Fig. S5A, B and E). By contrast, Nurr1 was much less efficient in the cultures derived from the mouse SVZ, and Ngn2 coexpression resulted in a striking enhancement of Nurr1-induced TH+ cell yield (Nurr1: 1.5 ± 0.3%; Nurr1 + Ngn2: 11.7 ± 1.3%, Suppl. Fig. S5C–E). All the findings seen above were obtained from the genes of mouse origin (mNurr1 and mNgn2). There is a possibility that Nurr1 and Ngn2 of different species could yield different results. However, it is not likely to be the case at least with regard to the Nurr1 effects, because the protein sequences of Nurr1 are extremely homologous among species (amino acid sequence homology of Nurr1 proteins between rat and mouse: 99.8%, rat and human 99.7%). Furthermore, similar species-dependent differences in rat and mouse DA neuron differentiation were also observed with hNurr1 and hNgn2 of human origins (Suppl. Figs. S1 and S2). Collectively, these findings indicate that the effects of Nurr1 and Ngn2 in DA neuron differentiation in vitro are not dependent on culture conditions, the culture types of the precursors, or the genes of different origins, but are species-dependent.

The observed contrasting roles in mouse and rat species are not likely to be easily accepted as a physiologically relevant phenomenon, especially because of a general belief that rats and mice belong to closely related species, between which developmental processes are largely the same. However, substantial discrepancies between these two species have been demonstrated in their phylogenic, ecologic, and genetic backgrounds. Notably, a recent study has reported intrinsic differences in the proliferative, biochemical, and differentiation properties of NPCs derived from rat and mouse brains [22]. Furthermore previous studies have demonstrated that the TH transactivation effect of Nurr1 is highly variable depending on cellular contexts. These findings support the cautious conclusion that the observed differential roles of Nurr1 and Ngn2 in in vitro DA differentiation may reflect real differences in the midbrain DA neuron development in these two species. However, confirmation is needed in in vivo systems

to assure that the species difference is a biologically relevant one.

Nurr1 gene manipulation in precursor or stem cells is particularly important in generating transplantable DA neurons in the cell replacement approaches for Parkinson's patients. However, it was recently reported that Nurr1, dissimilar to its action of DA cell differentiation in rodent cells, is unable to yield TH+ DA cells from human NPCs [23]. This is another example of the species-dependent differences of Nurr1 action, indicating that simple and direct application of Nurr1 exogenous expression is infeasible for generating human DA neurons. In this regard, further studies are needed to elucidate the mechanisms for the species-dependent roles of Nurr1 and Ngn2 that were observed in this study; these studies could provide possible clues for successful Nurr1-utilized generation of transplantable human DA neurons.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.01.018.

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