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Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain

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SUMMARY

Adult rat-derived hippocampal progenitor cells express many of the molecules implicated in midbrain dopaminergic determination, including FGF receptors 1, 2 and 3, the sonic hedgehog receptor components Smo and Ptc, and the region-specific transcription factors Ptx3 and Nurr1. Here we use undifferentiated progenitors to probe the events leading to the dopaminergic phenotype and find that the influences of Nurr1 can be temporally and mechanistically uncoupled from the patterning influences of sonic hedgehog and FGF-8 or the more generic process of neuronal differentiation itself. In gain-of-function experiments, Nurr1 is able to activate transcription of the tyrosine hydroxylase gene by binding a response element within a region of the tyrosine hydroxylase promoter necessary for midbrain-specific expression. This activation is mediated through a retinoid X receptor independent mechanism and occurs in all precursors, regardless of differentiation status. Overexpression of Nurr1 does not affect proliferation or stimulate neuronal differentiation and has no influence on the expression of other dopaminergic markers. This uncoupling of tyrosine hydroxylase expression from other dopaminergic markers suggests that the midbrain dopaminergic identity is dictated by a combination of pan-dopaminergic (e.g., Shh/FGF-8) and region-specific (Nurr1) mechanisms.

Key words: Nurr1, Tyrosine hydroxylase, Stem cells

INTRODUCTION

Dopaminergic neurons exist as a heterogeneous population of cells distributed in midbrain, hypothalamus, olfactory bulb and retina. Recently, explant culture analyses have shown that the intersection of Shh, which is expressed along the ventral neural tube, and FGF-8, which is locally produced at the mid/hindbrain boundary and in the rostral forebrain, defines induction sites for dopaminergic neurons in the midbrain and forebrain (Ericson et al., 1995; Wang et al., 1995; Crossley et al., 1996; Ye et al., 1998). Furthermore, transgenic founder analyses have shown that enhancer elements directing region-specific expression exist as separate and quite heterogeneous elements (Liu et al., 1997) within the tyrosine hydroxylase (TH) promoter. A hypothalamus regulatory domain was localized between –2.5 and –3.4 kb of the rat TH promoter and a midbrain-specific regulatory domain was localized between –0.8 and –2.5 kb. Olfactory bulb-specific elements appeared to reside outside the 6.0 kb that were evaluated. These results suggest that the individual attributes that define a dopaminergic neuron may be regulated by both subtype-specific and region-specific machinery.

Although the molecular mechanisms underlying the subtype specification and the regional specification of dopaminergic neurons are poorly understood, there are two transcription factors implicated in midbrain dopaminergic determination. These are *Nurr1*, an orphan receptor belonging to the nuclear receptor superfamily (Law et al., 1992; Zetterstrom et al., 1996a), and the bicoid-related homeobox factor *Ptx3*/*Pitx3* (Semina et al., 1997, 1998; Smidt et al., 1997). *Nurr1* is expressed at embryonic day (E) 10.5 in the ventral aspect of the mesencephalic flexure just prior to the appearance of TH at E11.5, and continues to be expressed into adulthood (Zetterstrom et al., 1996a,b). Although expression of *Nurr1* is not restricted to midbrain dopaminergic neurons, *Nurr1*-null mice lack only midbrain dopaminergic neurons (Zetterstrom et al., 1997; Saucedo-Cardenas et al. 1998; Castillo et al., 1998a). *Ptx3* is expressed in ventral midbrain starting at E11.5, soon after *Nurr1* begins to be expressed (Smidt et al., 1997; Saucedo-Cardenas et al., 1998). Despite strong evidence for a role for *Nurr1* and *Ptx3* in the developing midbrain, target genes that are regulated by *Nurr1* and *Ptx3* have yet to be identified via gain-of-function analyses. In addition, the exact role of *Nurr1* or *Ptx3* in dopaminergic phenotype determination versus the more generic neuronal differentiation process is not well understood.

Neural stem cells (reviewed by Gage et al., 1995b; Morrison et al., 1997; McKay, 1997; Stemple and Mahanthappa, 1997;

Johansson et al., 1999) may offer an effective platform for studying the regulation of cell phenotypes. Cloning experiments with cultured neural precursors have shown that FGF-2-responsive cells isolated from adult rat hippocampus include multipotent progenitor cells that are self-renewing and capable of generating both neurons and glia in vitro (Johe et al., 1996; Palmer et al., 1997). Although progenitor cells in the adult rat hippocampus generate only one type of neuron within the granule cell layer, they produce a variety of transmitter phenotypes when isolated from the hippocampus and expanded in the presence of FGF-2. When grafted into the rostral migratory pathway leading to the olfactory bulb, the adult hippocampus-derived progenitors (AHPs) generate mature olfactory bulb neurons, some of which express TH (Suhonnen et al., 1996). When induced to differentiate in vitro, these cells can also generate a wide variety of neurotransmitter phenotypes, including gamma-aminobutyric acid (GABA), acetylcholine esterase (AChE), TH, calbindin, choline acetyltransferase (ChAT) and substance P (Gritti et al., 1996; Takahashi et al., 1999). This plasticity suggests that the cells can respond to appropriate cues and may be an effective tool for studying the progression of events necessary to generate dopaminergic neurons.

In this study, we have shown that AHPs have the ability to generate dopaminergic neurons by mechanisms similar to those that exist in the developing ventral mesencephalon. In addition, we have shown that forced expression of Nurr1 was sufficient to induce endogenous TH expression in both differentiated and undifferentiated AHPs. However, Nurr1 did not trigger neuronal differentiation or affect the expression of other genes utilized in dopaminergic cells. Nurr1-dependent TH expression is mediated through a novel Nurr1 binding site (NBRE) located in a domain of *TH* promoter that is necessary for the region-specific expression of TH in midbrain dopaminergic neurons.

MATERIALS AND METHODS

Isolation and propagation of adult rat hippocampal progenitor cells

Neural precursors from adult rat hippocampal formations were isolated in culture as described by Gage et al. (1995a). Primary cultures were maintained on laminin-coated dishes (Ray et al., 1993) in DMEM:F12 (1:1) with N2 supplement (GIBCO) and 20 ng/ml recombinant human FGF-2 from *E. coli* (a gift from A. Baird). The bulk population (HC7) of AHPs used in this work has been characterized extensively (Palmer et al., 1997; Takahashi et al., 1999) and was used at passages 10 through 20. The stem cell-derived clone (C31) isolated from the HC7 population was also described previously (Palmer et al., 1997). Both polyclonal and clonal populations are capable of generating a variety of neuronal phenotypes including GABA, TH and AChE-positive neurons (Takahashi et al., 1999).

To induce differentiation, cells were initially plated onto 6 cm laminin-coated dishes or coated chamber slides at a density of 2×10^4 or 10^5 cells per cm^2, respectively. Cells were allowed to proliferate in N2-supplemented medium containing 20 ng/ml FGF-2 for 24 hours. FGF-2 was then withdrawn and cells were subsequently treated with N2 medium alone or with N2 medium containing 0.5 μM RA, 5 μM FK, or 40 ng/ml FGF-8. Medium was replaced every 48 hours.

Cloning of *Nurr1*, *Nurr1a*, *Ptx3* and *Shh-N*

cDNAs containing the full open reading frames of rat *Nurr1*, *Nurr1a*, *Ptx3* and *Shh-N* were cloned by RT-PCR from poly(A) RNA derived from Fischer 344 rat embryonic brain at embryonic day 13.5. Total RNA was isolated by the procedure developed by Okayama et al. (1987). Poly(A) RNA was purified using Oligo(dT)-cellulose (Pharmacia) column chromatography. First strand cDNA synthesis was carried out using 50 ng of poly(A) RNA, Superscript II reverse transcriptase (GIBCO BRL), and oligo(dT) primer followed by RNase H treatment. The resulting products were PCR amplified using Pwo polymerase (Boehringer Mannheim) and the following primers:

(1) 5′ primer for *Nurr1* and *Nurr1a* 5′-TCGGCTGAAGCCATGCCTTG-3′,
(2) 3′ primer for *Nurr1* and *Nurr1a* 5′-GACGTGCATGGGAGAAAGTC-3′,
(3) 5′ primer for *Ptx3* 5′-CATGGAGTTTGGGCTGCTTGG-3′,
(4) 3′ primer for *Ptx3* 5′-TCACACCGGCCGTTCCACG-3′,
(5) 5′ primer for *Shh-N* 5′-CGTACCAGCTCGCGCACAGAC-3′,
(6) 3′ primer for *Shh-N* 5′-GGGAATCAGCCGTCAGATTTG-3′.

The PCR products were subcloned into TOPO-TA cloning vector (Invitrogen) following the manufacturer's instructions. DNA sequencing confirmed that the clones contained the full-length fragments of the rat *Nurr1*, *Nurr1a*, *Ptx3*, and *Shh-N* coding regions.

Retroviral transduction and cloning

The NIT retroviral vector was constructed from LINX (Hoshimaru et al., 1996) by swapping positions of the tetracycline-controlled transactivator (tTA) and neomycin resistance genes. The neomycin resistance gene was replaced by E-GFP coding sequence to form GIT. Fragments containing *Nurr1*, *Nurr1a*, *Ptx3*, *Shh-N* and *E-GFP* (Clontech) were cloned into NIT vector and DNA was transfected to the producer cells (293gag pol; kindly provided from Nikunj Somia). RVEN3 was constructed from pCLMFG (kindly provided from Nikunj Somia) by deleting the first ATG between the viral splice acceptor site and multiple linker site. pCLMFG was derived from pMFG and contains a hCMV immediate early promoter in place of the MoMLV promoter within the U3 region of the 5′ LTR (Naviaux et al., 1996). To increase the degree of infection, viral preparations were pseudotyped with a vesicular stomatitis virus (VSV-G) coat protein by cotransfecting producer cells with pMD.G. Viral supernatants concentrated by centrifugation (Burns et al., 1993) were used to infect AHP suspensions for 30 minutes followed by plating to polyornithine/laminin-coated dishes. To generate stable expressing cells, HC7 cells were treated with NIT-based viruses (moi ~1) and cultured in the presence of 100 μg/ml G418. To improve cell survival during selection, medium was supplemented with conditioned medium from high density HC7 cell cultures. The RXRα (Human)-expressing retrovirus vector (LNCX-RXRα) was kindly provided by Steven Suhr and was based on the LNCX expression vector (Miller and Rosman, 1989).

RT-PCR and quantitative PCR

Total RNA was isolated from cell culture using RNAzol (Tel Test), and cDNA was made using the Superscript preamplification system (GIBCO BRL) from 4 μg of total RNA. First strand cDNA was diluted 3 fold and 2 μl of cDNA was used for each PCR reaction. RT-PCR products were analyzed in an agarose gel containing ethidium bromide (EtBr). DNA bands were photographed using an Eagle Eye II video system (Stratagene). The image was exported in a TIFF file and DNA bands were quantified using NIH Image 1.55 software for Macintosh. The quantification value of the band was designated the absorbance (Pixels). Measurements of absorbance using the above system were linear up to 120 pixels. For quantitative PCR, cycle numbers and template quantity were determined to be in the linear range for each gene. Kinetic analyses were also used to demonstrate linearity (Yokoi et al., 1993). Regression equations of the form $Y=A\times E^n$ (where Y is the yield of PCR products, E is the efficiency of amplification, and n is the number of cycles) were fitted to the data in the linear portion of the semilogarithmic graphs. We calculated the coefficient A for each reaction to estimate the relative amount of mRNA. We analyzed each sample at least three times, and the

difference in the obtained values was always less than 2%. For each condition, at least two independent experiments were carried out.

The following primers were used to amplify target cDNA:

(1) *G3PDH* 5′-ACCACAGTCCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTA-3′,
(2) *TH* 5′-CCTCCTTGTCTCGGGCTGTAA-3′, 5′-CTGAGCTTGTCCTTGGCGTCA-3′,
(3) *AADC* 5′CCTACTGGCTGCTCGGACTAA-3′, 5′-GCGTACCAGGGACTCAAACTC-3′
(4) *DBH* 5′-GTGACCAGAAAGGGCAGATCC-3′, 5′-CACCGGCTTCTTCTGGGTAGT-3′,
(5) *GFAP* 5′-GCAGACCTCACAGACGTTGCT-3′, 5′-AGGCTGGTTTCTCGGATCTGG-3′,
(6) *Phox2A* 5′-TGGCGCTCAAGATCGACCTCA-3′, 5′-CGTTAGGGTGGGATTAGCGGT-3′,
(7) *Nurr1* 5′-TAAAAGGCCGGAGAGGTCGTC-3′, 5′-CTCTCTTGGGTTCCTTGAGCC-3′,
(8) *Ptc* 5′-ACCTTTGGACTGCTTCTGGG-3′, 5′-AGTCGTAGCCCCTGAAGTGTT-3′,
(9) *Smo* 5′-GCCACCCTGCTCATCTGGA-3′, 5′-TTCCGGCCTAAACGCTTCTC-3′,
(10) *Gli1* 5′-CATGTGTGAGCAAGAAGGTTGC-3′, 5′-AAGTCGAGGACACTGGCTATAGG,
(11) *Shh* 5′-TTCTGGTGGCCCTTGCTTCCT-3′, 5′-TACTTGCTGCGGTCCCTGTCA-3′,
(12) *D2R* 5′-GCATCCTGAACCTGTGTGCCA-3′, 5′-GCAGCATCCTTGAGTGGTGTC-3′,
(13) *GFRα-1* 5′-GATTTGCTGATGTCCGCCGAG-3′, 5′-AATCAGTCCCGAGTAGGCCAG-3′,
(14) *c-Ret* 5′-AGACAGACCCAGGCTTCGCTA-3′, 5′-TTTCCGCTGATGCAATGGGCG-3′,
(15) *FGFR* 5′-TCNGAGATGGAGRTGATGAA-3′, 5′-CCAAAGTCHGCDATCTTCAT-3′,
(16) *VMAT2* 5′-GCAGTCACACAAGGCTACCAG-3′, 5′-AGCCCCATCCAAGAGCACCAA-3′,
(17) *RXRα* 5′-TTCCTGCCGCTCGATTCTCC-3′, 5′-GTTCAGCCCCATGTTTGCCTC-3′.

The PCR product of *Ptc* was subcloned into TOPO-TA vector and sequenced. Reaction products were confirmed by restriction enzyme digestion.

Immunofluorescent staining and fluorescent imaging

Staining was performed as previously described (Gage et al., 1995a). Briefly, after fixation with 4% paraformaldehyde in PBS, cells were incubated with primary antibodies overnight at 4°C. Then the cells were incubated overnight at 4°C with secondary antibodies (Jackson Immunoresearch) conjugated to fluorescein isothiocyanate, cyanin-3, or cyanin-5. Primary antibody concentrations used are as follows: (mo – mouse monoclonal, rb – rabbit polyclonal): mo α-MAP2ab, 1:500 (Sigma), mo α-TH, 1:500 (Boehringer Mannheim), rb α-TH, 1:500 (Eugenetech), mo α-AADC, 1:500 (Sigma). Labeled cells were visualized using a Bio-Rad MRC1000 confocal scanning laser microscope and color images were generated using Adobe Photoshop (Adobe System). The total cell numbers were scored using nuclear counterstaining with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). The relative proportions of each cell phenotype were determined by systematic sampling of 40× fields across the length and breadth of each well.

DNase I footprint assay

Labeled TH DNA fragments (–729 to –962) used for footprint analysis were generated by PCR using a plasmid containing 4.5 kb of rat *TH* promoter as template (kindly provided by D. Chikaraishi) and two oligo primers, one of which was ^{32}P end-labeled. The resulting PCR products were gel purified using 6% polyacrylamide gel. Nurr1 protein was produced in a TNT coupled reticulocyte lysate system (Promega). DNase I footprint reactions were carried out in 25 mM Hepes-KOH pH 7.5, 80 mM potassium chloride, 1 mM magnesium chloride, 20% glycerol, 0.05% NP-40, and 5% polyvinyl alcohol with a fixed amount of reticulocyte lysate. Dose response experiments were performed using different ratios of unreacted and reacted reticulocyte lysate. DNase I digestions were carried out with 0.1 unit of enzyme at room temperature for 1 minute. DNA sequencing was performed by the same labeled primer using Sequenase kit (Amersham).

Cell transfection and transactivation assay

Rat TH promoter/E-GFP fusion constructs were generated from 4.5 TH-GFPpA (gift from D. Chikaraishi). Two promoter fragments were used, –2502 to +10 or –4500 to +10, as numbered from the TH transcriptional initiation site. The TH reporter was co-transfected with CMV-β-gal vector into HC7 cells and HC7-Nurr1 cells using Effectene transfection reagents (Qiagen). About 2% to 5% of progenitor cells were transfected in using these conditions. Transfected cells were cultured for 5 days in the presence of FGF-2 and lysed by 1× reporter lysis buffer (Promega). The expression of GFP was quantified using a fluorimeter (Photon Technology International). Transfection efficiency was standardized by β-galactosidase activity.

RESULTS

AHPs cultured under FGF-2 can acquire a dopaminergic neuronal phenotype following FGF-2 withdrawal

Catecholamines are synthesized from the common cellular metabolite tyrosine in a series of three steps: tyrosine is converted to DOPA by the enzyme TH, DOPA to dopamine by aromatic L-amino acid decarboxylase (AADC), and dopamine to norepinephrine by dopamine β–hydroxylase (DBH). We have previously shown that FGF-2-responsive AHPs can differentiate over the course of two weeks to generate neurons, astrocytes and oligodendrocytes in vitro, and the addition of RA or FK potentiates the accumulation of neurons by 4 or 6 fold, respectively (Palmer et al., 1997). FGF-2 withdrawal in the presence of RA, serum and neurotrophin-3 (NT-3) or brain-derived neurotrophic factor (BDNF) yields neurons of which about 1% express TH (Takahashi et al., 1999). However, whether these TH-positive neurons are dopaminergic or adrenergic is not known. In the present work, AHP cells were allowed to differentiate in the absence of serum and neurotrophins and were evaluated for the presence of TH-immunoreactive neurons (Fig. 1A). A small proportion of the Map2ab-immunoreactive neurons was double labeled for TH (0.9±0.3% in the presence of RA and 1.5±0.4% in the presence of FK). Nearly all cells were immunoreactive for AADC but none contained detectable DBH (not shown). Consistent with the immunofluorescence data, RT-PCR showed an early upregulation of *TH* and *AADC* (at 6 days) in the absence of detectable *DBH* (Fig. 1B) or *Phox2a* (not shown) mRNA, which are specifically expressed in adrenergic neurons (Tiveron et al., 1996; Valarche et al., 1993). Taken together, these data indicate that TH-positive neurons generated in AHPs are dopaminergic rather than adrenergic or noradrenergic neurons.

Shh and FGFR3 signaling in AHP cells

To define the molecular mechanism underlying dopaminergic differentiation in cultured AHPs with regard to known developmental programs, we examined the expression of several key genes involved in dopaminergic fate determination. Shh and FGF-8 define the induction sites for dopaminergic neurons. Soluble FGF receptor 3c (FGFR3-IgG) has been shown to attenuate this differentiation, suggesting that FGFR3 activation is necessary for dopaminergic differentiation (Ye et al., 1998). In proliferating AHPs, *Shh* transcripts are not detected; however, transcripts for the Shh receptors *Ptc* and *Smo* and the Shh-responsive gene *Gli1* were detectable, suggesting some level of Shh-related signal transduction (Fig. 1B). In addition, we observed the expression of *FGFR1*, *FGFR2* and *FGFR3* in AHP cells (Fig. 1C). The high concentration of FGF-2 used to propagate AHPs (20 ng/ml) is known to activate all three FGF receptors (Ornitz et al., 1996), suggesting that FGFR3 is activated in proliferating cells.

To examine the role of Shh in AHPs, we introduced

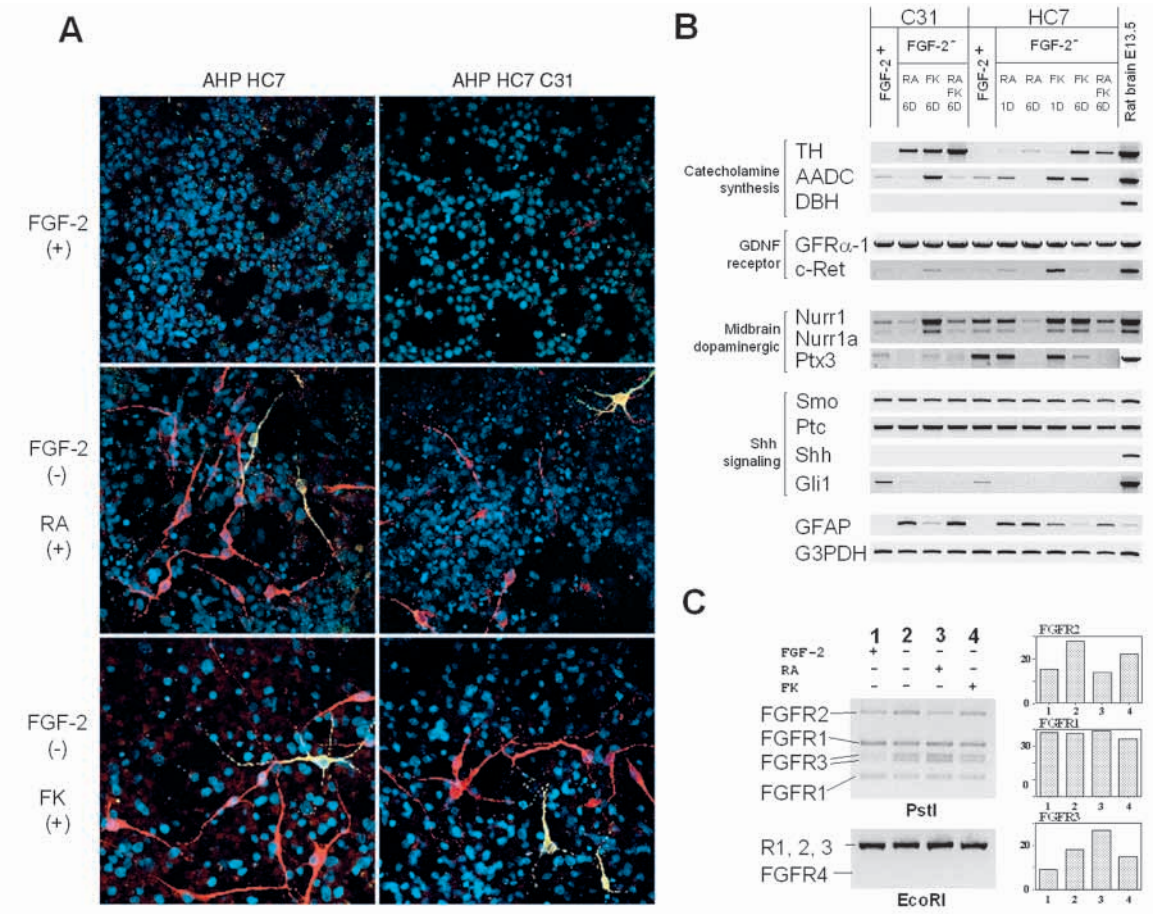

Fig. 1. Induction of TH in primary (HC7) and clonal (C31) cultures of adult hippocampus-derived stem cells by RA and FK. (A) Immunofluorescent detection of the neuron-specific marker Map2ab (red) and TH (green). Expression of TH in HC7 and C31 cells was evaluated in proliferating (FGF-2(+)) and differentiated cells. Differentiation was triggered by FGF-2 withdrawal followed by RA or FK treatment. In the presence of RA (FGF-2(–)RA(+)), cells were treated with 0.5 μM RA for 6 days, followed by N2 medium alone for an additional 8 days. In the FK condition (FGF-2(–)FK(+)), cells were treated with 5 μM FK for 14 days. Nuclei were counterstained with DAPI (blue). The overlap of red and green in Map2ab and TH double-positive cells appears yellow. Though not shown here, a typical RA- or FK-treated culture would also contain roughly 1% astroctyes and 1% oligodendrocytes. (B) Expression of genes related to catecholamine-producing neurons in AHP cells before and after differentiation. HC7 and C31 cells were cultured in the presence of FGF-2 (FGF-2$^+$) or differentiated by FGF-2 withdrawal followed by RA and/or FK treatments for 1 day or 6 days and evaluated by quantitative RT-PCR. Differentiation by FK induced *Nurr1*, *TH*, *AADC* and *c-Ret* in the absence of DBH. Although differentiation by RA also stimulated *TH*, the coordinated upregulation of Nurr1 and AADC seen with FK was absent. PCR products were separated in 1.5% agarose gel and detected by EtBr staining. Total RNA from rat embryonic brain E13.5 was used as a positive control. Evaluated groups included catecholamine synthesis enzymes (*TH*, *AADC*, *DBH*), GDNF receptor components (*GFRα1*, *c-Ret*), midbrain dopaminergic markers (*Nurr1*, *Nurr1a*, *Ptx3*), Shh signaling molecules (*Smo*, *Ptc*, *Shh* and *Gli1*) and the astrocyte marker (*GFAP*). (C) Expression of *FGFR1*, *FGFR2*, *FGFR3* and *FGFR4* transcripts in HC7 cells was analyzed by the single pair of PCR primers followed by *Pst*I or *Eco*RI digestion (McEwen and Ornitz, 1997). Total RNA was prepared from HC7 cells cultured as indicated for 24 hours. Expression of *FGFR1*, *FGFR2* and *FGFR3* was detected in both proliferating and differentiating AHPs.

a tetracycline suppressible *Shh-N* (amino-terminal autoproteolytic fragment of Shh) -expressing retrovirus (NIT-Shh-N) into AHPs and isolated a bulk drug-resistant population of stable *Shh-N*-expressing cells. These cells expressed a high level of *Shh-N* mRNA under control of a tetracycline repressible promoter.

First we examined the effects of *Shh-N* if expressed only during the terminal stages of differentiation. AHPs (C31) were infected with high titer *Shh-N*-expressing retrovirus immediately before differentiation. Surprisingly, *Shh-N* expression resulted in a mild depression of *TH* expression after 6 days of differentiation (Fig. 2). Similar depression was observed in the stable *Shh-N*-expressing cells when tetracycline was used to suppress *Shh-N* expression until just before differentiation (Fig. 2). In contrast, constitutive expression of *Shh-N* in proliferating cells for 10 days prior to differentiation showed 1.8-fold and 3.7-fold increases in *TH* expression after 6 days of differentiation in the presence of FK or RA, respectively (Fig. 2). Interestingly, constitutive Shh expression had no effect on TH expression in undifferentiated proliferating cells (not shown). Expression of *DBH* was not observed in any of these conditions (not shown). These results suggest that Shh may play an important role in the early patterning of proliferative precursors but may have an

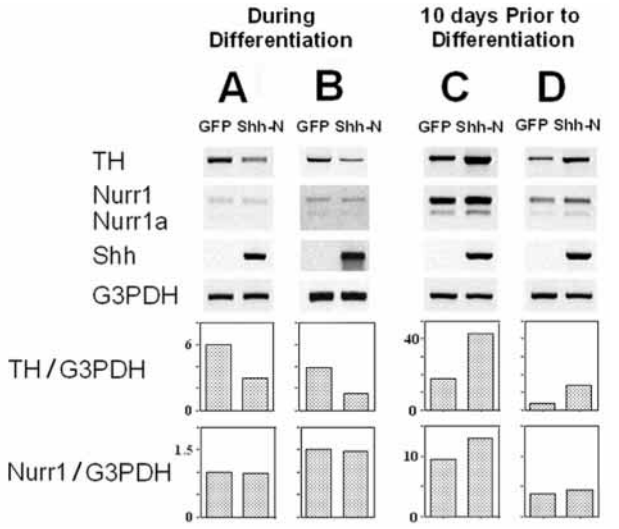

Fig. 2. Induction of *TH* by *Shh-N*. Quantitative RT-PCR demonstrating the effects of *Shh-N* expression on *TH*, *Nurr1* and *Nurr1a*. (A) C31 cells were infected by *Shh-N*- or *GFP*-expressing retrovirus 24 hours before differentiation. Differentiation was triggered by FGF-2 withdrawal followed by RA treatment for 6 days and total RNA was prepared. *Shh-N* depressed *TH* expression in this condition. (B) *Shh-N* or *GFP* stable expressing (HC7-Shh-N or HC7-GFP) cells were cultured in the presence of tetracycline to suppress transgene expression until just before differentiation. Differentiation was triggered by FGF-2 withdrawal followed by RA treatment for 6 days and total RNA was prepared. *Shh-N* depressed *TH* expression in this condition. (C,D) HC7-Shh-N and HC7-GFP cells were cultured in the absence of tetracycline for 10 days followed by FGF-2 withdrawal and the addition of tetracycline with FK (C) or RA (D) for 6 days. The abundance of TH and Nurr1 transcripts was analyzed by quantitative RT-PCR (kinetics analysis) and standardized by *G3PDH* (glyceraldehyde 3-phosphate dehydrogenase) (A-D). TH expression in HC7-Shh-N was 1.8-fold (C) and 3.7-fold (D) higher than control cells (HC7-GFP).

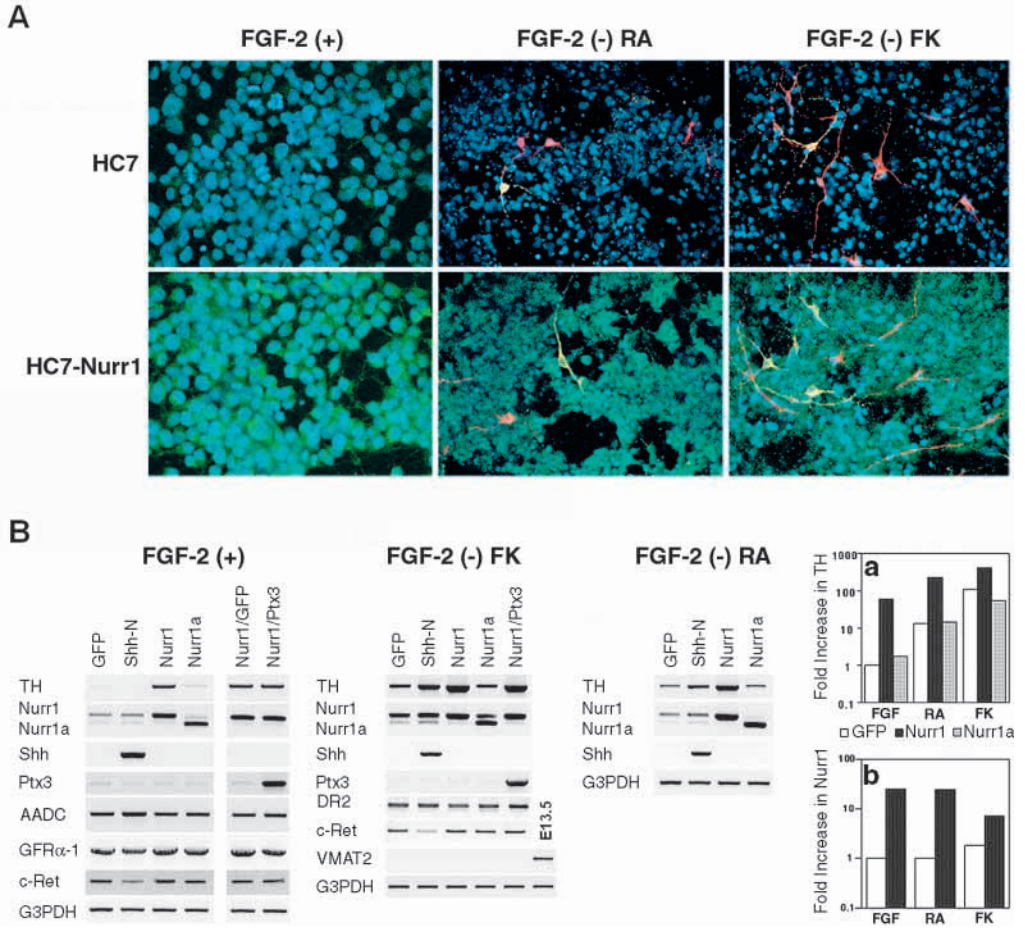

Fig. 3. Expression of *Shh-N*, *Nurr1*, *Nurr1a*, and *Ptx3* in the HC7 cells. (A) Immunofluorescent detection of Map2ab (red) and TH (green). Expression of TH was compared between parental cells (HC7) and *Nurr1* stable expressing cells (HC7-Nurr1). Nuclei were counterstained with DAPI (blue). Panels labeled FGF-2(+) show cells that were proliferating as undifferentiated precursors in the presence of FGF-2. Those labelled FGF-2(–)RA show cells treated with 0.5 μM RA in the absence of FGF-2 for 6 days, followed by N2 medium alone for an additional 8 days. Those labelled FGF-2(–)FK show cells treated with 5 μM FK for 14 days in the absence of FGF-2. Map2ab and TH double-positive cells are yellow. Ubiquitous green staining shows that *Nurr1* induces TH in all cells. (B) Changes in gene expression in HC7 cells following overexpression of *GFP, Shh-N, Nurr1, Nurr1a or Nurr1 + Ptx3*. HC7 cells were infected with NIT-based tetracycline suppressible retroviral vectors, selected in the presence of tetracycline and then stable cell lines switched to tetracycline-free medium to induce transgene expression. Total RNA was collected from proliferating cells 2 days later (FGF-2(+)) or from differentiated cells generated by culturing in FGF-2 for 10 days followed by 6 days in RA or FK containing medium (FGF-2 (–) RA and FGF-2 (–) FK, respectively). The effect of *Ptx3* expression in HC7 cells expressing Nurr1 was analyzed following transient transfection of *Ptx3*- or *GFP*-expressing retroviral plasmids into HC7-Nurr1 cells grown in the absence of tetracycline. In each case, *Nurr1* amplified *TH* expression; however, *Nurr1* had little effect on the expression levels of *Ptx3*, *AADC*, *c-Ret*, *GFR-α1*, *D2R*, or *VMAT2*, suggesting that the activation events mediated by *Nurr1* may be limited to the *TH* gene itself. *Ptx3* had no effect on any of the genes evaluated. Total RNA from rat embryonic brain E13.5 was used as a positive control. Quantification of *TH* and *Nurr1* transcripts in *Nurr1*, *Nurr1a* and *GFP* overexpressing cells (A,B) was obtained using a kinetic analysis on RNAs obtained from proliferating (FGF) or differentiated cells (RA, FK) as described in Materials and Methods.

inhibitory effect on *TH* expression when expressed during the terminal stages of differentiation.

Cells were also treated with FGF-8 during the terminal stages of differentiation; however, there was no detectable increase in *TH* expression above that presumably induced by the high levels of FGF-2 present just prior to differentiation (not shown). Although not necessarily definitive, the lack of effect during differentiation suggests that FGFR3 signal transduction may also have its strongest effects along with Shh-N signaling in proliferating precursor cells.

Forced expression of *Nurr1* is sufficient to induce TH expression in the absence of differentiation

Next we examined the roles of *Nurr1*, *Nurr1a* (a COOH-terminal truncation of Nurr1 formed by alternative splicing) and *Ptx3*, which are all expressed in midbrain dopaminergic neurons (Zetterstrom et al., 1997; Smidt et al., 1997). These transcription factor genes were subcloned into the retrovirus expression vector NIT and used to transduce AHPs cultures. Bulk populations of stably transduced cells were isolated under G418 selection. TH immunoreactivity was detected ubiquitously in HC7-Nurr1 cells in both proliferating and differentiating conditions (Fig. 3A). TH was detected at low levels in all proliferating Nurr1-expressing cells and was upregulated in differentiating conditions. In addition, TH expression was markedly higher in roughly 1% of the Map2ab-positive cells. These strongly TH-immunoreactive neurons were generated in similar numbers even in the absence of Nurr1 overexpression, suggesting that *Nurr1* overexpression may be sufficient to activate *TH* in undifferentiated cells yet not to the extent achieved during a fully activated neuronal differentiation program.

In HC7-Nurr1 cells, *Nurr1* mRNA was expressed at 25-fold higher levels than in non-transduced controls and *TH* expression was elevated 60 fold in proliferating cells and 7 fold above controls on the sixth day following FGF-2 withdrawal and FK treatment (Fig. 3B). Surprisingly, *Nurr1* had little effect on the proliferation of cells or on the expression levels of *Ptx3*, *AADC*, *c-Ret*, *GFR-α1*, *D2R* and *VMAT2*. This finding suggested that *Nurr1*'s primary effects were not differentiative. *Nurr1* expressed in the stem cell-derived C31 line generated similar results (not shown).

Overexpression of *Nurr1a*, the COOH-terminal truncated form of Nurr1, had little effect on *TH*, either in proliferating or differentiating cells (Fig. 3B). This finding indicated that the alternatively spliced form of Nurr1 does not function to activate *TH* or repress the spontaneous activation of *TH* in differentiating neurons.

To determine if Nurr1 expression alone was sufficient to induce *TH* in non-neural cells, full length *Nurr1* was introduced into the rat primary skin fibroblast cell line, FF12, and the human kidney cell line 293 using the RVEN3 retroviral vector. *TH* expression was not detected in either cell line (data not shown), suggesting that *Nurr1* alone is not sufficient to activate *TH* in non-neural cell lines.

DOPA production could not be detected by HPLC in HC7 cell lysate after 6 days of differentiation in the presence of FK. In contrast 49.2 ng/mg protein of DOPA was detected in HC7-Nurr1 cell lysate after 6 days of differentiation, suggesting that the *TH* expression detected in *Nurr1*-expressing cells led to the production of functional TH enzyme.

Forced expression of *Ptx3* had little effect on proliferation, differentiation or the acquisition of a TH-positive phenotype

Ptx3, a homeodomain transcription factor implicated in the generation of midbrain dopaminergic neurons, follows closely behind the expression of *Nurr1*, suggesting that Nurr1 may regulate *Ptx3* expression (Saucedo-Cardenas et al., 1998). However, Ptx3 expression was unaffected by the overexpression of Nurr1 in AHPs (Fig. 3B). During noradrenergic differentiation, the homeobox genes *Phox2a* and *Phox2b* are also implicated in activating *TH*, *DBH* and *c-Ret* gene expression. However, *Phox2a* and *Pho2b* genes are not expressed in dopaminergic neurons, suggesting that other homeobox genes would be involved in pan-dopaminergic gene expression. To examine the possible role of Ptx3 in the context of AHP differentiation, we overexpressed Ptx3 in Nurr1-stable expressing cells (HC7-Nurr1) using retroviral vectors. *Ptx3* mRNA was easily detected; however, the expression of *TH*, *AADC*, *c-Ret*, *GFRα-1*, and *D2R* was not affected. Neither was the expression of these genes affected by Ptx3 in the absence of excess Nurr1 (not shown) suggesting that Ptx3 plays a role other than the transcriptional activation of these dopaminergic-specific genes.

A unique Nurr1 binding element (NBRE) within the *TH* promoter is necessary for Nurr1-dependent *TH* expression

Nurr1 binds as a monomer to an extended half site sequence called the NGFI-B response element (NBRE, AAAGGTCA, Forman et al., 1995; Wilson et al., 1993). In addition, Nurr1 is able to form heterodimers with retinoid X receptor (RXR) (Perlmann and Jansson, 1995) which, in the presence of 9-*cis* retinoic acid (9cRA), bind selectively to a class of RA response elements composed of direct repeats spaced by 5 nucleotides (DR5, AGGTCANNNAAAGGTCA) (Perlmann and Jansson, 1995).

To distinguish whether the *Nurr1*-dependent *TH* expression observed in HC7-Nurr1 cells was mediated by monomeric Nurr1 or due to generation of Nurr1/RXR heterodimers, HC7-Nurr1 cells were differentiated in the presence of 9cRA for 24 hours. As shown in Fig. 4A, simple FGF-2 withdrawal increased *TH* expression 2 fold. Addition of all-*trans* RA (RA) had no additional effect on *TH* expression at this very early time in differentiation. In contrast, *TH* expression decreased in the presence of 9cRA in a dose-dependent manner. Since Nurr1 is overexpressed in HC7-Nurr1 cells, we expressed RXRα in HC7-Nurr1 cells using the retrovirus vector LNCX-RXRα to increase the potential for forming Nurr1 heterodimers. Forced expression of RXRα efficiently suppressed *TH* expression in HC7-Nurr1 cells (Fig. 4B), suggesting that the activating effects of Nurr1 on *TH* expression are not dependent on heterodimer formation with RXR.

To determine whether Nurr1-dependent *TH* expression resulted from a direct activation of *TH* promoter, we scanned 6 kb of sequences upstream of the *TH* start site elements similar to the reported NBRE and found a single putative half-site at positions −866 to −873 (AAAGGTCA). This site is contained within a region of the promoter known to be necessary for midbrain dopaminergic neuron-specific activation of *TH* (positions −776 to −2510) (Liu et al., 1997). To test if Nurr1 bound to this putative NBRE, we performed DNase I footprint

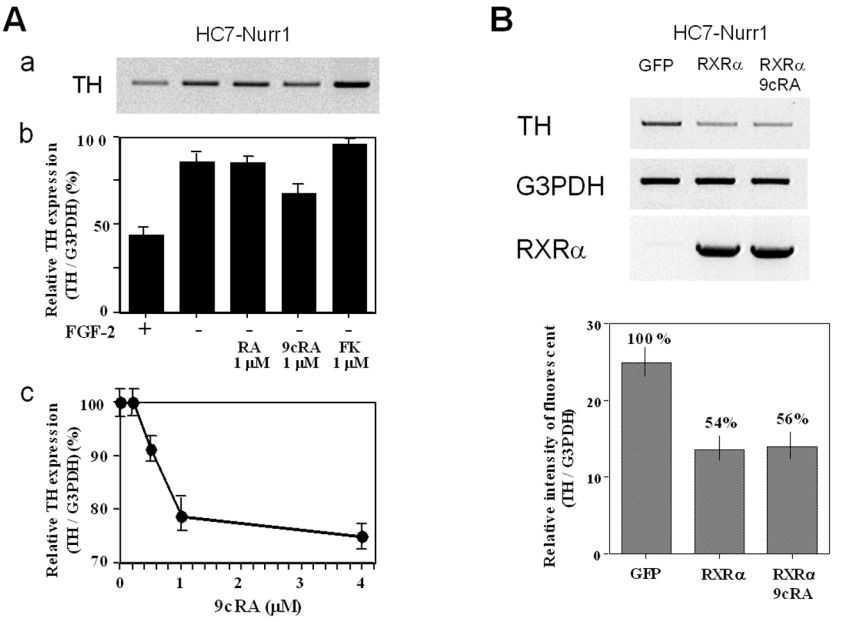

Fig. 4. Effects of 9-*cis* retinoic acid and RXR on Nurr1-dependent *TH* expression. (A)Effects of FGF-2 withdrawal (–), and addition of 0.5 μM all-*trans* RA (RA), 0.5 μM 9-*cis* RA (9cRA) or 5 μM FK on *TH* expression in HC7-Nurr1 cells. Total RNA was prepared after 24 hours in the indicated conditions. The abundance of *TH* transcripts was measured by quantitative RT-PCR analyses (a) and normalized to G3PDH (b). Simple FGF-2 withdrawal and combination of FK increased *TH* expression. Addition of all-*trans* RA had no additional effect. *TH* expression decreased in the presence of 9cRA in a dose-dependent manner (c). (B) Changes in *TH* expression following expression of human RXRα in Nurr1 stable expressing cells. Nurr1 stable expressing AHPs were infected with retroviruses-expressing RXRα, and cultured in the presence of FGF-2 for 2 days. Expression of *TH* and RXRα was analyzed by quantitative RT-PCR. Forced expression of RXRα significantly repressed *TH* expression in Nurr1 stable expressing cells.

analysis on a –962 bp to –729 bp rat *TH* promoter fragment. Titration of recombinant Nurr1 gave progressive protection of nucleotides spanning positions –873 to –866, which includes the putative NBRE (Fig. 5). Mutation of the putative NBRE sequence from AAAGGTCA to AAAGGCTA abolished protection in this region (not shown). These data confirmed that Nurr1 bound in a sequence-specific manner to the NBRE within the *TH* promoter. In contrast, Nurr1a showed only weak protection at the same site (data not shown), which is consistent with Nurr1a's reduced affinity for NBREs in gel shift assays (Castillo et al., 1998b).

Next we performed transactivation experiments using *TH* promoter/E-GFP fusion constructs to determine if the NBRE sequence within the TH promoter was able to act as a functional enhancer. Although 2.5 kb TH reporter construct showed constitutive expression in both HC7 cells and HC7-Nurr1 cells, the 4.5 kb TH reporter construct showed *Nurr1*-dependent E-GFP expression (Fig. 6). In addition, a mutation introduced into the NBRE sequence (wild: AAAGGTCA, mutant: AAAGGCTA) abolished *Nurr1*-dependent reporter gene expression (Fig. 6). Taken together, these data indicate that Nurr1 activates *TH* by binding directly to the NBRE sequence on the midbrain dopaminergic neuron-specific enhancer element and that this activation may, in part, be due to the derepression of unidentified negative regulatory elements contained within the larger 4.5 kb *TH* promoter fragment.

DISCUSSION

Progenitor cells in the adult hippocampus generate only one type of neuron within the granule cell layer, yet these cells display considerable phenotype plasticity when expanded in the presence of FGF-2 (Gritti et al., 1996; Suhonen et al., 1997; Takahashi et al., 1999). Cultured AHPs express a variety of homeobox genes, including Otx1, En1, GBX2, HoxA1, HoxA2, HoxD3 and HoxD4 (K. S., unpublished data), suggesting the potential to adopt a variety of anterior-posterior (A-P)/dorsal-ventral (D-V) identities. These observations suggest that expression of appropriate A-P transcription factors is important for the controlled conversion of cultured neural progenitor cells into specific classes of neurons. Consistent with this plasticity, we show for the first time that TH-positive neurons generated from hippocampus-derived progenitors in culture are dopaminergic.

Some of the factors that regulate dopaminergic determination in culture include Shh and FGF-8 signaling.

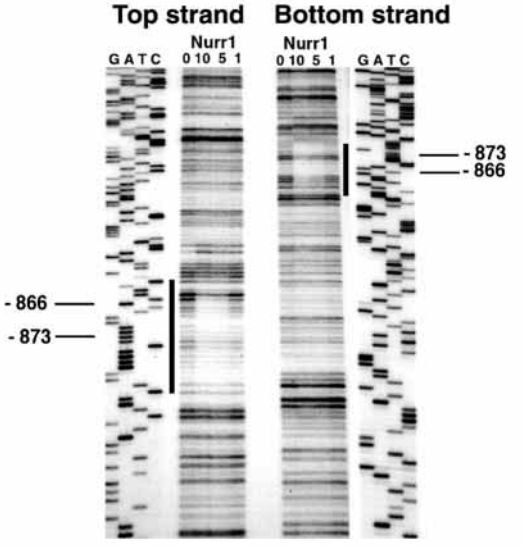

Fig. 5. Analyses of Nurr1 binding site within the rat *TH* promoter region. (A) Footprint analysis of the rat *TH* promoter region. A PCR fragment spanning –729 to –962 of the rat *TH* promoter was end-labeled with ^{32}P at either the –729 bp end (bottom strand, lane 5-8) or the –962 bp end (top strand, lane 9-12). The end-labeled fragments were incubated with 0, 10, 5, 1 μl of reticulocyte lysate reaction mixture of Nurr1 combined with 10, 0, 5, 9 μl of unreacted reticulocyte lysate, respectively. Protected areas suggest that Nurr1 binds to the NBRE sequence (AAAGGTCA) located between –873 and –866.

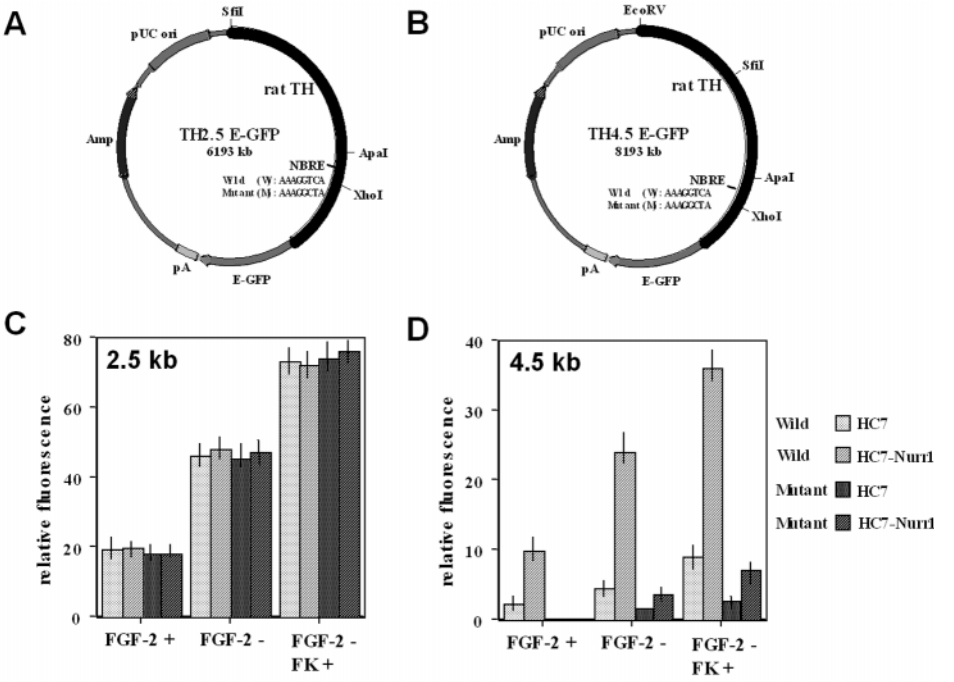

Fig. 6. Nurr1 transactivation of *TH* is dependent on the NBRE. (A,B) Structure of 2.5 kb (A) and 4.5 kb (B) TH promoter-reporter constructs and the mutations introduced into the NBRE sequence. (C,D) Both primary and Nurr1 stable expressing cells were co-transfected with 1 μg each of a CMV-β-galactosidase reporter and either wild type or mutated TH-EGFP fusion constructs. Results from the 2.5 kb or 4.5 kb upstream region of the *TH* promoter are shown in C and D respectively. GFP activity was normalized to β-galactosidase activity to correct for transfection efficiency.

Their respective receptors are present and overexpression of Shh-N caused an increase in *TH*, but only if expressed in proliferating cells prior to differentiation. It seems likely that this increase may have been due to either the differential expansion of precursors via the mitogenic effects of Shh-N (Wechsler-Reya and Scott, 1999) or to a ventral patterning influence of Shh that acts primarily in proliferative *vs* post-mitotic precursors (Ye et al., 1998).

Surprisingly, limiting expression of Shh-N to the terminal stages of differentiation decreased *TH* expression in the differentiated cells. The possibility that Shh may be inhibitory in late postmitotic dopaminergic differentiation is consistent with the previous observations demonstrating that, when ventral midline cells exit cell cycle, *Gli1* and *Shh* are downregulated at the midline but continue to be expressed by adjacent proliferating progenitors that remain sensitive to Shh signaling (Placzek et al., 1991; Hui et al., 1994). Although Shh signaling is required beyond the final cell cycle in cholinergic differentiation (Ericson et al., 1996), our data indicate that the later stages of dopaminergic differentiation may not depend on the continued presence of Shh.

One reason why Shh may be inhibitory at the later stages of differentiation may involve a conflict between Shh signaling and the protein kinase A (PKA) pathway. *TH* and *Nurr1* expression are potentiated by the accumulation of cAMP (Tinti et al., 1997; Castillo et al., 1997); it is also known that Shh inhibits adenylate cyclase (Hammerschmidt et al., 1997), suggesting that inhibition of the PKA pathway may account for some of the *TH*-repressing activity of *Shh-N*. Consistent with this competing pathway hypothesis, we noted that the expression of *c-Ret*, which is induced by FK in AHPs, was downregulated in the cells constitutively expressing *Shh-N* (HC7-Shh-N, Fig. 3B). In addition, FK is known to be a potent inhibitor of Shh signaling (Hynes et al., 1995) yet was clearly effective in promoting the generation of TH-positive neurons. This finding implies that the PKA and Shh pathways play important although sequential roles by necessity.

A role for *Nurr1* in dopaminergic differentiation of AHPs

Nurr1 and *Nurr1a*, encoding the COOH-terminal truncated isoform of Nurr1, are expressed in embryonic and postnatal rodent brain in a manner consistent with their involvement in dopaminergic determination (Fig. 1B; Castillo et al., 1998b). In the present study, we have shown that forced expression of *Nurr1* increased TH expression yet did not attenuate the proliferation of AHPs. One might argue that the Nurr1-dependent increase in TH expression could be due to preferential survival of TH-expressing cells. Although selection for TH-expressing cells may contribute to the effects seen in stable Nurr1 lines, we have also seen that *TH* is rapidly upregulated following transduction with the Nurr1 retrovirus in the absence of selection, and both endogenous TH and co-transfected TH reporter constructs are upregulated during the transient expression of Nurr1 following transfection with Nurr1-expressing plasmids (not shown). The upregulation of *TH* in these instances is more consistent with a direct activation of *TH* by Nurr1 than preferential survival of TH-expressing cells.

In contrast to Nurr1, Nurr1a had no measurable effect on any aspect of differentiation evaluated here. The lack of effect on proliferation or differentiation confirms that neither Nurr1 nor Nurr1a triggers terminal differentiation. In addition, the events mediated by *Nurr1* may be limited to activation of the *TH* gene alone since overexpression of *Nurr1* had little effect on any of the other dopaminergic-specific genes surveyed in AHPs.

Nurr1-dependent *TH* activation in AHPs appears to be mediated by Nurr1 binding to a NBRE sequence within a domain of the *TH* promoter. This domain has been shown to be necessary for the region-specific expression of TH in midbrain dopaminergic neuron. These observations, in combination with agenesis of midbrain dopaminergic neurons in Nurr1 null mice, indicate that over-expression of Nurr1 in AHPs reconstruct the role of Nurr1 in midbrain dopaminergic progenitor cells in developing brain.

It has been reported that Nurr1 may activate gene expression

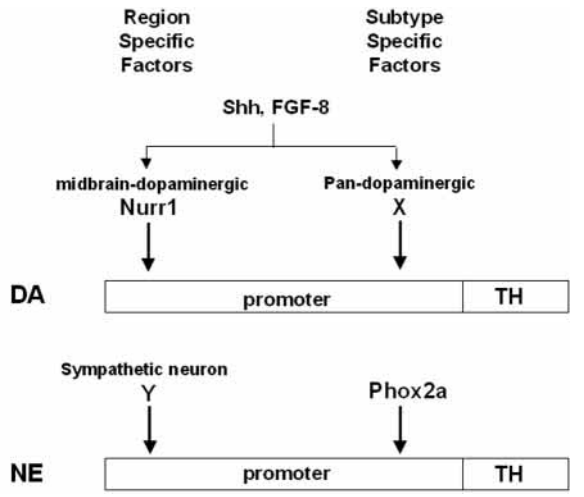

Fig. 7. A model for region-specific and cell-type-specific regulation of TH. Neurons that express TH are both regionally and phenotypically diverse and it is becoming clear that TH expression is not mediated by the same set of factors in each cell type. Shh-N and FGF-8 influence the generation of TH neurons in a number of regions and may represent global patterning signals for TH-expressing cells but the downstream events leading to TH activation appear to be different for each region. Nurr1 appears to be the first identified transcription factor to act in a strict region-specific manner since Nurr1-null mice only lack midbrain dopaminergic (DA) neurons. The fact that TH is still activated at other locations also suggests that other factors (Y) may play similar region-specific roles elsewhere. Phox2 and its action at another loci within the TH promoter in norepinephrin (NE) producing neurons represents a class of activator that may act at several regions in parallel to region-specific factors but only for specific sub-types of TH neurons. The overlap of these 'sub-type specific' regulators with region-specific factors may provide some of the signaling complexity required to produce TH neurons with unique attributes for each location.

in combination with RXR or as a monomer (Zetterstrom et al., 1996a). Our data using forced expression of RXRα in Nurr1 stable expressing cells suggest that Nurr1 acts in a RXR-independent manner on TH expression. In fact, the downregulation of *TH* in the presence of excess RXRα and Nurr1 may even suggest that the ratio of Nurr1 to RXR influences the cell-type specific function of Nurr1 during development.

The mechanism by which Nurr1 activates *TH* transcription remains to be determined but its early expression in proliferative precursors may play some role in activating previously silent domains. The influences of *Shh-N* and *Nurr1* in proliferating cells suggest that precursors may be particularly sensitive to instructional factors during proliferation. It has been suggested that changes in enhancer activity may be dependent on ease of chromatin remodeling afforded by DNA replication (reviewed by Blackwood and Kadonaga, 1998). The transient perturbation of chromatin structure that occurs during DNA replication may allow the binding of transcriptional enhancer proteins that otherwise could not access the DNA template. In the context of the TH promoter, our transactivation experiments suggest that elements included in the 2.5 kb upstream sequences actually contain a constitutively active element and that only with the addition of sequences extending to −4.5 kb does TH show appropriate regulation. This suggests that Nurr1 may suppress the actions of a negative regulatory element by binding through the NBRE element. Its major role, therefore, would not be to act as a stand-alone transactivator but as a derepressor of TH expression within the context of a neural progenitor cell.

In this study, we definitively place a region-specific factor and response element, Nurr1 and the NBRE, within a dopaminergic-specific differentiation program. Combinations of factors likely act together to regulate the TH gene (Fig. 7). The present data suggest that these factors may be grouped into independently acting region-specific and neuronal subtype-specific factors. Although subtype-specific transcription factors for dopaminergic neurons have not been identified, the fact that Shh and FGF-8 act as pan-dopaminergic inducers suggests that the downstream subtype-specific transcription factors (i.e., factors equivalent to Phox2a in the adrenergic lineage) should exist.

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