The *foxa2* Gene Controls the Birth and Spontaneous Degeneration of Dopamine Neurons in Old Age

Raja Kittappa¹, Wendy W. Chang¹*, Rajeshwar B. Awatramani², Ronald D. G. McKay¹*

1 Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, United States of America, 2 Department of Neurology, Northwestern University School of Medicine, Chicago, Illinois, United States of America

Parkinson disease affects more than 1% of the population over 60 y old. The dominant models for Parkinson disease are based on the use of chemical toxins to kill dopamine neurons, but do not address the risk factors that normally increase with age. Forkhead transcription factors are critical regulators of survival and longevity. The forkhead transcription factor, *foxa2*, is specifically expressed in adult dopamine neurons and their precursors in the medial floor plate. Gain-and loss-of-function experiments show this gene, *foxa2*, is required to generate dopamine neurons during fetal development and from embryonic stem cells. Mice carrying only one copy of the *foxa2* gene show abnormalities in motor behavior in old age and an associated progressive loss of dopamine neurons. Manipulating forkhead function may regulate both the birth of dopamine neurons and their spontaneous death, two major goals of regenerative medicine.

Introduction

Midbrain dopamine neurons play important roles in motor control, reward, addiction, attention, and cognition [1,2]. A progressive loss of dopamine neurons is a defining feature of Parkinson disease. This disease will cause increased hardship in many countries, as 30% of the population will be over the age of 65 y around 2025 [3]. Dopamine neurons are normally generated in limited numbers, for a restricted time, in a small region of the embryonic midbrain [4]. To improve access to these neurons, techniques have been developed to derive them from precursors dissected from the fetal midbrain and from pluripotent embryonic stem (ES) cell lines [5–11]. Despite this effort, our knowledge of the mechanisms controlling the birth and death of these cells is still limited.

*foxa2* is a forkhead transcription factor known to play a critical role in the early development of the endoderm and midline structures, including the notochord and floor plate [12–16]. FOXO transcription factors are closely related to the *foxa* genes and have a central role in cell survival, cancer, and the longevity of organisms [17–19]. Here, we show that midbrain dopamine neurons are derived from the floor plate and that *foxa2* plays a central role in specifying dopamine neurons. Late in life, *foxa2* heterozygous mice spontaneously develop significant motor problems and an associated late-onset degeneration of dopamine neurons. The initial deficit is asymmetric and preferentially affects dopamine neurons of the substantia nigra (SN) while leaving the ventral tegmental area (VTA) intact, a pattern of sensitivity also seen in Parkinson patients [20].

Results

Dopamine neurons can be identified by expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. In the nervous system, *foxa2* expression is restricted to the floor plate, a specialized ventral region that regulates the differentiation of nearby neurons by secreting the morphogenetic signal sonic hedgehog (SHH) [14,15]. The newly generated dopamine neurons form a band at the most ventral edge of the midbrain and express FOXA2 in their nuclei (Figure 1A and 1B). A panel of antibodies against transcription factors was used to define the domains of neuronal precursors in the ventral midbrain (Figure S1). The expression of the transcription factors LMX1b, NKX2.2, and PHOX2a between embryonic day (E)9.5 and 11.5 defines three adjacent ventral domains of neural progenitors before the first dopamine neurons are formed (Figure 1C). The transcription factor LMX1b is expressed in the most ventral domain, the medial floor plate, continuously from E9.5 through E11.5 (Figure S1C–S1E).

SHH is coexpressed with LMX1b in the floor plate (Figure 1D). From previous studies, it is unclear whether SHH-expressing cells are the direct precursors to dopamine neurons or if they are derived from a more lateral precursor that is induced by signals from the floor plate [21–23]. Genetic tracing using Cre-recombinase expressed from the shh regulatory sequences (shh-creR26R mice) allows the derivatives of these most medial cells to be identified [24].

Citation: Kittappa R, Chang WW, Awatramani RB, McKay RDG (2007) The *foxa2* gene controls the birth and spontaneous degeneration of dopamine neurons in old age. PLoS Biol 5(12): e325. doi:10.1371/journal.pbio.0050325

Academic Editor: Huda Y. Zoghbi, Baylor College of Medicine, United States of America

Received May 30, 2007; Accepted October 26, 2007; Published December 11, 2007

Copyright: © 2007 Kittappa et al. This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.

Abbreviations: E, embryonic day; ES, embryonic stem; GFAP, glial fibrillary acidic protein; shh, sonic hedgehog; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area

* To whom correspondence should be addressed. E-mail: mckay@codon.nih.gov

© These authors contributed equally to this work.
Author Summary

The restoration of dopamine neurons is a major focus of stem cell biology and regenerative medicine. The gradual loss of these neurons is a hallmark of Parkinson disease. Dopamine neurons in the midbrain convey important sensory and motor functions to the forebrain. We show that the transcription factor FOXA2 plays a central role in the birth and death of dopamine neurons in the midbrain. By defining their precursors in the ventral midbrain, we show that dopamine neurons are derived from organizer cells in the floor plate (the ventral cells of the neural tube, the embryonic foundation of the central nervous system). We also show that FOXA2 specifies the floor plate and induces the birth of dopamine neurons. Mice with only a single copy of the foxa2 gene acquire motor deficits and a late-onset degeneration of dopamine neurons. This spontaneous cell death preferentially affects neurons associated with Parkinson disease. This work provides new strategies to generate neurons in the laboratory and to block their death in old age.

When shh-cre mice were crossed with the R26R reporter strain, the expression of β-galactosidase is surprisingly found outside the midline (Figure 1E and inset). At E15.5, when the majority of dopamine neurons have become postmitotic, they express TH and also express β-galactosidase, definitively demonstrating that midbrain dopamine neurons are derived from the medial floor plate (Figure 1F–1H).

For the efficient ex vivo generation of dopamine neurons, it is important to understand the mechanisms that induce dopamine neuron identity. SHH gives increased numbers of dopamine neuron precursors in midbrain primary explants and in neuronal cultures derived from ES cells [8,22,23]. This effect is thought to reflect the morphogenetic induction of the dopaminergic fate. Access to reagents that define distinct precursor types allows the morphogenic effects of SHH in vitro to be reexamined. When dissociated cells from the developing ventral midbrain (E8.5) were exposed to increasing concentrations of SHH, the proportion of LMX1b\(^+\) cells did not change, but the proportion of the NKX2.2\(^+\) and NKX6.1\(^+\) cells increased while the more dorsal PAX7 expression decreased (Figures 2A and S2). This result shows that, under these conditions, SHH does not induce the precursors of dopamine neurons, whereas the proportion of more lateral precursors is altered in the graded way expected of a morphogenic signal. However, SHH is also a mitogen for neural precursors and this mitogenic effect may account for the increased number of dopamine progenitors (Figure 2B). These results suggest that growth control systems for dopamine neuron precursors are distinct from most other ventral midbrain neuron types.

A consequence of these results is that production of dopamine neurons in the laboratory will be limited by the number of floor plate cells. As foxa2 is known to control the generation of the floor plate, we asked whether this gene controls the number of TH-positive cells in vitro. Because foxa2-null embryos do not survive past E10.5, E8.5 midbrain explants from foxa2 homozygous mutant (n = 8) and control embryos (n = 12) were placed in culture. Neural progenitors migrate out from the explant and differentiate into Tuj1-positive neurons. In all of the wild-type explants, TH\(^+\) neurons were abundant (Figure 2C). In contrast, the mutant explants generated no TH\(^+\) neurons (Figure 2D). Using transcription factor expression, the neuron types in the ventral midbrain were distinguished (Figure S3). The islet1-positive oculomotor neurons were also absent, but more lateral neurons expressing GATA3 and LIM1/2 were generated in cultures from foxa2\(^-/-\) embryos (Figure 2E). These data show that the foxa2 gene is specifically required for the generation of dopamine and motor neurons, the two neuron types derived from the floor plate.

To ask whether expression of foxa2 would cause an increase in the number of dopamine neurons generated in vitro, a foxa2 expression plasmid was transfected into E10.5 midbrain cells. A 4-fold increase in the proportion of TH\(^+\) cells was observed (Figure 2F). A mouse embryonic stem (mES) cell line engineered to inducibly express a foxa2 transgene generated 7-fold more TH\(^+\) cells (Figure 2G). The SHH antagonist, cyclopamine, inhibited the production of TH\(^+\) neurons in vitro. This effect was reversed upon induction of foxa2 even in the presence of cyclopamine (Figure 2H). These results are consistent with an early role for shh in dopamine neuron specification demonstrated in mice carrying a conditional mutation in the SHH receptor smoothened [25]. Genetic analysis in zebrafish also demonstrates a role for foxa2 in the specification or patterning of ventral neurons in the midbrain and hindbrain [26]. Our data suggest that foxa2 specifies dopamine neurons in mammals.

Dopamine neurons in the adult brain continue to express foxa2 (Figure 3A). A foxa2 null allele was crossed into the C57BL/6 strain because this background is widely used in physiological and behavioral studies. Mice entirely lacking foxa2 die in early development. In all the data reported here, the effect of a single copy of the foxa2 gene, haploinsufficiency, was assessed in a C57BL/6 background. Haploinsufficient mice spontaneously developed major motor abnormalities. These behaviors were first observed at 18 mo of age when mice present with an asymmetric posture associated with a muscular rigidity that progresses from the tail, through the hind limbs, to the trunk. foxa2\(^-/-\) animals showed a slower speed of movement but reduced horizontal movement and a complete loss of vertical movement (Figure 3B–3D, foxa2\(^-/-\) n = 6, foxa2\(^-/-\) n = 4; Video S1). The mice demonstrate a unilateral constriction/torsion of the trunk so the spine becomes curved towards either the left or right side. In a group of affected animals, the spinal curvature was 15° measured by analysis of footprints (Figure 3E). Occasional episodes of high-frequency trembling have been observed. Rigidity and loss of mobility in the hindlimbs become so severe that the limb is abnormally extended or splayed (Figure 3G).

These defects could be caused by deficits in non-dopaminergic systems. A widely used and quantitative behavioral assay of dopaminergic function in rodents is amphetamine-induced rotation [27]. Rats or mice lesioned acutely and unilaterally with 6-OHDA circumambulate ipsilaterally in response to amphetamine, and the extent of rotational movement is directly correlated with the severity of the lesion. We assayed amphetamine-induced rotational behavior in old foxa2\(^-/-\) and wild-type C57BL/6 mice. A significant increase in rotational movement was observed in foxa2 mutants (Figure 3F). Rotations occurred in the clockwise or counterclockwise direction, depending on the individual mouse, and were ipsilateral to the “kinked” side of the mouse. In the ventral midbrain of 1-y-old heterozygous...
animals, there is a partial loss of FOXA2 protein, suggesting that a reduced level of the FOXA2 protein leads to a behavioral deficit that spontaneously appears in old age (Figure 3H).

The amphetamine-induced rotational behavior suggests an asymmetric loss of dopamine neurons. Immunohistochemistry for TH-positive cells was performed to shed light on potential cellular causes of the motor problems. Affected
Figure 2. The In Vitro Role of foxa2 and SHH in Cell Fate Specification in the Ventral Mesencephalon

(A) Effects of Shh on E8.5 mesencephalic precursors in vitro. In the absence of exogenous SHH, many LMX1b+ precursors (36.59 ± 6.10%) were observed. NKX2.2+ (19.83 ± 4.39%), NKX6.1+ (30.47 ± 6.72%), and PAX7+ precursors (16.33 ± 5.02%) were present. In the presence of 500 ng/ml of SHH protein, the proportion of NKX2.2+ (41.84 ± 5.84%) and NKX6.1+ (56.62 ± 7.04%) cells increased, whereas, the proportion of PAX7+ cells diminished (6.89 ± 2.54%). Despite these effects on other cell types, the proportion of LMX1b+ dopamine precursors was stable (32.48 ± 5.56%).

Higher concentrations of SHH would be expected to further ventralize the midbrain precursors at the expense of more dorsal fates. In the presence of 1 μg/ml of SHH protein, there was a further increase in NKX2.2+ and NKX6.1+ cells (nkx2.2+, 52.75 ± 6.34%; nkx6.1+, 69.12 ± 8.73%) and a greater reduction of PAX7+ cells (2.24 ± 0.81%). At this high concentration of SHH, the percentage of dopamine precursors again remained unchanged (33.04 ± 6.61%).

(B) SHH has a proliferative effect on mesencephalic precursors in cell culture. BrdU was added to day 3 cultures of mesencephalic precursors, grown in different concentrations of SHH protein. BrdU was added for 1 h before fixation and staining. The percentage of cells incorporating BrdU increased with the concentration of SHH in the culture.

(C) Wild-type mesencephalic explants differentiate to generate a large number of neurons (Tuj1, green) and a significant proportion of these neurons express tyrosine hydroxylase (TH, red).

(D) Mesencephalic explants from foxa2−/− embryos also generate many neurons, but TH expression is absent.

(E) foxa2−/− explants similarly do not yield islet-1–expressing motor neurons although the differentiation of GATA3- and LIM1/2-positive neurons born outside of the floor plate is unaffected. The developmental expression pattern of these proteins can be seen in Figure S3.

(F) Overexpression of foxa2 in cultured E10.5 mesencephalic explants results in an increase in TH-expressing dopamine neurons.

(G) Doxycycline induction of foxa2 in differentiating F4 mouse embryonic stem cells significantly increases the number of resulting dopamine neurons.

(H) Inhibition of SHH signaling by cyclopamine suppresses dopaminergic differentiation of ES cells. The induction of foxa2 by doxycycline overcomes cyclopamine suppression of dopamine neuron differentiation.

doi:10.1371/journal.pbio.0050325.g002
mice demonstrated an asymmetric loss of TH expression in midbrain dopamine neurons in the SN sparing the VTA (Figure 4A and 4B). No loss of dopamine neurons was seen in old foxa2 mutants without behavioral abnormalities (Figure 4C and 4D). In the SN of affected foxa2+/− animals, there are few neurons recognized by Nissl staining (Figure 4E). Serial sections through the midbrain dopaminergic system of a single animal show an almost total loss of TH+ neurons in the SN on one side of the brain and much less damage to the contralateral SN (Figure S4). In foxa2+/− animals (n = 3) showing abnormal motor behaviors, compared to foxa2+/+ age-matched controls, there is a specific loss of SN neurons.
This loss of dopamine neurons occurs in one third of animals over 18 mo old, and the asymmetric cellular loss explains the amphetamine induced rotation.

Within the SN, there are different types of dopamine neurons. The most ventral neurons specifically express retinaldehyde dehydrogenase-1 (RALDH1; [28]). In foxa2 mutant mice, there is a loss of RALDH1-positive neurons even when substantial numbers of dopamine neurons are still present in the SN. In the animal shown in Figure 4F, the SN on one side of the brain has a severe loss of dopamine neurons. In the contralateral SN, TH-positive cells are present, but there is a selective loss of RALDH1-positive cells.

**Figure 4.** Dopamine Neuron Loss in foxa2<sup>+/—</sup> Mice

(A and B) Dopamine neurons in a 24-mo-old wild-type mouse (A) and a foxa2<sup>+/—</sup> littermate (B). The mouse in (B) possessed a kinked posture and the other late-onset motor phenotypes. A significant loss of nigral dopamine neurons is easily observed on one side of the brain marked by a blue star in the foxa2<sup>+/—</sup> mouse.

(C and D) TH staining of the the ventral midbrain in older foxa2<sup>+/—</sup> animals without late onset behavioral abnormalities. Note that the dopaminergic system resembles the wild-type condition seen in (A).

(E) Nissl staining of wild-type and foxa2<sup>+/—</sup> mutant mice. Representative glia and neurons are highlighted with white and black arrows, respectively. Note the significant loss of normal neuronal morphologies in the mutant animal. Occasionally, a dysmorphic cell, highlighted here with a purple arrow, can be seen in the mutant, but not the wild-type brain.

(F) Ventral tier dopamine neurons, stained for RALDH1 (red), are selectively lost in foxa2<sup>+/—</sup> animals. In this animal, nigral dopamine neurons are almost completely lost on the left side (marked by the blue star), whereas the right side (marked by the red sun) is much less affected (note that these are coronal sections, presented as if the animal were facing the viewer, so that the left side of the animal is on the viewer’s right side).

(G) Quantitation of dopamine neurons in the SN and VTA of wild-type (n = 3) and foxa2<sup>+/—</sup> (n = 3) mice. The number of dopamine neurons in the mutant SN (2,765.0 ± 750.5) is substantially smaller than in the control SN (7,344.3 ± 197.9). In contrast, the number of the dopamine neurons in the VTA is roughly the same in wild-type (9,922.0 ± 375.7) and mutant mice (10,064.7 ± 355.4).

(H) The percentage of RALDH1-expressing neurons in the SN is reduced in foxa2<sup>+/—</sup> mutants as compared to wild-type controls.

doi:10.1371/journal.pbio.0050325.g004
(Figure 4F and 4H). Many of the affected animals show an asymmetric loss of dopamine neurons, and the most affected side could be either the right or left (marked by a star in Figures 4B and 4F). These data suggest the degenerative process is progressive, the RALDH1-positive neurons are the most vulnerable cells, and the SN on one side of the brain is affected first and then the disease progresses to the contralateral midbrain.

Lewy bodies, cellular inclusions immunoreactive for alpha-synuclein, ubiquitin, and other proteins, are often found in the basal ganglia and cortex of the human parkinsonian brain [29]. Pathological staining for alpha-synuclein was not observed in foxa2 mutant mice, but a small number of TH-expressing dopamine neurons that were highly immunoreactive for ubiquitin were seen in the SN of mutant but not in wild-type C57BL/6 mice. Gliosis is an activation of astrocytes that often occurs in neurodegenerative disease [30]. Accompanying the loss of dopamine neurons in foxa2 mutants, there is an increase in the number of activated glia measured by expression of the glial fibrillary acidic protein (GFAP) in the substantia nigra pars compacta, in the substantia nigra reticulata, and other regions of the ventral midbrain (Figure S5A–S5D). In contrast to neuron loss, this activation occurs in a symmetric manner.

Discussion

Rigidity, tremor, reduced and slow movement with asymmetric behavioral features, and dopamine neuron loss are characteristically found in Parkinson patients [31]. The age-dependent motor defects and the late loss of dopamine neurons seen in foxa2 mice are similar to symptoms of Parkinson disease. In mammalian cells, survival is controlled by phosphorylation of FOXO proteins that respond to oxidative stress for ubiquitin were seen in the SN of mutant but not in wild-type C57BL/6 mice. Gliosis is an activation of astrocytes that often occurs in neurodegenerative disease [30]. Accompanying the loss of dopamine neurons in foxa2 mutants, there is an increase in the number of activated glia measured by expression of the glial fibrillary acidic protein (GFAP) in the substantia nigra pars compacta, in the substantia nigra reticulata, and other regions of the ventral midbrain (Figure S5A–S5D). In contrast to neuron loss, this activation occurs in a symmetric manner.

This spontaneous model is important because it will allow us to understand the dynamics of dopamine neuron degeneration. In the SN of Parkinson patients, the RALDH1-expressing ventral dopamine neurons are particularly vulnerable to the etiology of Parkinson disease [47]. An initial asymmetric motor deficit, an asymmetric dopamine loss measured by positron emission tomography (PET) scanning, and dopamine neuron pathology are characteristic features of Parkinson disease [31,48]. Most studies on whole animals that address Parkinson disease use specific toxins to kill dopamine neurons [49]. Toxins are valuable, and they can specifically target the ventral tier neurons at risk in Parkinson disease, but they inadequately address the progressive nature of the disease and the increased sensitivity with age [50]. foxa2K animals give us access to the normal mechanisms that initiate and sustain loss of neurons in the SN. There would be great clinical benefit if the progression of disease could be slowed when patients are first diagnosed.

We present data that address the related questions: how to make dopamine neurons efficiently in the laboratory and how to support their survival in the brain. We present a precise map of the distinct origin of dopamine neurons that is fundamental to solving major current problems in cell therapies for Parkinson disease, the identity of dopamine neurons in the graft. However, our data have a significance that goes beyond the technology of cell therapy. Stem cell biology is often criticized for the slow transition to explicit clinical benefit. The work presented here illustrates how a developmental approach that is implicit in stem cell biology leads to a focus on the mechanisms that control the origin and survival of the cell of interest. In the case of dopamine neurons, our data suggest a central role for the PI3K/Akt/Fox survival pathway in defining the origin of dopamine neurons and developing an effective response to Parkinson disease.

Materials and Methods

Immunohistochemistry and histology. Timed pregnant CD-1 mice (Charles River Laboratories) were obtained and embryos were manually dissected and fixed in 4% paraformaldehyde (PFA) in PBS, transferred to 20% sucrose overnight, and embedded in O.C.T compound (Tissue Tek, Sakura). 12-μm sections were cut on a Microm MH 500 OM cryostat and processed for immunohistochemistry. Sections stained with the anti-SHH antibody were treated with 50 mM NH4Cl for 20 min at room temperature and washed twice with PBS prior to incubation with the primary antibody.

Adult mice were anesthetized with pentobarbital sodium (Nembutal) and perfused with 20 ml of PBS followed by 40 ml of 4% PFA. Brains were dissected out, transferred to 20% sucrose overnight, and 40-μm sections were cut on a cryostat and maintained as floating sections in PBS. Immunostaining was visualized on a confocal microscope. All animal work was carried out in accordance with National Institute of Neurological Disorders and Stroke (NINDS) Animal Studies Protocols 1204–05, 1205–05, and 1247–05, as approved by the NINDS Animal Care and Use Committee. Cell
counts were performed on every sixth section within the midbrain, directly at the microscope. In cases in which cell density made it difficult to easily discern individual cells, cell bodies stained for TH, RALDH1, and GFAP were matched to their DAPI-labeled nuclei. To estimate the absolute number of midbrain dopamine neurons, we multiplied the total number of cells by six (sampling frequency) and made the Abercrombie correction. We repeated this for midbrain dopamine neurons, both in the SN and VTA, and determined the mean nuclear diameter to be 8.69 μm, which we used for the Abercrombie correction.

For histological analysis, 40-μm brain sections were washed in distilled water and mounted on slides. Sections were treated with cresyl violet for 5 min and then washed again in distilled water. Slides were then run through an ethanol series (50%, 70%, 90%, 95%, and 100%) and finally dipped in xylene for 2 min, before mounting in Permount.

Antibodies. For immunohistochemistry of mouse embryonic sections and dissociated cells, the following antibodies were used: β-galactosidase (1:500, rabbit monoclonal; Biogenes); BrdU (1:200, rat monoclonal; Assay); FOXA2, Isl1e1, LIM1/2, HNKX2.2, PAX7, and SHH (1:10, mouse monoclonal; Developmental Studies Hybridoma Bank, University of Iowa); FOXA2 (1:1,000, rabbit polyclonal; gift of Ariel Ruiz I Altaba); FOXA2 (1:50, goat polyclonal; Santa Cruz Biotechnology); GATA3 (1:200, mouse monoclonal; Santa Cruz Biotechnology); GFAP (1:500, rabbit polyclonal; DAKO); LIMX1b (1:500, guinea pig polyclonal; gift of T. Muller); nestin (1:500, rabbit polyclonal; McKay lab); NKX2.2 (1:1000, rabbit polyclonal; RHo); RALDH1 (1:100, rabbit polyclonal; gift of Greg Duester); RALDH1 (1:100, rabbit polyclonal; gift of M. German); PTX3 (1:400, rabbit polyclonal; gift of B. Sosa Pineda); NKX6.1 (1:1,000, rabbit polyclonal; gift of M. German); PTX3 (1:400, guinea pig polyclonal; gift of B. Sosa Pineda); HNKX6.1 (1:1,000, rabbit polyclonal; gift of M. German); NUtxA (1:100, rabbit polyclonal; gift of Greg Duester); PHOX2a (1:500, rabbit polyclonal; gift of F. Brunet); SOX1 and SOX2 (1:500, rabbit polyclonal; gift of R. Lovell-Badge); and tyrosine hydroxylase (1:500, mouse polyclonal; Pel-free).

Cell culture. E8.5 embryos (9–13 somites) were dissected from timed pregnant CD-1 mice (Charles River Laboratories). Embryos were incubated in N2 medium containing 5% amylase (Sigma) for 1 h at 37°C, to facilitate manual dissection. After removal of ectoderm and brain mesenchyme, embryonic midbrains were moved to amylase-free N2 medium. Pooled tissue was washed 5× in PBS and then digested in N2 medium containing 0.005% Trypsin-EDTA (Gibco) for 5 min at room temperature. Dissociated mesencephalic cells were added to N2 medium containing Trypsin Inhibitor (Sigma), 100 ng/ml FGF2, 100 ng/ml FGF8, and 500 ng/ml Shh (R&D Systems). Three embryo equivalents of mesencephalic cells were plated per well in a 24-well plate (Costar), coated with poly-L-ornithine (Sigma) and fibronectin (R&D Systems). Upon cell attachment, the medium was changed to N2 medium (without trypsin inhibitor) containing 100 ng/ml FGF8 and Shh. Cells were fixed with 4% PFA after 4 d of expansion. To assay cell proliferation, cells were treated with 10 μM BrdU (Roche) for 1 h prior to fixation. Four independent experiments were performed in which each condition was analyzed in duplicate wells.

CNS explants were dissected from E8.5 foxa2+/− embryos and wild-type embryos. Explants were plated directly in N2 medium containing 100 ng/ml FGF8 and 500 ng/ml shh, as described above. After 4 d in culture with mitogens, the explants were grown for four additional days in the absence of exogenous growth factors to promote differentiation. After 4 d of differentiation, the explants were fixed with 4% PFA.

For overexpression of foxa2, E10.5 embryonic midbrain cells were harvested and plated in a manner identical to the E8.5 experiments, above, with the exception that two embryo equivalents of mesencephalic cells were plated per well in a 24-well plate (Costar). Cells were transfected, using the lipofectamine reagent (Invitrogen), with a CMV-foxa2 vector (generous gift of R.J. Matusik) and with a GFP expression vector (gift of T. Misteli, National Cancer Institute).

The mouse embryonic stem cell line, F4, which expresses foxa2 inducibly by the control of doxycycline, will be described in greater detail elsewhere (A. Kuzmichev and R. D. G. McKay, unpublished data). Briefly, these cells were generated by homologous recombination into the HPRT locus using a vector modified from a previously described vector [51] in which the actt transgene was replaced by foxa2. We differentiated the mES cells to neurons using a method modified from a previously described protocol [52]. Approximately 50,000 F4 cells were plated per well of a fibronectin-coated, 24-well plate into N2 medium. The cells were differentiated for 25 d in N2, in the absence of exogenous growth factors. foxa2-expressing mES cells were induced using doxycycline from days 6–25 of culture (no induction for the first and last 5 d of the differentiation).

Behavior. Spontaneous motor activity was analyzed using the VersaMax Animal Activity Monitoring System (AccuScan Instruments). Animals were placed into the 16.5° by 16.5° chamber for 60 min where vertical and horizontal movements detected by infrared beams were analyzed by VersaMax software. When wild-type and mutant mice were compared, there was no simple difference in the time spent in the center of the chamber. For amphetamine-induced rotation, mice were injected with 2.5 mg/kg of amphetamine intraperitoneally, and clockwise and counterclockwise rotations were measured using the Rota-count 8 system (Columbus Instruments) for 60 min.

Supporting Information

Figure S1. Expression of Transcription Factors in Ventral Midbrain Progenitors

(A–H) Transverse sections through mouse mesencephalon at E9.5 (C) and (F), E10.5 (A), (B), (D), and (G), and E11.5 (E) and (H), stained with anti-LMX1b (green in [A], [C], and [E]; red in [D]), anti-PHOX2a (red in [A]; green in [B] and [F–H]), anti-NKKX2.2 (red in [B]), and anti-FOXA2a (red in [C], [E], and [F–H]; green in [D]) antibodies. Double-staining is indicated by yellow.

(A) LMX1b expression (dopamine precursors) occurs in a single domain in the ventral midline. PHOX2a+ cells (calmodulin neuron precursors) are observed in an immediately adjacent, nonoverlapping domain.

(B) NKKX2.2-expressing cells (V3 neural precursors) occupy the domain immediately dorsal to the motor neuron precursor domain. Some expression of NKKX2.2 can be observed in PHOX2a+expressing domain.

(C–E) Dopamine precursors express the floor plate marker, FOXA2, and are found in the medial part of this domain. LMbx5+ cells are found in the medial FOXA2+ domain.

(F–H) PHOX2a+ cells are found in the lateral parts of the FOXA2+ domain. Numerous PHOX2a+ precursors coexpress FOXA2 at E9.5 (F). As these cells migrate to the pial surface, they down-regulate FOXA2 (G) and (H).

Found at doi:10.1371/journal.pbio.0050325.sg001 (1.1 MB PDF).

Figure S2. Expression of mhx Genes in Relation to the Mesencephalic Floor Plate

Transverse sections through mouse mesencephalon at E9.5 (B), E10.5 (A) and (C), and E11.5 (D), stained with anti-FOXA2 (green in [A]; red in [B–D]), anti-NKKX2.2 (red in [A]), anti-NKKX6.1 (red in [B–D]) antibodies. The NKKX2.2-expressing cells in (A) flank the FOXA2+ floor plate in the midbrain, just as they do in the developing spinal cord.

Found at doi:10.1371/journal.pbio.0050325.sg002 (695 KB PDF).

Figure S3. Expression of Transcription Factors in Neurons of the Ventrolateral Fetal Midbrain

(A) GATA3 expression is observed in a subset of neurons differentiating in the NKKX6.1 domain E10.5.

(B) and (C) At E11.5, GATA3+ neurons are located immediately adjacent to the NKKX6.1+ motor neurons (B). Like GATA3, LIM1/2 is expressed in neurons immediately adjacent to the NKKX6.1+expressing motor neurons (C). In (B) and (C), NKKX6.1 expression is lost as progenitors differentiate to GATA3+ and LIM1/2+positive neurons. (D) At E12.5, motor neurons are identified by PHOX2a expression, and more lateral neurons by LIM1/2 expression. LIM1/2 expression at E12.5 expands dramatically, compared to E11.5 (C).

Found at doi:10.1371/journal.pbio.0050325.sg003 (461 KB PDF).

Figure S4. Gliosis in foxa2−/− Mice

(A) Widespread gliosis is seen in the ventral midbrain of foxa2−/− mice (TH; green; GFAP, red).

(B) Increased density of glia is easily seen in the red nucleus of wild-type (B) and foxa2−/− mice (C).

(D) Quantitation of glia in wild-type and foxa2−/− dopaminergic system throughout the VTA, the SN pars compacta and reticulata, and red nucleus.

Found at doi:10.1371/journal.pbio.0050325.sg004 (2.3 MB PDF).

Figure S5. Severe Asymmetric Loss of Nigral Dopamine Neurons in One Mouse

Coronal sections through the midbrain of a wild-type (A), (C), (E), (G), and (I) and a foxa2−/− (B), (D), (F), (H), and (J) mouse at the indicated level. There is an almost complete loss of nigral dopamine neurons on
the left side, whereas the right side is relatively intact, quantitated in (K).

Found at doi:10.1371/journal.pbio.0050325.sg005 (684 KB PDF).

**Video S1. foxa2 Mutant with Late-Onset Motor Phenotype**

This is a 20-mo-old foxa2 heterozygote in a C57BL/6 background that has developed the late-onset motor problems characterized, here. Note the asymmetric kinked posture and the labored gait in this mouse.

Found at doi:10.1371/journal.pbio.0050325.s001 (1.3 MB MPG).

**Acknowledgments**

We thank J-F. Brunet, G. Duister, T. Jessell, R. Lovell-Badge, T. Muller, C. Birchmeir, A. Ruiz I Altaba, and B. Sosa-Pineda for generously providing antibodies and to A. Grifin-Linde for advice concerning anti-SHH immunohistochemistry. We thank K. Hochedlinger, R. Jaenisch, O. Kozich, A. Kuzmichev, and R. Matusik for providing constructs and for help with preparing them. We are grateful to C. Tabin for his gift of shh-crogh mice and to J. Rossant for her gift of foxa2 mutant mice. We thank N. Hitt and the staff of the Porter Neuroscience Research Center Shared Animal Resource Facility for excellent animal care. WWC is a member of the NIH-University of Cambridge Scholars Program.

**Author contributions.** RK and WWC conceived and designed the experiments. RK and WWC performed the experiments. RBA contributed reagents/materials/analysis tools. RK and RDGM analyzed the data and wrote the paper.

**Funding.** This work was supported by the Intramural Research Program of the National Institute of Neurological Disorders and Stroke at the National Institutes of Health, and by additional support from the Michael J. Fox Foundation, the National Parkinson's Foundation, and the Tuchman Foundation. WWC was supported in part by the Howard Hughes Medical Institute. RBA was supported by the American Parkinson's Disease Association and the Dana Foundation.

**Competing interests.** The authors have declared that no competing interests exist.
Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 44: 601–607.

43. Wolfrum C, Besser D, Luca E, Stoffel M (2003) Insulin regulates the activity of forkhead transcription factor Hnf-3beta/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytoplasmic localization. Proc Natl Acad Sci USA 100: 11624–11629.

44. Neff F, Noelker C, Eggert K, Schlegel J (2002) Signaling pathways mediate the neuroprotective effects of GDNF. Ann N Y Acad Sci 973: 70–74.

45. Kim RH, Mak TW (2006). Tumours and tremors: how PTEN regulation underlies both. Br J Cancer 94: 620–624.

46. Fallon L, Belanger CM, Corera AT, Kontogiannea M, Regan-Klapisz E, et al. (2006). A regulated interaction with the UIM protein Eps15 implicates parkin in EGF receptor trafficking and PI(3)K-Akt signalling. Nat Cell Biol 8: 834–842.

47. Galter D, Buervenich S, Carmine A, Anvret M, Olson L (2003) ALDH1 mRNA: presence in human dopamine neurons and decreases in substantia nigra in Parkinson’s Disease and in the ventral tegmental area in schizophrenia. Neurobiol. Dis 14: 637–647.

48. Cheesman AL, Barker RA, Lewis SJ, Robbins TW, Owen AM, et al. (2005) Laterisation of striatal function: evidence from 18F-dopa PET in Parkinson’s Disease. J Neurol Neurosurg Psychiatry. 76: 1204–1210.

49. Bove J, Prou D, Perier C, Przedborski S (2005) Toxin-induced models of Parkinson’s Disease. NeuroRx 2: 484–94.

50. Murase S, McKay RD. (2006) A specific survival response in dopamine neurons at most risk in Parkinson’s disease. J Neurosci. 26: 9750–9760.

51. Hochedlinger K, Yamada Y, Beard C, Jaenisch R (2005) Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell 121: 465–477.

52. Ying QL, Stavridis M, Griffiths D, Li M, Smith A (2003) Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol 21: 183–186.