Engrailed genes are cell-autonomously required to prevent apoptosis in mesencephalic dopaminergic neurons

Lavinia Albéri, Paola Sgadò and Horst H. Simon*

Department of Neuroanatomy, Interdisciplinary Center of Neuroscience, University of Heidelberg, Im Neuenheimer Feld 307, 69120 Heidelberg, Germany

*Author for correspondence (e-mail: horst.simon@urz.uni-heidelberg.de)

Accepted 17 February 2004

Development 131, 3229-3236
Published by The Company of Biologists 2004
doi:10.1242/dev.01128

Summary

The neuropathological hallmark of Parkinson's disease is the loss of dopaminergic neurons in the substantia nigra pars compacta, presumably mediated by apoptosis. The homeobox transcription factors engrailed 1 and engrailed 2 are expressed by this neuronal population from early in development to adulthood. Despite a large mid-hindbrain deletion in double mutants null for both genes, mesencephalic dopaminergic (mDA) neurons are induced, become postmitotic and acquire their neurotransmitter phenotype. However, at birth, no mDA neurons are left. We show that the entire population of these neurons is lost by E14 in the mutant animals, earlier than in any other described genetic model system for Parkinson's disease. This disappearance is caused by apoptosis revealed by the presence of activated caspase 3 in the dying tyrosine hydroxylase-positive mutant cells. Furthermore, using in vitro cell mixing experiments and RNA interference on primary cell culture of ventral midbrain we were able to show that the demise of mDA neurons in the mutant mice is due to a cell-autonomously requirement of the engrailed genes and not a result of the missing mid-hindbrain tissue. Gene silencing in the postmitotic neurons by RNA interference activates caspase 3 and induces apoptosis in less than 24 hours. This rapid induction of cell death in mDA neurons suggests that the engrailed genes participate directly in the regulation of apoptosis, a proposed mechanism for Parkinson's disease.

Key words: Transcription factors, Ventral tegmentum, Neurodegenerative disease, Neuronal survival, Neuronal differentiation

Introduction

The pathological hallmark of Parkinson's disease (PD) is the degenerative loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc). Although controversial, apoptosis has been discussed as a mechanism leading to the death of nigral DA neurons in PD (Andersen, 2001; Hartmann and Hirsch, 2001; Olanow and Tatton, 1999; Vila and Przedborski, 2003). The slow and progressive degeneration of the nigral dopamine system is more compatible with apoptosis than with any other mode of cell death. Molecular support for this hypothesis is provided by studies that show TUNEL-positive DA neurons in SNc of postmortem brains of individuals with PD (Mochizuki et al., 1996; Tatton et al., 1998; Tompkins et al., 1997) and after treatment of rodents with sub-chronic doses of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Tatton and Kish, 1997), a commonly used model of the human condition. More intriguingly, pro-apoptotic genes are upregulated in nigral DA neurons of individuals with PD (Hartmann et al., 2001) and the proportion of neurons positive for activated caspase 3, a final effector of apoptosis (Green, 1998), is five times higher than in healthy individuals (Hartmann et al., 2000). This suggests that nigral DA neurons of individuals with PD are more susceptible to apoptosis than are their healthy counterparts. Our knowledge about molecules that are involved in PD is very limited; however, the existing evidence strongly suggests that apoptosis is the mechanism leading to the death of nigral DA neurons in individuals with PD. Thus, apoptosis may be a suitable marker to identify genes with a potential link to PD.

The engrailed (En) genes are involved in regionalisation during early embryogenesis (Hidalgo, 1996; Joyner, 1996), and later in the specification of certain neuronal populations (Lundell et al., 1996; Simon et al., 2001). In mammals, two homologues of En have been identified, *En1* and *En2*. They are both expressed by all mesencephalic DA neurons (mDA) from early in development into the adult (Simon et al., 2001). Homologous recombinant mutant mice null for *En1* and *En2* show a large deletion in the midbrain and anterior hindbrain (Liu and Joyner, 2001; Simon et al., 2001). Despite this deficiency, the mDA neurons are generated, become postmitotic and express tyrosine hydroxylase (*Th*), the rate-limiting enzyme of dopamine synthesis. However, soon thereafter, the cells disappear, and at P0 the entire mDA system is absent.

The large deletion of mid-hindbrain tissue in the mutant raises the question whether the surrounding neuroepithelium provides essential support or whether the En genes are cell-autonomously required for the survival of mDA neurons. We have addressed this issue using in vitro cell mixing experiments and RNA interference technologies. We show that the En genes are cell-autonomously required for the survival of mDA neurons and that the loss of En expression in mDA neurons

induces apoptosis with a time course of less than 24 hours. These findings may open the paths to novel molecular links to PD.

Materials and methods

BrdU labeling

BrdU (Sigma, Germany) was intraperitoneally administered four times a day from E9.5 until E12.5 at a concentration of 50 mg/kg body weight dissolved in sterile PBS. To expose the BrdU, the paraformaldehyde cell cultures were treated with DNAse (Roche Diagnostic, Germany), 0.05 mg/ml in PBS supplemented with 5 mM $MgCl_2$ and 1 mM $CaCl_2$, for 5-10 minutes. BrdU incorporation was then detected using a standard immunohistochemistry protocol.

Primary cell culture

All primary cell cultures were performed using E12.5 mouse embryos. En double embryos were distinguished from their littermate counterparts by their midbrain/hindbrain morphology (16), which was occasionally verified by PCR. The embryonic neural tubes were dissected carefully removing meninges, followed by isolation of the ventral midbrains. The tissue was then dissociated using accutase (PAA Laboratories, Germany). The preparation of laminin- (Sigma, Germany) coated, membrane vesicle-coated and 3-D collagen matrix cultures are all described elsewhere (Krieglstein et al., 1995; Wizenmann et al., 1993; Yee et al., 1999). The medium was DMEM-F12 supplemented with 10% horse serum (HS), 5% fetal calf serum (FCS), 33 mM glucose, 50 U/ml penicillin and 50 μg/ml streptomycin. The cells were seeded at a concentration of 150,000 per well or coverslip and incubated from 1 hour to 5 days. In the cell mixing experiment, cell numbers of mutant and wild type were each 75,000 cells. For the RNA interference, we used always serum-free medium – DMEM-F12 medium containing 1×N2 supplement (Gibco), 33 mM glucose, 50 U/ml penicillin and 50 μg/ml streptomycin.

Immunohistochemistry

Cultured cells and all tissues were fixed with 4% paraformaldehyde in 100 mM phosphate buffer (pH=7.4). All immunostaining was performed as described (Simon et al., 2001) using rabbit and sheep anti-TH antibodies (AB152 and AB1542 Chemicon, Germany) at 1:1000, rabbit anti-activated caspase 3 (Catalog #9661 Cell Signaling, USA) at 1:500, rabbit anti-Pbx1/2/3 (sc888 Santa Cruz, USA) at 1:2000, mouse monoclonal anti-Engrailed 4g11 (Developmental Studies Hybridoma Bank) pure supernatant, and mouse monoclonal anti-BrdU (Catalog #1170376 Roche Diagnostic, Germany) at 1:50. Biotinylated or directly coupled species-specific antibodies were all obtained from Jackson Immuno Research, USA. The antibodies or the streptavidin were conjugated with Cy2, Cy3, Cy5 or horseradish peroxidase. After immunostaining, tissue and cultured cells were counterstained with DAPI (Catalog #236276 Roche, Diagnostic).

siRNA design and transfection

The siRNA duplexes were designed as described elsewhere (Elbashir et al., 2001b). In brief, the position of the 21 nucleotide siRNA duplexes were chosen at least 150 nucleotides 3′ to the first ATG of the coding region. The sense and antisense of each duplex were complementary at 19 nucleotides and had a two-nucleotide overhang at the 3′ terminus. We used the following RNA nucleotides for the experiments: *En1*a (NM_010133), CAUCCUAAGGCCCGAUUUCTT (sense) and GAAAUCGGGCCUUAGGAUGTT (antisense); *En1*b, GUUCCCGGAACACAACCCUTT (sense) and AGGGUUGUGUUCCGGGAACTT (antisense); *lamin A/C* (NM_019390), GCAGCUUCAGGAUGAGAUGTT (sense) and CAUCUCAUCCUGAAGCUGC (antisense); *Pbx1* (AF020196) CAGUUUUGAGUAUUCGGGGTT (sense) and CCCCGAAUACUCAAAACUGTT (antisense); and the randomly generated Scramble I Duplex (Dpharmacon, USA), CAGTCGCGTTTGCGACTGG (sense) and CCAGTCGCAAACGCGACTG (antisense). All RNA duplexes were purchased from MWG-Biotech, Germany. Three to five days after the dissociation, RNA oligo transfection was performed using Transmessenger transfection reagent (Qiagen, Germany). siRNA (0.1-0.3 μg per well) was condensed with 0.6 μl Enhancer R in 50 μl Transmessenger buffer, and complexed with 1.5 μl of Transmessenger reagent. The transfection complex was diluted in 500 μl DMEM/F12 1×N2 supplement, then added to the cells, 2 hours post-transfection the medium was replaced with fresh complete medium as described above.

Results

Time course and mechanism of cell death

First, we determined the ontogenetic time course for the disappearance of mDA neurons in En double mutant embryos. DA neurons in the midbrain are one of the first neuronal populations that become postmitotic (Altman and Bayer, 1981) and TH, as a marker for their neurotransmitter phenotype, is detectable as early as E10.5 (Di Porzio et al., 1990). At E12, the postmitotic TH-positive cells are found, as a cluster, in the ventral aspect of the mesencephalic flexure in wild-type and mutant embryos (Fig. 1A,A′). A notable feature of the mutants is the smaller size of the TH domain and a lack of axonal outgrowth in the direction of the basal telencephalon. Forty-eight hours later (E14), no DA neurons are present in the mutant midbrain, whereas the wild-type cells have continued with differentiation (Fig. 1B,B′). The progressive disappearance is closely correlated to the emergence of the En1 expression. At E12, only a small proportion of the wild-type mDA neurons expresses En1 (Fig. 1C-E), as demonstrated by the detection of the *En1/taulacZ* reporter (Saueressig et al., 1999). This is the same age at which mDA neurons are still detectable in the mutant embryos. Later at E14, when almost all wild-type mDA neurons express En1, the mutant cells are gone (Fig. 1F-H), suggesting that between E12 and E14 the En requirement for the survival of the cells has set in.

We have previously shown that re-specification of these neurons can be excluded (Simon et al., 2001). Therefore, we investigated the ventral midbrain of mutant embryos for evidence of cell death. Three modes of neuronal cell death are currently described; autophagic, apoptotic and necrotic (Pettmann and Henderson, 1998). Each mode can be distinguished by typical morphological and/or molecular criteria (Lockshin and Zakeri, 2002). At E13, 1 day before all mDA neurons are lost in the En double mutant embryos, the number of TH-positive cells in the ventral midbrain is strongly reduced, and depending on the individual specimens 10% to 30% of the left over TH-positive cells have an atypical rounded cell body with no processes and show signs of apoptosis – activation of the cysteine protease caspase 3, nuclear condensation and DNA fragmentation (Fig. 1I-K). By contrast, mDA neurons of the littermate controls do not exhibit any markers of cell death (data not shown).

Engrailed and axonal outgrowth

The death of mDA neurons in the En double mutant embryo might be related to the absence of axonal outgrowth, as the cells may lack essential molecular support from their innervation target. Involvement of En in axonal outgrowth has

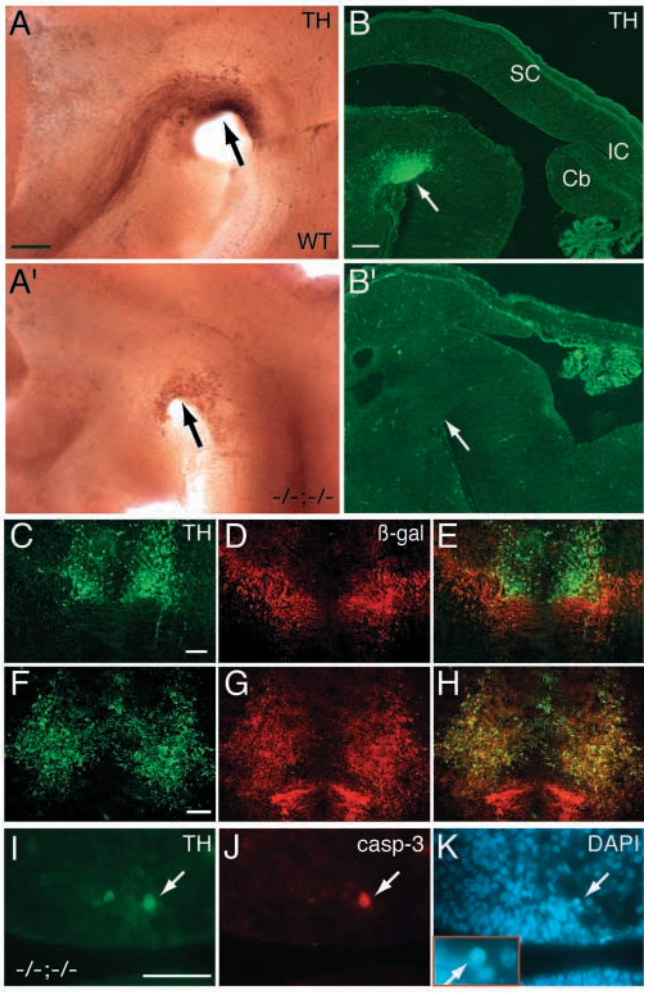

Fig. 1. Loss of midbrain dopaminergic neurons in engrailed double mutant embryo by apoptosis. (A) E12 whole-mount preparation of isolated neural tube. TH-positive neurons are located in the mesencephalic flexure (arrow) of wild-type (A) and mutant (A′) embryo. The TH domain in the mutant is smaller than the wild type and there are no axons heading in rostral direction. (B) Midsagittal sections of E14 embryos. In the wild type (B), mDA neurons have continued to differentiate and start to form the SNC and VTA (arrow). In the mutant embryos (B′), no TH-positive cells are detectable in the ventral midbrain. Additionally, the anlage for the cerebellum (Cb), inferior colliculus (IC) and superior colliculus (SC) are absent. (C-E) Transverse sections of E12 En1^{+}/tlz;*En2*$^{-/-}$ ventral midbrain immunostained against TH (green) and the En1 reporter, β-gal (red). (E) Merged image of C and D. The majority of TH-positive cells do not express En1. (F-H) 48 hours later at E14 at the same level, almost all TH (green)-positive cells express the En1 reporter (red). (H) Merged image of F and G. (I-K) Coronal section of ventral midbrain of E13.5 *En1*$^{-/-}$;*En2*$^{-/-}$ embryo. A rounded TH-positive cell body is detectable (I, arrow). This cell is positive for activated caspase 3 (J, arrow) and exhibits a condensed and fragmented nucleus (K, arrow) revealed by DAPI staining. (K, inset) Magnification of the pyknotic nucleus (arrow). A,B rostral is towards the right; C-K is dorsal towards the top. Scale bars: 200 μm in A,B; 50 μm in C-K.

been demonstrated in mammals and insects (Marie et al., 2002; Saueressig et al., 1999). To address this possibility, we placed dissociated E12 ventral midbrain into a three-dimensional collagen matrix with or without an explant of basal telencephalon of the same age. Alternatively, we used glass coverslips coated with laminin or membrane vesicles derived from E12 midbrain (Fig. 2A-C). During the first 24 hours in culture, the mutant mDA neurons differentiated normally. They acquired a spindle form shape and extended neurites. The mean length of their processes matched exactly that seen in the littermate controls (mixture of *En1*$^{+/-}$;*En2*$^{-/-}$ and *En2*$^{-/-}$) (Fig. 2D). However, cell death was only postponed, and after a further 48 hours of incubation almost all of the mutant DA neurons disappeared, regardless of culture condition (Fig. 2E, for survival of control cells see Fig. 3). The majority of the cells died between 24 and 48 hours after dissociation, showing the same signs of apoptotic cell death we observed on tissue sections, activation of caspase 3 and appearance of pyknotic nuclei (Fig. 2F-I). In the littermate control cultures, the loss of TH-positive cells after 72 hours in culture is not higher than 10% to 15% (see Fig. 3G for control experiment). These findings demonstrate that the lack of DA axonal outgrowth in En double mutants is not the reason for the cell death and that the mutant mDA neurons are viable and differentiate normally, until a requirement for the En genes sets in, which then becomes essential for their survival.

Cell-autonomous requirement of engrailed genes

The most pronounced morphological defect of the En double mutant embryos is the lack of cerebellum, inferior and superior colliculus, and ventral parts of the mesencephalon (Simon et al., 2001). These morphological deficiencies can be seen already at E9 (Liu and Joyner, 2001). At this age, isthmus and the surrounding neural tube are already absent. It is, therefore, possible that this missing tissue provides essential support for mDA neurons of a yet undefined molecular nature. In order to differentiate whether the death of mDA neurons in mutant mice reflects a cell-autonomous requirement of the En genes or is caused by the lack of surrounding midbrain tissue, we performed cell-mixing experiments in vitro. By mixing dissociated E12 mutant midbrain with wild-type cells of the same origin, we exposed mDA neurons of mutant and wild-type genotype to precisely the same environments. In such mixing experiments, a cell-autonomous function of the *engrailed* genes should be revealed by death of the mutant mDA neurons, and a requirement of the surrounding mid-hindbrain tissue by their survival. To distinguish cells from mutant animals, we injected pregnant dams with the nuclear marker BrdU (four times daily from E9 to E12). This regime resulted in a labeling efficiency of greater than 90% of all mDA neurons (Fig. 3A-C). BrdU injections had no significant effect on the survival rate of mDA neurons derived from control littermates for the duration of our in vitro experiments (Fig. 3G). By contrast, the mutant mDA neurons started to disappear after an initial delay of 24 hours (Fig. 2E), and were lost 72 hours after dissociation regardless of their placement in an isogenetic or heterogenetic in vitro environment (Fig. 3A-G). These experiments strongly suggest that *En1* and *En2* are cell-autonomously required for the survival of mDA neurons.

Rapid induction of cell death after ablation of En

Despite all signs of viability that the En double mutant mDA neurons showed during the first 24 hours in culture (Fig. 2), it was possible that the cells were already committed to cell

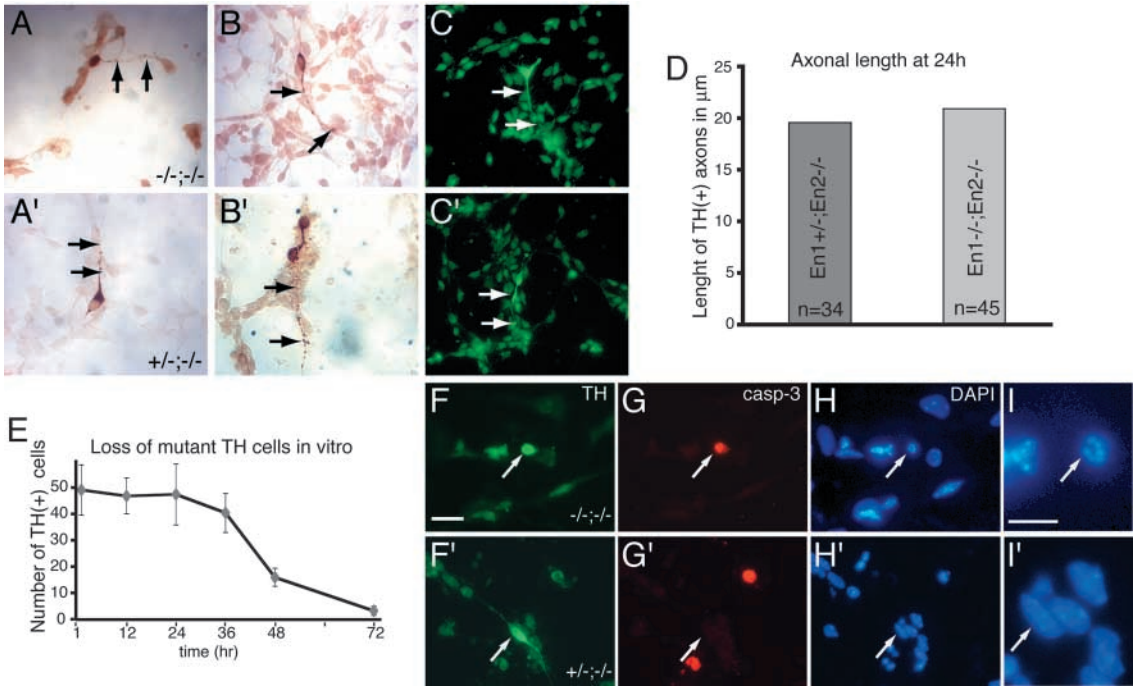

Fig. 2. Axonal outgrowth and survival in vitro. Dissociated E12 ventral midbrain of control (mixture of *En1*$^{+/-}$*;En2*$^{-/-}$ or *En2*$^{-/-}$) and En double mutant in 3D collagen matrix (A), on coverslips coated with laminin (B) and a membrane carpet derived from wild-type E12 midbrain (C) after 1 day in culture stained against TH. For the first 24 hours in vitro, dissociated En double mutant mDA neurons are viable and extend axonal processes. (D) The mean length of the TH-positive processes was the same for mutant and littermate controls. The difference was not statistically significant (Student's *t*-test, *P*=0.35). Error bars are not shown, as axonal outgrowth varied between 0.3 and 78 μm. *n*=number of cells measured out of four independent experiments for each genotype. (E) Average number of TH-positive En double mutant cells counted at several time points. After dissociation, disappearance of mDA neurons was arrested for 24 hours. Thereafter, the mutant cells follow their in vivo counterparts such that almost no TH-positive cells are left 72 hours post dissociation. *n*=5 independent cell culture experiments for each time point. Error bars indicate s.d. (F-I) Cell culture at about 48 hours post dissociation stained against TH (F,F′, green) and activated caspase 3 (G,G′, red). Each sample was counterstained with DAPI (H-I′) to identify cell nucleus. Mutant mDA neurons (arrows) retract their processes, round up and are positive for activated caspase 3. An additional sign for apoptosis is the pyknotic nuclei. (F′-I′) By contrast, TH-positive cells derived from littermate controls possess elongated processes and show no signs of apoptosis (arrows). (I,I′) Magnification of H,H′. Scale bars: 20 μm for A-C,F-H; 10 μm in I.

death when the tissue was dissociated. Thus, the selective loss of mutant mDA neurons during the cell mixing experiments (Fig. 3) might only reflect this commitment. To address this possibility, we silenced the En expression in mDA neurons by RNA interference (RNAi). Recent experiments have shown that the application of small interfering RNA duplexes (siRNA), 21-22 nucleotides in length, leads to sequence-specific mRNA degradation in mammalian cell lines (Elbashir et al., 2001a). We performed all RNAi experiments in primary cell culture of ventral midbrain derived from homozygous null mutants for the *En2* (*En2*$^{-/-}$), in order to increase the silencing efficiency. This strategy was possible, as rescue experiments have shown that *En2* can functionally replace *En1* (Hanks et al., 1995) and *En2*$^{-/-}$ mutants show no phenotype with respect to the mDA neurons (Simon et al., 2001). After isolation and dissociation of the E12 ventral midbrain, cells were left growing in vitro for 72-96 hours and then transfected with siRNA duplexes. At this point, mDA neurons exhibited an elaborate network of neurites and they all expressed *En1*. Ninety-six hours after transfection with two different *En1*-specific RNA duplexes, the number of mDA neurons was reduced by about 25% compared with the mock-transfected control cultures (Fig. 4A). Furthermore, we analyzed the si*En1* transfected cultures at successive post-transfection time points (Fig. 4B). The first En1-negative DA neurons were detectable 12 hours after transfection, but the numbers of TH-positive cells remained unchanged. We saw the first loss of DA neurons at around 24 hours. The proportion of En1-negative cells increased further, reaching a peak at around 48 hours post-transfection. The number of TH-positive cells gradually declined until 96 hours and stabilised at around 75%. At this stage, En1-negative cells were rarely seen (Fig. 4B). The mode of cell death appeared to be the same as in the En double mutant embryos. Dying En1-negative mDA neurons had rounded cell bodies containing condensed, fragmented nuclei and showed a complete retraction of their neurites and the presence of activated caspase 3 (Fig. 4C-J). The amount of cell loss after 96 hours, when no further cell death was observed, suggested a transfection efficiency of 25%; however, the proportion of En1-negative cells was never higher than 13% at any time point of the experiment. There are two possible explanations for this phenomenon: (1) the turnover rate of the En protein, or the onset of RNA degradation, may be different in individual cells; or (2) suboptimal silencing, where En1 is still detectable by immunohistochemistry, is sufficient to induce cell death. The

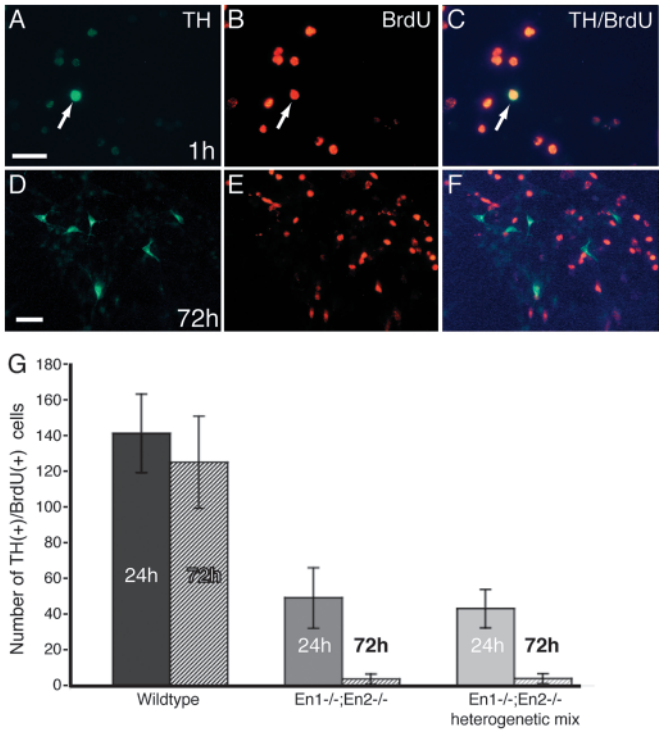

Fig. 3. Cell-mixing experiment. (A-C) After multiple intraperitoneal BrdU injections, almost all cells derived from En double mutants were positive for BrdU (B) when attached to a coverslip after 1 hour. (C) Example of double-labelled TH-positive neurons (arrow) (TH, green; BrdU, red). (D-F) In the mixture of En double mutant cells and control, no TH-positive, BrdU-labelled cells were present after 3 days. (G) Number of TH-positive cells after 24 hours and 72 hours in vitro: control (wild type), mixture of heterozygote ($En1^{+/-};En2^{-/-}$) and homozygote ($En2^{-/-}$). In controls, the number of mDA neurons decreased only slightly between 24 hours and 72 hours. Regardless of culture conditions, the numbers of TH-positive cells derived from engrailed double mutants decreased almost to zero after 72 hours from a baseline of an average of 45 TH-positive mutant cells at 24 hours. n=12 for each bar (Student's t-test, P<0.001). Error bars indicate s.d. Scale bars: 20 μm.

later possibility is unlikely, because we never observed an apoptotic cell that was En1 positive.

To demonstrate that the induction of apoptosis in mDA neurons was specific to the transfection with siRNA duplexes directed against *En1*, we used several others as controls. A set of scrambled siRNA, a set directed against *Lamin A/C* (Elbashir et al., 2001a), and siRNAs targeting *Pbx1*, another homeobox transcription factor expressed by all mDA neurons (P.S. and H.H.S., unpublished). None of these RNA duplexes caused a reduction in the number of mDA neurons (Fig. 4A). Furthermore, the silencing efficiency and time course for Pbx1 was the same as for En1. However, after the application of *Pbx1*-specific siRNA duplexes, the mDA neurons maintained their neurites and there were no signs of apoptosis detectable (Fig. 4K-N).

Discussion

The molecular mechanisms that are responsible for the maintenance and survival of mDA neurons are just beginning to be unraveled. In previous studies, we had demonstrated that the survival of mDA neurons depend on the expression of the En genes. Here, we extended these studies addressing the issue of whether this loss of mDA neurons in the mutant mice is caused by a cell-autonomous requirement for En or by the large mid-hindbrain deletion. Our cell mixing experiments in combination with RNA interference demonstrated that the death of mutant mDA neuron is caused by a cell intrinsic requirement and not a deficit in the embryonic environment. Furthermore, the death of the cells is caused by apoptosis, which is rapidly induced if En is silenced in mDA neurons.

With the exception of the apoptotic cell death, the most pronounced feature of mDA neurons, which are deficient of the En genes, is the lack of axonal outgrowth. This let us to speculate that the En genes cell-autonomously regulate this process. Previous studies had shown that axonal pathway finding is interrupted in a subpopulation of interneurons if *En1* is not present (Saueressig et al., 1999). In the case of mDA neurons, the in vivo phenotype of the En double mutants (Fig. 1A) suggested a general inability of the mutant neurons to extend processes. However, this is unlikely, as En double mutant mDA neurons from E12 embryos grow out neurites for the first 24 hours, if dissociated and placed into culture. Length and number of these neurites are indistinguishable from littermate control cells. More likely, the deficit in the embryonic environment, deletion of the neural tube rostral and caudal to the isthmus, underlies the inability of the mDA neurons to extend an axon in vivo. However, there is an alternative explanation for this observation. The dissociation by enzymatic digestion may cause the reorganisation of cell surface proteins leading to a delay in cell death. Axonal outgrowth, which is mostly dependent on the assembly of intracellular cytoskeletal components, is probably not affected by the digestion. Therefore under in vitro conditions, axonal outgrowth is initiated and continues for the first 24 hours until apoptosis sets in. Thus, the failing of axonal outgrowth in vivo may be just a manifestation of the imminent programmed cell death and not related to deficits in the environment of mDA neurons. This notion also fits the time course of the cell death after silencing of *En1* by the application of siRNA duplexes (Fig. 4A). A way to test this hypothesis would be the application of inhibitors of RNA transcription or protein translation when the mutant cells are placed into culture or when the siRNA duplexes are administered.

All experiments were performed in an $En2^{-/-}$ background in order to increase the number of mutant embryos per litter and to restrict the silencing by RNAi to just one gene, which was essential because the transfection efficiency, if measured by the rate of induced cell death, was ~25%. For this reason, we cannot directly conclude that En2 is also required for the prevention of apoptosis in mDA neurons. However, there is strong evidence that this must be the case. In homozygote mutants null for *En1* or *En2*, alterations in the midbrain dopaminergic system are minimal. The complete loss of mDA neurons only occurs when all four alleles are deleted, strongly suggesting the two genes are functionally interchangeable. The same can be said for the development of the entire CNS. The replacement of *En1* by *En2* leads to the rescue of the *En1* phenotype with virtually no brain defect (Hanks et al., 1995). However, the two genes are not completely identical. The limb abnormalities of *En1* mutants are neither suppressed, placing

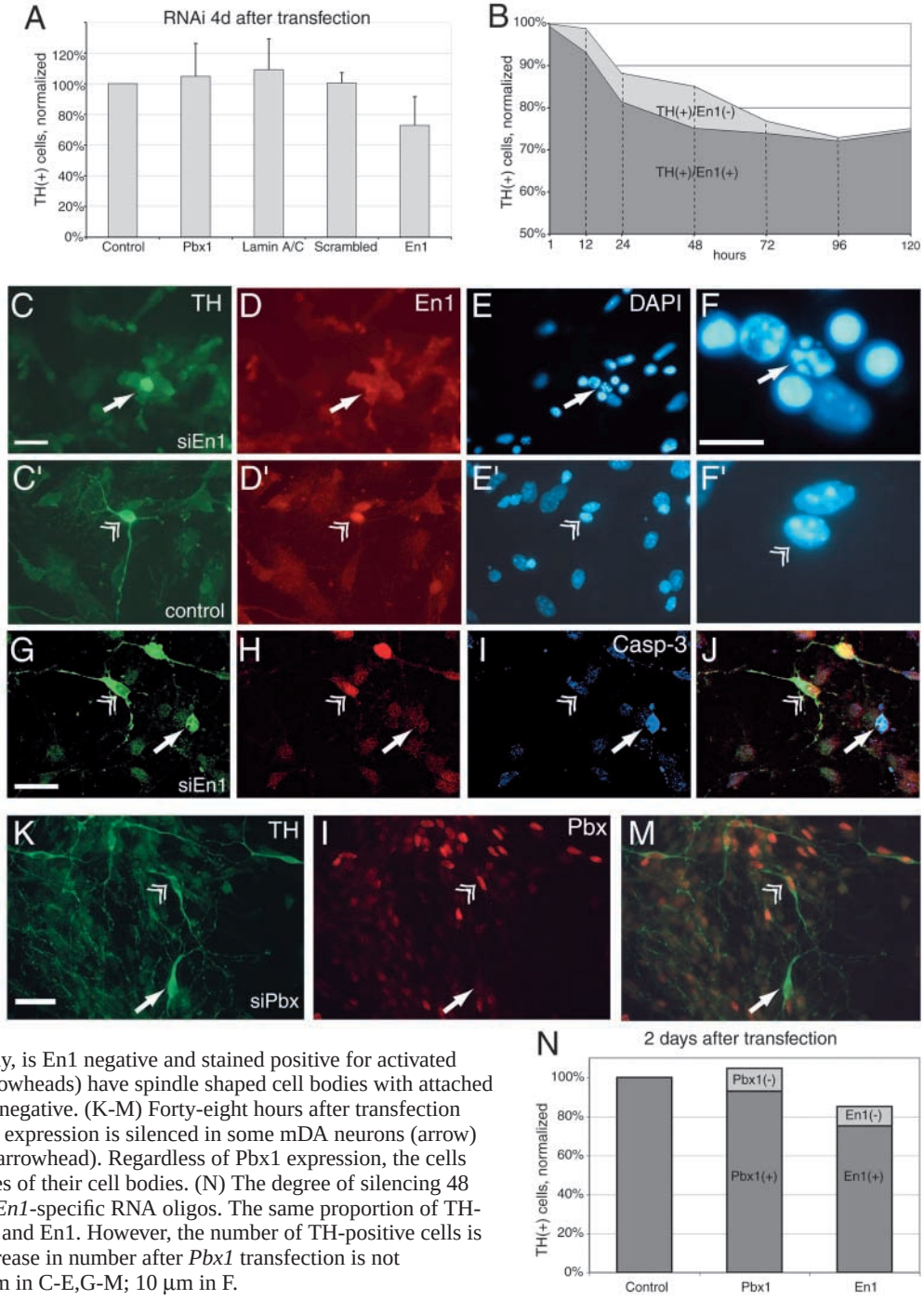

Fig. 4. RNA interference on primary cell cultures derived from *En2*$^{-/-}$ ventral midbrain. (A) Numerical chart of RNA interference normalised against mock-transfected control. Only the transfection with En1-specific siRNA oligo duplexes reduced the numbers of TH-positive cells after 96 hours. *n*=10 independent experiments for each transfection (Student's *t*-test, *P*=0.001 for mock control versus En1 transfection). Error bars indicate s.d. (B) Time course for the loss of mDA neurons after transfection with En1 oligos normalised against mock-transfected controls. Twelve hours after transfection, there were no changes in the numbers of TH-positive neurons; however, a significant proportion of En1-negative cells were present. Number of TH-positive cells gradually decreased until 96 hours when the amount of DA neurons stabilised. Maximum amount of TH-positive and En1-negative cells was detectable at 48 hours. (C-F) Immunohistochemistry on En1 transfected cultures after 48 hours. The transfected TH-positive cells (arrow) are round, have no processes, are En1 negative (red) and possess a fragmented, pyknotic nucleus (DAPI). (C′-F′) The mock-transfected control cells (double arrowhead) maintained their neurites and are En1 positive. (F,F′) Magnification of DAPI staining in E,E′. (G-J) Confocal image of En1 transfected culture. Three TH-positive neurons are present in this field; one (arrow) has a rounded cell body, is En1 negative and stained positive for activated caspase 3 (blue). Two others (double arrowheads) have spindle shaped cell bodies with attached neurites, are En1 positive and caspase 3 negative. (K-M) Forty-eight hours after transfection with *Pbx1*-specific RNA duplexes, Pbx1 expression is silenced in some mDA neurons (arrow) but is preserved in the majority (double arrowhead). Regardless of Pbx1 expression, the cells maintained their processes and the shapes of their cell bodies. (N) The degree of silencing 48 hours after transfection with *Pbx1*- and *En1*-specific RNA oligos. The same proportion of TH-positive cells, 12%, is negative for Pbx1 and En1. However, the number of TH-positive cells is reduced only after En1 transfection (increase in number after *Pbx1* transfection is not statistically significant). Scale bar: 20 μm in C-E,G-M; 10 μm in F.

En2 into the *En1* locus, nor in a different genetic background (Bilovocky et al., 2003). Furthermore, *En1*$^{+/-}$;*En2*$^{-/-}$ mutant mice are viable and fertile and there is no defect in the midbrain dopaminergic system observable at P0. This is in contrast to the opposite genotype, *En1*$^{-/-}$;*En2*$^{+/-}$; here, the cluster of mDA neurons is reduced to a small domain (Simon et al., 2001). Thus, with respect to SN and VTA one *En1* allele is sufficient to produce a P0 wild-type phenotype, but one *En2* allele is not.

Several null mutations of transcription factors lead to the prenatal loss of mDA neurons. In homologous recombinant mutant mice for *Nurr1* (*Nr4a2* – Mouse Genome Informatics) (Wang et al., 2003; Zetterström et al., 1997), mDA neurons fail to express their neurotransmitter phenotype and begin to disappear at E15 (Wallen et al., 1999). The *aphakia* mice, which have a spontaneous null mutation of *Pitx3*, (Semina et al., 2000) exhibit a specific loss of nigral DA neurons (Nunes et al., 2003; Smidt et al., 2004; Van den Munckhof et al., 2003). In *Lmx1b* null mutants, the entire population of mDA neurons is lost by E17 (Smidt et al., 2000). Furthermore, the null mutation for the trophic factor TGFα (Blum, 1998) leads to a reduction of DA neurons in the SNc. The differential time

courses and the different degrees of neuronal loss suggest that the molecular bases for the reduction in each of the mutant strains are probably unrelated. In this context, the En double mutant phenotype is of particular interest as the mDA neurons disappear the earliest and the entire population of mDA neurons is affected, suggesting that the En genes exert fundamental control over a mechanism that assures the survival of these cells.

Apoptosis is the mechanism leading to the loss of mDA neurons in many genetic and experimental models of PD. Apoptotic profiles in the ventral midbrain were observed in null mutants for *Nurr1* (Saucedo-Cardenas et al., 1998; Wallen et al., 1999) and in *aphakia* mice (Van den Munckhof et al., 2003). Apoptotic cell death is induced in nigral dopaminergic neurons by axotomy of the median forebrain bundle (El-Khodor and Burke, 2002), striatal excitotoxic injury (Macaya et al., 1994) or by treatment with specific neurotoxins such as MPTP (Tatton and Kish, 1997) and 6-hydroxydopamine (He et al., 2000). Additionally, apoptosis plays a role in the regulation of the numbers of mDA neurons during the first 14 days after birth (Chun et al., 2002; Jackson-Lewis et al., 2000), probably reflecting a dependency of neurons on GDNF during this period (Burke et al., 1998; Granholm et al., 2000). Apoptosis in mDA neurons is triggered in so many different experimental paradigms, during normal development and during the pathological degeneration of mDA neurons, it suggests that a common molecular pathway may be the cause. Our RNA interference experiments showed that the activation of caspase 3, and consequent cell death by apoptosis, occurs in some cells within 24 hours after the application of the RNA duplex and silencing of *En1*. This is a similar time scale as seen in 6-hydroxydopamine- and MPTP-induced degeneration of the nigrostriatal DA system (Jeon et al., 1995; Sundstrom et al., 1988; Zuch et al., 2000) or the induction of cell death in sympathetic neurons after withdrawal of NGF (Deckwerth and Johnson, 1993) or activation of the low-affinity NGF receptor, p75 (Freidin, 2001). Each of these experimental models leads to the induction of apoptosis via the mitochondrial (intrinsic) pathway (Vila and Przedborski, 2003). The rapid induction of apoptosis, when En1 is silenced in the mDA neurons by RNAi, make it plausible that the intrinsic pathway is also triggered.

The total loss of mDA neurons in En double mutant mice, as early as E14, and the speed with which apoptosis is induced in mDA neurons after silencing of En expression, suggests that an essential molecular mechanism is affected. It is possible that the degeneration of neurons seen in individuals with PD and the loss of cells in the En double mutants have a common molecular origin. In the En double mutant, large alterations in the level of gene expression downstream of *En1* and *En2* are probably the reason for the death of mDA neurons. During PD, the difference between pathological and healthy levels of gene expression is probably small. This may be the reason why most cases of PD cannot be traced back to a genetic mutation, despite the fact that twin studies suggest a substantial genetic contribution (Piccini et al., 1999). Inherently, human mutant studies are less successful if they try to identify small alteration in regulatory elements of a given gene. Recently, two mono-allelic point mutations 5′ to the coding region of *Nurr1* have been shown to be associated with PD. The mutation leads to a reduction of *Nurr1* expression that, in turn, seems to affect the level of TH expression (Le et al., 2003). In light of these studies, it is possible that a minor alteration in the expression level of one of the En genes or small changes in the promoter region of a downstream gene, which they regulate, leads to the slow degeneration of nigral DA neurons in PD.

This work was supported by a grant from the German Federal Secretary for Education and Research, BMBF Biofutur 98. We thank Martyn Goulding for the *En1/taulacZ*, and Alex Joyner and Wolfgang Wurst for the *En2* mutant mice. Furthermore, we thank Gabi Döderlein for technical support, and Richard Dyck and Jochen Röper for fruitful discussions and corrections of the manuscript.

References

Altman, J. and Bayer, S. A. (1981). Development of the brain stem in the rat. V. Thymidine-radiographic study of the time of origin of neurons in the midbrain tegmentum. *J. Comp. Neurol.* **198**, 677-716.

Andersen, J. K. (2001). Does neuronal loss in Parkinson's disease involve programmed cell death? *BioEssays* **23**, 640-646.

Bilovocky, N. A., Romito-DiGiacomo, R. R., Murcia, C. L., Maricich, S. M. and Herrup, K. (2003). Factors in the genetic background suppress the engrailed-1 cerebellar phenotype. *J. Neurosci.* **23**, 5105-5112.

Blum, M. (1998). A null mutation in TGF-alpha leads to a reduction in midbrain dopaminergic neurons in the substantia nigra. *Nat. Neurosci.* **1**, 374-377.

Burke, R. E., Antonelli, M. and Sulzer, D. (1998). Glial cell line-derived neurotrophic growth factor inhibits apoptotic death of postnatal substantia nigra dopamine neurons in primary culture. *J. Neurochem.* **71**, 517-525.

Chun, H. S., Yoo, M. S., DeGiorgio, L. A., Volpe, B. T., Peng, D., Baker, H., Peng, C. and Son, J. H. (2002). Marked dopaminergic cell loss subsequent to developmental, intranigral expression of glial cell line-derived neurotrophic factor. *Exp. Neurol.* **173**, 235-244.

Deckwerth, T. L. and Johnson, E. M., Jr (1993). Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. *J. Cell Biol.* **123**, 1207-1222.

Di Porzio, U., Zuddas, A., Cosenza-Murphy, D. B. and Barker, J. L. (1990). Early appearance of tyrosine hydroxylase immunoreactive cells in the mesencephalon of mouse embryos. *Int. J. Dev. Neurosci.* **8**, 523-532.

El-Khodor, B. F. and Burke, R. E. (2002). Medial forebrain bundle axotomy during development induces apoptosis in dopamine neurons of the substantia nigra and activation of caspases in their degenerating axons. *J. Comp. Neurol.* **452**, 65-79.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494-498.

Elbashir, S. M., Lendeckel, W. and Tuschl, T. (2001b). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**, 188-200.

Freidin, M. M. (2001). Antibody to the extracellular domain of the low affinity NGF receptor stimulates p75(NGFR)-mediated apoptosis in cultured sympathetic neurons. *J. Neurosci. Res.* **64**, 331-340.

Granholm, A. C., Reyland, M., Albeck, D., Sanders, L., Gerhardt, G., Hoernig, G., Shen, L., Westphal, H. and Hoffer, B. (2000). Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. *J. Neurosci.* **20**, 3182-3190.

Green, D. R. (1998). Apoptotic pathways: the roads to ruin. *Cell* **94**, 695-698.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B. and Joyner, A. L. (1995). Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. *Science* **269**, 679-682.

Hartmann, A. and Hirsch, E. C. (2001). Parkinson's disease. The apoptosis hypothesis revisited. *Adv. Neurol.* **86**, 143-153.

Hartmann, A., Hunot, S., Michel, P. P., Muriel, M. P., Vyas, S., Faucheux, B. A., Mouatt-Prigent, A., Turmel, H., Srinivasan, A., Ruberg, M. et al. (2000). Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. *Proc. Natl. Acad. Sci. USA* **97**, 2875-2880.

Hartmann, A., Michel, P. P., Troadec, J. D., Mouatt-Prigent, A., Faucheux, B. A., Ruberg, M., Agid, Y. and Hirsch, E. C. (2001). Is Bax a mitochondrial mediator in apoptotic death of dopaminergic neurons in Parkinson's disease? *J. Neurochem.* **76**, 1785-1793.

He, Y., Lee, T. and Leong, S. K. (2000). 6-Hydroxydopamine induced apoptosis of dopaminergic cells in the rat substantia nigra. *Brain Res.* **858**, 163-166.

Hidalgo, A. (1996). The roles of engrailed. *Trends Genet.* **12**, 1-4.
Jackson-Lewis, V., Vila, M., Djaldetti, R., Guegan, C., Liberatore, G., Liu, J., O'Malley, K. L., Burke, R. E. and Przedborski, S. (2000). Developmental cell death in dopaminergic neurons of the substantia nigra of mice. *J. Comp. Neurol.* **424**, 476-488.
Jeon, B. S., Jackson-Lewis, V. and Burke, R. E. (1995). 6-Hydroxydopamine lesion of the rat substantia nigra: time course and morphology of cell death. *Neurodegeneration* **4**, 131-137.
Joyner, A. L. (1996). Engrailed, Wnt and Pax genes regulate midbrain–hindbrain development. *Trends Genet.* **12**, 15-20.
Krieglstein, K., Suter-Crazzolara, C., Fischer, W. H. and Unsicker, K. (1995). TGF-beta superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. *EMBO J.* **14**, 736-742.
Le, W. D., Xu, P., Jankovic, J., Jiang, H., Appel, S. H., Smith, R. G. and Vassilatis, D. K. (2003). Mutations in NR4A2 associated with familial Parkinson disease. *Nat. Genet.* **33**, 85-89.
Liu, A. and Joyner, A. L. (2001). EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region. *Development* **128**, 181-191.
Lockshin, R. A. and Zakeri, Z. (2002). Caspase-independent cell deaths. *Curr. Opin. Cell Biol.* **14**, 727-733.
Lundell, M. J., Chu-LaGraff, Q., Doe, C. Q. and Hirsh, J. (1996). The engrailed and huckebein genes are essential for development of serotonin neurons in the Drosophila CNS. *Mol. Cell Neurosci.* **7**, 46-61.
Macaya, A., Munell, F., Gubits, R. M. and Burke, R. E. (1994). Apoptosis in substantia nigra following developmental striatal excitotoxic injury. *Proc. Natl. Acad. Sci. USA* **91**, 8117-8121.
Marie, B., Cruz-Orengo, L. and Blagburn, J. M. (2002). Persistent engrailed expression is required to determine sensory axon trajectory, branching, and target choice. *J. Neurosci.* **22**, 832-841.
Mochizuki, H., Goto, K., Mori, H. and Mizuno, Y. (1996). Histochemical detection of apoptosis in Parkinson's disease. *J. Neurol. Sci.* **137**, 120-123.
Nunes, I., Tovmasian, L. T., Silva, R. M., Burke, R. E. and Goff, S. P. (2003). Pitx3 is required for development of substantia nigra dopaminergic neurons. *Proc. Natl. Acad. Sci. USA* **100**, 4245-4250.
Olanow, C. W. and Tatton, W. G. (1999). Etiology and pathogenesis of Parkinson's disease. *Annu. Rev. Neurosci.* **22**, 123-144.
Pettmann, B. and Henderson, C. E. (1998). Neuronal cell death. *Neuron* **20**, 633-647.
Piccini, P., Burn, D. J., Ceravolo, R., Maraganore, D. and Brooks, D. J. (1999). The role of inheritance in sporadic Parkinson's disease: evidence from a longitudinal study of dopaminergic function in twins. *Ann. Neurol.* **45**, 577-582.
Saucedo-Cardenas, O., Quintana-Hau, J. D., Le, W. D., Smidt, M. P., Cox, J. J., de Mayo, F., Burbach, J. P. and Conneely, O. M. (1998). Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc. Natl. Acad. Sci. USA* **95**, 4013-4018.
Saueressig, H., Burrill, J. and Goulding, M. (1999). Engrailed-1 and netrin-1 regulate axon pathfinding by association interneurons that project to motor neurons. *Development* **126**, 4201-4212.
Semina, E. V., Murray, J. C., Reiter, R., Hrstka, R. F. and Graw, J. (2000). Deletion in the promoter region and altered expression of Pitx3 homeobox gene in aphakia mice. *Hum. Mol. Genet.* **9**, 1575-1585.
Simon, H. H., Saueressig, H., Wurst, W., Goulding, M. D. and O'Leary, D. D. (2001). Fate of midbrain dopaminergic neurons controlled by the Engrailed genes. *J. Neurosci.* **21**, 3126-3134.
Smidt, M. P., Asbreuk, C. H., Cox, J. J., Chen, H., Johnson, R. L. and Burbach, J. P. (2000). A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat. Neurosci.* **3**, 337-341.
Smidt, M. P., Smits, S. M., Bouwmeester, H., Hamers, F. P., Van Der Linden, A. J., Hellemons, A. J., Graw, J. and Burbach, J. P. (2004). Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. *Development* **131**, 1145-1155.
Sundstrom, E., Luthman, J., Goldstein, M. and Jonsson, G. (1988). Time course of MPTP-induced degeneration of the nigrostriatal dopamine system in C57 BL/6 mice. *Brain Res. Bull.* **21**, 257-263.
Tatton, N. A. and Kish, S. J. (1997). In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience* **77**, 1037-1048.
Tatton, N. A., Maclean-Fraser, A., Tatton, W. G., Perl, D. P. and Olanow, C. W. (1998). A fluorescent double-labeling method to detect and confirm apoptotic nuclei in Parkinson's disease. *Ann. Neurol.* **44**, S142-S148.
Tompkins, M. M., Basgall, E. J., Zamrini, E. and Hill, W. D. (1997). Apoptotic-like changes in Lewy-body-associated disorders and normal aging in substantia nigral neurons. *Am. J. Pathol.* **150**, 119-131.
Van den Munckhof, P., Luk, K. C., Ste-Marie, L., Montgomery, J., Blanchet, P. J., Sadikot, A. F. and Drouin, J. (2003). Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons. *Development* **130**, 2535-2542.
Vila, M. and Przedborski, S. (2003). Neurological diseases: Targeting programmed cell death in neurodegenerative diseases. *Nat. Rev. Neurosci.* **4**, 365-375.
Wallen, A., Zetterstrom, R. H., Solomin, L., Arvidsson, M., Olson, L. and Perlmann, T. (1999). Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice. *Exp. Cell Res.* **253**, 737-746.
Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N. P. and Perlmann, T. (2003). Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature* **423**, 555-560.
Wizenmann, A., Thanos, S., von Boxberg, Y. and Bonhoeffer, F. (1993). Differential reaction of crossing and non-crossing rat retinal axons on cell membrane preparations from the chiasm midline: an in vitro study. *Development* **117**, 725-735.
Yee, K. T., Simon, H. H., Tessier-Lavigne, M. and O'Leary, D. M. (1999). Extension of long leading processes and neuronal migration in the mammalian brain directed by the chemoattractant netrin-1. *Neuron* **24**, 607-622.
Zetterström, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L. and Perlmann, T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248-250.
Zuch, C. L., Nordstroem, V. K., Briedrick, L. A., Hoernig, G. R., Granholm, A. C. and Bickford, P. C. (2000). Time course of degenerative alterations in nigral dopaminergic neurons following a 6-hydroxydopamine lesion. *J. Comp. Neurol.* **427**, 440-454.