Induction of a midbrain dopaminergic phenotype in *Nurr*1-overexpressing neural stem cells by type 1 astrocytes

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The implementation of neural stem cell lines as a source material for brain tissue transplants is currently limited by the ability to induce specific neurochemical phenotypes in these cells. Here, we show that coordinated induction of a ventral mesencephalic dopaminergic phenotype in an immortalized multipotent neural stem cell line can be achieved in vitro. This process requires both the overexpression of the nuclear receptor *Nurr*1 and factors derived from local type 1 astrocytes. Over 80% of cells obtained by this method demonstrate a phenotype indistinguishable from that of endogenous dopaminergic neurons. Moreover, this procedure yields an unlimited number of cells that can engraft in vivo and that may constitute a useful source material for neuronal replacement in Parkinson's disease.

Keywords: cell therapy, differentiation, Parkinson's disease, progenitor cells, tyrosine hydroxylase

Functional replacement of specific neuronal populations through transplantation of neural tissue represents an attractive therapeutic strategy for treating neurodegenerative disorders such as Parkinson's disease[1]. Recent advances in neural stem cell biology have shown that multipotent neural progenitors can be isolated, expanded, and used as source material for brain transplants[1–8]. However, although multiple studies demonstrate that implanted neural progenitors successfully engraft and assume legitimate neural phenotypes, when transplanted in the intact adult brain these cells seem biased toward astroglial and oligodendroglial fates[6,7,9]. Given that most neurodegenerative diseases affect neuronal populations of a specific neurochemical phenotype, an ideal source material for transplantation would be an expandable cell that could be instructed to completely assume the desired neuronal phenotype upon differentiation. Indeed, this strategy would circumvent ethical and practical issues surrounding the use of human fetal tissue for transplantation. However, the complete and coordinated induction of a specific neuronal phenotype in multipotent neural precursors in vitro has proved elusive. Our goal in this study was to define the factors required by neural stem cells to produce a dopaminergic phenotype, the major cell type lost in human Parkinson's disease.

One such factor may be *Nurr*1, a transcription factor of the thyroid hormone/retinoic acid nuclear receptor superfamily. *Nurr1* is specifically required for the induction of midbrain dopamine neurons, which fail to develop in *Nurr*1-null mutant mice[10–12]. It is unclear, however, whether *Nurr*1 is also sufficient to specify this neurochemical phenotype. To test this hypothesis, we stably overexpressed *Nurr*1 in a clone of multipotent neural stem cells, which do not normally give rise to dopaminergic neurons in vitro. Thus, any induction of dopaminergic phenotype by *Nurr*1 could be attributed to an inductive process and not to a modulatory role of *Nurr*1 on an ongoing developmental program. For our studies, we chose a neural stem cell line termed C17.2 (ref. 2), which was initially derived from developing mouse cerebellum. These cells contain a *lacZ* reporter, possess the ability to differentiate into neurons, astrocytes, and oligodendrocytes in vitro and in vivo, and upon transplantation into the developing brain, adopt regionally appropriate neuronal phenotypes[2,13]. Moreover, the same single factors that direct the differentiation of primary stem cells from the fetal and adult central nervous system[14] direct the differentiation of C17.2 cells in vitro, suggesting that C17.2 neural stem cells fulfil all necessary criteria to identify the factors sufficient to reconstitute dopaminergic neuron development in vitro and to generate a renewable source material for transplantation.

Results and discussion

Properties of *Nurr*1 transfected C17.2 cells. C17.2 cells were stably transfected, and 50 *Nurr*1 clones were analyzed for transgene expression by RNase protection assay (RPA; Fig. 1A). Several *Nurr*1 clones overexpressed the transgene, of which the five highest expressors were chosen for analysis, along with eight randomly chosen, mock control clones. All *Nurr*1 clones behaved similarly to the parental and mock clones in serum-containing media, with no obvious differences in growth rate or morphology. To define differentiative capacity and phenotypic fate of *Nurr*1 clones, we examined their behavior after low-density passage into serum-free defined media (SFM). In this condition, the parental cell line begins to differentiate, so that after 4–5 days in vitro (DIV), 80–85% of the population is postmitotic—that is, 5-bromodeoxyuridine-negative ($BrdU^-$) after extended pulse—with approximately 20–30% of the postmitotic cells adopting a neuronal fate, judged by the expression of β-tubulin III ($TuJ1^+$, Fig. 1B). The effects of *Nurr*1 on the fate of postmitotic

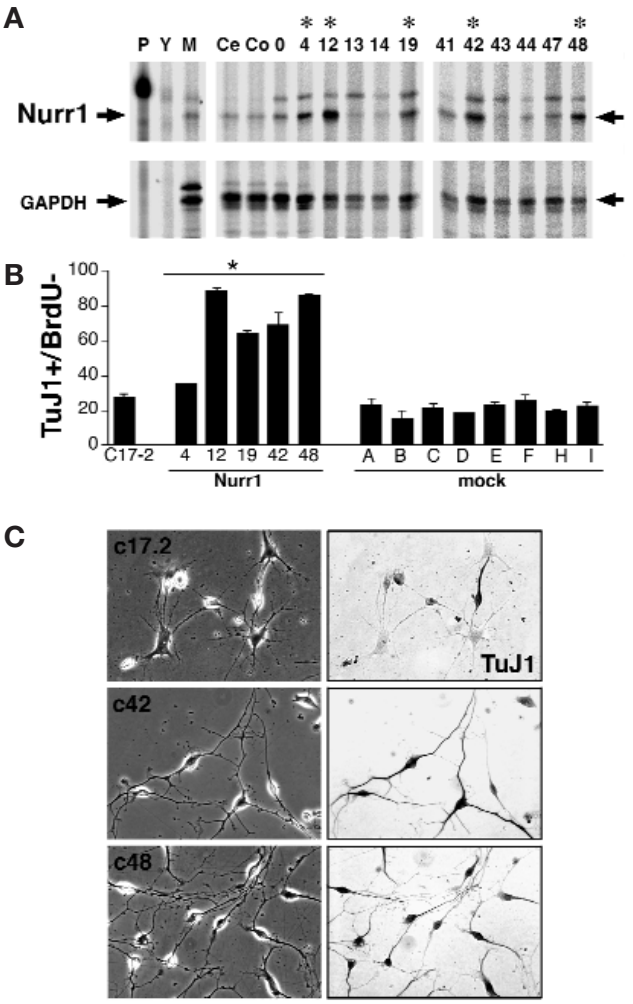

Figure 1. ***Nurr*1 overexpression induces the differentiation of C17.2 neural stem cells into nondopaminergic neurons. (A) RNase protection assay of the levels of expression of *Nurr*1 mRNA in different brain regions (M, ventral mesencephalon (VM); Ce, cerebellum; and Co, cortex), in the parental C17.2 cells (0) and the C17.2-*Nurr1* clones (from 4 to 48). A GAPDH probe was run to standardize for mRNA loading. The selected (*) C17.2-*Nurr*1 clones expressed much higher levels of *Nurr*1 than the VM or any other cells. P, undigested *Nurr1* probe; Y, yeast tRNA. (B) Expression of the *Nurr*1 transgene significantly increased neuronal fate in SFM, as judged by expression of β-tubulin III (TuJ1) at six DIV, to an average of 68% across all clones (global effect of *Nurr*1 transgene vs. mock, *$p < 0.0001$ by two-way ANOVA), but none of these cells were spontaneously dopaminergic. (C) Representative photographs of typical morphologies displayed by the parental (C17.2) and two *Nurr*1 clones (c42 and c48) from top to bottom, respectively. TuJ1 immunocytochemistry (second column of panels) revealed both increased neuronal fate and morphological differentiation in the *Nurr*1-overexpressing clones.**

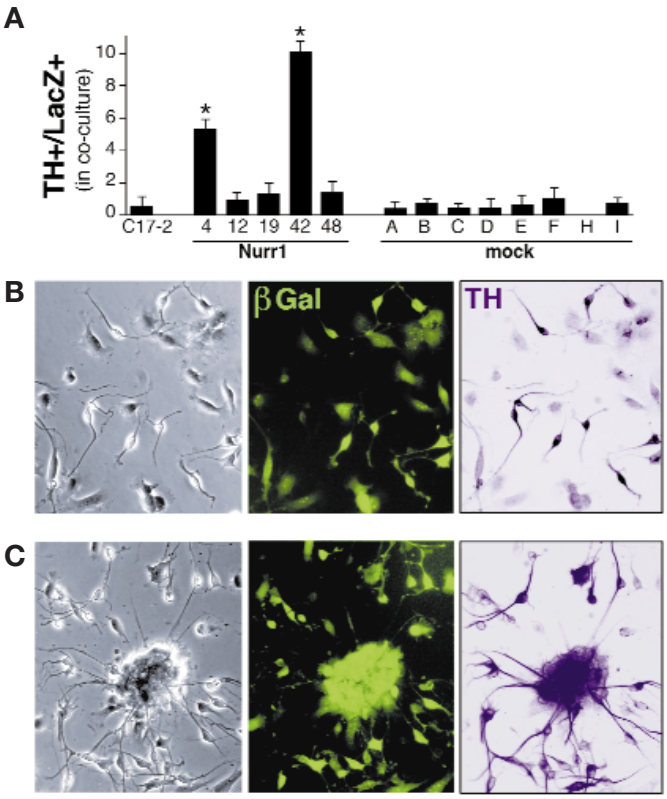

Figure 2. The VM is the source of a signal required for the induction of TH-positive neurons from *Nurr*1-overexpressing neural stem cells. (A) Co-cultures of E16 VM with the *Nurr*1 clones induced a significant increase in the number of TH-positive cells in clones 4 and 42 (*$p < 0.0001$, interaction of transgene vs. clone by two-way ANOVA), suggesting that local factors from the VM are required for the induction of this process. (B) Phase-contrast photograph of E16 VM-*Nurr*1-c42 co-culture at nine DIV. A subset of β-gal–positive cells (stained with FITC, second panel) also demonstrate immunoreactivity for TH (peroxidase stained, third panel). (C) Occasionally, large proliferative clusters of c42-derived TH-positive cells were observed in the co-cultures.

cells were clear and robust (Fig. 1C). In four of five *Nurr*1 clones, the vast majority of postmitotic cells adopted a neuronal fate, a phenomenon not seen in any of the mock clones. However, although *Nurr*1 appears to restrict the C17.2 cell line to neuronal lineages, no significant tyrosine hydroxylase (TH, a marker for dopaminergic neurons) immunoreactivity was detected in any of the *Nurr*1 clones under these conditions (data not shown), suggesting that the fate of these neurons is not dopaminergic.

As *Nurr*1 possessed lineage-restricting capacity in our clonal lines but alone was unable to direct dopaminergic characteristics, we treated the *Nurr*1 lines with a variety of trophic factors, mitogens, cytokines, and other agents known to be important in proliferation (basic fibroblast growth factor, bFGF; epidermal growth factor, EGF; insulin-like growth factor, fetal calf serum), differentiation (sonic hedgehog, Shh; retinoic acid; forskolin; dopamine), and survival of endogenous dopaminergic neurons (glial cell line–derived neurotrophic factor, GDNF; neurturin; brain-derived neurotrophic factor, BDNF; neurotrophin-3 (NT-3); ciliary neurotrophic factor, CNTF). None of these factors, alone or combined, induced TH expression in any of the clones (data not shown).Finally, to test whether as yet unidentified, regionally specific local factors were required, we co-cultured the *Nurr*1 clones with primary cultures derived from E16 rat ventral mesencephalic (VM), the age and region where endogenous dopaminergic neurons of the substantia nigra have just been born[15]. Under these conditions a small, yet significant, percentage of isolated cells, and occasionally mitotic clusters, from two of the *Nurr*1 lines demonstrated measurable amounts of TH immunoreactivity (Fig. 2), whereas little or no TH staining was seen in other *Nurr*1 lines, the C17.2 parent, or any of the mock control lines.

Collectively, these observations suggested that a factor derived from the primary cultures interacted directly or indirectly with *Nurr*1 to induce TH-expressing neurons. However, because TH expression was limited to a minority subpopulation within a fraction of the *Nurr*1 clones, some property of the primary cultures or of the *Nurr*1 clones themselves must have been limiting. We next examined the effects of *Nurr*1 on the differentiative capacity of C17.2 cells, measured by acute BrdU incorporation after passage into SFM, and found varied levels of proliferation (Fig. 3A). Although there was a

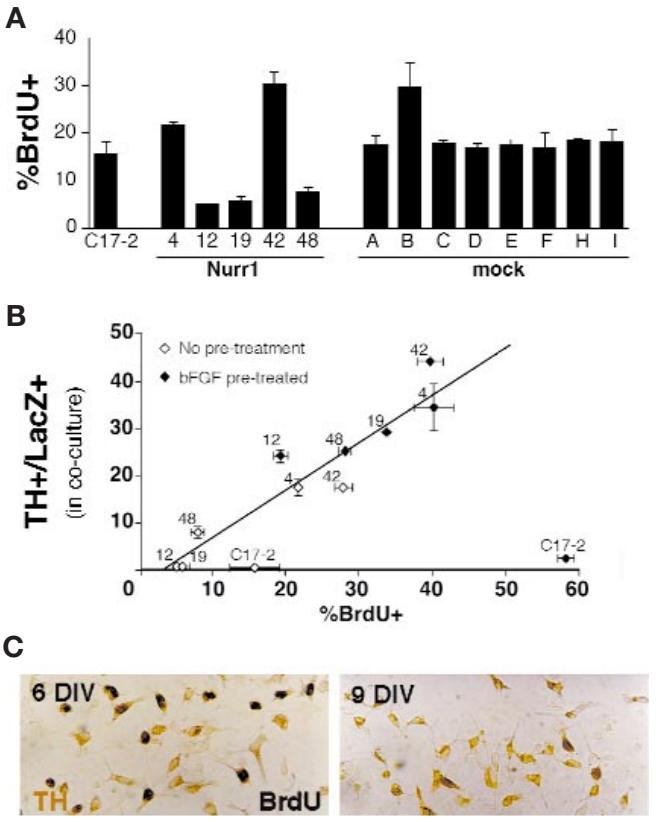

Figure 3. High proliferative rates in *Nurr*1-overexpressing clones correlate with high induction of TH-positive neurons from C17.2-*Nurr1* in VM co-cultures. (A) Residual proliferative rate of the parental-, *Nurr*1-, or mock-C17.2 clones demonstrated that C17.2-*Nurr1* clones with higher residual proliferation also showed the higher number of TH-positive neurons (clones 4 and 42, compare with Fig. 2). (B) A significant linear relationship (r^2 = 0.890 by regression) between the proliferative capacity of the *Nurr*1-overexpressing clones at the initiation of co-culture (with or without bFGF pretreatment) and the expression of TH after differentiation was found, suggesting that *Nurr*1-overexpressing clones must be mitotic during the initial phases of contact with primary VM cells for optimal induction of TH-positive neurons. This was further supported by the observation that, (C) at the time when most TH-expressing c42 cells begin appearing in co-culture (six DIV), nearly all of them are still mitotic (BrdU positive); however, these cells rapidly withdraw from the cell cycle by nine DIV.

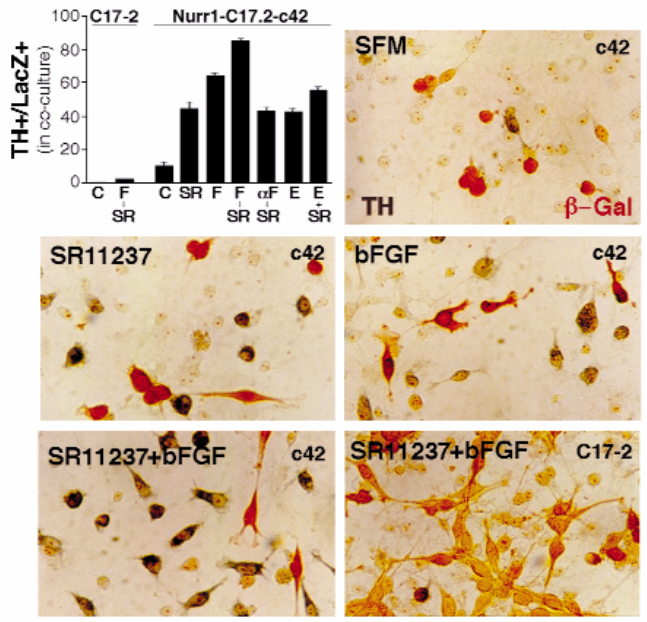

Figure 4. Retinoids and bFGF increase the number of TH-positive neurons derived from C17.2-*Nurr1*, but not from C17.2 cells, in VM co-cultures. Addition of SR11237 (SR, 1 μM) and bFGF (F, 10 ng/ml) to VM/C17.2 co-cultures (C) in SFM did not enhance the differentiation of parental C17.2 cells into TH-positive neurons. Instead, both SR and bFGF increased the number of TH-positive neurons originated from the C17.2-*Nurr1*-c42 cell line (c42) in co-cultures in SFM at seven DIV. Moreover, the effects of bFGF could be substituted by EGF (E), and were additive with those of SR.

trend toward increased differentiation within the *Nurr*1 clones, no clear effect of the transgene on this process was seen. However, we noted that *Nurr*1 clones with the highest residual proliferation in SFM (4 and 42) also demonstrated significant TH expression in co-culture (compare Figs 3A and 2A). We then reasoned that increasing the proliferation of other *Nurr*1 clones should also increase the number of TH-expressing neurons. Thus, we pretreated *Nurr*1 clones with bFGF for five DIV (a procedure that elevates baseline proliferation of most clones after passage into SFM alone) before splitting and replating into primary ventral mesencephalic cultures. Such a procedure allowed us to selectively examine the effects of bFGF on the *Nurr*1 clones and eliminated the mitogenic and indirect trophic effects of bFGF on the primary cultures. As expected, BrdU labeling of bFGF-pretreated *Nurr*1 clones was increased at 24 h after passage of cells into co-culture compared with cells directly split out of serum-containing medium (rightward shift of individual clones on x-axis, Fig. 3B). Concomitant with this increase in proliferation, proportional increases in the percentage of TH-positive cells were observed in all of the *Nurr*1 clones (upward shift of clones on y-axis, Fig. 3B), reaching as high as 45% TH positive (clone 42). In fact, regression analysis indicated a significant linear relationship between mitosis at the beginning of culture and propensity to express TH within, as well as between, individual clones after nine DIV. The C17.2 parental cell line, which responded robustly to bFGF pretreatment, demonstrated minimal increases in TH expression. Thus, our results suggest that an important determinant of dopaminergic fate within individual *Nurr*1 clones is their proliferative capacity.

Previous studies examining the adoption of phenotypic fate during cerebral cortical[16] and spinal cord[17] development have demonstrated that exposure of noncommitted neuroblasts to spatially restricted local factors induces specific phenotypes within these populations, but this induction is contingent upon continuous exposure up to and including the terminal S phase of the neuroblast. A similar mechanism may underlie our observations as most TH-expressing cells are also BrdU positive after acute pulsing at six DIV (Fig. 3C), a time at which the greatest numbers of TH-positive cells begin to appear. However, after acute BrdU treatment of co-cultures at nine DIV, the vast majority of TH-positive cells are BrdU negative, suggesting withdrawal from the cell cycle following induction of measurable TH expression. Taken together, these observations indicate that exposure of a mitotic *Nurr*1 clone to the primary cultures is prerequisite for the determination of dopaminergic fate.

Charcterization of inductive signal. To examine whether molecules important for dopaminergic development[18–20] (e.g., Shh, retinoic acid, or bFGF) could be the inductive signal derived from the ventral mesencephalon (VM), co-cultures of VM and C17.2-*Nurr1* cells were treated with blocking antibodies to bFGF or Shh, or specific retinoic acid receptor (RAR) and retinoic X receptor (RXR) antagonists (data not shown). However, none of these molecules prevented the basal induction of TH expression in C17.2-*Nurr*1-c42 (c42) in co-culture (i.e., ~10%; see Fig. 2). We next tested whether

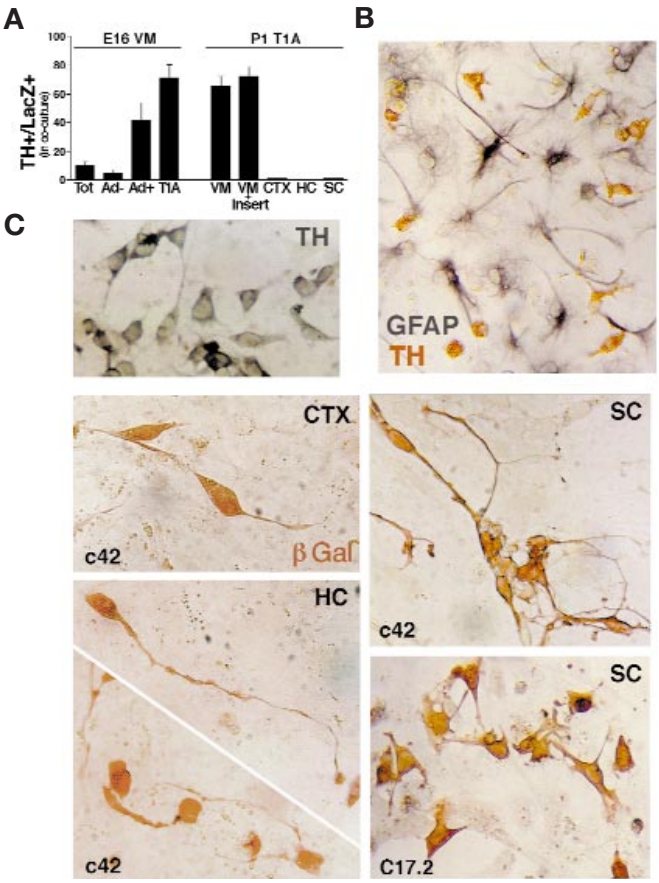

Figure 5. VM type 1 astrocytes specifically induce a dopaminergic phenotype on C17.2-*Nurr1* neural stem cells in SFM. (A) E16 VM adherent cells (Ad+) enhanced the induction of TH in the *Nurr*1-c42 line compared with total (Tot) primary cells or nonadherent (Ad-) cells. Moreover, T1A, which are enriched in the Ad+ fraction, induced a dramatic increase in the number of dopaminergic cells derived from the *Nurr*1-c42 line in the absence of any added factor. The same level of activity was also possessed by postnatal day 1 (P1) T1A from the VM (A and B). This activity was very labile and could not be recovered from conditioned media or a membrane fraction from T1A, but could be recovered when *Nurr*1-c42 cells were cultured separated by a microporous insert (A and C), suggesting that a diffusible factor was involved. (D) In co-cultures of *Nurr*1-c42 cells (c42) with T1A from other brain regions, including cortex (CTX), hippocampus (HC), and spinal cord (SC), *Nurr*1-c42 cells acquired unique, regionally distinct neuronal morphologies, as assessed by lacZ immunocytochemistry, but no induction of dopaminergic phenotype was observed (A and D). The induction of these phenotypes was specific to the *Nurr*1-c42 cells and was not observed in parental C17.2 cells co-cultured with T1A from areas other than the VM, suggesting a remarkable regionally specific inductive potential of T1As.

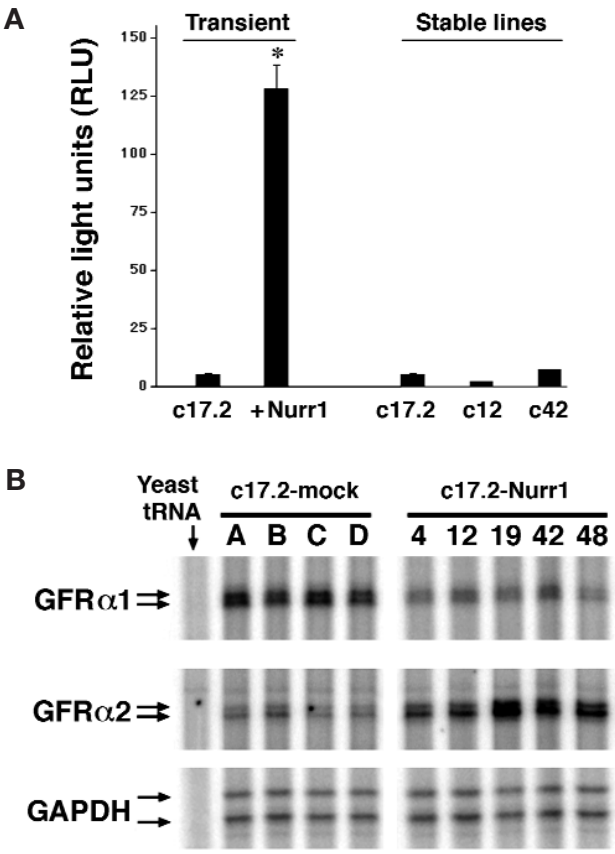

Figure 6. Early activity of *Nurr*1 produces long-lasting changes in gene expression in C17.2 cells. (A) A massive increase in *Nurr*1 transactivation was observed in C17.2 cells 36 h after cotransfection with *Nurr*1 and an NBRE-luciferase reporter (see Experimental protocol). However, no significant increase in basal *Nurr*1 transactivational activity was noted in *Nurr*1 clones 12 or 42 after transfection with the reporter. (B) RNase protection assay of GFRα1 and GFRα2 mRNA levels in mock and *Nurr*1 clones. *Nurr*1 clones had significantly downregulated GFRα1 and significantly upregulated GFRα2, compared with the mock clones, suggesting that early periods of *Nurr*1 activity had significantly altered long-term patterns of gene expression in the *Nurr*1 clones.

treating c42/VM co-cultures with combinations of the previously mentioned proliferative, differentiation, and trophic factors could increase the number of TH-positive neurons. Treatments to the c42/VM cocultures were generally ineffective, with three important exceptions: bFGF, EGF, and the synthetic retinoid analog SR11237 (Fig. 4), each of which induced TH expression in 40–60% of the population after six DIV. Furthermore, the effects of SR11237 and bFGF or EGF were additive at saturating doses (up to 90% lacZ positive were TH positive), suggesting that these molecules may act through distinct mechanisms: increased proliferation, as both bFGF and EGF are directly mitogenic to the C17.2 cells[21]; and RXR receptor stimulation, as SR11237 specifically stimulates RXR receptors[22], which heterodimerize with *Nurr*1 and/or other nuclear receptors to form transcription-initiating complexes[23]. Thus, these results suggest that FGF and retinoids, although not inductive signals in our system, act to enhance the efficiency of TH induction. Thus, the only obligate requirement for the induction of a dopaminergic phenotype seemed to be exposure of *Nurr*1-expressing neural stem cells to signals derived from primary VM cells.

What is the nature and cellular source of such an inductive signal? Primary VM cultures typically contain a mixture of dopaminergic and other neurons, astrocytes, and oligodendrocytes, as well as assorted nonneural elements such as microglia, endothelial cells, and fibroblasts. To begin identifying the source of this TH-promoting activity, we performed a crude separation of the primary cells based on adhesion. The rapidly adherent population was enriched for glial and nonneural elements, whereas the nonadherent population consisted mainly of neurons, oligodendrocyte precursors, and a few astrocytes. After co-culture of these fractions with c42, we determined that the majority of TH-inducing activity was contained within the rapidly adherent population (Fig. 5A). As the major neural constituent of this population was type 1 astrocytes (T1A), we prepared purified cultures of these cells from E16 VM and were able to verify that T1A were indeed the source of TH-inducing activity. Furthermore, this activity was not restricted to early development, as astrocytes isolated from newborn rats induced equivalent numbers of TH-positive c42 cells (~70%, Fig. 5A). In order to determine if this activity was due to a soluble or a membrane-bound factor, we treated C17.2-*Nurr1*-c42 cells with type 1 astrocyte–conditioned

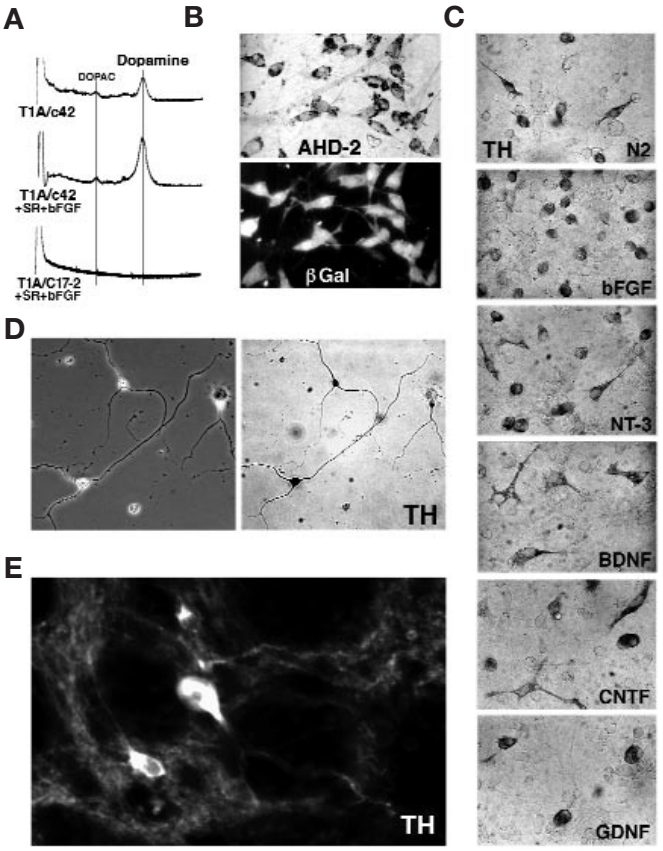

Figure 7. Characterization and long-term stability of the dopaminergic phenotype acquired by *Nurr*1-c42 cells after differentiation. (A) HPLC traces show that supernatants collected from KCl-depolarized *Nurr*1-c42 cells (c42), but not the parental C17.2 cells, contained dopamine and its metabolite DOPAC. Moreover, factors enhancing dopaminergic differentiation, such as bFGF and SR11237, similarly enhanced the amount of dopamine release, in c42 cells, but were ineffective in C17.2 cells. (B) After dopaminergic differentiation, almost all *Nurr*1-c42, but not parental cells, expressed the enzyme aldehyde dehydrogenase-2 (AHD-2), a marker associated with endogenous dopaminergic neurons of the midbrain. (C). *Nurr*1-c42 cells induced to differentiate into dopaminergic neurons respond to multiple neurotrophic fators similarly to midbrain dopaminergic neurons. bFGF (10 ng/ml) and NT-3 (30 ng/ml) dramatically increased the number of TH-positive cells in the culture, compared with the N2 control condition. Moreover, BDNF (30 ng/ml), CNTF (10 ng/ml), and GDNF (10 ng/ml) induced neuritogenesis and/or hypertrophy on *Nurr*1-c42-derived dopaminergic neurons, suggesting that the dopaminergic phenotype acquired by C17.2-*Nurr1*-c42 represents a specific ventral mesencephalic dopaminergic fate. Finally, TH-positive *Nurr*1-c42 cells generated in co-culture possess a stable dopaminergic phenotype (D and E). Intense TH expression was noted in a small but significant number of highly differentiated cells passaged into N2 medium for 14 days in vitro (D) and 12 days after transplantation into adult mouse corpus striatum in vivo (E).

media or membrane fragments, respectively. However, neither of these treatments induced significant increases in the number of TH-expressing cells, suggesting that this activity was highly labile. To circumvent this problem, we co-cultured astrocytes and C17.2-*Nurr1*-c42 cells, but spatially separated the two populations via a microporous insert, which allowed free passage of macromolecules but prevented contact between the two populations. In this environment, TH expression was induced in C17.2-*Nurr1*-c42 cells at a level equivalent to direct co-culture (Fig. 5A and C), suggesting that ventral mesencephalic type 1 astrocytes, in addition to providing both trophic support[24] and morphogenetic cues[25] to dopaminergic neurons during development, secrete a highly labile diffusible factor that interacts with *Nurr*1-overexpressing stem cells to generate TH-positive neurons.

We next examined whether the TH-inductive activity is restricted to type 1 astrocytes from the VM. Type 1 astrocytes were isolated from several brain regions that contain populations of *Nurr1*-expressing cells during development[26]. No increase in the number of TH-immunoreactive cells was observed in c42, compared with parental C17.2 cells, when co-cultured with cerebrocortical, hippocampal, or spinal cord astrocytes (Fig. 5A), indicating that a putative TH-inducing factor is selectively produced by ventral mesencephalic type 1 astrocytes. However, c42 cells were not completely unaffected by astrocytes from other regions; cells of this line developed distinct, neuronal-like morphologies unique to each region (Fig. 5D). Under the same conditions, however, the C17.2 parental line tended to display a uniform mixture of polygonal, and bi- and tripolar morphologies. Collectively, these data raise the intriguing possibility that astrocytes from distinct brain regions secrete unique, or unique combinations of, factors that interact with *Nurr*1-expressing cells to produce specific, and perhaps regionally appropriate, neuronal phenotypes. Confirmation of this hypothesis awaits detailed phenotypic analysis of multiple *Nurr*1 lines under such conditions.

Mechanism. Perhaps the simplest mechanism for dopaminergic induction would involve the secretion by astrocytes of a putative ligand for *Nurr*1, which in turn activates appropriate intracellular machinery involved in the expression of the specific TH-positive phenotype. We found that significant *Nurr*1 transactivational activity was detected 36 h after cotransfection of our *Nurr*1 expression vector and a *Nurr*1-responsive luciferase reporter, but not after transfection of the reporter into the stable, proliferating C17.2-*Nurr*1 clones (Fig. 6A), as well as differentiated clones (data not shown). Because *Nurr*1 was not active during the neuronal differentiation of stable C17.2-*Nurr*1 clones, our findings suggest that earlier transient high levels of *Nurr*1 activity may have conferred upon the *Nurr*1 clones long-lasting competence to be dopaminergic. To test this hypothesis we examined changes in gene expression in the *Nurr*1 clones. We observed that the C17.2 and all mock clones examined expressed high levels of the GDNF receptor GFRα1 mRNA and very low levels of the related receptor GFRα2 mRNA. In contrast, all *Nurr*1 clones demonstrated the inverse profile, that is, very low GFRα1 and very high GFRα2 mRNA levels (Fig. 6B), suggesting that in C17.2 cells, *Nurr*1 regulates target genes before actual induction of a TH-positive phenotype. In such a way, *Nurr*1 may bestow competence upon multipotent cells to respond to specific factors, including those derived from ventral mesencephalic astrocytes.

Specificity. We examined whether the gain of TH expression represented the adoption of a legitimate dopaminergic phenotype within the C17.2-*Nurr1*-c42 line. By convention, the ability to release dopamine in response to membrane depolarization is the vital criterion for designation of a neurochemical phenotype as dopaminergic. Thus, we acutely treated co-cultures of parental or c42 lines and VM astrocytes with 50 mM KCl and assayed the supernatants for monoamine content with HPLC. Significant levels of dopamine and DOPAC, a major dopamine metabolite, were detected in supernatants from co-cultures containing c42, with increased release detected in co-cultures treated with SR11237 and bFGF, thereby correlating dopamine release to the number of TH-expressing cells (Fig. 7A). No dopamine release was observed in the parental line. Thus, TH-expressing C17.2-*Nurr1*-c42 cells may be considered dopaminergic. However, is the phenotype of this line of a *ventral mesencephalic* dopaminergic type? As complete criteria for this distinction within endogenous precursor populations remain undefined, one cannot fully ascertain whether our *Nurr*1 lines adopt a complete mesencephalic phenotype. Nonetheless, C17.2-*Nurr1*-c42 cells in co-cul-

ture also acquired immunoreactivity for aldehyde dehydrogenase-2 (AHD-2; Fig. 7B), an enzyme selectively expressed in developing dopaminergic precursors within the VM[27] and expressed c-*ret* mRNA (data not shown), the signaling receptor for GDNF[28,29] that is present in dopaminergic neurons[28]. Furthermore, TH-positive c42 cells demonstrated similar responses to factors with known neurotrophic effects on dopaminergic progenitors[8] and primary ventral mesencephalic dopaminergic neurons in vitro[30,31] (Fig. 7C). Thus, inasmuch as the behavior of TH-expressing c42 cells parallels that of endogenous dopaminergic neurons of the substantia nigra, these cells should be considered ventral mesencephalic-like dopaminergic neurons.

Stability of the iduction. After eight days in insert co-cultures, c42 cells were removed from the inserts and replated into defined media without additional factors for 14 days. Although significant cellular attrition was observed two weeks after removal from co-culture, a small but significant number of cells displayed a highly mature dopaminergic phenotype, including long, elaborate processes, hypertrophic cell bodies, and intense levels of TH immunoreactivity (Fig. 7D). c42 cells from parallel co-cultures also were surgically injected into adult mouse corpus striatum and allowed to mature for 12 days, in the absence of any additional trophic factors or supportive cells (i.e., astrocytes, oligodendrocytes, or other neurons). Although many cells were lost in this condition, a few c42-derived dopaminergic cells displayed a high level of differentiation and apparent integration into the host tissue (Fig. 7E). No TH-positive cell derived from the C17.2 parental line were found in either of the long-term in vitro or in vivo experiments. Thus, the observation that some c42 cells maintained, or even increased, TH expression after removal from T1A-derived factors shows that, after dopaminergic induction, their phenotype is stable. However, since few surviving TH-positive cells could be detected, exogenously applied trophic factors or supporting cells may be required for long-term survival.

In conclusion, our results suggest that *Nurr*1 induces neuronal differentiation and confers competence to respond to extrinsic signals that induce dopaminergic fate. Moreover, the current study demonstrates that the source of such signals are VM astrocytes, which release a soluble factor that induces *Nurr*1-expressing neural stem cells to develop into typical dopaminergic neurons of the VM. Our findings further suggest that primary astrocytes may be the source of signals required for the induction of regionally appropriate neuronal phenotypes in multiple brain structures. Finally, the procedure we describe, taking advantage of the multipotential capacity of neural stem cells, selector genes such as *Nurr*1, and primary astrocytes, might be used to engineer neurons with the desired neurochemical phenotype as a source material for neuronal transplantation in the treatment of neurodegenerative diseases. In this respect, the induction of an unlimited number of midbrain dopaminergic neurons in vitro, that can engraft in vivo, could prove particularly useful in cell replacement strategies to treat Parkinson's disease.

Experimental protocol

*Nurr*1-overexpressing cell lines. C17.2 cells were cotransfected with a CMX-*Nurr*1 expression vector[17] and the PGK-puromycin resistance plasmid[23]; mock controls received PGK-puromycin alone. For reporter assays, C17.2 parental cells were transfected with the *Nurr*1 expression vector, the reporter plasmid[23] (NGFI-B-binding response element [NBRE] triplet upstream of TK minimal promoter fused to firefly luciferase) and a pRSV-alkaline (Clonetech, Palo Alto, CA) phosphatase reference plasmid at a 2:1:2 ratio. A total of 50 stable *Nurr*1-transfected clones were selected for puromycin resistance, isolated, amplified, and *Nurr*1 mRNA expression was analyzed by RPA. Assays were performed using the RPAII Ribonuclease Protection Assay Kit (Ambion Inc., Austin, TX), following the manufacturer's recommendations. A 288 bp antisense *Nurr*1 cRNA probe spanning nucleotides 1,798–2,086 of the mouse *Nurr*1 cDNA sequence[32] was transcribed with T7 (from a m*Nurr*1 cDNA cloned into the *Eco*RI/*Xho*I site of the pBSKS expression vector [Stratagene, La Jolla, CA] linearized with *Eco*RI) and labeled with (α-^{32}P)CTP (Amersham Intl., Little Chalfont, UK). Protected cRNA fragments were separated on 4% PAGE under denaturing conditions. The intensity of the signal was analyzed with a phosphoimager MD storm 840 (Molecular Dynamics), and *Nurr*1 signal was standardized to the content of glyceraldehide 3-phosphate dehydrogenase (GAPDH) in every sample. GFRα expression was analyzed similarly, using probes already described[33].

Cell culture and treatments. C17.2 neural stem cells were maintained and passaged as previously described[4]. Ventral mesencephala from E16 rat embryos were dissected, mechanically dissociated, and plated at a final density of 1×10^5 cells/cm^2 on poly-D-lysine–coated culture wells in SFM (N2, consisting of a 1:1 mixture of F12 and DMEM containing 10 ng/ml insulin, 100 μg/m transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, 6 mg/ml glucose, and 1 mg/ml bovine serum albumin, BSA). After 24 h in vitro, co-cultures were initiated by directly plating $2.5–5 \times 10^4$ C17.2-derived cells into the primary cultures; all ages of cultures given use this point as zero DIV. This sequence of plating and ratio of primary/C17.2 cells resulted in the healthiest cultures, although varying the numbers of C17.2 cells over a 10-fold range did not significantly affect the proportion of TH-positive cells observed (data not shown). Purified type 1 astrocytes were obtained from mixed glial cultures derived from various regions of P1 rats according to a standard protocol[34]. After replating into 6- or 12-well plates, astrocytes were grown to confluency in serum-containing media and changed to N2 medium. After three to five DIV, co-cultures were initiated in fresh N2 as described. All factors were added once, at the initiation of co-culture (concentrations are noted in figure legends), with the exception of BrdU, which was added 4–6 h before fixation. Cultures were maintained in a humidified 5% CO_2, 95% air incubator at 37°C and fixed after given time periods with 4% paraformaldehyde for 45 min before immunocytochemical analysis.

Immunocytochemical analysis and HPLC. Fixed cultures were incubated with one of the following antibodies, diluted appropriately in PBS containing 1% BSA and 0.3% Triton X-100: mouse anti-BrdU, 1:50 (DAKO, Glostrup, Denmark), mouse anti-β-tubulin, type III (TuJ1), 1:250 (Sigma, St. Louis, MO), mouse anti-TH, 1:1,000 (Incstar, Stillwater, MN), rabbit anti-β-galactosidase, 1:250 (Cappel, Durham, NC)) rabbit anti-glial fibrilary acidic protein (GFAP), 1:500 (DAKO), rabbit anti–AHD-2, 1:4,000 (generous gift of Dr. R. Lindahl, University of South Carolina, School of Medicine). Incubations were either carried out at 4°C overnight, or at room temperature for 1 h. Both processes yielded similar results. After washing, cultures were incubated for 1–3 h with appropriate secondary antibodies (biotinylated horse anti-mouse IgG or goat anti-rabbit IgG, Vector, Burlingame, CA), 1:100, in the same dilution buffer. Immunostaining was visualized with the Vector Laboratory ABC immunoperoxidase kit, using either (red) AEC, grey (SG) , or violet (VIP) substrates. Fluorescent double-labeling of β-galactosidase (β-gal) was performed by substituting the biotinylated secondary with fluorescein isothiocyanate (FITC)-conjugated antibody (Vector), 1:100. Quantitative immunocytochemical data represent means and standard errors from 100 to 500 cells counted in each of three to six separate wells from two to four experiments. For quantitation of TH expression in co-cultures of primary and C17.2–derived cells, TH was visualized with the bright-field AEC or VIP substrate, while β-gal was assessed using FITC; thus, all data expressed as "percent TH positive" represent the number of TH-positive/β-gal-positive divided by total β-gal-positive cells. The bright-field staining of these markers in Figure 4 was performed for presentation purposes only.

For analysis of dopamine release, large (10 cm) co-cultures containing ~1 million C17.2-*Nurr1*-c42 or C17.2 parental cells with P1 VM T1A were treated with 200 μl of 50 mM KCl in PBS/0.1 M sodium citrate for 5 min while swirling. The supernatants were immediately assayed for dopamine content using HPLC. Samples were separated with a reverse-phase C-18 column, eluted with acetonitrile, and detected electrochemically. Results were verified with a standard containing dopamine, DOPAC, serotonin, and 5-hydroxylindoleacetic acid.

Long-term cultures and transplantation. Parental C17.2 cells or C17.2-*Nurr*1-c42 cells were grown in large (10 cm) insert co-cultures with VM type 1 astrocytes for eight days in the presence of bFGF (10 ng/ml) and SR11237 (1 μM). Cells were then trypsinized off of the insert, pelleted, and resuspended, at a concentration of 100,000 cells/μl, in their own conditioned media; an aliquot of this mixture was then plated into poly-D-lysine–treated six-well tissue culture plates containing N2 media, while the remainder were used for transplantation. Adult (25–30 g) CD1 mice (Charles River, Uppsala, Sweden), housed and treated according to the guidelines of the European Community (86/609/EEC), were anesthetized with pentobarbital (60 mg/kg intraperitoneally). A total of 25,000 cells were stereotaxically injected into the

striatum at each of the two following coordinates (in mm): AP (bregma) = 0.56, L = 1.9, DV(dura) = -2.55 and -2.75, with the incisor bar at -3. Twelve days after grafting, mice were transcardially perfused with 4% paraformaldehyde. Brains were postfixed for 2 h, embedded in 10% sucrose for more than one day, and frozen in dry-ice cooled isopentane. We then processed 14 μm cryostat sections for TH immunohistochemistry using a mouse anti-TH (Incstar) 1:1,000, and a donkey anti-mouse rhodamine (Jackson, West Grove, PA) 1:100, antibodies. TH-positive cells were photographed using a Hamamatsu camera attached to a Zeiss Axioplan 2 microscope.

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