Restriction of Neural Precursor Ability to Respond to Nurr1 by Early Regional Specification

Chiara Soldati1,2, Emanuele Cacci1,2, Stefano Biagioni1, Nicoletta Carucci1,2, Giuseppe Lupo1,2, Carla Perrone-Capano3,4, Isabella Saggio5,6,7, Gabriella Augusti-Tocco1*

1 Department of Biology and Biotechnology-Neurobiology Research Unit, and “Daniel Bovet” Neurobiology Research Center, “Sapienza” University of Rome, Rome, Italy, 2 Istituto Pasteur Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome, Italy, 3 Department of Biological Sciences, University of Naples Federico II, Naples, Italy, 4 IGB “A. Buzzati Traverso”, Naples, Italy, 5 Department of Biology and Biotechnology, “Sapienza” University of Rome, Rome, Italy, 6 Istituto Pasteur Fondazione Cenci Bolognetti, “Sapienza” University of Rome, Rome, Italy, 7 Institute of Molecular Biology and Pathology, CNR, Rome, Italy

Abstract

During neural development, spatially regulated expression of specific transcription factors is crucial for central nervous system (CNS) regionalization, generation of neural precursors (NPs) and subsequent differentiation of specific cell types within defined regions. A critical role in dopaminergic differentiation in the midbrain (MB) has been assigned to the transcription factor Nurr1. Nurr1 controls the expression of key genes involved in dopamine (DA) neurotransmission, e.g. tyrosine hydroxylase (TH) and the DA transporter (DAT), and promotes the dopaminergic phenotype in embryonic stem cells. We investigated whether cells derived from different areas of the mouse CNS could be directed to differentiate into dopaminergic neurons in vitro by forced expression of the transcription factor Nurr1. We show that Nurr1 overexpression can promote dopaminergic cell fate specification only in NPs obtained from E13.5 ganglionic eminence (GE) and MB, but not in NPs isolated from E13.5 cortex (CTX) and spinal cord (SC) or from the adult subventricular zone (SVZ). Confirming previous studies, we also show that Nurr1 overexpression can increase the generation of TH-positive neurons in mouse embryonic stem cells. These data show that Nurr1 ability to induce a dopaminergic phenotype becomes restricted during CNS development and is critically dependent on the region of NPs derivation. Our results suggest that the plasticity of NPs and their ability to activate a dopaminergic differentiation program in response to Nurr1 is regulated during early stages of neurogenesis, possibly through mechanisms controlling CNS regionalization.

Introduction

During development, lineage commitment is a multistep process requiring the activation and repression of sets of genes at successive stages, leading from an embryonic stem (ES) to a tissue-specific stem cell identity as neural stem cells (NSCs). NSCs are capable of giving origin to glial cells and different populations of neurons. How and when the different phenotypic features that underlie cell diversity are specified is an important issue in developmental neuroscience. Cell fate specification appears to occur early during development and is governed by a complex combination of intrinsic and extrinsic factors. Positional identity is assigned to NSCs via gradients of signaling molecules secreted throughout the dorsoventral and rostro-caudal axes of the neural tube during defined temporal windows. Once neuronal fates have been specified and restricted by these extrinsic cues, intrinsic signals direct differentiation into mature neurons. [1,2]. Despite major progresses in this field, the molecular mechanisms as well as the intrinsic and extrinsic factors underlying lineage commitment in NSCs remain only partially understood [3–5]. In this context, an important issue is to understand when positional identity is acquired by neural precursors (NPs) and whether it can be maintained when NPs are removed from their environment and cultured in vitro under defined experimental conditions.

Cells displaying properties of NPs have been isolated and cultured in vitro by exposing cells derived from different regions of the Central Nervous System (CNS) to growth factors, which maintain NPs in a proliferative and undifferentiated state; upon growth factor deprivation NPs can undergo differentiation and give rise to a mixed population of neurons and glial cells. Despite some contradictory data reported in previous studies, regional identity seems to be, at least partially, conserved after NP expansion in vitro [6–9]. Preservation of positional identity has recently been re-examined and shown to be maintained over 20 passages in vitro by NPs derived from 14.5 mouse cortex [10]. These findings raise the question of whether acquisition of positional identity may restrict the ability of NPs to respond to transcription factors that direct or cooperate to the acquisition of specific neuronal identities. A well-known example is represented by midbrain (MB) dopamine (DA)-producing neurons, whose
specification requires the expression of a set of specific transcription factors [11–15]. Among these, Nurrl has a critical importance for the differentiation and maintenance of MB DA neurons. In mice, high Nurrl expression is detected at E10.5 in newly born postmitotic DA neurons [16,17] and is maintained in the adult. Nurrl controls the expression of key genes involved in DA neuron function such as tyrosine hydroxylase (TH), vesicular monoamine transporter 2, DA transporter (DAT), the co-receptor for the GDNF family Ret and the neurotrophin BDNF [17–19]. Nurrl overexpression can positively influence the ability of embryonic stem (ES) cells and NPs to differentiate into DA neurons [20–23]. Previous reports on the ability of Nurrl to activate TH expression used short-term cultures of NPs [24] and only a few studies have investigated the ability of multipassaged NPs to generate dopaminergic neurons in vitro [22,25]. However, the use of long-term expanded NP lines provides a significant advantage for establishing NPs properties and their possible use in transplantation experiments.

In this work, we have used growth factor-immortalized cells to test Nurrl1’s ability to activate dopaminergic differentiation in relation to the regional character of NPs. Our data show a differential ability to respond to Nurrl1 overexpression of NPs from different CNS regions; this observation supports the existence of mechanisms that restrict NP plasticity, possibly due to the specification and maintenance of “positional identity” cues. To the best of our knowledge this is the first study comparing the ability of long-term expanded murine NP cell lines from different CNS areas to generate TH-positive neurons under uniform experimental conditions.

**Results**

**Neural Precursors from embryo and adult CNS**

Nurrl1 ability to activate expression of dopaminergic markers was analyzed in NP lines adapted to in vitro cell culture as previously described [26]. NPs were isolated from various areas of the mouse E13.5 embryo CNS, namely cortex (CTX), ganglionic eminence (GE), spinal cord (SC), midbrain (MB), and from the adult subventricular zone (SVZ). Cells were propagated as adherent cultures in the presence of bFGF and EGF, as described in the Materials and Methods section. Nestin immunoreactivity was found in 100% of cells when NPs were cultured under proliferative conditions, confirming NP identity (Fig. 1A). NPs were also characterized following in vitro culture (6–7 passages) for the expression of molecular markers of the regions of derivation. In particular, FoxG1 is expressed in the developing mouse telencephalon (CTX and GE), Dlx2 and Six3 are expressed in the developing GE, HoxB6 and HoxB9 are expressed in the developing SC, while Ngn2 is expressed in both CTX and SC [7,8,10,27–30]. Real-time PCR analysis showed that CTX-, GE- and SC-derived NPs maintain in vitro expression profiles coherent with the corresponding CNS regions (Fig. 1B). These expression profiles were reproducible in NP lines derived from independent dissection experiments (data not shown). These observations are consistent with a recent study showing that region-specific molecular signatures are preserved in NPs derived from different areas of the developing mouse CNS [10], indicating that positional identities are at least partially maintained by NPs used to test Nurrl1 ability to promote dopaminergic cell fates.

As described in the Materials and Methods section, we either transiently infected NPs with Nurrl1-expressing lentivirus or generated NP transgenic lines with stable Nurrl1 expression following electroporation with a plasmid carrying Nurrl1 and antibiotic selection. In either case, NPs expressing Green Fluorescent Protein were used as a control. Expression of Nurrl1 was evaluated by Real-time PCR 72 hours after infection or in transgenic lines, showing that with both approaches Nurrl1 mRNA levels were markedly increased in all NPs lines compared to control cells and reached levels comparable or even higher than those in E12 MB (Fig. 2 and Fig. S1). NP cells were then exposed to a differentiation protocol, as described in Materials and Methods and characterized by immunostaining and Real-time PCR. The presence of neuronal (beta-III-tubulin) and glial (GFAP) markers was evaluated in either controls or Nurrl1-expressing cells after 7 days of differentiation (Fig. 3 A, B and Fig. S1). Although the extent of neuronal and glial differentiation was somewhat different among NPs derived from various CNS regions, ranging from 20% to 40% of beta-III-tubulin positive cells and 60–80% glia, Nurrl1 overexpression did not change the percentage of beta-III-tubulin positive cells in NPs derived from various CNS regions.

To investigate whether Nurrl1 overexpression enhanced dopaminergic marker expression, control and Nurrl1-expressing NPs were differentiated and subsequently analysed by RT-PCR and/or Real-time PCR for TH, DAT and Pitx3 expression and immunostained for TH. In these assays, we found that TH mRNA levels was higher in control NPs from GE and MB, compared to control NPs from CTX and SC, consistently with the spatial distribution of DA neurons in vivo. Importantly, compared to control NPs, Nurrl1 overexpression caused a significant increase in TH expression only in transduced GE- and MB-derived NPs (3–5 fold), but not in CTX- and SC-derived NPs (Fig. 4 and Fig. S1). In addition, Nurrl1 also promoted TH expression in NPs derived from mouse ES cells (see below). The presence of TH-positive cells under differentiating conditions in both control and Nurrl1-expressing NPs derived from GE and MB, but not from CTX and SC, was revealed by immunostaining (Fig. 5 A). Notably, the number of TH-positive cells was 2-fold higher in Nurrl1-overexpressing cultures from GE and MB, when compared to control cultures (Fig. 5 B). Similar results were also obtained with DAT expression (Fig. 6A and B). Previous studies have shown that Nurrl1 can directly activate Pitx3 expression and cooperate with Pitx3 in the specification of dopaminergic cell fates [31,32]. Thus, it was interesting to see that Nurrl1 overexpression specifically upregulated Pitx3 in GE- and MB-derived NPs (4–5 fold) and that its expression was not detected in the CTX NPs overexpressing Nurrl1 (Fig. 6C). In addition to NPs derived from the embryonic CNS, adult NPs (aNPs) derived from the murine SVZ were subjected to the same experimental procedures. Multipassaged aNPs expressed nestin (Fig. 1 A) and retained the ability to generate both neurons and glial cells (Fig. 3). Nurrl1 expression following lentivirus infection yielded overexpression levels comparable to those obtained in embryonic NPs infected with the same vector (Fig. 2). Nonetheless, Nurrl1 overexpression was not sufficient to cause formation of TH-positive cells (Fig. 5B) and activate DAT expression (Fig. 6A in aNPs. In conclusion, these results show that, by stage E13.5 of mouse development, Nurrl1 is able to promote dopaminergic cell fates specifically in NPs derived from GE and MB, but not in NPs from non-dopaminergic neural areas, such as CTX, SC and adult SVZ.

**ES-D3 cells**

As a positive control for our overexpression approaches, we also examined the ability of Nurrl1 to promote dopaminergic cell fates in NPs derived from mouse ES cells (ES-D3), as previously described [33,34]. ES-D3 cells were first induced to differentiate by culturing them as embryoid bodies, followed by selection and expansion of Nestin-positive NPs (see Materials and Methods). As
Figure 1. Immunodetection of Nestin in NPs lines derived from different brain regions and RT-PCR detection of regionally expressed genes. A) Immunostaining for nestin (green) on NPs derived from a) cortex (CTX), b) lateral ganglionic eminence (GE), c) spinal cord (SC), d) midbrain (MB), e) subventricular zone (SVZ), f) mouse blastocyst (ES-D3). Nuclei were stained with DAPI (blue). Scale bar = 20 μm. B) Real time
shown in Fig. 1 A, following the differentiation procedure all cells in the culture became Nestin-positive. When NPs derived from ES-D3 cells were infected with Nurr1 lentivirus or GFP lentivirus, we detected by real time PCR a significant increment of Nurr1 mRNA levels 72 hours after infection (Fig. 2). Control (GFP infected cells) and infected ES-D3 were then differentiated upon bFGF deprivation, obtaining about 40% of neurons (beta-III-tubulin-positive cells) and 50% of glial cells (GFAP-positive cells) in both control and infected ES-D3 cells (Fig. 3B). Real-time PCR and immunostaining analyses of infected ES-D3-derived NPs, maintained under differentiating culture conditions, showed a significant increase of TH mRNA levels compared to control cultures, (Fig. 4) and the presence of TH positive cells only in Nurr1-expressing cultures (2–3% of total cells, Fig. 5). Importantly, expression of the dopamine transporter DAT was detected by RT-PCR in Nurr1-infected cells (Fig. 6A), further confirming the induction of the dopaminergic phenotype.

Discussion

Expandable cells are a useful tool that can be used in transplantation because of the advantage of standardized cell populations, in terms of proliferation and differentiation potential [35–38]. In this respect, a question of interest is whether NPs from different brain regions retain the ability to respond to transcription factors involved in the differentiation of specific neuronal populations, once they have acquired a positional identity. To address this issue, we compared the ability of NPs derived from different CNS regions to respond to the transcription factor Nurr1 by activating a dopaminergic phenotype.

NPs from embryonic nervous system and adult neurogenic areas can proliferate in culture in the presence of bFGF and EGF and, under appropriate culture conditions, can generate both neurons and glial cells [39–41].

Our results show that NPs derived from E13.5 mouse embryos retain at least in part their positional identity, as shown by preservation of regulated expression profiles of region-specific transcription factors, such as FoxG1, Dlx2, Six3, HoxB6, HoxB9 and Ng2 and also recently reported for E14.5 NPs [10]. Our data also show that NPs derived from E13.5 mouse embryos respond to Nurr1 overexpression by giving rise to a small number of dopaminergic neurons, but this ability is critically dependent on their area of origin. In fact, Nurr1 overexpression caused an increase of TH mRNA levels and the percentage of TH-positive neurons specifically in GE- and MB-derived NPs, but not in CTX-, SC- or SVZ-derived NPs. Similar effects were detected with other DA-cell markers, such as DAT and Pitx3, indicating that they are not limited to the TH gene but reflect the acquisition of a dopaminergic phenotype. Pitx3 region-specific induction following Nurr1 overexpression is interesting, since Pitx3 expression has been reported to be directly regulated by Nurr1 in mesencephalic cells, due to the presence of a Nurr1 binding region on the Pitx3 promoter [31]. Our results suggest that additional mechanisms modulate the ability of Nurr1 to activate Pitx3 transcription, consistently with the fact that in vivo Pitx3 is expressed only in a subset of Nurr1-positive cells [32]. The ratios of beta-III-tubulin-positive and GFAP-positive cells showed that Nurr1 does not recruit larger numbers of NPs to neurogenic versus gliogenic fates, but rather suggest that the increase of TH-positive cells might be ascribed to NP assignment to a dopaminergic fate. These effects were reproduced with NPs derived from independent dissections and with different experimental approaches to achieve Nurr1 overexpression. Together with the fact that Nurr1 was also able to promote dopaminergic cell fates in ES cells, as previously described [20], these observations strongly argue against the idea that the lack of dopaminergic specification in CTX-, SC- and SVZ-derived NPs in due to insufficient Nurr1 activity in these cells and suggest instead that these differential effects are closely related to the pre-existing regional identity of NPs.

While DA neuronal populations are well known to reside in the ventral midbrain, striatal neurons expressing TH have also been reported in several mammalian species and in humans [42,43], and the existence of striatal DA neurons, which are similar to MB DA neurons, has also been demonstrated [44]. These observations could explain the fact that Nurr1 is able to promote dopaminergic fates also in GE-derived NPs, besides MB-derived NPs.

Considerable controversy exists concerning the degree to which NPs retain in vitro the positional information acquired in their region of origin. Some studies indicated that NPs maintained their regional specification even after several passages in vitro as neurospheres [5,9,45,46], while others reported a progressive loss of regional identity due to the downregulation of dorsal and ventral markers in culture [29,47]. It should be mentioned that different properties of NPs depend on the culture conditions used, even at early passages [48], or on the species of origin, e.g. rat [29] or mouse [46]. Armando et al. [45] showed that mouse embryonic CTX, STR and SC derived neurospheres, even after 8 passages, generate in vitro neural progenies that show, after differentiation, region-specific behaviour. Moreover, NPs derived from human fetal CTX and STR exhibit region-specific differentiation in vitro, survive, migrate, and form mature neurons after intracerebral transplantation in newborn rats [9]. Kim et al. [49] also demonstrated that human neurospheres isolated from different CNS compartments express distinctive molecular markers of regional identity and maintain these patterns of region-specific gene expression during long-term passages in vitro, as also recently reported for mouse cortex NPs [10]. While all these studies have
been very informative in showing that some region-dependent traits of NPs can be maintained in vitro, there is limited information available on whether this pre-existing specification can be overcome by forced expression of transcription factors controlling specific cell fates. Our work thus confirms and extends previous studies in this field by showing that NPs cultured in vitro maintain at least some of their original positional information, not only in terms of molecular marker expression or differentiation properties, but, importantly, also in terms of their competence to respond to Nurr1 overexpression by activating a dopaminergic differentiation programme. These results suggest that Nurr1 needs a specific cellular environment and a pattern of gene expression that supports and/or promotes dopaminergic function in some brain regions only and that the presence or absence of this positional signature can be at least partially preserved in vitro. Induction of dopaminergic neurons from human foetal forebrain has been reported also by Christophersen et al. [50]; however in this case TH expression appears to be dependent on the presence of specific factors in the medium, which are required for TH expression. On the other hand, experiments on ES-D3 cells demonstrate that NPs derived from pluripotent uncommitted embryonic ES-D3 can give rise to dopaminergic neurons following Nurr1 overexpression. These results are consistent with previous

Figure 3. Evaluation of neuronal and glial cells in differentiated NPs lines. A) Immunostaining for beta-III-tubulin (green) GFAP (red) on differentiated cells in control conditions (A, C, E, G, L) and after infection with Nurr1 lentivirus (B, D, F, H, N). (A, B) CTX, (C, D) GE, (E, F) SC, (G, H) MB, (I, L) adult SVZ, (M, N) ES-D3. Nuclei were stained with DAPI. B) Percentage of beta-III-tubulin and GFAP positive cells after differentiation of NPs. The reported values are the mean ± SEM of 10 observations from two independent experiments Scale bar = 20 μm. The error bars represent standard deviation.
doi:10.1371/journal.pone.0051798.g003

Figure 4. Induction of TH expression by Nurr1 overexpression: RT-PCR analysis. TH mRNA level detected by Real Time PCR in differentiated CTX, GE, MB and ES-D3 NPs and in cells infected with Nurr1. TH mRNA was not detectable in other analyzed NPs. TH mRNA level in mouse MB (E MB) is reported as positive control. The error bars represent standard deviation (n = 3). *** P<0.001.
doi:10.1371/journal.pone.0051798.g004
findings showing the ability of Nurr1 to elicit differentiation of dopaminergic neurons in ES cells [20–23]. The higher increase of TH positive cells induced by Nurr1 overexpression in GE and MB NPs compared to the limited increase in ES-D3 cells suggests that the regional specification either prevents (as in CTX, SC and SVZ NPs) or permits/facilitates (as in GE and MB NPs) the activation of a dopaminergic program regulated by Nurr1. The lack of Pitx3 expression in control NPs from midbrain is consistent with the observation that Pitx3 transcripts are present only in committed and differentiated MB dopaminergic neurons [51] and shows that Nurr1 overexpression is not sufficient to turn on this gene, at least in our culture conditions. On the other hand, the increase of TH-positive neurons on MB NPs overexpressing Nurr1, Activation of Pitx3 expression in GE and MB NPs is consistent with the observation that during embryonic development the induction of TH expression in the ventral midbrain requires Nurr1 which in turn activates Pitx3 expression [14]. In conclusion, although interplay between Nurr1 and Pitx3 have been reported to exert a cooperative effect in the induction of MB dopaminergic differentiation in vivo and in vitro [52,53] Nurr1 overexpression appears sufficient to promote the expression of a subset of midbrain DA neuron markers, such as TH and DAT, possibly via Pitx3 activation.

As far as SVZ derived NPs are concerned, we did not find TH positive neurons from either uninfected or Nurr1 infected adult SVZ cells. On the other hand Shim and colleagues reported that SVZ cells do not generate TH-positive neurons in vitro, even if exposed to cytokines involved in dopaminergic differentiation; however, they obtained functional dopaminergic neurons by overexpressing Nurr1 [54]. The discrepancy with our study could be explained by the fact that Shim’s experiments were performed in unpassaged SVZ cells rather than in multipassaged cells. Notably it has been shown that neural precursors from ventral mesencephalon almost completely lose their ability to generate dopaminergic neurons after in vitro expansion [55], which could account for the differences between our and Shim’s study.

Figure 5. Induction of TH expression by Nurr1 overexpression: immunocytochemistry analysis. A) Immunostaining for TH (green) on differentiated cells in control conditions and after infection with Nurr1 lentivirus. Nuclei were stained with DAPI (blue). (a) ES-D3, (d) Nurr1 infected ES-D3, (b) MB, (e) MB Nurr1 infected (c) GE, (f) Nurr1 infected GE. B) Percentage of TH positive cells (vs total cell number). Scale bar = 30 μm. doi:10.1371/journal.pone.0051798.g005
addition differences between species of origin (rat versus mouse neural precursors) could also account for the different responsiveness to Nurr1, as also observed for maintenance of region specific transcription factor expression [29,46].

In conclusion we demonstrated that Nurr1 is not sufficient to drive dopaminergic differentiation in all neural precursors, suggesting that cell ability to respond to this transcription factor might depend on the regional specification acquired during early steps of the neural tube regionalization.

**Materials and Methods**

**Materials**

Cell culture media and fetal calf serum (FCS) were obtained from SIGMA; growth factors (LIF, EGF, bFGF) from R & D Systems; Media Supplements (N2, B27) from Gibco; polyornithine, laminin, fibronectin and other reagents from SIGMA.

**ES cells**

The mouse blastocyst-derived ES cell line D3 (ES-D3) obtained from A.T.C.C. (Rockland, MD), was propagated and maintained as previously described [56]. In brief, undifferentiated ES cells were cultured on gelatin-coated dishes in Dulbecco’s modified minimal essential medium (DMEM), supplemented with 2 mM glutamine, 0.001% β-mercaptoethanol, non essential amino acids, 15% fetal bovine serum, and 2000 U/ml human recombinant LIF. Formation of embryoid bodies was obtained upon culture in non adherent bacterial dishes for four days [57]. The resulting embryoid bodies were then plated in adhesive tissue culture dishes. After 24 h in culture, selection for NP cells was initiated in serum-free medium [57]. After 6–10 days of selection, NP were plated on

---

**Figure 6. Activation of the dopaminergic markers DAT and Ptx3 by Nurr1 overexpression.** A) Representative samples of RT-PCR amplified products for DAT B) and C) real-time PCR of DAT (B) and Ptx3 (C) mRNA on differentiated cells in control conditions and after Nurr1 overexpression HPRT was used as normalizing gene. The error bars represent standard deviation (n = 3). **P<0.02.

doi:10.1371/journal.pone.0051798.g006
polyornithine (15 mg/ml) and fibronectin (1 mg/ml) coated coverslips in N2 medium supplemented with 1 mg/ml laminin and 10 ng/ml bFGF. After expansion bFGF was removed to induce differentiation.

**Neural Precursors**

All animal-related procedures were conducted in accordance with European Communities Council Directive No. 86/609/EEC. Animals were anaesthetized and sacrificed by decapitation.

NPs were obtained from E13.5 mouse embryonic cortex (CTX), spinal cord (SC), midbrain (MB) and ganglionic eminence (GE) and adapted to cell cultures as previously described [26,50]. Tissues were incubated in Dulbecco’s modified Eagle’s Medium (DMEM) for 20 min at 37°C and then mechanically dissociated to single cell suspensions by trituration. The cells were transferred to basal medium (DMEM/F12, 1% penicillin/streptomycin, 0.1 M L-glutamine (Gibco), 23.8 mg/100 ml Heps, 7.5% NaHCO₃, 0.6% glucose), with the addition of 20 ng/ml human recombinant EGF, 10 ng/ml bFGF, and 1% N2 supplement. This medium was termed embryonic expansion medium. Living cells were counted in the presence of trypan blue and plated in uncoated T25 plastic flasks in embryonic expansion medium. After several days, spontaneous floating clusters were harvested, enzymatically dissociated (Accutase; Sigma) and single cell suspensions were plated in flasks coated with 10 mg/ml poly-ornithine and 5 mg/ml laminin at a density of 0.4–0.6 ×10⁶ cells/cm²; cells between passages 6 and 14 were used. Adult NPs (aNPs) were obtained. Both embryonic and adult NPs were maintained under proliferative conditions at 37°C in a 5% CO₂ atmosphere. Infection of NPs was performed by transfection of three elements: 1) a transfer vector, 2) the promoter containing the specific transfer construct (20 µg/ml polybrene, Sigma) and 3) the IRES puromycin resistance gene allowing strong selection for transgene expression. Early passage (p<6) NPs from CTX, GE and SC were transfected with pTP6-Nurr1 with Amaxa Nucleofector and Neural Stem Cell Nucleofector kit (Lonza) according to manufacturer instructions. Two days after transfection, NPs with stable integration of the pTP6-Nurr1 transgene were selected and expanded by adding puromycin (0.66–1 µg/ml) to the culture medium. Control NP lines were generated with pTP6-GFP to test infection efficiency all cells were infected with lentiviral vectors carrying GFP and analyzed by FACs 72 hours after infection. Accordingly lentivirus carrying Nurr1 was used at MOI 2, which yielded a 26–30% infection efficiency for both ES cells and NPs; higher lentivirus concentrations could not be used as they lead to extensive cell death. Transduction experiments were performed in the presence of 8 µg/ml polybrene, (Sigma). After viral addition, cells were centrifuged at 1,800 rpm, 75 minutes at 32°C, and incubated for 45 minutes at 37°C, under 5% CO₂. Medium was then replaced and the cells were cultured until further analysis.

**Generation of NP lines with stable Nurr1 expression**

Nurr1 open reading frame was amplified by PCR using the following primers containing the MfeI restriction site (Forward primer: 5'—ACT GCA CAA TTG ATG CCT TGT GTG CAG GCC CAG TGT-3'; Reverse primer: 5'—AGA GCT CAA TTT CTT AGA AAG GTA AGG TGT CCAG-3') and subcloned into the EcoRI site of the pTP6 expression vector [59]. pTP6 vector contains the CAGG promoter followed by the Nurr1 cDNA and an IRES puromycin resistance gene allowing strong selection for transgene expression. Early passage (p<6) NPs from CTX, GE and SC were transfected with pTP6-Nurr1 with Amaxa Nucleofector and Neural Stem Cell Nucleofector kit (Lonza) according to manufacturer instructions. Two days after transfection, NPs with stable integration of the pTP6-Nurr1 transgene were selected and expanded by adding puromycin (0.66–1 µg/ml) to the culture medium. Control NP lines were generated with stable expression of the previously described pTP6-hrGFP construct [60]. Transgenic NPs were cultured as described above, except that they were maintained in puromycin when grown in proliferating conditions.

**Immunocytochemistry**

Cells were fixed for 10 min in 4% para-formaldehyde in PBS, pH 7.4 at room temperature and then washed in PBS containing 1% bovine serum albumin (BSA). Dishes were pre-incubated first in 0.1 M glycine in PBS and then in PBS containing 5% normal rabbit or goat serum and 0.025% Triton X-100 for 30 min at room temperature. Incubation with the primary antibody was carried out overnight at 4°C. Antibodies were diluted in PBS containing 5% normal rabbit or goat serum and 0.025% Triton X-100 as follows: mouse anti-beta-III-tubulin (Promega) diluted 1:200, mouse anti-nestin (Chemicon) diluted 1:100, rabbit anti-GFAP (DAKO Cytomation) 1:200, mouse anti-TH (Chemicon) 1:200, rabbit anti-TH (Chemicon) 1:100. Dishes were then washed and incubated for 60 min at room temperature with FITC-conjugated rabbit anti-goat IgG, diluted 1:100, or TRITC conjugated goat anti-rabbit IgG diluted 1:100, as secondary antibodies (Jackson). After washing, samples were mounted in DABCO (FLUKA). No staining was observed when primary
antibodies were omitted. DNA staining was performed by incubating cells with 1 μg/mL DAPI (SIGMA) in PBS for 10 min at room temperature. Immunofluorescence was observed using either a Zeiss Axiosphot microscope or a Leica confocal microscope. Immunostained cells were counted on at least 10 randomly selected microscopic fields of three independent experiments and reported as percentage of DAPI stained cells.

RNA extraction and RT-PCR analysis
Total RNA was extracted from cell cultures using the Trizol extraction procedure (Invitrogen) and digested with DNase I (Ambion). For each RNA sample, 2 μg of total RNA were reverse transcribed for 60 min at 37°C with random hexamers (Promega) as primers and M-MLV reverse transcriptase (Promega). A 2 μg aliquot was treated identically except that reverse transcriptase was omitted. A control was also included containing all reagents (including reverse transcriptase) except RNA. After reverse transcription, GoTaq DNA Polymerase (Promega) primers (final concentration 0.4 μM each primer) and the appropriate buffer were added (final volume 25 μl) to the tubes. PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 30 cycles with the following profile: 95°C for 30–45 sec, 55°C for 30–45 sec, 72°C for 30–60 sec, final extension at 72°C for 5 min. 25 μl of each PCR reaction product were run on a 0.8% agarose gel containing 1 μg/ml ethidium bromide to reveal DNA bands. The specificity of the PCR product was verified by sequencing.

Real-Time/RT-PCR and quantification of gene expression
Real-time PCR amplifications were performed on reverse transcription (RT) products with the SYBR Green JumpStart Taq ReadyMix (Qiagen, Valencia, CA, USA) in a Lightcycler apparatus (Bio-Rad, Hercules, CA, USA), following the manufacturer’s instructions. All samples were run in duplicate, and each well contained 25 μl as a final volume, including 2.5 μl as cDNA, 0.2 μM forward primer, 0.2 μM reverse primer, and 12.5 μl SYBR Green JumpStart Taq ReadyMix and 0.2 μl internal reference dye. The PCR started with 94°C for 2 min and then continued with 35-40 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C, and 15 s at 80°C. A melting curve was obtained for each PCR product after each run to confirm that the signal corresponded to a unique amplicon of the predicted size. The specificity of the PCR product was verified by sequencing. Expression levels were normalized to the housekeeping genes encoding for Actin, HPRT or PBGD, obtained for every sample in parallel assays of two to four independent experiments. The mRNA levels for each gene were compared between cells infected with either the Nurrl containing lentivirus vector or the corresponding empty vector by using the DDCT method (Primers sequence are available under request).

Supporting Information
Figure S1 Nurrl and TH expression in in NP lines with stable expression of Nurrl. NP lines were selected for stable expression of Nurrl as described in Materials and Methods. A and B, real-time PCR of Nurrl (A) and TH mRNA (B) on differentiated cells in control conditions after Nurrl overexpression, HPRT was used as normalizing gene. C. Percentage of beta-III-tubulin and GFAP positive cells after differentiation of NPs. The error bars represent standard deviation (n = 3). ** P<0.02. (TIIF)

Acknowledgments
We wish to acknowledge Fondazione Cenci Bolognetti, Viviana Orlando for technical assistance with PCR experiments, Dr Ludovic Vallier for plasmids and Dr. F. Leckie for language revision of the text.

Author Contributions
Conceived and designed the experiments: GAT SB CPC GL. Performed the experiments: CS EC IS NC. Contributed reagents/materials/analysis tools: GAT SB CPC EC IS GL. Wrote the paper: CS GAT.

References
1. Edlund T, Jessell TM (1999) Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell 96: 211-224.
2. Ruiz I, Altaha A (1992) Planar and vertical signals in the induction and patterning of the Xenopus nervous system. Development 115: 67-80.
3. Bertrand N, Castro DS, Guillemot F (2002) Proneural genes and the specification of neural cell types. Nat Rev Neurosci 3: 531–541.
4. Kelly TK, Karsten SL, Geshwind DH, Kornblum HI (2009) Cell lineage and regional identity of cultured spinal cord neural stem cells. Mol Cell Neurosci 40: 241-253.
5. Chopp M, Arai K, Motomizu K, Saito H, de Vellis J, et al. (1995) Human Neural Stem Cells Generate Region-Specific Neurons In Vitro and In Vivo. Neuromarketing 16: 1829–1838.
6. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 different mouse central nervous system compartments. Cell Mol Life Sci 68: 1769–83.
7. Kelly TK, Karsten SL, Geshwind DH, Kornblum HI (2009) Cell lineage and regional identity of cultured spinal cord neural stem cells and comparison to brain-derived neural stem cells. PLoS ONE 4: e4213.
8. Castillo SO, Baffi JS, Palkovits M, Goldstein DS, Kopin IJ, et al. (1998) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson’s disease. Nature 418: 50–56.
9. Wallén Å, Zetterström RH, Funakoshi T, Lehman JS, Le WD, Smith MP, Cox JJ, et al. (1998) Nurrl is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc Natl Acad Sci U S A 95: 4013-4018.
10. Zetterström RH, Funakoshi T, Lehman JS, Zetterström J, Funakoshi T, et al. (1997) Nurrl regulates midbrain dopaminergic neuron development. Int J Dev Biol 44: 679-687.
11. Luskin MB, School of Medicine, Stanford University, Stanford, CA, USA. A retina-specific enhancer of the Pax6 gene is a downstream target of Nurr1 transcription factor in rat midbrain neurons in vitro. J Neurosci 31: 1401–1413.
12. Saxena-Cardenas O, Quintana-Hau J, Le WD, Smith MP, Cox JJ, et al. (1998) Nurrl is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc Natl Acad Sci U S A 95: 4013-4018.
13. Perreone-Capanno C, Do Porzio U (2000) Genetic and epigenetic control of midbrain dopaminergic neuron development. Int J Dev Biol 44: 679-687.
14. Smith MP, Asbeck CH, Cox JJ, Chen H, Johnson RL, et al. (2000) A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. Nat Neurosci 3: 357–341.
15. Simon HH, Saueressig H, Wurst W, Goulding MD, O’Leary DD (2001) Fate of midbrain dopaminergic neurons controlled by the engrailed genes. J Neurosci 21: 3126–3134.
16. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2397-2402.
17. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2397-2402.
18. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2397-2402.
19. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2397-2402.
20. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2397-2402.
21. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2397-2402.
22. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2397-2402.
23. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2397-2402.
24. Zappone MV, Galli R, Carena R, Meani N, De Biasi S, et al. (2000) Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2397-2402.
22. Kim JY, Koh HC, Lee JY, Chang MY, Kim YC, et al. (2003) Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression. J Neurochem. 85: 1433–1454.

23. Sonntag KC, Simantov R, Kim KS, Iascone O (2004) Temporally induced neural commitment of regionalized cell type in embryonic stem cell differentiation. Eur J Neurosci. 19: 1141–1152.

24. Park GH, Kang JS, Yoon EH, Shim JW, Kim HS, et al. (2008) Pronuclear bHLH neurogenin 2 differentially regulates Nurr1-induced dopamine neuron differentiation in rat and mouse neural precursor cells in vitro. FEBS Letters 582: 537– 42.

25. Andersson EKI, Irvin DK, Ahlsio J, Parmar M (2007) Ngls2 and Nurr1 act in synergy to induce midbrain dopaminergic neurons from expanded neural stem and progenitor cells. Exp Cell Res 13: 1172–80.

26. Pollard SM, Conti L, Sun Y, Goffredo D, Smith A (2006) Adherent Neural Stem (NS) Cells from Fetal and Adult Forebrain. Cerebral Cortex 16: 112–120.

27. Schuurmans C, Arntz O, Nieto M, Stemman JM, Britz O, et al. (2004) Sequential phases of cortical specification involve Neurogenin-dependent and independent pathways. EMBO J 23: 2892–2902.

28. Mizuguchi R, Sugimoto M, Takebayashi H, Kosako H, Nago M, et al. (2001) Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and sub-type-specific properties of motoneurons. Neuron 31: 757– 771.

29. Bithell A, Finch SE, Hornby MF, Williams BP (2008) FGF2 maintains the neurogenic capacity of embryonic neural progenitor cells in vitro but changes their neuronal subtype specification. Stem Cells 26: 1563–1574.

30. Hack MA, Sugimoto M, Lundberg C, Nakafuku M, Gotz M (2004) Regionalization and fate specification in neuroepithelium: the role of Olig2 and Pax6. Mol Cell Neurosci 25: 664–678.

31. Vojvodic F, De Gregorio R, Pukranzo S, Perrone-Capano C, di Porzio U, et al. (2012) Direct regulation of Pitx3 expression by Nurr1 in culture and in developing mouse midbrain. PLoS One. 7: e30661.

32. Nunes I, Tovmasian LT, Silva RM, Burke RE, Goff SP (2003) Pitx3 is required for development of substantia nigra dopaminergic neurons. Proc Natl Acad Sci U S A 100: 4245–4250.

33. Jacobs FM, van Erp S, van der Lnden AJ, von Orthel L, Burbach JP, et al. (2007) How to make a mesodiencephalic dopaminergic neuron. Nat Rev Neurosci. 8: 21–32.

34. Martinat C, Bacci JJ, Leete T, Kim J, Vantti WB, et al. (2006) Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. Proc Natl Acad Sci U S A 103: 2074–2079.

35. Jacobs EM, van Esp S, van der Leden AJ, von Orthel L, Burbach JP, et al. (2009) Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression. Development 136: 531–40.

36. Shire JW, Park GH, Bae YC, Bar JY, Ghigna S, et al. (2007) Generation of Functional Dopamine Neurons from Neural Precursor Cells Isolated from the Subventricular Zone and White Matter of the Adult Rat Brain Using Nurr1 Overexpression. Stem Cells 25: 1252–62.

37. Thompson LH, Andersson E, Jensen JB, Barrand P, Guillenot F, et al. (2006) Neurogenin 2 identifies a transplantable dopamine neuron precursor in the developing ventral mesencephalon. Exp Neurol. 198: 183–198.

38. Pliester U, Kirchey A, Torper O, Wood J, Nelder J (2011) Direct conversion of human fibroblasts to dopaminergic neurons. Proc Natl Acad Sci U S A 108: 10345–10350.

39. Fricker RA, Carpenter MK, Windler C (1999) Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. J Neurosci. 19: 5900–6005.

40. Roy NS, Benaissa A, Wang S (2000) Promoter-targeted selection and isolation of neural progenitor cells from the adult human ventricular zone. J Neurosci Res 59: 321–331.

41. Villa A, Snyder K, Yescova A (2000) Establishment and properties of a growth factor-dependent, perpetual neural stem cell line from the human CNS. Exp Neurol 161: 67–84.

42. Consolati M, Bedard A, Parent A (2003) Dopaminergic neurons in human striatum and neurogenesis in adult monkey striatum. Ann NY Acad Sci 991: 346–349.

43. Consolati M, Parent A, Lévesque D (2004) Tyrosine hydroxylase-positive neurons intrinsic to the human striatum express the transcription factor Nurr1. Eur J Neurosci 20: 2809–28095.

44. Ho J, Parent A (2007) Dopaminergic neurons intrinsic to the striatum. J Neurochem 101: 1441–1447.

45. Armando S, Lebrun A, Huqg JP, Ridolfi C, Saumier M, et al. (2007) Neurosphere-derived neural cells show region-specific behaviour in vitro. Neuroreport 18: 1359–429.

46. Nakagawa Y, Kamei T, Ogura T, Suzuki T, Torii M, et al. (2006) Roles of cell-autonomous mechanisms for differential expression of region-specific transcription factors in neuroepithelial cells. Development 122: 2449–64.

47. Gabay L, Lowell SB, Rubin LL, Andersson DJ (2003) Deregression of dorsoventral patterning by FGF confers unilineage differentiation capacity on CNS stem cells in vitro. Neuro 40: 485–499.

48. Jens P, Parmar M (2006) Strengths and limitations of the neurophoreses culture system. Mol Neurobiol 34: 153–161.

49. Kim HJ, Sugimoto M, Nakafuku M, Swenden JN (2007) Control of neurogenesis and tyrosine hydroxylase expression in neural progenitor cells through bHLH proteins and Nurr1. Exp Neuro 203: 394–405.

50. Christophersen NS, Meijer X, Jorgensen JR, Englund U, Gronborg M, et al. (2006) Induction of dopaminergic neurons from growth factor expanded neural stem/progenitor cell cultures derived from human first trimester forebrain. Brain Res Bull 70: 457–466.

51. Smit D, Burzarh JP (2007) How to make a mesodiencephalic dopaminergic neuron. Nat Rev Neurosci. 8: 21–32.

52. Marinat C, Bacci JJ, Leete T, Kim J, Vantti WB, et al. (2006) Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. Proc Natl Acad Sci U S A 103: 2074–2079.

53. Jacobs EM, van Esp S, van der Leden AJ, von Orthel L, Burchar JP, et al. (2009) Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression. Development 136: 531–40.

54. Shire JW, Park GH, Bae YC, Bar JY, Ghigna S, et al. (2007) Generation of Functional Dopamine Neurons from Neural Precursor Cells Isolated from the Subventricular Zone and White Matter of the Adult Rat Brain Using Nurr1 Overexpression. Stem Cells 25: 1252–62.

55. Chung S, Shin BS, Hwang M, Lardaro T, Kog UJ, et al. (2006) Neural precursors derived from embryonic stem cells, but not those from fetal ventral mesencephalon, maintain the potential to differentiate into dopaminergic neurons after expansion in vitro. Stem Cells 24: 1363–93.

56. Deacon T, Dinmore J, Costantini LC, Ratliff J, Isacson O (1998) Blastula-derived stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation. Exp Neurol. 149: 28–41.

57. Okabe S, Forsberg-Nilsson KA, Spiro C, Segal M, McKay R (1996) Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech Devel 59: 89–102.

58. Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, et al. (2005) Niche-dependent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol 3: e283.

59. Pratt T, Sharp L, Nichols J, Price DJ, Mason JO (2000) Embryonic stem cells and transgenic mice ubiquitously expressing a tau-tagged green fluorescent protein. Dev Biol 229: 19–28.

60. Vallier L, Rieh-Gunn PP, Bouhon IA, Andersson FK, Sadler AJ, et al. (2004) Enhancing and diminishing gene function in human embryonic stem cells. Stem Cells 22: 2–11.