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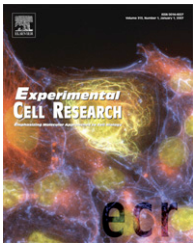

Research Article

Ngn2 and Nurr1 act in synergy to induce midbrain dopaminergic neurons from expanded neural stem and progenitor cells

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ABSTRACT

Parkinson's Disease (PD) is a debilitating motor function disorder due primarily to a loss of midbrain dopaminergic neurons and a subsequent reduction in dopaminergic innervation of the striatum. Several attempts have been made to generate dopaminergic neurons from progenitor cell populations in vitro for potential use in cell replacement therapy for PD. However, expanding cells from fetal brain with retained potential for dopaminergic differentiation has proven to be difficult.

In this study, we sought to generate mesencephalic dopaminergic (mesDA) neurons from an expanded population of fetal mouse ventral midbrain (VM) progenitors through the use of retroviral gene delivery. We over-expressed Ngn2 and Nurr1, two genes present in the ventral midbrain and important for normal development of mesDA neurons, in multi-passaged neurosphere-expanded midbrain progenitors. We show that over-expression of Ngn2 in these progenitors results in increased neuronal differentiation but does not promote mesDA formation. We also show that over-expression of Nurr1 alone is sufficient to generate tyrosine hydroxylase (TH) expressing cells with an immature morphology, however the cells do not express any additional markers of mesDA neurons. Over-expression of Nurr1 and Ngn2 in combination generates morphologically mature TH-expressing neurons that also express additional mesencephalic markers.

Introduction

Mesencephalic dopamine (mesDA) neurons are generated in the mouse ventral midbrain (VM) during a restricted time period starting at embryonic day E10 [1]. Typically, dopamine neurons are detected by the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in the dopamine (DA) pathway. In the midbrain, the dopaminergic neurons and their progenitors also express genes such as Nurr1, Lmx1a, Lmx1b, Pitx3, En1, En2, and Ngn2, which are necessary for correct specification and/or survival in vivo (reviewed in [2,3]) and in vitro [4–6].

Previous studies have demonstrated that over-expressing Nurr1, an orphan nuclear receptor that is essential for DA differentiation, may regulate TH expression in cultured cells [6], including embryonic stem (ES) cells, primary neural

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precursors derived from cortex, midbrain and developing striatum [7–9], and in non-neural cells [10]. However, this potential has not yet been demonstrated in multi-passaged VM progenitor cultures. Additionally, the Nurr1-induced TH-expressing neurons from fetal cells are often phenotypically and functionally immature [9] and expression of other genes and proteins that are characteristic of a midbrain identity has not been firmly established in these cells.

Transcription factors belonging to the basic helix–loop–helix (bHLH) gene family promote neuronal differentiation (for review see [11]). In addition to acting as neuronal determinants, the proneural bHLH genes play a role in cell fate specification in several areas of the developing CNS [12]. Several members of the proneural gene family have recently been reported to be expressed in dividing mesDA progenitors residing in the developing ventral midbrain [13,14] but only Ngn2 is required for the correct development of the mesDA neurons [13,15]. While the potential for Ngn2 to promote neuronal fates from neural progenitors in vitro is well established [16], its ability to promote a mesDA fate from expanded VM progenitors has not been elucidated.

In this study, we analyzed the ability of Ngn2 and Nurr1 to induce formation of mesDA neurons from multi-passaged neurosphere cultures derived from fetal VM. Neural stem and progenitor cells are expanded in these cultures. However, while they are isolated from the VM at a developmental time-point when mesDA neurons are actively generated, the neurons generated upon differentiation from these expanded VM progenitors never express TH or other markers characteristic of a dopaminergic phenotype (own observation and also reported in [17–20]).

We show that over-expression of either Ngn2, Nurr1 or both combined, have distinct effects on neuronal differentiation in VM progenitor cell cultures. When over-expressing Ngn2 alone in multi-passaged VM-derived neurospheres we observed a significant increase in neuronal differentiation, however, the neurons did not express TH. On the other hand, over-expression of Nurr1 alone produced TH-positive cells with relatively immature neuronal morphologies that did not express any additional mesDA markers. In contrast, when Ngn2 and Nurr1 were over-expressed in the same culture, morphologically mature TH-positive neurons that also expressed additional dopaminergic and mesencephalic neuronal markers were generated. These data suggest that expanded VM progenitors that have lost their potential to generate mesDA neurons can be promoted to generate these cells by viral gene delivery in vitro.

Materials and methods

Neurosphere cultures

Embryos of gestational age E11.5 were collected from timed pregnant NMRI mice. The embryonic brains were dissected free of mesenchymal tissue followed by sub-dissection of ventral mesencephalon in L15 medium (Gibco). The tissue pieces were incubated in DNAse/trypsin solution (Sigma, R&D) at 37 °C for 15 min after which the trypsin was inactivated with DMEM/10%FBS and removed. A single-cell suspension was obtained by mechanical dissociation in a small volume of neurosphere medium. Live cells were counted by the Tryphan blue exclusion method and plated in neurosphere medium with added growth factors in NUNC flasks at a density of approximately 500000 cells/ml. The neurosphere medium consisted of DMEM/F12 medium (Gibco) supplemented with L-glutamine, $NaHCO_3$, Glucose, penicillin/streptomycin and B27 (1:50, Gibco). Fresh medium and basic fibroblast growth factor, bFGF (10 ng/ml), epidermal growth factor EGF (20 ng/ml, R&D) were added approximately every other day. Heparin (40 U/ml, Sigma) was added for stabilization of bFGF. Once the spheres were formed (passage 0), they were passaged to single cell suspension by mechanical dissociation every 7th day, and the cells were replated at a density of 100000 cells/ml.

For differentiation, spheres were collected by centrifugation and plated as whole spheres on poly-L-Lysine (PLL)/laminin coated plastic chamber slides in basic neurosphere medium containing 1% FBS (differentiation medium). The spheres were allowed to differentiate for 4 days, replacing half the medium after 2 days.

Retroviral gene delivery

Vesicular Stomatitus virus G-protein (VSV-G) pseudotyped retrovirus containing cDNA encoding Ngn2 (Ngn2–IRES2–eGFP, [16]) or Nurr1 (Nurr1–IRES2–eGFP) were generated as previously described [21]. All viral vectors were derived from the Maloney Murine Leukemia virus, Murine Stem Cell Virus-based (Clontech). These bi-cistronic retroviruses encode an internal ribosomal entry sequence (IRES2, Clontech) and enhanced green fluorescent protein (eGFP, Clontech). Control retrovirus contained no additional cDNA (IRES2-eGFP). All viruses used in this study had a titre of $1–3\times10^8$ TU/ml.

Single-cell neurosphere cells were spun down (1000 rpm, 10 min) 24 h after third passage and incubated with virus (1 TU/cell) in a small volume for 4 h in the presence of protamine sulfate (4 μg/ml). The cells were re-suspended in the previously removed medium and plated in non-coated plastic 4-well chamber slides (1 ml/well; ≈100000 cells/ml) under proliferating conditions. Fresh medium and growth factors (EGF and bFGF) were added every 2–3 days. After a total of 7 days in proliferating conditions, the expansion media and any free-floating spheres were removed, spun down and gently resuspended (not to single-cell suspension) in differentiation media (1% FBS, no growth factors) which was then added back to the same plastic culture slides and let to differentiate for 4 or 7 days. GFP-transduced control cultures were expanded and differentiated in the same way. Non-transduced control cultures were in this case differentiated on uncoated plastic to match the differentiation conditions for the transduced cultures.

Immunohistochemistry

Differentiated cells were fixed in 4% paraformaldehyde (PFA) for 20 min, or 4% PFA/0.4% glutaraldehyde (for GABA staining), rinsed with phosphate buffered saline (PBS) and processed for immunohistochemistry. Slides were pre-incubated in KPBS (potassium phosphate buffered saline) with

10% normal donkey serum (NDS) and 0.1% triton-X for 1 h and then incubated over night with primary antibody diluted in blocking solution. Following rinses in KPBS, the cells were incubated with secondary antibody for 2 h in the dark. Cell nuclei were counterstained with DAPI (4′-6-diamidino-2-phenylindole) nuclear stain (1:1000, Sigma) and the slides were coverslipped using PVA-DABCO (poly-vinylalcohol diazabicyclo octane). Primary antibodies used were: mouse anti-β-III-tubulin (1:333, Sigma), mouse anti-CNPase (1:100, Sigma), rabbit anti-GFAP (1:1000, DAKO), chicken anti-GFP (1:5000, Chemicon), rabbit anti-TH (1:1000, Pelfreeze), mouse anti-TH (1:1000, Sigma), rabbit anti-VMAT2 (1:1000, Chemicon), rabbit anti-Dlk1 (1:5000, gift from C.H. Jensen), rabbit anti-En1/2 (1:500, gift from A. Joyner), rabbit anti-Pitx3 (1:1000, gift from P. Burbach), rabbit anti-GABA (1:2000, Sigma). Secondary antibodies used were Cy2-donkey anti-mouse, Cy3-donkey anti-mouse, Cy5-donkey anti-mouse, Cy2-donkey anti-chicken, Cy3-donkey anti-rabbit, Cy5-donkey anti-rabbit (all 1:200, Jackson lab).

Cell counts and statistical analysis

For quantification of neurons (β-III-tubulin immunopositive cells) and oligodendrocytes (CNPase immunopositive cells) from differentiated neurospheres, total cell number (DAPI stained nuclei) and immunostained cells were counted in randomly chosen fields from four wells per staining, at 100× magnification. On average 1500 cells/well were counted. Three separate cell cultures were evaluated and the average from the three separate experiments calculated. This was repeated for passage 3, 4 and 5.

For quantification of β-III-tubulin-positive cells and Nurr1-positive cells in transduced cultures, 5–10000 cells/well were counted in randomly chosen fields in three separate wells and the average fraction of total cells (DAPI stained nuclei) was calculated.

For quantification of TH- and GFP-positive cells in Nurr1 transduced cultures and Nurr1/Ngn2 co-transduced cultures, total cell number (DAPI stained nuclei) and immunostained cells were counted in randomly chosen fields at 40× magnification. On average 4000 cells/well were counted. Two to three wells for each separate experiment were examined and all experiments were repeated three times. For statistical analyses the data from the separate experiments were pooled.

Statistical significance was tested for TH-cell counts in Nurr1 transduced cultures compared to Ngn2/Nurr1 transduced cultures and β-III-tubulin-cell count in Ngn2 transduced cultures compared to GFP transduced cultures. All comparisons were carried out using an un-paired *t*-test. All values are reported as mean ± 1 S.E.M.

To estimate the number of triple labeled cells the average number of cells positive for VMAT2, PITX3 and EN1/2 in a set area (a 4-chamber slide well) was calculated and compared to the average number of TH-positive cells in the same area. As immunoreactivity for either marker and TH overlapped in more than 90% of the cells (as determined by confocal microscopy on randomly selected cells) the percentage of TH-positive cells that also express another marker could be estimated. The total number of immunoreactive cells for each marker was counted in 2–3 wells.

Morphological analysis

Images were taken from 15 randomly selected fields per group. 3–4 focal planes per area were captured and later collapsed in order to put the whole cell and its processes in focus. The number of processes and extent of branching was counted for each cell and the length of the processes was measured using Canvas™. A total of 200 cells per group were analyzed. The data is reported as value ± S.E.M. and significance was tested for using ANOVA post hoc test followed by Bonferroni–Dunn analysis with a p value of less than 0.05.

Results and discussion

With the aim to establish neurosphere cultures from the VM, we subdissected ventral mesencephalic tissue from E11.5 mice embryos and expanded the dissociated cells in proliferation medium containing growth factors EGF and bFGF. The spheres were passaged weekly by mechanical dissociation and the number of cells was counted. After the first passage, the cultures generated on average an eight-fold net increase in total cell number at each passage (Fig. 1A).

In order to determine the differentiation potential of multi-passaged spheres, whole neurospheres from passage 3–5 cultures were collected and plated on PLL/laminin coated surface in differentiation medium containing 1% serum. The neurosphere cultures generated neurons, oligodendrocytes and astrocytes upon differentiation as indicated by β-III-tubulin, CNPase and GFAP immunostaining respectively (Figs. 1B–D). At these early passages the majority of the differentiated cells were astrocytes, but the cultures also contained neurons (~5%) and oligodendrocytes (~1%) (Fig. 1E). However, there was no detectable staining of mesDA neuron markers such as TH, NURR1, PITX3 or EN1/2. The lack of TH was also confirmed by RT-PCR (data not shown).

These data show that VM neural progenitors can be expanded to generate neurons, oligodendrocytes and astrocytes for several passages. However, their potential to generate mesDA neurons is lost after passaging.

Nurr1 expression promotes generation of TH-positive cells in expanded VM-derived neurospheres

Nurr1 is expressed in post-mitotic dopaminergic neuroblasts prior to TH expression [22,23] and is involved in establishing the dopaminergic neurotransmitter identity in mesDA neurons [22,24–26]. Other studies have shown that over-expression of Nurr1 enables neural stem cell lines to respond to extrinsic signals such as SHH, FGF8 and Wnt5a and thus differentiate into TH-expressing cells [6,27], and that it can elicit TH expression in rat embryonic neural progenitor cells [9].

In order to determine the effect of Nurr1 over-expression in multi-passaged VM-derived neurosphere cultures, passage 3 cells were transduced with a replication deficient murine leukemia retrovirus containing a Nurr1–IRES2–eGFP construct. An IRES2–eGFP retrovirus was used as a control.

The transduced cells were expanded for 7 days and then differentiated for 4 days. After differentiation, 10.5 ± 0.7%

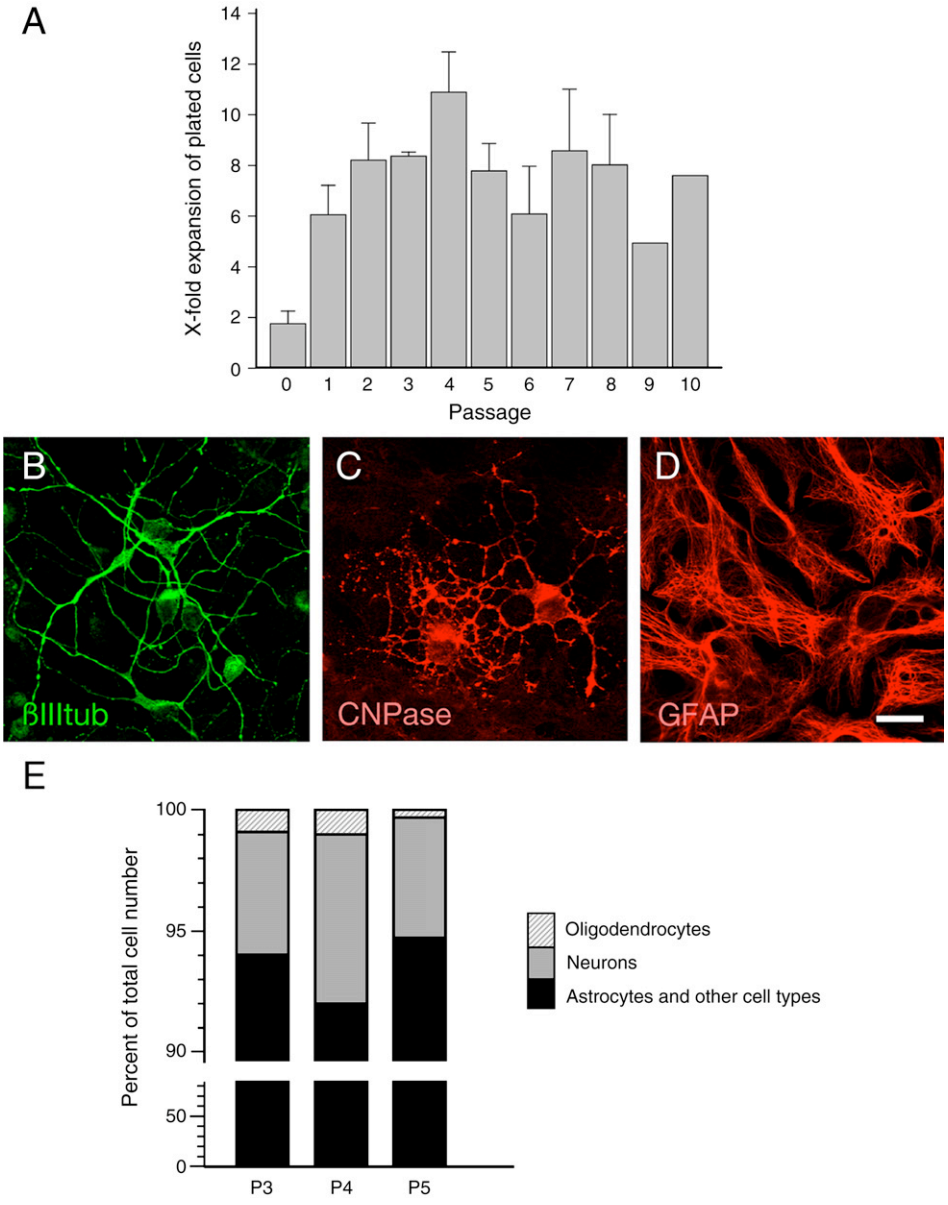

Fig. 1 – Cell expansion and differentiation of neurosphere cultures. (A) Primary cells grown as neurosphere will expand to form 1.4 times the number of originally plated cells during the first week. However, once established as a neurosphere culture, the yield is on average 8 times the number of plated cells at each passage. The bar chart represents the average expansion at each passage based on three separate continued cultures. For passage 9 and 10 measures from only one culture is represented. Error bars show 1 S.E.M. (B–D) Neurospheres were differentiated on PLL/laminin for 4 days and subsequently stained for neuronal marker β-III-tubulin (B), oligodendrocyte marker CNPase (C) and astrocyte marker GFAP (D). (E) Neurospheres of early passage (P3–P5) give rise to 5–7% neurons and around 1% oligodendrocytes. The majority of the remaining cells are astrocytes. Note that the *x*-axis is broken and unevenly distributed. Scale bar: 10 μm (D).

expressed GFP and 11.3±2.5% of all cells expressed Nurr1, the majority of which co-expressed GFP. In contrast, Nurr1-positive cells were never present in GFP-transduced control cultures although a comparable number of cells expressed GFP. When examined for TH immunoreactivity, we found that 4.3%±0.8% of GFP-expressing cells in the Nurr1 transduced cultures were TH-positive after differentiation whereas no TH-positive cells could be identified in either the GFP transduced or non-transduced control groups (Fig. 2A).

A subset of the TH-expressing cells co-expressed β-III-tubulin but many appeared morphologically immature with mainly short processes, if any (Figs. 2A, D–G). In order to determine if any of the TH-expressing cells were of a midbrain phenotype we stained for mesDA markers PITX3 and EN1/2. No cells immunopositive for these markers were found in Nurr1 transduced or control cultures (Table 1). We also differentiated some cultures an additional 3 days (in total 7 days of differentiation). In these cultures, the TH-

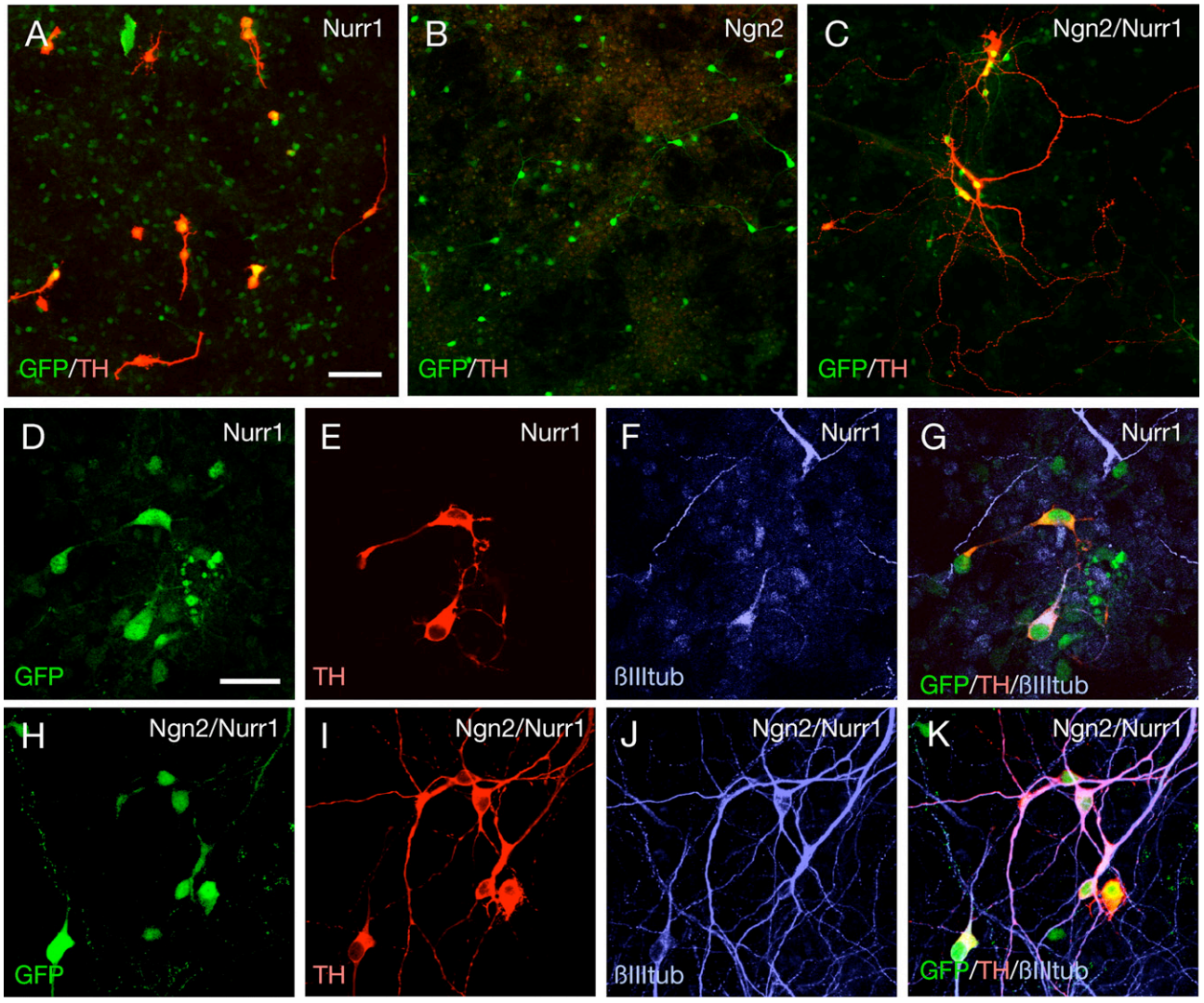

Fig. 2 – TH-expression is seen in neurosphere cultures after over-expression of Nurr1. Neurosphere cultures were retrovirally transduced to over-express Ngn2, Nurr1 and Ngn2/Nurr1 combined. (A) Cells transduced with retrovirus encoding Nurr1–IRES2–eGFP yielded TH-expressing cells that were also GFP-positive, showing that they were derived from transduced cells. The TH-positive cells had mainly an immature morphology with few processes. (B) Transduction with Ngn2–IRES2–eGFP retrovirus gave rise to many GFP-positive transduced cells but no TH-positive cells. (C) However, when cultures were simultaneously transduced with both Ngn2–IRES2–eGFP retrovirus and Nurr1–IRES2–eGFP retrovirus, TH-positive cells with mature neuronal morphologies and elaborate extensions were seen. (D–G) Only some of the TH-positive cells in Nurr1 transduced cultures co-expressed β-III-tubulin, indicating that only a portion were neuronal. (H–K) In comparison, the majority of TH-positive cells in co-transduced cultures expressed β-III-tubulin. Scale bars: 100 μm (A), 50 μm (D).

positive neurons acquired a slightly more mature morphology but still did not express the mesDA marker PITX3 (not shown). Thus, longer differentiation than 4 days did not lead to further subtype differentiation of Nurr1 transduced TH-positive cells.

Taken together, these data indicate that the potential to generate TH positive cells upon differentiation that is lost in multi-passaged VM derived cells can be regained by over-expressing Nurr1. However, the TH-positive cells generated have a relatively immature morphology and do not express additional mesDA proteins.

Ngn2 increase neurogenesis in expanded VM-derived neurosphere cultures but does not promote the generation of TH-positive cells

The basic helix–loop–helix transcription factor Ngn2 is involved in neuronal differentiation in various regions of the CNS and PNS (for review see [11]). Ngn2 is expressed in the midbrain ventricular zone progenitors that give rise to mesDA neurons, where it promotes the generation of DA precursors and their differentiation into DA neurons [14,15]. In order to determine if expression of Ngn2 could induce a mesDA fate in

Table 1

	GFP	Nurr1	Ngn2	Nurr1/Ngn2
β-III-tubulin	+	+	+	+
TH	0	~4%[a]	0	~4%[a]
VMAT	N/A	5%[b]	N/A	20%[b]
EN1/2	N/A	0[b]	N/A	<1%[b]
PITX3	N/A	0[b]	N/A	<1%[b]

[a]Of total cell number, [b]number of TH positive cells that co-express other marker, N/A=not applicable since no TH expressing cells present.

Table 2 – Morphological analysis of TH expressing cells

	Nurr1	Nurr1/Ngn2
Number of processes	3.4±0.2	2.9±0.2
Branching	1.0±0.2	2.0±0.4*
Total process length (mm)	0.66±0.1	2.2±0.2*

* Denotes significant difference ($p<0.05$) from corresponding Nurr1 only group.

the expanded cells, we transduced neurosphere expanded cells with a retrovirus containing a Ngn2–IRES2–eGFP construct [16].

Expression of Ngn2 in these expanded VM progenitors increased the number of neurons by 10-fold upon differentiation, from 0.34+0.03% to 3.9+0.2%, $p<0.01$ (Fig. 3A, compare to Fig. 3B), an effect that has also been reported when Ngn2 is over-expressed in adult neural stem cells [16]. The majority of the neurons were GFP-positive (of 103 randomly selected β-III-tubulin-positive cells, 96 had a strong expression of GFP) confirming that they were indeed derived from Ngn2 transduced cells (Figs. 3B–E).

However, transducing the cells with Ngn2 did not induce any detectable protein expression of TH (Fig. 2B, Table 1) or any other dopaminergic marker tested (Table 1). Thus, when over-expressed in VM-derived neurosphere expanded cells, Ngn2 promotes neuronal differentiation and maturation, but is not sufficient to induce a dopaminergic phenotype in the cells.

Co-transduction of Ngn2 and Nurr1 promotes differentiation of mature DA neurons

Given its function during normal midbrain development, it is possible that Ngn2 may confer a competency to respond to, or act in synergy with developmental control genes/proteins in other differentiation cascades, including Nurr1. We aimed at determining if expression of both Ngn2 and Nurr1 together in expanded VM-derived progenitor cultures could generate a more mature and phenotypically correct mesDA neuron upon differentiation, compared to TH-positive cells generated from over-expression of Nurr1 alone. Thus we over-expressed both Nurr1 and Ngn2 in the same cells by co-transduction.

Co-transduction with Nurr1 and Ngn2 generated TH-positive cells but there was no significant difference in the number of TH-positive cells in Ngn2/Nurr1 co-transduced cultures compared to Nurr1 transduced cells ($p>0.05$). However, we observed that there was a striking change in morphology of the TH-positive cells in cultures transduced with both Nurr1 and Ngn2. The co-transduced TH-positive cells had both longer and more elaborate projections compared to those in Nurr1 transduced cultures (Figs. 2A, D–G). A larger proportion also expressed β-III-tubulin (Figs. 2C, H–K). A detailed morphological analysis of TH-expressing cells in both groups showed that the cells had the same number of processes on average, but the processes branched twice as much and were significantly ($p<0.05$) longer in the group that had received both Nurr1 and Ngn2 in combination (Table 2).

Co-transduction of Nurr1 and Ngn2 promotes mesDA phenotype in a subset of neurons

To characterize the TH-expressing neurons in the co-transduced cultures at a molecular level, we performed triple

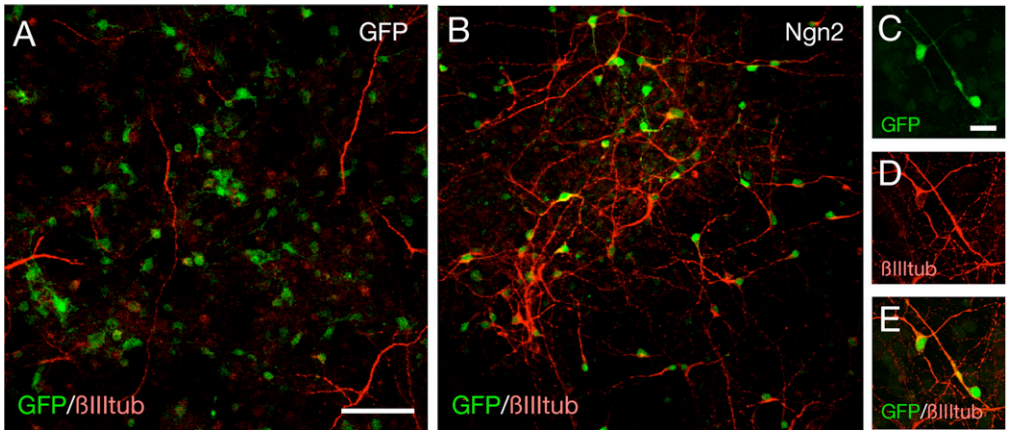

Fig. 3 – Over-expression of Ngn2 in neurosphere cultures gives rise to significantly more neurons. Neurosphere expanded cells were transduced with a retrovirus encoding Ngn2–IRES2–eGFP 1 day after the third passage. Upon differentiation the cells gave rise to 10-fold more neurons (B) than cells transduced with IRES2–eGFP control virus (A). (C–E) Most β-III-tubulin positive neurons in Ngn2 transduced cultures co-expressed GFP showing that they were derived from transduced cells. Scale bars: 100 μm (A), 20 μm (C).

immunohistochemistry for TH, GFP and VMAT2, EN1/2, PITX3, DLK-1, or GABA, respectively.

VMAT2 (Vesicular monoamine transporter) is part of the neurotransmittor phenotype of dopaminergic neurons and in mesDA neurons it is turned on around E14 [28]. Expression of VMAT2 expression would indicate mature dopaminergic neurons that are also capable of processing dopamine. We found many VMAT2+/TH+/GFP+ cells in Ngn2/Nurr1 co-transduced cultures (Fig. 4A, Table 1); approximately 20% of TH-positive cells were also VMAT2-positive. Weak expression of VMAT2 could also be detected in a few cells (<5% of TH-positive cells) in the Nurr1 transduced cultures but not in GFP transduced control cultures (Table 1).

Next we sought to determine whether the TH-positive cells had the required mesencephalic phenotype and not just a general dopaminergic phenotype, by analyzing the expression of DLK1, EN1/2 (Engrailed) and PITX3 (Pituitary homeobox gene).

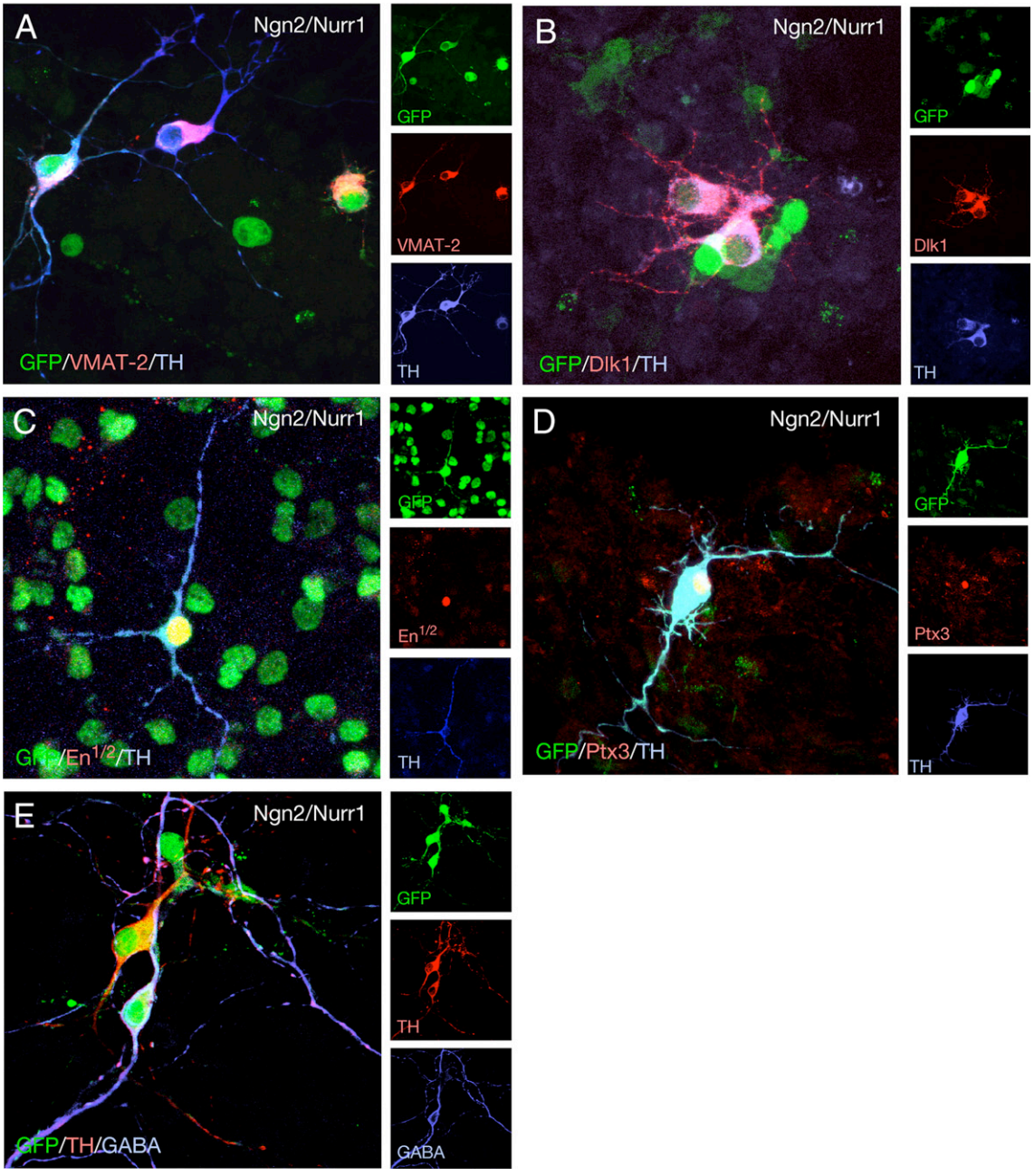

Fig. 4 – TH-expressing cells in Nurr1/Ngn2 co-transduced cultures also express other mesDA neuron markers. Cultures that had been transduced with both Nurr1–IRES–eGFP and Ngn2–IRES–eGFP were analyzed for expression of other mesDA neuron markers. (A) The TH-positive cells also labeled for other proteins involved in neurotransmittor phenotype as shown by co-expression with VMAT2. (B) DLK-1 positive cells often had a stubby appearance but co-labeled with TH and GFP. (C) EN1/2-positive and (D) PITX3-positive cells that co-labeled with TH and GFP were found exclusively in double transduced cultures. (E) While some TH-positive cells co-labeled for GABA, the majority did not.

DLK-1 (Delta-like1) is co-expressed with TH in post-mitotic mesDA neurons during development (unpublished data) and in the adult brain [29]. Cultures transduced with Nurr1/Ngn2 and Nurr1 alone both contained DLK-1-positive cells, but no such cells were seen in GFP transduced control cultures. However the TH-positive cells that stained for DLK-1 frequently had an immature morphology with only short processes (Fig. 4B).

En1 and En2 is expressed early during development in both the midbrain and hindbrain and are maintained in mesDA neurons in the adult [30,31]. In cultures co-transduced with Nurr1 and Ngn2 we detected EN1/2-immunopositive cells that also co-labeled with TH and GFP (Fig. 4C, Table 1), but they were not detected in Nurr1 transduced cultures or in the IRES2–eGFP transduced control cultures (Table 1).

Within the CNS, expression of Pitx3 is restricted to mesDA neurons in both the embryonic and adult brain [32], and thus is a specific marker for post-mitotic mesDA neurons of any developmental stage [32,33]. Examples of PITX3+/TH+/GFP+ cells were found in Nurr1/Ngn2 co-transduced cultures (Fig. 4D) but were not detected in Nurr1 transduced cultures or GFP transduced control cultures. Although the number of both EN1/2 and PITX3 labeled neurons were very low (less than 1% of the number of TH-positive neurons in all experiments), the results were clear and reproducible.

GABA is co-expressed with TH in the dopaminergic neurons in the olfactory bulb while other subcategories of DA neurons do not express GABA [34]. We examined the presence of GABA in our expanded VM cultures and found that GABAergic neurons that are not dopaminergic are generated from expanded cultures derived from the midbrain. Among the Nurr1/Ngn2 induced TH-positive cells we found a few cells that co-express both neurotransmitters (Fig. 4E) but most of the TH-expressing cells did not express GABA, which is in accordance with a midbrain character.

Taken together, the expression profiling of the TH-expressing cells generated after Nurr1 and Nurr1/Ngn2 co-transduction show that the expression of both Nurr1 and Ngn2 in combination is required for the generation of dopaminergic neurons with midbrain characteristics from multi-passaged, expanded neural progenitors from the ventral midbrain. Over-expression of Nurr1 alone, although it gave rise to TH-positive neurons, does not promote a midbrain phenotype, as we did not see expression of PITX3 or EN1/2 in any of these cells.

Other studies have demonstrated the possibility of over-expressing multiple genes by retroviral gene delivery to promote a dopaminergic phenotype from primary and briefly (e.g. <7 days) expanded neural progenitor cells [35,36]. However, these progenitors still have the potential to generate dopaminergic neurons to a limited degree. In this study, we show that expanded VM neural progenitors that have lost the ability to generate dopaminergic neurons regain this potential when both Ngn2 and Nurr1 is re-introduced into the cells. In the co-transduced cultures, we see more mature dopamine neurons with longer, more elaborate processes and a larger percentage of TH-expressing cells that also express VMAT2. Also, in these cultures, at least some of the dopaminergic neurons are likely to be of a midbrain phenotype as they express Pitx3 and En1/2. On the other hand, a few are likely to be of an olfactory bulb phenotype as they co-express GABA. However, it is a small population of the TH expressing cells that express GABA or Pitx3 and En1/2, thus the bulk of the dopamine cells are not of a clear phenotype or subcategory. It remains to be determined if they represent a specific subtype or of they are unspecified “generic” dopamine neurons.

Summary

Our experiments show that over-expression of both Ngn2 and Nurr1 in combination is required to promote the generation of mesDA neurons that express TH, VMAT, Dlk1, En1, and Pitx3, from expanded multi-passaged neurosphere cultures originating from the ventral midbrain. This suggests that developmental cascades parallel to the one initiated by Nurr1 expression are active in the neurosphere-expanded cells transduced with both Nurr1 and Ngn2, indicating that these factors act in synergy to induce midbrain dopaminergic neurons. Further, as midbrain identity is key for functional integration of transplanted dopaminergic neurons [37], this method may be developed further to generate such neurons for transplantation.

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