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

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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., 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 MgCl₂ and 1 mM CaCl₂ 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 (Kriegstein 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 1:200, 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¢ from 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: En1a (NM_010133), CAUCCUAAGGCGCGAUUCC (sense) and GAAAGCGGCGGCUAAGGAGT (antisense); En1b, GUUCCCCGAAACAACCUCCTT (sense) and AGGGUGUGUUGGCGGAACTT (antisense); lamin A/C (NM_019390), GCAUCUUGAGGAUGAGT (sense) and CAUCCUGAAGCUUGUC (antisense); Pbx1 (AF020196) CAGUUUUGAUGCAUUGGUGAGT (sense) and CCCCCAUCUACUAAAACUGTT (antisense); and the randomly generated Scramble I Duplex (Dharmacon, USA), CAGTCCGCTTGCGACTGG (sense) and CCAATCCGCAAGCAGCAGTG (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).

Engrafted 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

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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 midbrain 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 isogenic 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 death.
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 siEn1 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 (Figs 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
of scrambled siRNA, a set directed against (Fig. 4K-N).

Furthermore, the silencing efficiency and time course for Pbx1 caused a reduction in the number of mDA neurons (Fig. 4A).

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 that 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
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 (Walten 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 Munchhof 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 (Piccinii 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 monoallelic 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 promotor 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, BMF Biofutur 98. We thank Martyn Goulding for the Enh/tau1lacZ, 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 correcions of the manuscript.

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