DISSERTATION

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Presented by

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born in Trieste 24.07.1974

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ENGRAILED GENES ARE CELL AUTONOMOUSLY REQUIRED FOR THE SURVIVAL OF THE MESENCEPHALIC DOPAMINERGIC NEURONS

Referees: Prof. Dr. Beyreuther Prof. Dr. Witzemann
"A new dawn, a new world."

- - -

A new dawn, a new world.
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DISCUSSION

I. mDA neurons undergo apoptosis when ablated of En-1 and En-2 expression
II. Engrailed does not regulate axonal outgrowth of mDA neurons
III. mDA neurons require En-1 and En-2 cell autonomously for their survival
IV. En-1 RNAi induces rapid apoptosis of En-2 -/- mDA neurons
V. En-1 acts as repressor for p75NTR expression
VII. En-1 has no direct influence on dopaminergic progenitors

Conclusions

REFERENCES
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>AADC</td>
<td>L-Aromatic amino acid decarboxylase</td>
</tr>
<tr>
<td>AHD-2</td>
<td>Aldehyde dehydrogenase 2</td>
</tr>
<tr>
<td>AP</td>
<td>Anterior-posterior</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<tr>
<td>BME</td>
<td>Basal medium eagle</td>
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<tr>
<td>BMP</td>
<td>Bone morphogen protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2′-deoxyuridine</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cicloxygenase</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
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<tr>
<td>DAPI</td>
<td>4′,6-Diamidino-2-phenyldole dilactate</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>D.i.v.</td>
<td>Days in vitro</td>
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<tr>
<td>D.p.c</td>
<td>Days post transfection</td>
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<td>Deoxyribonucleic acid</td>
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<td>DMEM</td>
<td>Dulbecco modified eagle medium</td>
</tr>
<tr>
<td>dNTP</td>
<td>Desoxyribonucleotide</td>
</tr>
<tr>
<td>DV</td>
<td>Dorsal-ventral</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetracetic acid</td>
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<tr>
<td>En</td>
<td>Engrailed</td>
</tr>
<tr>
<td>En-1</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FP</td>
<td>Floor plate</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>GP</td>
<td>Globus pallidus</td>
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<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line derived factor</td>
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<tr>
<td>HB</td>
<td>Homogenization buffer</td>
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<td>Hamburger Hamilton stage</td>
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<td>HS</td>
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<td>IL-1</td>
<td>Interleukin-1</td>
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<td>INF-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>L-15</td>
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<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
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<tr>
<td>MHB</td>
<td>Mid-hindbrain boundary</td>
</tr>
<tr>
<td>MPDP+</td>
<td>1-methyl-4-phenyl-2,3-dihydropyridinium</td>
</tr>
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<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
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<td>MPTP</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NSC</td>
<td>Neural stem cells</td>
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<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxy dopamine</td>
</tr>
<tr>
<td>P75(NTR)</td>
<td>Low affinity neurotrophin receptor p75</td>
</tr>
<tr>
<td>Park-1</td>
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</tr>
<tr>
<td>Park-2</td>
<td>Parkin-2</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PD</td>
<td>Parkinson disease</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PORN</td>
<td>L-polyoornithine</td>
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<tr>
<td>RCAN</td>
<td>Retroviral vector containing an acceptor null</td>
</tr>
<tr>
<td>RCAS</td>
<td>Retroviral vector containing an acceptor sequence</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RRF</td>
<td>Retrorubral field</td>
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<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>Si</td>
<td>Small interfering</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
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<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>SNpc</td>
<td>Substantia nigra compacta</td>
</tr>
<tr>
<td>Snpl</td>
<td>Substantia nigra lateralis</td>
</tr>
<tr>
<td>Snpr</td>
<td>Substantia nigra reticulata</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxilase</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLZ</td>
<td>Tau lac z</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Tdt-mediated dUTP nick end labelling</td>
</tr>
<tr>
<td>UTP</td>
<td>Uracyl triphosphate</td>
</tr>
<tr>
<td>VMAT</td>
<td>Vesicular monoamine transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>zVAD-fmk</td>
<td>Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone</td>
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</tbody>
</table>

-/-   Homozygous for null mutation of the gene of interest
-/+   Heterozygous only one allele of the gene of interest is mutated
%    Percentage
°C   Celsius degree
g    gram
hr   hour
l    liter
µ    micro
M    molar
ml   milliliter
µl   microliter
mm   millimeter
µm   micrometer
U    unit
The dopaminergic neurons of the substantia nigra and ventral tegmentum are the main source of dopamine in the central nervous system. The progressive loss of dopaminergic neurons in the substantia nigra compacta over several decades leads to one of the most prominent human neurodegenerative disorder, Parkinson’s Disease. Accumulating evidence suggest that these neurons die by apoptosis in afflicted patients. Still little is known about the molecular mechanisms that lead to full differentiation and maintenance of these neurons. This neuronal population expresses the homeobox transcription factors En-1 and En-2, briefly, after the cells become postmitotic onwards. In mutant mice deficient for both genes, the neurons are induced at the proper location, express TH, but at a time, when En expression sets in, these neurons rapidly undergo apoptosis, so that at E14 the whole population is lost. Another remarkable feature of those neurons is the lack of axonal projection towards the telencephalon. We first addressed the question whether these neurons, lacking En, were dying because of their inability to project and interact with trophic clues. In our study we report that En genes are not regulating axonal outgrowth of mDA neurons, and that these neurons if cultured onto permissive substrate elongate neurites of the same length in comparison to the wild-type. Secondarily we addressed the question whether the large deletion of midbrain tissue in En-1/-, En-2/- mutant embryos deprives these cells of an essential trophic support released by the surrounding neuroepithelium or whether the En genes are cell-autonomously required for the survival of mDA neurons. To address this, we performed cell-mixing experiments of En1/2 double mutant and wild type cells in primary cell culture of E12 ventral midbrain and we observed that En-1/-, En-2/- mDA neurons, both in homotypic and heterotypic cultures, are lost within 72 hours of culture. To complement these experiments and verify whether the absence of any rescue effect of the mDA neurons from the wild-type neuroepithelium was not a result of a pre-commitment to cell death, at the time of dissociation, we silenced En-1 by RNAi in En-2/- ventral midbrain cultures. We observed, thereby, that in En-1 interfered cultures the number of mDA neurons 4 days after transfection was always diminished about 25% and that interfered En-1 mDA neurons rapidly undergo apoptosis. These results taken together
demonstrate that En genes are cell-autonomously required for the survival and the maturation of the dopaminergic neurons in the substantia nigra compacta and the ventral tegmentum. Finally overexpression experiments in chick demonstrate that En-1 is not involved in the specification of the dopaminergic precursors, since midbrain tissue, transiently overexpressing En-1, did not show any increase in the number of mDA neurons. Our data suggest that genes downstream of En-1 and En-2 are essential for the maturation and maintenance of this neuronal population and that these transcription factors may be involved in Parkinson’s Disease.
INTRODUCTION
1. General introduction

The midbrain dopaminergic neurons are the main source of dopamine in the mammalian CNS. The catecholamine, dopamine, is a neurotransmitter responsible for several neuronal functions, including motor integration, cognition (Backman and Farde, 2001), attention (Nieoullon, 2002), emotion and reward (Schultz, 2001), as well as sexual function (Giuliano and Allard, 2001).

The midbrain dopaminergic neurons are a relatively restricted population and are divided into three distinct nuclei, the substantia nigra (SN), the ventral tegmental area (VTA), and the retro rubral field (RRF) (Nelson et al., 1996). The nigral neurons project to the dorsal striatum, and are innervated by different structures of the diencephalon and telencephalon. The ascending nigrostriatal pathway regulates motor control. The VTA neurons on the other hand, project to the limbic system and cortex forming the mesolimbical pathways, which controls emotion and reward behavior (Schultz, 2001). Alteration to this system are linked to schizophrenia, addictive behavior and attention-deficit hyperactivity disorder (ADHD) (Watanabe et al., 1998; Floresco et al., 2001).

The dopaminergic neurons are formed at stereotypic location in the ventral midbrain. The induction and differentiation of the midbrain dopaminergic neurons is the result of a various display of factors. Data arising from tissue transplantation and genetic studies indicate that the mDA neurons are formed at the midbrain neural plate upon the induction of two intersecting signals, the fibroblast growth factor-8 (FGF-8), arising from the Mid-Hindbrain Boundary, and Sonic hedgehog (Shh), arising form the floor plate (Ye et al., 1998). The inductive signal are believed to activate a cascade of other signaling molecules and transcription factors such as Lmx-1b, Ptx-3, Nurr-1, Engrailed-1 and –2 that finally lead to the full differentiation of this neuronal population (Zetterstrom et al., 1996; Smidt et al., 2000; Simon et al., 2001; Hwang et al., 2003).

Once the neurons have completed the last cell cycle and have become postmitotic, they start to elongate their axons rostrally to reach their respective target; nigral neurons project to the caudate putamen and tegmental neurons to the nucleus accumbens, cortex, and olfactory tubercule.
This neuronal population is heterogeneously affected during Parkinson disease, where loss of dopaminergic neurons in the SNpc leads to the impairment of the nigral striatal pathway, which results in bradikynesia, rigidity, resting tremor, and ataxia. The disease can have genetic and/or environmental etiology. Different mechanism of cell death have been proposed, necrotic (Kosel et al., 1997), apoptotic, and autophagic (Anglade et al., 1997). Although still controversial recent evidence suggest the apoptotic pathway as the principle mechanism responsible for the demise of this neuronal population (Hartmann and Hirsch, 2001)

My Ph.D. project was focused on understanding the type of the requirement and role of En-1 and En-2 for the midbrain dopaminergic neurons. As experimental mode I used En-1 +/- En-2/- mouse where the midbrain dopaminergic neurons are induced but than shortly after disappear. I investigated the cell death time course both in vivo and in vitro and unraveled the cell death mechanism leading to their disappearance.

2. The dopaminergic system

2-1. Location of the midbrain DA neurons in the rodent brain

The midbrain DA neurons are morphologically divided into three major cell groups A9, A10 and A8. The area A9 identifies the substantia nigra (SN), which is further subdivided in substantia nigra pars compacta (SNpc), substantia nigra pars reticulata (SNpr), and substantia nigra pars lateralis (SNpl), the SNpc represent the main dopaminergic subpopulation among the three. The area A10 indicates the ventral tegmental area VTA, and the area A8 the RRF (Fig I.1). The SNpc is densely packed with DA pyramidal-like neurons, whose dendrites invade ventrolaterally the SNpr (Bjorklund and Lindvall, 1975) and there release dopamine (Cheramy et al., 1981). More basal dendrites of the SNpc neurons, which spread mediolaterally, invade overlying dorsal sheet of the SNpc and VTA. Another kind of fusiform neurons is localized in the more dorsal aspect of the SNpc, and give rise to dendrites, which protrude ventrolaterally into the SNpr. The neurons of the VTA are characterized by a wide variety of shapes and sizes, 80% of the neurons are dopaminergic the rest non-DA neuron (Halliday and Tork, 1986) at laterally contiguous to the VTA, develops the SNpc. The cells from the two DA populations have a very similar fusiform shape. Other DA neurons have radiating dendrites. More
ventrally at the mid-anterior level the neurons are small and densely packed together (Paxinos, 1985). The neurons of the RRF appear to be a caudal extension of the SNpc and SNpl, are of middle size, and project to the striatum (Fallon and Moore, 1978).

Fig I.1 3D reconstruction of the DA neurons in the ventral midbrain. Caudal to rostral (B-C). Group A8 in blue depicts the RRF(B), group 9 in yellow the SNC (C), and group 10 in red the VTA(D) From German and Manaye 1993.

2-2. The dopaminergic afferent projections to the striatum

The ventral and intermediate sheets of the SNpc and ventrolateral VTA give rise to dopaminergic projections, which are directed towards the caudate putamen, and form the nigrostriatal pathway. Whereas dorsal and middorsal VTA project to nucleus accumbens and olfactory tubercule. VTA, lateral SNpc and SNpl in turn innervate the amigdala. The lateral septum is innervated by ventral VTA, and adjacent-medial SNpc. The neocortex receives the input from the dorsal-most sheet SNpc and VTA neurons forming the mesocorticolimbical pathway (Fig I.2). Minor dopaminergic projections are directed to the cerebellum, the hypothalamus, the raphe, the hippocampus, the ventral pallidum and the locus coeruleus (Kizer et al., 1976; Fallon and Moore, 1978; Simon et al., 1979; Gerfen et al., 1982; Swanson, 1982; McRae-Degueurce and Milon, 1983; Gasbarri et al., 1991; Ikai et al., 1992; Prensa and Parent, 2001).
2-3. The afferent projection to the mDA neurons

The γ-aminobutyric acid (GABA)-ergic neurons of the striatal patch give rise to two pathways that connect the striatum to the output nuclei of the basal ganglia, namely the globus pallidus pars interna (Gpi) and the SNpr. Striatal projections from the caudate putamen innervate the Gpi and SNpr to form the direct inhibitory pathway. Those striatal neurons bear Dopamine D1 receptor, coexpress substance P and dynorphin and provide a direct inhibitory effect on their target. Striatal projection, directed via subthalamic nucleus (STN) and globus pallidus pars externa (GPe) to the GPi and SNpr, form the indirect inhibitory/excitatory pathway. These striatal neurons can be recognized by differential expression of D2 dopamine receptor, Enkephalin. Via their GABAergic output they inhibit the Gpe, activate the glutamatergic neurons from the STN and thereby excite the Gpi and SNpr (Fig I.3). Thus direct and indirect pathways have antipodal effect on the dopaminergic target (Alexander and Crutcher, 1990) (Gerfen, 1992). Moreover afferent projection from the amigdala, hypothalamus, preoptic area and cortex are directed to the SNpc and VTA (Wright and Arbuthnott, 1980; Hurley et al., 1991; Wallace et al., 1992).
2-4. Function of the midbrain DA system

The midbrain dopaminergic neurons targeting a wide range of structure form a complex network where any changes result in a dramatic alteration of many neuronal functions.

The nigral neurons (SNpc) project to the caudate putamen forming the nigrostriatal pathway, which indirectly controls voluntary motor movement by modulating the striatal output neurons (Gerfen, 1992). The dorsal and middorsal VTA and medial SNpc neurons innervate the nucleus accumbens and olfactory tubercule, whereas the more ventral VTA and adjacent medial SNpc neurons project to the lateral septum forming the limbic system, and eventually the dorsal-most sheet VTA neurons project the neocortex to form the mesocorticoclimbical pathway. The latter pathway together with nigrostriatal pathway controls locomotor activity, mediates emotion, cognition,
and memory processing (Le Moal and Simon, 1991).
Since different nuclei are involved in this system, a variety of functions is displayed.
Damage in this network to a specific neuronal subpopulation in this network will lead
to different symptoms, i.e. hyperactivaction of the mesolimbical pathway causes
abnormal behavior and schizophrenia (Jentsch et al., 2000; Floresco et al., 2001). In
turn progressive degeneration of the DA neurons of the SNpc results both in motor
and behavioral deficiencies, which are apparent in Parkinson’s disease.

3. Parkinson’s disease

3-1. Epidemiology and Clinical traits

Parkinson’s disease (PD) is a progressive neurodegenerative disorder, affecting 2% of
the population over 60 years of age (Elbaz et al., 2002) Positive family history
doubles the risk of developing PD to about 4%. Recent studies indicate that PD with a
early onset is more likely to be genetically related than PD cases with a late onset
(Tanner et al., 1999). Pathologically PD is characterized by a progressive loss of DA
neurons of the SNpc and ventrolateral VTA resulting in a deficiency of the striatal
and nigral dopamine. However due to a compensatory mechanism, such as
supersensitivity of DA receptor, bradikynesia and rigidity, are first encountered when
the dopamine concentration in the putamen is decreased to 75%, and when 60% of
nigral dopaminergic neurons are already been lost (Bernheimer et al., 1973). In
addition, the degeneration of dopaminergic neurons is associated with loss of neuromelanin normally present in the human DA neurons (Kastner et al., 1992)
accompanied by strong glyosis (Folkerth and Durso, 1996). In PD, intracytoplasmatic
ubiquinated inclusion, called Lewy Bodies (LBs), are usually present in many of the
surviving neurons (Gibb and Lees, 1989). Lewy bodies are characterized by fibrillar
aggregates of α-synuclein, this molecular form of α-synuclein contrasts sharply with
normal α-synuclein, which is an abundant soluble presynaptic protein in brain
neurons (Dev et al., 2003). Nevertheless not all form of PD are characterized by the
presence of Lewy Bodies, brain of PD patients with autosomal recessive juvenile
parkinsonism (AR-JP), an early onset of PD, show only nigral degeneration but no
Lewy Bodies (Shimura et al., 2000).
The clinical symptoms result from the imbalances in the normal activity of the direct
and indirect pathway. A decrease in striatal dopamine leads to an increase in inhibitory GABA-ergic output activity in the basal ganglia, this increased output coming from the basal ganglia conducts to an excessive inhibition leading to a blackout of the thalamic and brainstem nuclei because of overinhibition by the internal GP and SNpr through GABA. That causes a repression of the cortical motor system, resulting in motor behavioral abnormalities, as bradikinesia, hypokinesia, rigidity and tremor, and postural deficiency resulting from the inhibition of the brain stem nuclei (Lang and Lozano, 1998; Hornykiewicz, 2001). PD patients suffer also from autonomic dysfunction as dysautonomia including orthostatic hypotension, gastrointestinal, urogenital, sudomotor and thermoregulatory dysfunction, pupillary abnormalities and sleep and respiratory disorders (Micieli et al., 2003), psychiatric symptoms as mood disorder and depression are also often diagnosed (Wolters, 2001).

3-2. Causes and pathogenesis of PD

The specific etiology of Parkinson´s disease is not known. Epidemiological studies indicate that the disease can have environmental and/or genetic origin. The environmental hypothesis implies that neurodegeneration in PD results from the exposure to neurotoxin affecting the dopaminergic system, and progression of the neuronal degeneration may be produced by chronic neurotoxin exposure triggering a perpetuating molecular cascade, which leads ultimately to cell death. Among the environmental causes, the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is the best studied. The meperidine derivate induces parkinsonian syndrome in humans with specific loss of the nigral dopaminergic neurons (Langston et al., 1983). In addition, exposure to other mitochondrial poisons such as the pesticide, rotenone (McCormack et al., 2002), or herbicide, paraquat, increases the risk of developing PD (Tanner, 1992). In addition, reactive oxygen species, generated from the catabolism of DA, may generate a harmful oxidative stress and function as endogenous neurotoxin for the mDA neurons. This would explain why long-term consumption of L-DOPA in PD treatment results in a toxic oxidative effect for the nigral remaining DA neurons (Soliman et al., 2002). The formation of free radicals such as dopamine or hydrogen peroxide triggers a proteotoxic insult to the cells. This eventually results in -synuclein misfolding, ubiquitin and proteosome impairment.
and protein-aggregate formation, which are found in the typical LBs (Ostrerova-Golts et al., 2000). Moreover products of oxyradicals are highly reactive species and leads to oxidative damage resulting in abnormal lipid peroxidation in the substantia nigra and widespread increase in protein and DNA oxidation can be detected in the brain of Parkinson's disease patients (Jenner, 1998). In addition oxidative stress can lead to mitochondria dysfunction related to reduced activity of complex I of the electron-transport chain. Complex I impairment, in turn, leads to electron accumulation and reduced ATP production which aggravate oxidative stress and results in apoptosis and neuronal cell demise. Such mechanisms have been implicated in the pathogenesis of both mitochondrial permeability transition pore-induced Parkinsonism and idiopathic PD (Greenamyre et al., 1999). Moreover the inflammatory process, surrounding the area of nigral loss in PD, leads to the release of proinflammatory cytokines, with apoptogenic potential versus the surviving neurons, thereby perpetuating neuronal degeneration and accelerating the progression of the disease (Hirsch et al., 2003). To confirm the role of the contribution of the surrounding microglia in the progression of the disease, it is reported that rotenone neurotoxicity is potentiated by the activation of the surrounding neuroglia (Gao et al., 2002).

Recent studies have identifies genes, which are related to PD: Nurr-1 (Le et al., 2003), parkin, [-synuclein, and most recently discovered but yet not fully unraveled UCHL-1 (ubiquitin C-terminal hydrolase1)(Hoenicka et al., 2002). The three latter genes are involved in the ubiquitin-proteosome pathway and that is of particular interest since the LB aggregates are one of the pathological features of PD (Giasson and Lee, 2001; Tanaka et al., 2001). Two missense mutation Ala53→ Thr and Ala30→ Pro in [-synuclein cause dominantly inherited PD (Polymeropoulos et al., 1997). Clinical and pathological feature typical of PD have been found in patients with either mutation (Kruger et al., 1998). Parkin was first discovered in patients with early PD onset. Parkin related PD is characterized by the loss of SNpc but it is not associated with LBs. Recent studies suggest that polymorphism at the parkin and synuclein loci may contribute to the risk of idiopathic PD (Farrer et al., 2001).

3-3. Mechanisms of cell death in the nervous system

Neuronal cell death occurs through different mechanisms, which are distinguished as
apoptosis, necrosis and autophagy. Apoptosis is the best understood mechanism for neuronal cell death. It is characterized by cytoplasmatic condensation, nuclear pyknosis, chromatin condensation, DNA fragmentation, cell rounding, membrane blebbing, cytoskeletal collapse, and the formation of membrane bound apoptotic bodies that are rapidly phagocytosed and digested by the neighboring scavenger cells. Apoptosis has been observed during neuronal development, neural cell death caused by acute injury such as ischemia and in neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease and Huntington’s disease and amyotrophic lateral sclerosis (Yuan and Yankner, 2000). Necrosis is characterized by early swelling of intracellular organelles followed by loss of plasma and nuclear membrane integrity and finally cell edema. Necrosis is mostly associated with pathological neuronal cell death following injury as in ischemia and stroke (Zheng et al., 2003). In addition, certain developmental cell death exhibits feature of necrosis (Clarke, 1990). Although apoptosis and necrosis are two different cell death mechanisms mediated by distinct pathways, a mixture of the two or a transition from apoptosis to necrosis is not a rare phenomenon (Moskowitz and Lo, 2003).

Autophagy is characterized by the appearance of cytoplasmatic autophagic vacuoles of lysosomal origin, followed by mitochondrial dilatation and enlargement of the ER and Golgi apparatus. Autophagy plays an important role in cellular homeostasis, and in the turnover of intracellular organelles and proteins. This type of cell death has been described in neurons during neuronal development (Xue et al., 1999) and in association with degenerative disease such as Parkinson’s Disease (Anglade et al., 1997).

3-4. Apoptosis and Parkinson Disease

Although still controversial, there are several reports of apoptosis as the principle mechanism leading to the death of nigral DA neurons in PD (Olanow and Tatton, 1999; Andersen, 2001; Hartmann and Hirsch, 2001; Vila and Przedborski, 2003). The feature of apoptosis implies nuclear DNA fragmentation caused by endonuclease activation. This characteristic hallmark has been confirmed in studies on postmortem brains of PD patients where five brains out of seven showed TUNEL positive DA
neurons in SNpc (Mochizuki et al., 1996; Tompkins et al., 1997; Tatton et al., 1998). The data was confirmed in rodents after treatment with sub-chronic doses of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Tatton and Kish, 1997), a commonly used for the human condition. More intriguingly, pro-apoptotic genes are upregulated in nigral DA neurons of PD patients (Hartmann et al., 2001a) 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 PD patients are more susceptible to apoptosis than their healthy counterparts. Furthermore, aggregates of a-synuclein, which are also found in Lewy bodies of PD patients (Spillantini et al., 1997), promote apoptotic cell death in cell culture (El-Agnaf et al., 1998). To conclude contradictory results on the apoptotic origin of cell death in PD (Kosel et al., 1997; Banati et al., 1998) could be explained by the relative low rate of cell loss in PD patients (McGeer et al., 1988), on the contrary, the disappearance of apoptotic cell occurs in the range of only a couple of hours (Raff et al., 1993). To further mitigate the discussion regarding the mode of cell death in PD there is evidence that also other non-apoptotic forms of cell death take part in the progression of PD (Sperandio et al., 2000).

3-5. Key molecules in neuronal apoptosis

Mammalian apoptosis is regulated by several key molecules i.e. the Bcl-2 family proteins, the adaptor protein APAF-1, and the cysteine protease caspase family, homologous of the C. elegans cell death gene product CED-9, CED-4 and CED-3 (Hengartner, 2000).

Neurons share the same basic of apoptosis program with all other cell types, however distinct type of neurons, and neurons at different developmental stages express different combination of Bcl-2 and caspase family members, suggesting a certain specificity for apoptosis regulation (Yuan and Yankner, 2000). The Bcl-2 protein family has a crucial role in intracellular apoptotic signal transduction. This protein family include both proapoptotic and antiapoptotic proteins that have in common one or more Bcl-2 homology domain (Merry and Korsmeyer, 1997). The major anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-xL are localized at the mitochondrial outer membrane, endoplasmatic reticulum, and perinuclear membrane.
Gain of function studies show that transgenic mice overexpressing Bcl-2 in the nervous system are protected against neuronal cell death during development (Martinou et al., 1994), and show increased resistance to MPTP (Yang et al., 1998) and to 6-OHDA intoxication (Offen et al., 1998). In addition overexpression of Bcl-2 can rescue sympathetic neurons in case of neurotrophin withdrawal (Garcia et al., 1992).

Bcl-x<sub>L</sub>, the other anti-apoptotic member of the Bcl-2 protein family, is continuously expressed throughout adulthood (Gonzalez-Garcia et al., 1995). Bcl-x<sub>L</sub>/- null mice dye around E13 with massive cell death in the developing nervous system (Motoyama et al., 1995). Bcl-x<sub>L</sub> may therefore be essential for the survival of immature neurons before they have established any synaptic connection.

Bax is a pro-apoptotic member of the Bcl-2 protein family that is widely expressed in the nervous system. Bax deficient mice have increased superior cervical ganglia and facial nuclei. In the Bax/- the midbrain dopaminergic neurons are more resistant to MPTP intoxication (Vila et al., 2001) as well as neonatal sympathetic neurons and facial motor neurons to neuronal cell death induced by NGF deprivation (Deckwerth et al., 1996).

Downstream the Bcl-2 family member another essential molecule in apoptosis is Cytochrome C, this molecule is released from the mitochondria to the cytosol where it binds to Apaf-1 and caspase-9 and transmit the death signal from the mitochondria to the effector caspases.

Apaf-1 is a mammalian homologue of <i>C.elegans</i> cell death gene product CED-4. Apaf-1/- mutant mice die during late embryonic development, they exhibit reduced apoptosis in neural precursor, which leads to morphological defects as early as day E9.5, and a marked enlargement of the periventricular proliferative zone (Cecconi et al., 1998).
Similarly to cytochrome c, Smac/Diablo and AIF are mitochondrial internal protein responsible for activation of the apoptotic cascade. Once in the cytosol Smac/Diablo interacts with several inhibitors of apoptosis (IAPs), which disinhibit apoptosis initiators and effectors, respectively caspase-9 and caspase-3 (Chai et al., 2000; Du et al., 2000), whereas AIF triggers cell death inducing directly chromatin condensation and DNA fragmentation (Susin et al., 1999; Daugas et al., 2000).

The cysteine proteases, caspases, are the master players in apoptosis. Mammals have 14 different caspases, which are organized into parallel and sometimes overlapping pathways that are triggered by different stimuli. Caspases are expressed as catalytically inactive proenzymes composed of an amino-terminal pro-domain, upon which they are classified, a large and a small subunit. Caspases with death-effector domain, including caspase-8 and caspase-10, are activated by interaction with the intracellular domains of the death receptors such as the CD95 (Apo-1/Fas), and tumor necrosis factor (TNF) receptors. Caspases with short prodomains, such as caspase-3, can be activated by most caspase pathways, whereas caspase-11, and 12 are activated only under pathological conditions (Kang et al., 2000; Nakagawa et al., 2000). The two major caspases involved in neuronal cell death are caspase-3 and -9. Both homozygous null mice for caspase-3 (Kuida et al., 1996) and caspase-9 (Kuida et al., 1998) mice show a similar defect in developmental neuronal cell death of progenitor cells. Ectopic masses in the cerebral cortex, hippocampus, and striatum of the
caspase-3-/ and a marked expansion of the periventricular zone very much resembling the phenotype of the Apaf-1 null mice. The prominent neuronal apoptosis defect in all three mutant mice supports the requirement of neural progenitor cell death in the normal neuronal development in vertebrate (Raff et al., 1993)

3-6. Molecular pathways for neuronal cell death in PD

Despite the recent evidence for the apoptotic nature of cell death in PD and PD model, yet a mixture of necrotic and apoptotic cannot be excluded. Apoptosis, called also programmed cell-death (PCD), is an active, genetically-controlled, intrinsic program of cell "suicide" and it is characterized by morphological hallmark as cell shrinkage and chromatin condensation (Ziv et al., 1997). Death by Apoptosis results in a precise sequence of event:

I. An induction phase in which death can be stimulated by extracellular or/and intracellular signals
II. An effector phase which translates the death inducing signals into the ultimate decision to die
III. An end stage degradation phase in which morphological and biochemical changes characteristic for apoptosis become apparent.

Apoptosis can be triggered via two different pathways: an external and an internal cascade (Fig I.5). Nevertheless both pathways can impair the function of the mitochondria is believed to be a primary mediator of cell death in Parkinson’s disease (Fiskum et al., 2003).

The death receptor or extrinsic apoptotic pathway is involved in the inflammatory process that takes place during PD (Hirsch et al., 2003). This pathway is activated upon binding of different cytokines such as INF-γ, IL-1β, and TNF to cell surface death receptors respectively Fas/CD95 or TNF receptor, moreover there is evidence that p75 the low affinity neurotrophin receptor (p75NTR), bares a death domain recalling the TNF receptor domain and it is able to induce cell death during
development in sympathetic neurons (Frade et al., 1996), and of oligodendrocytes upon NGF treatment (Casaccia-Bonnefil et al., 1996). Despite this similarities it became recently clear that the molecular cascade signaling for p75NTR follows a pathway, which differs from the TNF-cascade (Barrett, 2000; Dobrowsky and Carter, 2000; Troy et al., 2002). The binding of TNF or Fas ligand to their specific receptor triggers activation of the intracellular “death domain”. This domain associates then with an adaptor protein, containing a “death effector domain” respectively FADD for Fas, and TRADD for TNF family receptors. These adaptor proteins recruit by autoproteolitical activation caspase-8. This aspartic protease in turns cleaves, initiator caspases, caspase-1, caspase-6, caspase-7, or the effector caspase-3, which ultimately elicit the morphological hallmark of apoptosis. However caspase-8 can also mediate translocation of BID, a proapoptotic member of Bcl-family, to the mitochondria and the subsequent release of cytchrome c from mitochondrial intermembrane space into the cytosol (Slee et al., 1999). Cytochrome c eventually triggers Caspase-3 activation. Thus inflammation appears to be a feature of neurodegeneration with growing pathogenic significance (Wyss-Coray and Mucke, 2002). Evidence for the involvement of caspase-8 in PD arises from human postmortem study, and after subchronical MPTP-treatment where a significantly high percentage of dopaminergic (DA) of the SNpc neurons displayed caspase-8 activation (Hartmann et al., 2001b).

Several lines of evidence support that mitochondrial dysfunction contribute to the
etiology of Parkinson´s disease (Schapira, 1999). It has been shown that genetic mutations in the parkin gene on chromosome 6 in PD patients results in an impairment of the electron chain transport I (Kosel et al., 1998). In addition sequence analysis of the mitochondrial genome show that mitochondrial point mutations are involved in PD pathogenesis (Vives-Bauza et al., 2002). One further line of evidence comes from recent studies on cybrid lines, which contain a normal nuclear genome but mitochondrial DNA form PD patients, and show a higher cytotoxicity to MPP⁺ (Swerdlow et al., 1996).

All these results indicate that the complex I defect in PD has a genetic cause and that it may arise from mutations in the mitochondrial DNA. Studies on Parkinson etiology report that disruption of the complex I can be induced by reactive oxygen species (ROS), generated by a number of different systems through the action of monoamine oxidase-B (MAO-B). As an example MAO-B is essential for i) the activation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to 1-methyl-4-pheny-2,3-ldihydropyridinium (MPDP⁺) ii) for the enzymatic conversion of dopamine and 6-OHDA to hydrogen peroxide (H2O2), and iii) for the activation of other potential toxins such as isoquinolines and beta-carbolines (Jenner and Olanow, 1996). It has become clear overtime that the redox state of the mitochondria, namely the activity of enzymatic reducer as glutathione, superoxid dismutase, and catalase, is extremely important for the extent of mitochondrial injury. Inhibition of the mitochondrial complex I represent the molecular basis for the MPP⁺, Rotenone, and 6-OHDA neurotoxicity in the dopaminergic neurons, and thereby these substances are widely used as neurotoxin in PD model (Feger et al., 2002). Studies on human PD postmortem brains confirm this feature and report that inhibition of mitochondrial alpha-KGDH and complex I, but not other respiratory complexes, is lower in the parkinsonian SN (Shen et al., 2000). In addition recent studies show that production of free radicals by mitochondria complex I can be activated by the sphingomyelin-dependent transduction pathway. Convergent data from postmortem brain suggest that this signaling pathway may be activated in the dopaminergic neurons disappearing during Parkinson's Disease (France-Lanord et al., 1997).

Impairment of mitochondrial complex I induces the translocation of pro-apoptotic molecules such as Bax to the mitochondria and leads to the release of mitochondrial apoptotic protein including cytochrome c and apoptosis initiating factor (AIF) into the
cytosol. Protein release from the mitochondria occurs either i) by physical disruption of the mitochondrial outer membrane, due to osmotic swelling of the matrix caused by accumulation of Ca\(^{2+}\) in the mitochondria (Friberg and Wieloch, 2002), or ii) by pore formation promoted by BH3/Bax/lipid interaction and which is directly inhibited by Bcl-x\(_I\) (Kuwana et al., 2002).

The endoplasmatic reticulum (ER) is the other organelle, which senses cell stress. It may be also in the pathogenesis of PD. Bax and Bak are "multidomain" proapoptotic proteins, which are also localized to the endoplasmic reticulum (ER). Similarly to the mitochondria, internal stimuli induce the translocation of pro-apoptotic molecules such as Bax to the ER. Bak translocation leads to the disruption of calcium homeostasis and accumulation of unfolded proteins in the ER, which result in activation of caspase-12, caspase-9 and finally cell death (Scorrano et al., 2003). In addition PD “in vitro” models induced by 6-hydroxydopamine, MPP\(^+\), and Rotenone show a striking increase in transcripts associated with the unfolded protein response, phosphorylation of the key endoplasmic reticulum stress kinases IRE1alpha and PERK (PKR-like ER kinase) and induction of their downstream targets (Ryu et al., 2002). The potential importance of Bax in PD is strengthened by the observation that Bax null mice are resistant to nigrostriatal cell death induced by MPP\(^+\)(Vila et al., 2001).

3-7. Models for Parkinson disease MPTP, 6-OHDA, and Rotenone

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine to 1-methyl-4-phenylpyridine (MPTP) is a by-product of the chemical synthesis of meperidine analogue with a potent heroin-like effect that can induce parkinsonian syndrome in humans with specific loss of the nigral dopaminergic neurons (Langston et al., 1983). Since the discovery of MPTP, as a Parkinsonism causing substance in humans and primates, and other mammalian species, this neurotoxin has been extensively used as a model for Parkinson disease. From neuropathological data, MPTP administration appear to cause damage to the nigrostriatal pathway recapitulating the progressive dopaminergic loss in PD. Postmortem analysis of PD brains, reveal a selective impairment of the mitochondrial electron transport chain, which is affected by MPTP (Gluck et al., 1994). Following systemic administration, MPTP rapidly crosses the
blood brain barrier and it is metabolized to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺) by the enzyme monoamine oxidase-B (MAO-B) in non-DA cells, and then by spontaneous oxidation it is transformed to 1-methyl-4-phenylpyridinium (MPP⁺), which is taken up by the dopamine transporter. Once inside the DA neurons, MPP⁺ is concentrated through an active process in the mitochondria. There it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain. Inhibition of the complex I blocks the flow of electrons along the mitochondrial electron transport chain, which results in an increased production of ROS leading to DNA damage. Cell damage is sensed by p53 (Mandir et al., 2002), which in turn up-regulates Bax (Thornborrow et al., 2002), Bax is then translocated to the mitochondria, propagating the apoptotic cascade. Administration of 6-hydroxydopamine (6-OHDA) was the first PD animal model. Its toxicity is selective for monaminergic neurons, resulting from its preferential uptake of by the dopamine transporter. As 6-OHDA cannot cross the blood brain barrier it has to be administered by intracranial injection, it is then oxidized by molecular oxygen and/or MAO B, and leads to the production of intracellular \( \text{H}_2\text{O}_2 \), and reactive oxygen species. Indeed overexpression of glutathione peroxidase, the enzyme, which metabolizes hydrogen peroxide to water, can prevent or slow down neuronal injury in 6-hydroxydopamine-induced toxicity (Bensadoun et al., 1998). In addition 6-OHDA can cause impairment of mitochondrial respiratory chain via complex I inhibition (Beal, 2001). Recently studies report that 6-OHDA is

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**Fig.1.6 Cascade triggered by the neurotoxin affecting the DA neurons.** The toxicity to dopamine neurons is exerted via mitochondrial dysfunction and oxidative damage. 6-OHDA is taken up by the dopamine transporter and then generates free radicals. MPTP is converted into MPP⁺ by monoamine oxidase MAO B on oligodendrocytes, is then taken up by the dopamine transporter, accumulates into the mitochondria where causes complex I inhibition and generation of free radicals, or by the vesicular monoamine transporter thereby reducing its toxicity. Rotenone acts by directly inhibiting complex I. MPTP and Rotenone cause LBs formation. (Adapted from Beal, 2001)
also formed endogenously in patients suffering from Parkinson's Disease (Glinka et al., 1997).

Rotenone is a common pesticide used in vegetable gardens, and to select fish population in lakes or reservoir. Chronic infusion of Rotenone in rats has shown to recapitulate most of the pathological hallmarks of Parkinson’s disease. Once given systematically it crosses rapidly the blood-brain barrier and binds and inhibits complex I uniformly through out the brain, but induces activation of the mitochondrial apoptotic cascade only in the mDA neurons. This reinforce the concept that nigral DA neurons display a particular sensitivity to complex I inhibition (Betarbet et al., 2000). Both oxidative damage and cytochrome c release subsequent to complex I inhibition enhance [square-bracket]-synuclein aggregation in nigral neurons, and formation of cytoplasmatic inclusion reminiscent of LBs in PD (Perier et al., 2003; Trojanowski, 2003). In addition recent studies reported that rotenone is activating resident microglia triggering an inflammation response, which may contribute to the progressive neuronal demise in PD (Gao et al., 2002, 2003). This inflammatory response is also observable in other animal model for PD and postmortem brain of PD patients, which suggest that this phenomenon may take actively part in the progression of the pathology (Orr et al., 2002; Hirsch et al., 2003).

3-8. Targeting apoptosis in Parkinson disease

A closer insight of the apoptotic cascade in PD and PD models has offered a concrete possibility for the treatment of Parkinson disease. Approaches aiming to inhibit apoptosis at the level of the effector caspase were yet unfruitful, whereas blockade of the cascade upstream caspase activation resulted more successful. A broad-spectrum caspase inhibitor Val-Ala-Asp-fluoromethylketone (zVAD-fmk) attenuates the loss of DA neurons exposed to MPP+ in culture but does not restore neurites regression (Bilsland et al., 2002).

Preventing only the death of the nigral cell body without preventing the degeneration of their axonal processes has not resulted an helpful therapeutic strategy, but suggest us that the molecular pathways that lead to the demise of the some are different from those governing axonal degeneration.

Additionally attempts to prevent the progression of the pathology have been pursued
by use of antinflammatory agents, since inflammation in Parkinson’s disease is recognized to contribute to further dopaminergic degeneration. Inhibition of either COX-1/COX-2 by acetylsalicylic acid or preferentially COX-2 by meloxicam provided a clear neuroprotection against MPTP-toxicity on the striatal and nigral levels (Teismann and Ferger, 2001). In addition thalidomide, a sedative, immunosuppressive, and anti-inflammatory agent, which inhibits the TNF transduction pathway, showed a dose-dependent protection against the MPTP-induced decrease in DA (Boireau et al., 1997). Prevention of apoptosis in PD models was also pursued through genetic targeting. Transgenic mice overexpressing p35, a general caspase inhibitor for both initiator and effector caspase, shows attenuated MPTP-induced cell death and striatal DA depletion (Viswanath et al., 2001).

3-9. Clinical treatment of PD

Since the mid-1960s a symptomatic therapy that replaces the neurotransmitter with its precursor L-DOPA has been the most common treatment for PD. This is usually administered with a peripheral decarboxylase inhibitor to prevent formation of dopamine in the peripheral tissue and a catechol-O-methyltransferase (COMT) to increase the plasma half-life of levodopa. This therapy is very efficient in the early phase of the treatment however in the later phases about 60% of the patients develop severe complications such as motor fluctuation and diskynesia (Bezard et al., 2001). Other drugs like dopamine agonists (Rascol et al., 2000) are also potential therapeutic agents that don’t show a wear off effect as levodopa but are more likely to cause hallucinations confusion, particularly, in elderly.

Intracerebral transplantation of embryonic ventral mesencephalic tissue is a potential treatment for patients with Parkinson's disease for whom medical management is unsatisfactory. Neural transplantation for Parkinsonism has been studied experimentally in animal models of Parkinson's disease for more than two decades. These animal studies have shown significant graft survival, synapse formation, graft induced-dopamine release, and behavioral recovery in transplanted animals (Ramachandran et al., 2002). 300 patients worldwide have undergone neural transplantation of embryonic ventral
mesencephalic tissue. Among those, only in two bilaterally transplanted patients, graft survival and host reinnervation was achieved and the patients showed clinical improvement (Kordower et al., 1995), however in all cases functional recovery was incomplete. Logistical and ethical issues have impeded up to now large scale clinical trials and have increased the search for alternatives to human fetal cell grafts including engrafts, stem cells, genetically engineered cells, immortalized cell lines, and paraneural cells secreting specific neurotrophic and growth factors (Borlongan and Sanberg, 2002). Since surgical therapy is now becoming increasingly available, stereotaxic brain stimulation of the subthalamic nucleus reduces significantly bradikynesia, rigidity and tremor and is regarded by now as the most successful therapy against Parkinsonism (Limousin et al., 1998).

4. Development of the midbrain DA neurons

4-1. Midbrain patterning and specification

The central nervous system arises from the neuroectoderm, which is generated briefly after gastrulation. The neuroectoderm begins to thicken and subsequently rolls up along it’s anterior-posterior axis (AP) to form the neural tube which partitions at its rostral end into the basic subdivisions of the vertebrate brain, the fore-, mid- and hindbrain (Lumsden and Krumlauf, 1996; Rubenstein and Beachy, 1998), clearly distinguishable by day E9.5. This rostrocaudal patterning is followed by the dorso-ventral (DV) polarization of the neural tube (Lee et al., 1999 and Simon et al.1995), which is induced by two antipodal signals, sonic hedgehog, Shh, released by both the floor plate (FP) and notochord, and bone morphogen proteins, BMPs, first released by non-epidermal ectoderm and later by roof plate cells (Lee and Jessell, 1999). At this time transcription factors expressed along the AP axes start to define the region were the DA neurons are formed. At E7.5 the anteriorly located Otx-2 expressing tissue and the posteriorly located Gbx-2 confront each other along the rostrocaudal axis, defining the midbrain hindbrain boundary (MHB) or isthmus, which represent the border between mesencephalic and metencephalic compartment, (Wassarman et al., 1997). Thereafter two other transcription factors, Pax-2, followed by Pax-5, appear at the site of confrontation (Wassef and Joyner, 1997). Parallel to Pax-2 the expression pattern
of the two secreted molecules Fgf-8 and Wnt-1 become restricted to the adjacent transverse band of the Otx-2/Gbx-2 border (Fig I.7) (Wilkinson et al., 1987; Crossley and Martin, 1995). Homozygous mutant mice null for Otx-2, Wnt-1 and the double mutant for Pax-2/5 all show the same phenotype with respect to the midbrain, a complete deletion of it (McMahon and Bradley, 1990; Acampora et al., 1995; Ang et al., 1996; Schwarz et al., 1997). At E9, 5, En-1 and En-2, similarly to Pax-5, span an expression domain at the midbrain-hindbrain boundary (MHB) (Liu and Joyner, 2001a).

**Fig I.7 Simplified scheme of Otx2, Pax2, Pax5 and Wnt-1 expression pattern of at E8 and E9 in mouse.** Otx2, Pax2, Pax5 and Wnt-1, which are represented by bars above the embryo, appear in temporal order starting from Otx2, and show an expression overlap in the midbrain region rostral to the isthmus. F = forebrain, M = midbrain, H = hindbrain.

### 4-2. Induction of the midbrain dopaminergic neurons

In mammalian nervous system individual neurons develop at stereotypic positions defined by their coordinates along the anterior-posterior (AP), dorsal–ventral (DV) (Tanabe and Jessell, 1996). The three organizing centers, the midbrain hindbrain boundary (MHB), the floor plate (FP), and the anterior neural ridge (ANR), are positioned along the two main axes and specify the neuronal location and the dopaminergic fate of the progenitor cell within the grid of Cartesian coordinates (Rubenstein et al., 1994). This is also true for the mDA neurons (Hynes and Rosenthal, 1999). The midbrain and forebrain DA neurons are specified by the intersection of two signals arising respectively from the floor plate and MHB. Ectopic induction of a second floor plate in dorsal location is accompanied by generation of a cluster of DA neurons (Hynes et al., 1995b). The inductive potential FP tissue resides in its secreted molecule Shh, in fact recombinant Shh, alone, is able to induce ectopic expression of mDA neurons (Hynes et al., 1995a) and antibody directed against Shh ablate its effect (Ye et al., 1998).
Along the dorso-ventral axis, the MHB (Gardner and Barald, 1991; Martinez et al., 1991) and the ANR are the main source of Fgf-8 and provide inductive signals for the mDA neurons and the forebrain DA respectively. The isthmus, itself, can induce DA neurons in ectopic ventro-caudal forebrain explants (Ye et al., 1998) and blocking the activity of Fgf-8 in mid-forebrain explants prevents the development of both the midbrain-forebrain DA neurons (Ye et al., 1998). In addition, mice knocked out for Fgf-8 lack any DA neurons (Ye et al., 1998).

Thus, the two inducing signal Shh along and Fgf-8 can control the dopaminergic fate along the AP and DV axis respectively, but concurrent activation of both signals seems to necessary and sufficient requirement for the induction of the DA neurons at multiple site along the anterior neural tube (Fig I.8) (Ye et al., 1998).

The first postmitotic DA neurons are detectable at E9.5 as scattered faintly stained TH positive cells along the ependymal layer at the medio-basal part of the mesencephalon and upon formation, start to migrate first ventrally along tenascin bearing radial glial processes, then laterally along neurofilament and L1 positive tangential fibers (Kawano et al., 1995; Ohyama et al., 1998). By E12 the mDA neurons are distributed throughout the entire length of the mesencephalon (Kawano et al., 1995). As soon as the mDA are induced they start protruding an axon by responding short range guiding molecules, such as the extracellular matrix protein (ECM) laminin, fibronectin and tenascin (Lander, 1987), membrane bound molecules that can be cleaved and act a soluble signal as L1 (Walsh and Doherty, 1997), glycosaminoglycans (Mace et al., 2002) and astrocytes secreted factors (Johansson and Stromberg, 2002). Recent studies have reported that the primary neurites extension of the mDA neurons in the mesencephalon is dictated specifically by the permissive substrate provided by lateral (L) radial glia and it is hemmed by the medial (M) radial glia and the repulsive
molecule netrin at the midline (Cavalcante et al., 1996; Garcia-Abreu et al., 2000). A few days later around E14, the nigral dopaminergic axons have reached the striatum and the dopamine transporter DAT start to be expressed. The mechanism for the axonal attraction is unknown, however from studies in vitro and vivo it has been shown that the striatum can function as an attractive cue for the developing axons (Manier et al., 1997) and that striatal glia induce axon elongation rostrally (Rousselet et al., 1990). At E15 the nuclei of the SN laterally and the VTA more ventrally to the neural tube are distinguished (Paxinos, 1995). The two nuclei have developed projection towards different areas, the SN project to the caudate putamen and VTA to the nucleus accumbens and olfactory tubercule forming respectively the nigrostriatal and mesocorticolimbical pathway. The specificity for this pathfinding-choice between the two populations is determined by the differential expression of the receptor EphB1 and the ligand ephB2. Ligand and receptor are expressed in complementary pattern in the midbrain dopaminergic neurons and their targets, which is consistent with their repulsive interaction. The receptor EphB1 is expressed at high level in the SN but at low levels in the VTA. In turn the ligand eph-B2 is expressed at high levels in the nucleus accumbens and olfactory tubercule, but at low levels in dorsal striatum. Thus SN dopaminergic neurons, that express high level of the receptor EphB1, project to the caudate putamen (dorsal striatum), which, in turn, express low level of ephrin B2. Conversely VTA neurons, which express low levels of the receptor, project to the ventromedial striatum where high levels of ephrin B2 are transcribed (Yue et al., 1999). At E15 the first functional synaptic connections are being established and dopamine is first detected in the striatum (Perrone-Capano and Di Porzio, 2000). In addition a mutual trophic interaction between the striatal target tissue and the nigral dopaminergic sets on. Evidence for a retrograde trophic activity of the striatum on the afferent dopaminergic neurons is provided by in vivo experiments where striatal excitotoxic lesion in immature rat induces apoptotic cell death in the SN (Macaya et al., 1994) and axon-sparing lesion of the striatum during development is associated with an induction of apoptotic cell death in the substantia nigra (SN)(Kelly and Burke, 1996).
4-3. The maturation phase

4-3-I. Extracellular factors

Once the midbrain dopaminergic neurons have become postmitotic other factors responsible for the maturation and later specification come into play.

Nerve growth factors such as brain derived neurotrophic factor (BDNF) (Alonso-Vanegas et al., 1999), neurotrphin-3,-4 and 5 (Hyman et al., 1994; Studer et al., 1995), bFGF (Ferrari et al., 1989), members of the transforming growth factor, TGF-α (Blum, 1998) and TGF-β (Farkas et al., 2003), GDNF protein families (Horger et al., 1998; Strelau et al., 2000), and Thrombin (Debeir et al., 1998) support survival and regulate axonal processing and branching. The SN of TGF-α knockout mice contained 50% fewer dopaminergic neurons than the control littermates, whereas VTA neuron number remained unchanged. This suggests that TGF-α takes part in the differentiation of these dopaminergic nuclei (Blum, 1998). Other factors, such as pituitary adenylate cyclase-activated polypeptide (Takei et al., 1998) calcitonin gene related polypeptide (Burvenich et al., 1998) endothelin (Webber et al., 1998), neurotensin (Sotty et al., 1998), estrogen and progesterone (Kritzer and Kohama, 1998) whose receptor are expressed in the mDA neurons may play a role in the late specification of these neuronal subtype or serve as mitogenic factors for DA progenitor maturation and survival support of this neuronal population.

4-3-II. Intracellular mediator

More recent studies have been focusing in understanding the role of the intracellular signals. Until now five transcription factors have been identified: Nurr-1, Lmx-1b, Ptx-3, En-1 and En-2, and they are known to be actively involved in the development of the mDA neurons, however none of those alone can induce DA neurons in the midbrain.

When the midbrain DA progenitor are still mitotically active at E7.5 they start expressing Lmx-1b (Smidt et al., 2000) and further on development, at E9.5, aldeyde dehydrogenase-2 (AHD-2) is detected (Wallen et al., 1999). After the first mDA neurons have become postmitotic and begin to differentiate upon the induction of Shh and Fgf-8, they express the transcription factor Nurr-1 (Zetterstrom et al., 1997) and
just one day later at E11, the homeodomain transcription factor *Pt*-*x*-3 (Smidt et al., 1997), and thereafter at E11.5 the homologous transcription factors *En*-1 and *En*-2. At this stage the neurons start to express TH and L-aromatic amino acid decarboxylase (AADC), as well as other protein that are necessary for the dopamine function, the dopamine Transporter (DAT) and the vesicular monoamminno transporter (VMAT)(Foster et al., 1988; Fujita et al., 1993; Lee et al., 1999).

**Nurr-1**

*Nurr*1 (also known as RNR1, Not or HZF-1) is a member of the steroid/ thyroid hormone nuclear receptor family, and acts as a ligand-activated transcription factor. Binding to NBRE-like motifs (i.e. NL1, NL2 and NL3) proximal to the TATA box of the TH promoter leads to *Nurr-1* transactivation and TH gene transcription (Kim et al., 2003). *Nurr-1* expression can be induced by Fgf-8 alone(Ye et al., 1998). *Nurr-1* is expressed in at least 95% of all TH positive midbrain neurons (Backman et al., 1999). It is not responsible for the induction of this neuronal phenotype(Hynes and Rosenthal, 1999), but it plays a key role in the determination of the dopaminergic phenotype, by regulating expression of TH, and AADC, DAT and VMAT. Knock out mice for *Nurr1* don’t express any of those genes , seem to develop normally during embryogenesis, and die briefly after birth, since they are unable to feed (Zetterstrom et al., 1997; Castillo et al., 1998; Sakurada et al., 1999). The use of *Nurr1* independent markers for mDA neurons, such as *AHD2, En1, Ptx-3* and a *Nurr1* riboprobe 5’ to the deletion, revealed that the cells otherwise develop normally in the mutant embryos until E15.5. At birth there are still some DA neurons left, principally in the VTA, as shown by continued expression of the markers (Wallen et al., 1999). Studies on the mutant phenotype remain contradictory, one group report that the neurons in the Nurr1 mutants are abnormally distributed, no axons are traceable from the striatum and a high amount of apoptotic nuclei is present in the ventral midbrain (Wallen et al., 1999).The other group undergoing the same phenotypical analysis was unable to observe any of the above mentioned alterations (Witta et al., 2000). An explanation for these different observations could be that the investigated mutant mice are the results of two separate experiments using different constructs. The first group removed exons 2, 3 and part of 4 whereas the second group removed only exon 3. It
is, therefore, possible that a partial *Nurr-1* protein is still translated in the latter case resulting in a weaker phenotype.

**Lmx-1b**

*Lmx-1b* is a member of the lim-homeodomain family of proteins. It is an essential regulator for dorso-ventral patterning of the developing limbs (Johnson and Tabin, 1997), and participates in the regionalization of the midbrain-hindbrain (Adams et al., 2000). *Lmx-1b* loss of function mutation lead to nail patella syndrome (Chen et al., 1998; Knoers et al., 2000). It is expressed in the neural tube as early as E7.5, including the region of the ventral midbrain which gives rise to the mDA neurons and its expression in this neuronal population is maintained throughout life (Smidt et al., 2000). In the E12.5 *Lmx1b/-* mutant embryos few cells, corresponding to the VTA, express TH and *Nurr-1*, but not *Ptx-3*, indicating that *Ptx-3* is not necessary for TH expression, further on in development by E16.5 the VTA neurons are lost. However TH expression is maintained in other region of the brain in the DA and neuroadrenergic system. The absence of *Ptx-3* expression in the TH positive neurons suggests that *Lmx-1b* is essential for the proper specification of the midbrain DA neurons. In the *Lmx-1b/-* mice lack of necessary molecular signals seem to impair survival of the mDA neurons during development.

It appears that the molecular pathway of *Lmx1b*, together with the one *Ptx-3*, is distinct form the one of *Nurr-1*.

Yet the published data is rather limited and the question remains open whether the ablation of the mDA neurons in the mutant is due to a cell-autonomous requirement of *Lmx-1b* in the neurons or an effect of the large midbrain deficit also observed in the mutant animals. In chicken, the expression of *Lmx1b* is followed with a slight delay by the expression of *Wnt1* and gain of function experiments using a replication-competent retroviral vector (Lmx1b/RCAS) suggest that *Lmx1b* is required for the onset and maintenance of the *Wnt-1* expression (Adams et al., 2000) If this interaction also exists in mouse, then the *Lmx-1b* mutant phenotype with respect to the mDA neurons is likely to be a consequence of the lack of *Wnt1* expression in the midbrain speaking against the notion of a cell autonomous requirement of *Lmx-1b* in the mDA neurons.
**Ptx-3**

Ptx3, also known as Ptx3, (paired-like homeodomain transcription factor 3 or pituary homeobox 3) is a homeodomain containing transcription factor with binding activity to DNA similar to the drosophila bicoid. It is uniquely expressed in the brain by the mDA neurons from E11.5 onwards and is maintained throughout the entire life of the animal (Smidt et al., 1997). Recent studies report that in the Pitx3-deficient afrontosia mice subsets of mDA neurons are progressively lost by apoptosis during fetal (substantia nigra, SN) and postnatal (ventral tegmental area) development, resulting in very low striatal DA and akinesia (Hwang et al., 2003; van den Munckhof et al., 2003). This suggests that Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons.

**Engrailed-1 and 2**

The mammalian Engrailed genes, En-1 and En-2, are homeodomain transcription factors, which were originally cloned on the basis of their sequence similarity to Drosophila engrailed or invected (Joyner et al., 1985; Joyner and Martin, 1987). As in insects, they have two distinct ontogenetic roles. During early embryogenesis, they take part in the regionalization of the embryo (Kornberg, 1981; Wurst et al., 1994; Tabata et al., 1995) whereas during later embryogenesis they are involved in the specification of neuronal phenotypes (Condron et al., 1994; Lundell and Hirsh, 1998; Saueressig et al., 1999; Simon et al., 2001).

Both En-1 and En-2 genes start to be expressed at E8 in the anterior mouse neuroectoderm as a marked band of cells, which will later give rise to the MHB. The two transcription factor are patterning genes for the midbrain in different animal models, such as chicken, mice and zebrafish (Wurst et al., 1994; Araki and Nakamura, 1999; Scholpp and Brand, 2001). The mechanism proposed for the role for En in the maintenance of the midbrain identity suggests a negative regulation exerted by En on repressors of mesencephalon-related genes (Araki and Nakamura, 1999). Between E11 and E12, the two genes emerge in the postmitotic mDA neurons and are then continuously expressed throughout the entire life of the cells (Fig I.9)
The dopaminergic phenotype

**Fig I.9** En-1 and En-2 are expressed specifically in the mDA neurons. In situ hybridization (A,C) and double immunostaining (C,D) respectively on P0 mouse brain sagittal and coronal sections. A,B adjacent section of a P0 wild-type mouse hybridized with riproprobess for En-1(A) and En-2(B), reveal the distribution of Engrailed at transcript level, En-1 is expressed at high levels both in VT and SN, whereas En-2 is highly expressed only in a small subset of cells and in the cerebellum. C,D Immunohistochemistry on P0 wild-type coronal sections using a the Enhb antibody, recognizing both En-1 and En-2 proteins (C,red) and an antibody against TH(D, green). En and TH are coexpressed in the mDA neurons of the SN and VTA. Engrailed is nuclear and TH is located in cell somata and axonal processes.

**Fig I.10** Loss of the midbrain dopaminergic neurons in the En double mutant. Nissl staining (A,D), immunohistochemistry on sagittal section of P0 mouse brain using an antibody against TH to identify the DA neurons (B,C,D,E) of wild type (WT) and En double mutant (En-1/-/- En2/-/) mouse, TH immunostaining reveals the natural distribution of the midbrain DA neurons. At the midline (B) the VTA neurons are labeled and in a lateral section (C) those of the SN are labeled. In the double mutant animal for Engrailed neither the VTA (E) neurons nor the SN(F) are to be detected by TH immunostaining, however DA neurons of the dorso-medial hypothalamic nucleus (DMH)remain to be observed (E) clearly indicating that their DA phenotype is independent of En-1 and En-2 genes. (Adapted from Simon et al., 2001)
Mice homozygous for an En1 null mutation die at birth and lack the inferior colliculus and parts of the cerebellum (Wurst et al., 1994). The mutant mice for En2 are viable and fertile, and show only a minor defect in cerebellar foliation (Joyner et al., 1991a; Millen et al., 1994).

In chick, retrovirus-mediated misexpression of En genes (Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996; Logan et al., 1996; Shamim et al., 1999), within the developing mesencephalon results in a defect of the cytoarchitectonic organization of the optic tectum, abnormal arborization, and mistargeting of nasal retinal ganglion cell (RGC) axons and complete degeneration of the temporal RGC axons.

It has been previously described by Simon et al., 2001 that both genes are involved in the development of the mDA neurons, and that their function is redundant in respect to the generation and maintenance of this cell population. The requirement for the Engrailed genes is apparent in the absence of both En-1 and En-2. In En double mutant the mDA are generated, express TH fail to differentiate and disappear (Fig I.10)(Simon et al., 2001). Furthermore, the mutant analysis also provided evidence that the expression of a-Synuclein is missing when both En genes are knocked out, suggesting a direct regulation of the transcription factors on the expression of a-Synuclein (Simon et al., 2001). This is particularly interesting since two human point mutations in the a-Synuclein gene have been recently linked to familial form of PD (Polymeropoulos et al., 1997; Kruger et al., 1998).

### 4-4. The dopaminergic phenotype

Once the midbrain dopaminergic neurons have become postmitotic they continue differentiating and start expressing functional markers asynchronously. The first phenotypical marker to be expressed is tyrosine hydroxylase TH, followed by the synaptic vesicle monoamine transporter VMAT2, the glial derived factor receptor alpha, GFR-α1, receptor kinase, c-ret. TH is the rate-limiting step of DA synthesis, which transforms L-tyrosine into L-DOPA. VMAT2 belongs to a larger family of vesicular monoamine transporter and is responsible for the storage of monoamines into a dense core of vesicles in most monoaminergic neurons (Amara and Arriza,
1993). GFR-1 colocalize with the, c-ret, which is activated upon binding with GDNF and promotes survival and sprouting of the mDA neurons (Sauer et al., 1995; Tomac et al., 1995; Gash et al., 1996; Akerud et al., 1999).

DAT modulates DA uptake, and is the site of action of various psychostimulant drugs and neurotoxin. Homozygous DAT null mice show spontaneous locomotion due to protracted persistence of DA in the extracellular space and are insensitive to the action of amphetamine, cocaine and the specific DA toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)(Drago et al., 1998; Bezard et al., 1999) From data in vitro it appears that DAT expression and DA uptake are modulated by the target striatal cells which further support the hypothesis that the striatal target would contribute to the maturation and support of the dopaminergic neurons (di Porzio and Estenoz, 1984; Perrone-Capano and di Porzio, 1996).
MATERIALS and METHODS
1. Generation and maintenance of mice

The generation of the En-2 deficient mice by targeted gene deletion has been previously described (Joyner et al., 1991b). The En-1 tau-lacZ mice were generated by a knock in strategy were the first 71 codons, including the first codon, are replaced by a Tau-lacZ sequence TLZ (Callahan and Thomas, 1994), and resulted in the En1 null allele. Construct and procedure are described by Sauressig et al., 1999. Parental lines both En-1TLZ/+ En-2/- produce the double null mutant mice for En-1 and En-2.

The initial tail genotyping for En-2 wildtype and mutant alleles was performed by PCR using the three primers to detect in one reaction wild-type and mutant alleles (neo). `Common´ primer 5´-TTGAGAAGAGAGGCCCTGTA-3´, `wild-type´ primer 5´-CTGGAACAAAAGGCCAGTGT-3´, `neo´ primer 5´-TCTCATGCTGGAGTTTCTTGTCG-3´. PCR parameters: 5 min at 94°C, 36 cycles (45 sec at 94°C, 1 min at 54°C, 1 min at 72°C), 5 min at 72°C, PCR conditions: 2.5 mM MgCl₂. The tails genotyping for the En-1TLZ allele was done by PCR using two primers `TLZ´ 5´-GTGTCCGGCAGCTTGGTCTT-3´, `EN1s´ 5´-TTCGCTGAGGCTTCGCTTTG-3´ PCR parameters: 4 min- 94°C, 30 cycles (45 sec at 94°C, 1 min at 54°C, 1 min at 72°C), 4 min at 72°C, PCR conditions: 2.5 mM MgCl₂.

2. Genomic DNA extraction

Tail biopsies from 2-3 weeks old mice were obtained and stored at –20°C. The tissue for genomic DNA extraction was first digested overnight at 55°C in proteinase K buffer [50mM Tris pH 8, 100mM EDTA, 100mM NaCl, 1%SDS supplemented with 5mg/ml proteinase K (Applichem, Germany)]. DNA extraction was performed in high salt solution. 200µl 5M Nacl were added, the mixture was shaken vigorously and centrifuged for 10min at RT. The supernatant was then collected and one volume of cold 95% Ethanol was added, the precipitated DNA was spooled out and placed in a new Eppendorf tube. Precipitated DNA was rinsed with 70%Ethanol and then resuspended into 100-500µl TE (10 mM Tris pH=8, 1mM EDTA). The DNA was finally heated at 65°C from 2 hours to overnight and used for genotyping.
3. Derivation of En-1/- En2/- stem cells

3-1. Preparation of feeder layer cells from mouse embryonic fibroblasts

E12-E14 mouse embryos were dissected under the hood in a dish containing fresh L-15 (Cambrex, Belgium), Head, limbs and internal organs were removed. Once having removed the plunger, the carcasses were sucked with a 5ml syringe and homogenized through an 18-gauge needle L-15, until clumps were broken up. The slurry was then transferred into two 50 ml falcon tubes containing 25 ml of Trypsin/EDTA (Cambrex, Belgium). The tubes were incubated for 5-10 min at 37°C, and mixed occasionally by inversion, until no clumps were visible. Trypsin was, then, inactivated by adding 25 ml of DMEM plus 10% of fetal bovine serum to each tube. The solution was pipetted up and down several times with a 10 ml pipette. 100-200µl 5mg/ml sterile stock DNAse I (Roche, Germany) was added to each 50 ml tube, incubated for 5min at 37°C and mixed by inversion, until the DNA precipitate was no longer visible. Cells were then spun down by centrifugation and resuspended in 5-10 ml of feeder medium [DMEM, 10%fetal bovine serum (FBS), 50µg/ml penicillin and streptomycin, 2mM glutamine]. The cells were plated into 10 cm tissue culture dish per embryo. The medium was changed after 24 hours at 37°C and 5%CO₂. Cell were let grow to confluency and then frozen into DMEM supplemented with 20% FBS and 10% dimethylsulfoxide (tissue grade culture, Sigma-Aldrich, Germany) in aliquots of 1ml at a concentration of 4x10⁶ cells per aliquot.

3-2. Mytomicin C treatment of fibroblast feeder cells

In order to prepare mitotically inactive fibroblast feeder layers, we used mitomycin C (tissue grade culture Sigma, Germany). Confluent dishes of mouse embryo fibroblasts on 0,1% gelatin were treated with DMEM plus 10% new born calf serum and 10 µg/ml of mytomicin C for 2 hours at 37°C and 5% CO₂. The dishes were then washed three times with PBS, and fresh ES medium [DMEM (high glucose, without pyruvate), 15% fetal FBS ES cell tested (PAN, Germany), 2mM glutamine, 0,1mM non-essential amino-acids (Invitrogen, Germany), [-mercaptoethanol (Invitrogen, Germany), 50µg/ml penicillin and streptomycin, 2mM glutamine and leukemia-inhibitory factor (LIF) 200U/ml (Chemicon, Germany)] was added at least 2hr at
37°C before placing the stem cells. The rest of the mytomicin C treated mouse embryo fibroblasts, were frozen down for future experiment in DMEM supplemented with 20% FBS and 10% dimethylsulfoxide (Tissue grade culture Sigma-Aldrich, Germany) in aliquots of 1ml at a concentration of 6x10⁶ cells per aliquot.

**3-3. De novo isolation of embryonic stem cells from E3.5 blastocysts**

The uterine horns were removed from pregnant *En-1-/+ En-2-/-* mothers, and surrounding fat was removed. The content of the horns was flushed using a 1ml disposable syringe with M2 medium (Invitrogen, Germany) into a sterile concave glass plate under a stereo dissecting microscope. The individual recovered blastocysts were then aspirated with a drawn-out Pasteur pipette held in a mouth-controlled tube, and placed into a 6 well plate precoated with feeder cells and ES medium. The embryos hatching from the zona pellucida, attached within 24-36hr by growth of the trophoblast cells. The spreading of the trophoblast component onto the substrate resulted into the ICM component, visible in the tissue culture. The blastocysts were left undisturbed for a period of 4-6 days of continuous culture. During this time ICM cell clumps proliferated and gave rise to a small clump of cells. The ICM was then disaggregated under a stereo dissecting microscope using a 200µl pipette, and the disaggregated ICM was transferred into 3cm sterile dish with a drop of 200µl of Accutase (PAA laboratories, Austria), pipetted gently and placed for 3 min at 37°C. A drop of culture medium (200µl) was then added to neutralize the digestive enzyme and with an extremely fine capillary, pulled from a Pasteur pipette. The ICM clumps were repetitively sucked up and down to separate them into single cells. The content of the drop was then transferred into the centre of the well in a 6 well plate precoated with fresh feeder cells and containing ES medium. ICM-derived cellular aggregates attached to the feeder layers and give rise to small colonies of cells within 4 days of culture. Colonies were inspected and then classified into several broad types on the bases of their morphology. Stem cell colonies colonies are typically small, have a large nucleus and minimal cytoplasm, and are densely packed together into small nests with individual cells at the edge of the colony.
4. Immunohistochemistry

4-1. Processing of the embryonic specimen

Mouse and Chicken embryo heads were fixed at 4°C overnight in 4% PFA in 100mM phosphate buffer (PB), pH 7.4. The heads were cryoprotected with 30% sucrose in 100mM PB, and cut at the cryostat (Microm).

4-2. Processing of the whole mounts

The neural tube was dissected from E12.5 embryos and fixed in 4% PFA at 4°C overnight. The day after the whole mounts were rinsed in blocking solution [1% Triton PBS (PBT), 10% NCS] twice for 10 min, the specimen were then incubated for 1 hour in blocking solution supplemented with 0.1% H$_2$O$_2$. Thereafter they were rinsed three times for 10 min in PBT and incubated overnight at 4°C with the primary antibody, rabbit anti TH, (AB152, Chemicon, Germany), diluted 1:1000 in blocking solution. The next day the neural tube was washed three time for 1 hour with PBT and incubated overnight at 4°C with the secondary antibody, biotinilated goat anti rabbit (Dianova, Hamburg), diluted 1:500 in blocking solution. The next day whole mounts were washed three times with PBT for 1 hour and incubated overnight at 4°C with streptavidin peroxidase (Dianova, Hamburg) diluted 1:1000 in PBT. On the next day the neural tube was rinsed twice for 10 minutes in 100mM phosphate buffer pH 7.4, and preincubated for 10 min with in 0.005% DAB in 100mM phosphate buffer pH 7.4. The color reaction was developed by adding a 100 mM pH 7.4 phosphate buffer solution containing 0.005% DAB and 0.01% H$_2$O$_2$. The reaction was then blocked by washes in 100mM phosphate buffer pH 7.4. Following staining the neural tube was cut at the midline and flattened in glycerol/PBS (9:1).

4-3. Immunohistochemistry

All immunostaining on cryostat sections, tissue and cell cultures were performed using Rabbit anti TH (AB152, Chemicon, Germany) at the dilution 1:1000, Sheep anti TH (AB1524, Chemicon, Germany) at the dilution 1:250, Rabbit anti-activated Caspase-3 (9661 Cell Signaling, USA) at at the dilution 1:500 for cell culture and
1:50 for tissue, Rabbit anti Pbx 1/2/3 (sc888 Santa Cruz, USA) at the dilution 1:2000, Rabbit anti Nurr-1 (AB 5778, Chemicon, Germany) at the dilution 1:500, mouse monoclonal anti-Engrailed 4g11 (Developmental Studies Hybridoma Bank) as pure supernatant, and mouse monoclonal anti-BrdU (1170376 Roche Diagnostic, Germany), mouse monoclonal anti L1 (557, Gift of Schachner M.) as pure supernatant, mouse monoclonal anti α;β-Tubulin (1111876, Boehringer Mannheim GmbH, Germany). Coverslips and cryostat sections were postfixed in ice-cold acetone for 5-10 min, washed with PBS three times for 2 min, and incubated for 30 min in blocking solution (10%HS in PBS). Collagen tissue cultures were just blocked in 10% HS 0,01% Triton PBS (blocking solution) for 1 hour at room temperature. Collagen tissue culture coverslips and cryostat sections were incubated overnight at 4°C with the primary antibody, diluted in blocking solution. On the next day coverslips and slides were first washed for 5 min three times with PBS, for collagen cultures washes were always performed with 0,01% Triton PBS. All samples were then incubated for 1-2 hours with the secondary antibody diluted in blocking solution. Biotinilated or directly coupled species-specific antibodies (Jackson Immuno research, USA) with Cy2, Cy3, Cy5 or Horseradish peroxidase. Following incubation with biotinilated secondary antibody, cell cultures, cryostat sections and collagen cultures were washed and further incubated for 1 hour at room temperature with Cy2, Cy3, Cy5 or Horseradish peroxidase streptavidin (Jackson Immuno research, USA). After immunostaining, cultured cells on coverslips and tissue sections were counterstained with DAPI (236276 Roche Diagnostic, Germany).

5. In situ Hybridization

5-1. Synthesis of mouse En-1 riboprobe

The mouse DIG En-1 riboprobe was synthesized from En-1 pBsk, linearized by enzymatic digestion with Hind III. The digested DNA was incubated for 1-2hr at 37°C with T7 polymerase (Invitrogen, Germany), Trans 5x buffer (Promega, Germany), 10x DIG labeling mix (Roche, Germany), 0,01 M DTT (Promega, Germany), 1U/µl of RNase inhibitor (Promega, Germany). After transcription had taken place 10U/µl DNAse RNAse free were added for 15min at 37°C to get rid of DNA residues. The DIG-probe was, then, precipitated adding 100µl TE, 1M LiCl,
and 300µl 100% ethanol, for 30 min in dry ice and then centrifuged at maximum speed for 10 min. The pellet was washed with 80% ethanol, and resuspended in TE/formamide (50µl: 50µl), let for few minutes at 60°C or longer at room temperature. The DIG-probe was checked on a 1% Agarose gel, and the concentration at the spectrophotometer (RNA 230 and 260 nm), and eventually stored at –20°C.

5-2. In situ hybridization with mouse En-1 riboprobe

Chicken neural tubes were disected in PB pH 7.4 carefully removing surrounding mesoderm. Fixed in 4% PFA overnight and next day washed with methanol/0.1%Tween-20 PBS (1:1), and twice with absolute methanol. At this point the embryos can be put at –20°C for long-term storage. The neural tubes were rehydrated, and postfixed in 4% PFA, 0.1% glutaraldehyde. Thereafter they were submerged in the hybridization mix buffer containing 50% formamide, 0.5% CHAPS, 0.2% Tween-20, 3x SSC, 5mM EDTA, 50µg/ml Yeast RNA, 100µg/ml Heparin for 1hr at 70°C, after this preincubation the neural tubes were transferred in a new hybridization mix containing 1µg/ml of the DIG labeled mouse En-1 probe incubated overnight at 70°C. On the following day the whole mounts were washed twice with prewarmed hybridization buffer, once in 50% hybridization buffer and 50% Aqueous solution containing 1.3 M NaCl, 20mM KCl, 0.25 M Tris-HCl pH 7.5, 0.1% Tween (TBST), and twice only with TBST at room temperature. Neural tubes were then blocked in TBTS supplemented with 10% new born calf serum (10%NCS-TBST) for 1 to 3 hours, and, then, incubated overnight with anti-DIG antibody (Roche Diagnostic, Germany) diluted 1:5000 in 10%NCS-TBST. The following day they were washed twice with TBST, and three times with an aqueous solution containing 20mM NaCl, 50mM Tris-HCl pH 7.5, 25 mM MgCl₂, 1% Tween (NTMT). The color reaction was developed by adding NBT/BCIP (Roche Diagnostic, Germany) in NTMT, and stopped at the desired staining intensity with 1M TE pH 8. The whole mounts were dried and stored in 2-4% PFA in 0.1 M PB.
6. BrdU labeling

BrdU (Sigma, Germany, 50mg/kg body weight) was injected intraperitoneally at pulses of 6 hours per day from E10.5 to E12.5. To demask the BrdU, the PFA fixed cultures were treated with DNAse (Roche Diagnostic), 0.05 mg/ml in PBS supplemented with 5mM MgCl₂ and 1mM CaCl₂, for 5-10 min. BrdU incorporation was detected using mouse monoclonal anti-BrdU (170376 Roche Diagnostic, Germany) and following the standard immunohistochemistry protocol (4-3).

7. Coverslip coating

12mm glass coverslips (Neolab, Germany) were sterilized by flambation after submerging them in 99% ethanol. Each coverslip was placed into a 24 well plate (Corning, Germany). PORN/Laminin coating was performed by submerging the coverslips in 10μg/ml polyornithine (Sigma, Germany), dissolved into sterile water. The coverslips were then checked for uniform submersion and then left for 1 hour at room temperature under the hood. They were then washed three times with sterile water, in the meanwhile aliquots of 1mg/ml laminin were slowly thawed on ice, diluted 1:1000 into DMEM or DMEM-F12 (Cambrex, Belgium), and added the coverslips in the wells.

For the coating with membrane vesicles from wildtype ventral midbrain: 12 ventral midbrain were dissected from E12.5 embryos in L-15 (Cambrex, Belgium). The tissue was then transferred into 1.5 ml eppendorfs and most of the medium removed. 500μl homogenization buffer supplemented with protein inhibitors, (HB inh) Aprotinin 10μg/ml and Pepstatin 1μl/ml (both from Sigma, Germany) was then added, and homogenized with a 1 ml syringe( 0.4 mm x 25 mm length) to a clear solution. The solution was layered on a sucrose step gradient of 350μl 50% and 150μl 5% sucrose dissolved in HB inh inside a 3ml polycarbonate tubes (Beckman- Coulter, Germany). The tubes were centrifuged at 50000 g for 10 min in an ultracentrifuge Beckmann TL, rotor TLS 55. After centrifugation the membrane fraction at the border line, between 50% and 5% sucrose, was collected with a micropipette, resuspended in 1ml PBS, and spinned at high speed at 4°C. The supernatant was then discarded and the pellet resuspended in 1ml PBS. Protein content was determined by diluting membrane
suspension in 2% SDS 1:10, extinction factor was determined at 220 nm against 2% SDS using a Beckman Coulter spectrophotometer. Membrane solution was then diluted to 0,1mg/ml. 50 µl/ml red fluorescent covaspheres (Duke Scientific, S.Antonio, California, USA), were added to one of the diluted membrane suspension in order to verify by fluorescence labeling of the ventral midbrain membrane vesicles. Membrane fraction were stored at –80°C. 150µl membrane suspension was then layered onto PORN precoated coverslips, left 1-2hr at 37°C and then washed with medium previous to cell plating.

8. Threedimensional collagen gels from rat tails

Frozen rat tails were put into 70% ethanol to slowly thaw. Tendons were then removed and put in a large 6mm sterile dish, washed twice with 70% Ethanol. Tails were, then, put in a flask, and weighted, washed twice with water, dissolved in 0,1% Acetic acid and steered for 3 days at low speed to complete dissolution. The solution was poured in 50 ml tubes, which were then centrifuged at 15000rpm for 2hr at 4°C. The supernatant was collected and poured in dialysis tubes, placed in 1l flask, containing 1:10 BME10x (Gibco, Germany) in pH=4 water, and left to dialyze for at least 3 days at 4°C with a stir bar. The collagen was then stored up to one year into 5-10 ml aliquots.

The collagen gels were obtained by placing 9 parts of dialyzed collagen into a 1.5ml eppendorf tube on ice together with 1 part of BME10x and 0,09 parts of pH=7.5 sodium bicarbonate (Sigma, Germany). The mixture was pipetted up and down always on ice. Pellet of dissociated ventral midbrain was diluted to 1000 cells/µl into the collagen gel. 150µl cell- collagen drops containing the cells were pipette into 4 well plates (Corning, Germany). Cell medium was added after 15-30 min, to allow thickening.

9. Primary cell culture

All primary cell cultures were performed using E12.5 En-1 -/-, En-2/- double mutant mouse embryos. Mutant embryos were distinguished from their control littermates by the characteristic midbrain hindbrain morphology (Simon et al., 2001).
embryonic ventral midbrain was carefully dissected into fresh L-15 (Cambrex, Belgium) and meningis were removed. The ventral midbrain from the En double mutant and control littermate were placed into separate tubes and dissociated using Accutase (PAA laboratories, Austria). Cells were plated at a concentration of 150000 cells per well on precoated glass coverslips with Poly-ornithine/Laminin (both from Sigma, Germany), membrane vesicles or 3-D collagen matrix. The medium was DMEMF12 (Cambrex, Belgium) supplemented with 10% Horse Serum (HS) (Gibco, Germany), 5% fetal calf serum (Gibco, Germany), 33mM glucose (Sigma, Germany), 50U/ml penicillin and 50μg/ml streptomycin (Cambrex, Belgium). Cells were cultured from one hour to five days. In the cell mixing experiment, cell numbers of mutant were each 75000 to a final concentration of 150000 as for the homotypic cultures. For the RNA interference experiment, we always used serum-free medium-DMEM-F12 supplemented with 1xN2 (Gibco), 33mM glucose, 50U/ml penicillin and 50μg/ml streptomycin.

10. Neurointoxication

Ventral mesencephalic culture were first cultured for three days on PORN/Laminin glass coverslips in DMEM-F12 (Cambrex, Belgium) supplemented with 10% horse serum (HS)(Gibco, Germany), 5% fetal calf serum (Gibco, Germany), 33mM glucose (Sigma, Germany), 50U/ml penicillin and 50μg/ml streptomycin (Cambrex, Belgium), the third day of culture the medium was changed to serum-free medium-DMEMF12 supplemented with 1xN2 (Gibco). At the fifth day of culture, cells were exposed to 10μM MPP⁺ (Sigma, Germany), 100μM 6-OHDA, and 100μM Rotenone. Cells were fixed 48 hours after intoxication and counterstained for En, Pbx, Nurr-1, Lmx-1b, Ptx-3, and TH.

11. RNA interference in vitro

11-1. siRNA design

21 nucleotide siRNA (small interfering RNA) were designed for En-1, lamin A/C, Pbx-1, in order to contain uridine residues and 2-nucleotide 3’overhang (Elbashir et al., 2002). The specific sequence within the coding region were produced and used for
siRNA interference: si En1 5’-CAUCCUAAGGCCGAUUUUCTT-3’ and antisense 5’-G A A A U C G G G C C U U A G G A U G T T -3’, si scrambled 5’-CAGTCGCGTTTGCGACTGG-3’ sense and 5’-CCAGTCGCAAACGCGACTG-3’ antisense. The oligos, except for the scrambled oligos, were pursued by mwg-biotech, International as 2’-acetylated single strand (option A2). Deprotection was performed at arrival using 400µl deprotection buffer provided. The oligos were first incubated at 60°C for 45 min and then 30 min at room temperature. Annealing was performed adding 40µl 10mM ammonium acetate (enclosed) and 1.5 ml ethanol to the 400µl siRNA duplex solution, then vortexed. The RNA duplex was then placed at −20°C for more than 16 hours or at −70°C for 2 hours. The duplex was centrifuged at maximum speed for 30 min at 4°C, the supernatant pipetted away and the pellet rinsed with 95% cold ethanol. The pellet is dried in a speed vacuum and resuspended at the appropriate concentration in 1x annealing buffer provided. The solution can be stored frozen in aliquots at −20°C.

11-2. siRNA transfection

At the third day of culture of En2-/- ventral midbrain, siRNA oligos transfection was performed using Transmessenger transfection reagent (Qiagen, Germany). siRNAs (0.3 µg per well) were condensed with Enhancer R, and complexed with 1.5 µl of Transmessenger reagent, according to the manufacturer instruction. The transfection complex was diluted into 400 µl of N2Medium, and added to the cells. 2hours post transfection the medium was replaced with fresh N2Medium. Cells were stained following a time course after 1, 2, 3, 4, 5 days.

12. Caspase inhibition

En1-/- En2-/- and control littermate ventral midbrain were separately dissociated and plated on to PORN/laminin precoated coverslips, cultured for 6 hours into DMEM-F12 supplemented with 10x N2 (Gibco, Germany), 33mM glucose (Sigma, Germany), 50U/ml penicillin and 50µg/ml streptomycin (Cambrex, Belgium), in order to allow primary processing and conditioning of the cells. Medium was then changed to fresh serum free medium-DMEM-F12 supplemented with 1xN2 (Gibco), containing 300 µM zVAD-fmk (Calbiochem, USA) dissolved in DMSO (Sigma,
Germany), or an equivalent volume of DMSO for control cultures. Cells were fixed 72 hours after plating, and stained for TH and DAPI and cells counted.

13. Real time PCR on E12.5 and E14.5 ventral midbrain tissue

13-1. RNA isolation

The ventral midbrain from En-1/2/- E12.5 embryo and control littermate were first dissected in fresh L15 medium (Cambrex, Belgium) taking care of removing mesodermal tissue and meninges, and placed in RNA later solution (Qiagen, Germany), at 4°C overnight, and the next day at –20°C for longer storage. At the time of RNA extraction all the tissue pieces from the double mutant and control littermate were collected, RNA later was sucked out, and lysis buffer provided with the RNA extraction Kit (Qiagen, Germany) was applied. Each sample was passed through a Qiashredder (Qiagen, Germany) and applied on an RNAeasy column (Qiagen, Germany). The RNA was then treated with with RNase free DNase (Invitrogen, Belgium) in order to get rid of DNA traces and repurified on RNeasy column. The average yield of RNA was 1.5µg per ventral midbrain. Aliquots of 3 µg were snap frozen in liquid nitrogen to avoid repeated thawing and freezing.

13-2. Reverse transcription

cDNA was prepared from 3 µg RNA obtained from En1/2/-/-, control littermate ventral midbrain, En1EGFP+dox N2A clone, and En1EGFP-dox N2A clone. First strand cDNA synthesis was performed with random hexamer primer by using Superscript Preamplification System for First Strand cDNA Synthesis (Invitrogen, Belgium) using a thermocycler. Reverse transcription was initiated using 1-5µg of RNA, 50-250 ng of random hexamers, 1mM dNTP, incubated at 65°C for 5 min and then at 4°C for at least 1 min. Thereafter the reaction containing 1x reverse transcription buffer, 2.5mM MgCl₂, 0.01DTT, and 50 U RNaseOUT recombinant ribonuclease inhibitor was added to the solution and incubated at 25°C for 2 min, then the reaction mix was incubated with 50 U of SuperScript™II reverse transcriptase at 25°C for 10 min. The tube was then transferred to 42°C and incubated for 50 min. The reaction was terminated at 70°C for 15 min. Chilled at 4°C. The reaction solution was centrifuged briefly and 50U of RNaseH were added to the tube and incubated for
20 min at 37°C. Reverse transcription efficiency was verified by PCR using primers for β-Actin sense 5´- CAACTGGGACGACATGGAGA-3´ and antisense 5´- TTTCATGGATGCCACAGGAT-3´. PCR parameters: 5 min at 94°C, 36 cycles (45 sec at 94°C, 1 min at 57°C, 1 min at 72°C), 5 min at 72°C, PCR conditions: 2.5 mM MgCl₂.

13-3. Real time PCR

Quantification of the gene target, Trp53 and p75NTR in the sample En1-/-, En-2-/- ventral midbrain, and En1EGFP+dox N2A was performed using the Standard curve method (User Bulletin #2, Applied Biosystem). En-2-/- ventral midbrain and En1EGFP-dox N2A clone were used as calibrator to build up a standard curve, of known step dilution, from which, each quantity of the sample and calibrator are derived. Endogenous control for mouse phosphoglycerokinase 1 (PGK1), and mouse transcription factor IID/TATA-box binding protein (Tbp) were used to normalize quantization of a target mRNA for difference in amount of total RNA added to each reaction. We used ready-to-use 5´fluorogenic nuclease assay for the target genes and endogenous control assay for mouse phosphoglycerokinase 1 (PGK1), and mouse transcription factor IID/TATA-box binding protein (Tbp) (Assays-on-Demand from Applied Biosystem, Germany, www.appliedbiosystem.com). Gene expression quantification was performed using a two-step reverse transcription-polymerase chain reaction (RT-PCR, default PCR parameters were 2 min 50°C, 10 min 95°C 45 cycles (15 sec-95°C, 1 min-60°C), in which PCR step is coupled with the respective 5´fluorogenic nuclease assay, 5ng/µl of cDNA from En1-/-, En-2-/- ventral midbrain, En1EGFP+dox N2A, and calibrator cDNA En-2-/- ventral midbrain, and En1EGFP-dox N2A, were added to a reaction mix specific for each Assay [2x TaqMan Universal PCR Master Mix, No AmpErase UNG, 20X Assays-on-Demand™ Gene Expression Assay Mix (all products by Applied Biosystem)] and RNase-free water to a final volume of 30-50 µl per well for a 96 well format. 30-50µl of each reaction mixture were transferred to well of an optical reaction plate (Applied Biosystem, Germany). Usually duplicates or triplicates of a sample were made. Standard dilution curves with dilution values 1, 0.5, 0.25, 0.125 were set up for both target genes and endogenous control using the calibrator cDNA. The plate was then covered with an
optical adhesive cover, and centrifuged to spin down the content and eliminate any air bubble. The reaction plate was then placed in an ABI prism 7000 sequence detector system (Applied Biosystem, Germany) and the run started. The amplification trend was followed during the run by viewing the amplification plot for the entire plate. Once the run was terminated baseline and threshold were set. Data elaboration was performed as following: the relative quantities of the target gene and the calibrator, calculated both from the standard curve, were always normalized to the respective standard curve derived target quantity of the endogenous control essay. The quantity of the target gene of the sample was then divided by the target quantity of the calibrator, so that all quantities were expressed as n-fold difference relative to the calibrator.

14. Promoter analysis

The mouse p75 promoter sequence was first retrieved from the GeneBank ID by using http://www.ncbi.nlm.nih.gov, copied then to "Human gene sequence retrieval tool " http://siriusb.umdnj.edu in the box "Value" and submitted for the region ranging from –5000bp to +1000; the obtained sequence containing also the promoter region was then analyzed for consensus sequence recognized by transcription factors using the"MatInspectorV2.2" http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl. The same promoter analysis was performed for the human p75 promoter. Strongly conserved sequence between human and mouse >95% were found between –140 and -340bp. In this conserved sequence highly matching consensus sequence for the Drosophila engrailed at –140-180bp and the mammalian En-1 at –250-340bp respectively were localized.

15. Retroviral construction and infection

The mouse En-1 coding region plus the first 19bp of the 3’ untranslated region (Joyner and Martin, 1987; Logan et al., 1992) was subcloned into the Cla12Nco adapter plasmid using appropriate restriction sites within the polylinker. A ClaI fragment was then purified following partial digestion of this vector with ClaI and cloned into the
retroviral vector RCAS (BPA) and RCAN (BPA) generating RCAS En-1, and RCAN En-1, respectively. RCAN (BPA) is a variant of RCAS (BPA) from which the splice acceptor upstream the Clal site has been removed, preventing translation of the inserted gene. It acts as control for unspecific effects resulting from viral infection.

Chick embryo fibroblast and concentrated viral stocks were prepared as previously described by Fakete and Cepko (Fekete and Cepko, 1993). Viral titers ranged from $2 \times 10^8$ to $10^9$. Fertilized Hubbard broiler hen eggs, containing endogenous retrovirus were incubated at $37^\circ$C for 1.5 days. Concentrated viral supernatant was electroporated in ovo by Cairine Logan (Canada) on the top of the neuroepithelium at HH stages 8-10 at the left side using two electrodes (4 mm apart). A pulse of 25 Volts, for 25 msec, was charged 6-10 times with 1 s intervals. Animals were harvested and allowed to develop until a specific stage, at which they were dissected and fixed by immersion with 4%PFA for 6-12hr.

**16. Image processing**

All images were captured with a CoolSnap Photometrics camera through a Zeiss Axiophot, a Leica Macrophot, or a Leica Confocal microscope and processed using Adobe Photoshop 10.
RESULTS
1. Cell autonomous requirement of En1/2 in the midbrain DA neurons

1-1. The midbrain DA neurons are induced in the En-1/2-/-, but fail to survive

En-1 and En-2, are two homeodomain transcription factors, involved early during embryogenesis in regionalization (Hidalgo, 1996; Joyner, 1996), and later in the specification of neuronal phenotypes (Lundell and Hirsh, 1998; Simon et al., 2001). In mammals the engrailed genes are both expressed in a domain spanning the posterior midbrain and anterior hindbrain. The domain involves part of the neural tube generating dorsal structure, such as cerebellum and culliculi, as well as ventral midbrain nuclei including the midbrain dopaminergic neurons. The two transcription factors have overlapping domain and redundant function so that one (En-2) can functionally replace the other (En-1)(Hanks et al., 1998). At birth, both single mutant for En-1 and En-2 have no phenotype in respect to the mDA neurons (Simon et al., 2001) Homologous recombinant mutant mice null for En1 and En2 show a large deletion in the midbrain and anterior hindbrain, the mice die at birth due to the inability to suck and no TH positive are present in the midbrain (Liu and Joyner, 2001a; Simon et al., 2001). It has been demonstrated that albeit the large deletion at the MHB organizer in the En-1-/-, En-2-/-, mDA neurons are formed at E12 and are identified as a TH positive cluster, in the ventral aspect of the mesencephalic flexure (Simon et al., 2001). One notable feature of the mutants is the smaller size of the TH domain and a lack of axonal projection in rostral direction. This is in contrast to control littermate specimen, where the mDA neurons as soon as they are induced they start to elongate their axons towards the basal telencephalon (Fig 1.A,A’).

Two days later, by E14, no TH positive neurons are anymore detected in the En double mutant, while in the wild-type they continue to differentiate, reach the striatum (Fig 1. B,B) and ,there, establish functional connections (Perrone-Capano and Di Porzio, 2000). At P0 the whole midbrain dopaminergic system is missing, whereas the dopaminergic neurons of the dorsal hypothalamic nucleus continue to be present (Simon et al., 2001), further supporting the specific requirement of Engrailed in the mDA neurons.
Fig 1. Developmental disappearance of the midbrain dopaminergic neurons in En1-/- En2-/- double null mutant mice. Comparison of wild-type and engrafted embryos (En1-/-;En2-/-) at E12 and E14. Rostral is to the right. (A) E12 whole mount preparation of isolated neural tube. TH-positive neurons are located in the mesencephalic flexure (arrow) of wild-type and mutant embryo. The TH domain in the mutant is smaller than the wild-type and there are no axonal projections in rostral direction. (B) Midsagittal sections of E14 embryos. In the wild-type, mDA neurons have continued to differentiate and start to form the SNC and VTA (arrow). In the mutant embryos, 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 in the mutant. Scale bars A,B = 200µm.

Fig 2. Developmental expression of β-gal in mDA neurons. Immunohistochemical TH/β-gal labeling on E12 En-1 TLZ+/+, En2-/- (A-C), En-1 TLZ/TLZ, En2-/- (D-F), E14 En-1 TLZ+/+, En2-/- (G-I) coronal sections. At E12 in En-1 TLZ+, En2-/- midbrain only few TH positive cells dorsally located to the ventricle (A) express beta-gal (B), at the same stage En-1 TLZ/TLZ, En2-/- no TH positive cell (D) express β-gal (E). Two days later at E14 in the En-1 TLZ+, En2-/- almost all TH positive cells forming the anlage of the SN and VTA (D) express β-gal (E) with the exception of some TH positive cells located at the midline. Scale bars A-F= 100µm
It has been previously demonstrated that no respecification of the phenotype occurs. No expression of the reporter gene, [β]-gal, which is cloned in the *En-1* locus, and other dopaminergic markers such as c-ret, GDNFRa, ADC, and *Nurr-1* is detectable at P0 in the *En-1 tlZ/tlZ, En2/-* (Simon et al., 2001). Thus, since in the *En* double mutant by E14 no TH expressing cells are found, it means that these cells disappear shortly after induction.

**1-2. Developmental expression of *Engrailed* in the mDA neurons**

*En* expression is developmentally regulated. *En-1* and *En-2* genes start to be expressed at E8 in the anterior mouse neuroectoderm as a mark a band of cells, corresponding to the MHB. Later on development, the postmitotic mDA neurons start to express both transcription factors and maintain their expression through out life (Davis and Joyner, 1988; Gardner and Barald, 1991; Simon et al., 2001). The generation of mDA neurons in the *En-1 -/-, En2/-* suggests that Engrailed is not required for the specification of the precursor cells, but for the later postmitotic fate of the cells. At this point we wanted to understand when *En* start to be expressed in the mDA neurons and if its requirement is consistent with its developmental expression. Since *En-1* null allele was achieved by a “knock-in” strategy in which the first 71 codons were replaced by a *Tau-lacZ* sequence, we tracked the expression of *En* during development by analyzing that of the reporter gene, [β]-galactosidase. E12 *En-1 TLZ/+, En2/-* and *En-1 -/-, En2/-* mice and E14 *En-1 TLZ/+, En2/-* only were analyzed as by E14 that time no mDA neurons are left in the *En-1 -/-, En2/-* mesencephalon. In the hetero at E12.5 only a few, laterally located, mDA neurons, are positive for [β]-galactosidase (Fig 2). It is likely that the *En* expressing neurons are those cells which first exit the cell cycle and while migrating from the ventricle to the their final position (Kawano et al., 1995) switch on *En* (Fig2. A-C). At the same stage in the *En-1 TLZ/TLZ, En2/-* no mDA neuron express [β]-galactosidase, which indicates that these neurons have just become postmitotic, express TH but are still to immature to express *En-1* (Fig 2.D-F). Two days later at E14 in the *En-1 TLZ/+, En2/-* almost all mDA neurons forming the anlagen of the SN and VTA are [β]-galactosidase positive with the exception of some at the midline (Fig 2.G-I) and P0 when all midbrain dopaminergic neurons express the two homeodomain transcription factors as
illustrated in Fig I.10. This confirms that En is not required for the induction of the mDA neurons (Simon et al., 2001) and further suggests that the expression of the En-1 is switched on in the mDA neurons some time after the neurons have become postmitotic. Thus it appears that En-1 requirement sets in progressively overtime in the midbrain tissue and it is essential for the proper maturation and survival of the midbrain dopaminergic neurons, so that, in the En-1 TLZ/TLZ, En2-/-, the neurons fail to survive at the time when this requirement should set in.

1-3. The demise of the midbrain dopaminergic neurons occurs by apoptosis

At this point, we were interested to unravel the mechanism beyond the disappearance of the midbrain dopaminergic neurons in the En-1 TLZ/TLZ, En2-/- where the requirement of En was missing. Recently, growing interest has been cast on the mode of neuronal cell death. Three mode of cell death are distinguished: apoptosis, necrosis, and autophagy. Although still controversial, apoptosis appears to be the cell death mechanism that leads to the progressive loss of mDA neurons in Parkinson’s disease. Thereby we were interested to understand whether in the En double mutant, the mDA neurons were also lost via an apoptotic mechanism, implying that the mDA neurons undergo cell death through a common mechanism upon different insults. At E13, one day before complete disappearance of the mDA neurons in the in the En double mutant embryo, very few TH positive cells are left, and most have retracted axons, are rounded up, and bare typical signs of apoptosis as activated cysteine protease, caspase-3, and a chromatin condensed nucleus (Fig 2.A-C). It is likely that in absence of En, at this point, the mDA neurons are ´improperly geared´ for further maturation and thereby activate a suicide program. Moreover we could observe scavenger cells embracing dieing neurons (Fig3.F), which is in line with the normal cell clearance after an apoptotic event (Yuan et al., 2003). It has been demonstrated that the scavenging occurs also during PD, with detrimental effect for the progression of the disease. In fact, dieing mDA neurons trigger an inflammatory response in the surrounding microglia, the activated microglia, in turn, release cytokines and NO, which makes the remaining cells vulnerable accelerating their demise (Hirsch et al., 2003). This may also be the case for the En-1-/-, En2-2-/- mutant mice (Fig 1.B-B`).
The apoptotic demise of the mDA neurons in the En-1-/-, En-2-/- raises different hypothesis for the requirement of these two transcription factor: I. the midbrain tissue, which in the En double mutant is missing, provides normally trophic support for this neuronal population. II The developing telencephalon, which is not reached by the mDA neurons in the mutant embryo, may provide also cues of trophic support. III. Ablation of the engrailed genes would deprive the mDA neurons of essential components for their maturation and survival. We investigated all three different possibilities.

2. Axonal outgrowth of the mDA neurons in En-1-/-, En-2-/- mice

2-1. Engrailed does not regulate axonal outgrowth of the mDA neurons

The lack of dopaminergic projection in the En-1-/-, En-2-/- mutant embryos the mDA neurons, and rapid cell demise to two days after induction raised the question whether those neurons were not able to reach their target from which they could receive trophic support (Oo et al., 2003). This possibility is though rather improbable, considering that only by E14 the mDA neurons reach the striatum (Perrone-Capano...
and di Porzio, 1996), and mDA neurons are already lost at this age in the En-1-/-, En-2-/- mice. It remained, however, possible that Engrailed was regulating the ability of the mDA neurons to extend an axon and the lack of projections may have deprived the cells of local survival support leading, thus, to neuronal degeneration. As soon as the mDA neurons are induced, they grow out extensions from the cell body by polymerization of tubulin in microtubule, which protrude progressively in rostral direction by responding to short-range guiding molecules, and interact with receptors present on the surface of their growth cone. These local cues are represented by the extracellular matrix protein (ECM) laminin and fibronectin and tenascin (Lander, 1987), permissive and non permissive glycosaminoglycans (Ca- al., 1996; Mace et al., 2002), astrocytes secreted factors (Johansson and Stromberg, 2002), and membrane bound molecules that can be cleaved and act a soluble signal as L1 (Walsh and Doherty, 1997). En is an essential patterning gene for the midbrain-hindbrain region and it possible that ablation of the two transcription factors leads to a reorganization of the midbrain matrix. In such a case, the absence of any axonal outgrowth in the mDA neurons of the En-1-/-, En-2-/- mice may result from the lack of local permissive cues. In addition there is evidence that engrailed is directly involved in axonal pathfinding and guidance of interneurons in vertebrate spinal cord (Saueressig et al., 1999) and Drosophila CNS (Siegl and Jia, 1999). Specifically, in Drosophila, it has been demonstrated that engrailed negatively regulate expression of the cell adhesion molecules, Connectin and Neuroglian, in interneurons. If this occurs also in mammals it is likely that En is responsible for the ability of the mDA neurons to extend an axon. In order to investigate whether En genes are essential for the axonal outgrowth of mDA neurons, we cultured mutant ventral midbrain onto different permissive substrate such as 3-D collagen matrix, glass coverslips coated with laminin or membrane vesicles derived from E12 wild-type midbrain (Wizenmann et al., 1993). In all three conditions, the mutant TH positive had developed processes, with a similar cytoskeletal pattern to the littermate controls (Fig. 4.A-C, A’C’). Moreover the En-1-/-, En-2-/- mDA neurons, cultured on PORN/laminin coated coverslips, showed a normal pattern for β;γ-tubulin (Fig 5.D-F) in comparison to the control littermate culture (Fig 5.A-C), suggesting thereby a normal cytoskeletal organization of the En double mutant dopaminergic fibers.
Fig 4. *En-1-/− En2-/−* mDA neurons have the ability to project an axon if cultured on permissive substrate. Dissociated E12 ventral midbrain of control (mixture of *En1+/-, En2-/−* or *En2-/−*) and En double mutant in 3D collagen matrix (A), on cover slips 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 24h *in vitro*, dissociated *En* double mutant mDA neurons (A-B) are viable and extend axonal processes similarly to the control culture (A’-C’). (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, since axonal outgrowth varied between 0.3 and 78µm.

Fig 5. *En-1-/− En2-/−* mDA neurons have a pattern of α:β-tubulin expression comparable to the control littermate DA neurons. Confocal images of dissociated E12 of control littermate (mixture of *En1+/-, En2-/−* or *En2-/−*) (A-C) and *En1-/−, En2-/−* ventral midbrain (D-F) plated on polyornithine/laminin coated coverslips after 1 day in culture, stained against TH and α:β-tubulin. The TH positive fibers of the mDA neurons, from both Control (A) and *En* double mutant (D) ventral midbrain cultures, show a comparable staining for α:β-tubulin (B, E respectively) indicating that both cultured mDA neurons are capable of active microtubule polymerization in their processes extensions. Scale bar= 63µM
The extension of the mDA neurons were measured on captured layers of En-1/-, En-2/- and control littermate ventral midbrain cultured on PORN/laminin coated coverslips, since a bi-dimensional view was more appropriate for the measurement. The axonal extension resulted of the same length of the controls littermate (Fig 4.D). Despite this primary differentiation in vitro, under all conditions, after 72 hours the En-1/-, En-2/- mDA neurons were lost completely whereas control littermate mDA neurons were still viable and continued to differentiate in vitro (data not shown).

![Fig 6. Time course of cell death for En1/2 double mutant mDA neurons in culture. Average number of TH positive](image)

By tracking the number of surviving cells over time, we established that the En double mutant cells were starting to disappear 24 after plating with a delay in comparison to the in vivo condition, probably due to a survival supporting activity of the extracellular matrix (ECM) protein (Pixley and Cotman, 1986). The highest loss was then detectable between the 24- 48 hours of culture (Fig 6). These findings demonstrate that the lack of DA axonal outgrowth in En double mutants is not due to the inability of the dopaminergic neurons to extent an axon and that the mutant mDA neurons are viable and differentiate normally in the first 24 hours of culture. However, shortly after a requirement for the En genes sets in, the cells are lost. From the present results it is more likely that these transcription factors are involved in the patterning of the midbrain substratum, where normally permissive clues to axonal extension resides. Studies in this direction are going on and aim to
unravel the composition of the midbrain glia in the En double mutants, which in the wild-type midbrain represent the first instructury clue for the axonal outgrowth.

2-2. L1 expression is not under Engrailed regulation

Along with the studies aimed to understand the nature of the non-permissivity of the midbrain in the En-1-/-, En-2-/-, we investigate L1 expression. L1 has multiple roles in axonal growth and guidance (Walsh and Doherty, 1997). It is well known that L1 is responsible for migration of the mDA neurons along the mesencephalic radial glia (Ohyama et al., 1998), in fact L1-/- mutant mice presents an abnormal distribution of midbrain dopaminergic neurons of the SN and VTA moreover the axons projecting from the substantia nigra to the caudate putamen also exhibited an abnormal targeting pattern (Demyanenko et al., 2001). In contrast Ohyama and colleagues report that L1 expression was detected only on the tangential fibers along which the mDA neurons migrate. However there is evidence that L1 is expressed from E12 on the mDA neurons and it acts as a survival-promoting factor for this cells (Hulley et al., 1998). Furthermore Neuroglian, a member of the immunoglobulin superfamily closely related to the mouse adhesion molecule L1 (Bieber et al., 1989), is transcriptionally repressed by engrailed in Drosophila, and that L1 promoter contains a consensus DNA with high affinity for Hox 1.3, bicoid and engrailed, and that En-1 is active on the L1 promoter in vitro (Kohl et al., 1992).

In light of these results, we hypothesized that engrailed could regulate axonal outgrowth of the postmitotic mDA neurons in rostral direction by repressing expression of the adhesion molecule L-1. If En would acts as a repressor of L1 expression, it should be overexpressed in the En-1-/-, En-2-/- . It could be thus speculated that perturbed homophilic and or heterophilic interactions of this protein with resident neurons or glia are established (Friedlander et al., 1994; Maurel et al., 1994). This might hinder thereby the normal outgrowth of the neurites in the mesencephalic environment.

By culturing mutant and wild type littermate ventral midbrain on the permissive substrate PORN/laminin, we observed that mDA neurons from both phenotypes were expressing L1 with a similar pattern (Fig 7).
The adhesion molecule on the mDA neurons is distributed along the cell membrane of the cell body contours and along the axons length (arrows). This is in line with the role of this adhesion molecule playing in cell-to-cell interaction and promoting and driving axonal growth during development (Walsh and Doherty, 1997).

Thus, no differential L1 expression between the En-1-/-, En-2-/- and control mDA neurons would speak against any direct involvement of L1 in the axonal impairment and would argue that En is not acting as a transcriptional repressor of L1 expression in mammals.

2-3. Differentiation of En-1-/-, En2-2- /- mDA neurons in vitro does not prevent these cell to die by caspase-mediated apoptosis.

As shown above in the En-1-/-, En2-2- /- mDA neurons are viable by E12 and differentiate normally, until a requirement for the En genes sets in, which then
becomes essential for their survival. In light of the evidence that in the En1/2 double mutant programmed cell death is switched on in the mDA neurons some time after induction, we wanted to confirm the same mode of cell death was also taking place in vitro, sustaining the reliability of our cell culture strategy.

We chose the time range when the highest rate of cell demise was occurring (Fig 6) and investigated for typical hallmarks of apoptosis. 48 hr after culture, we saw that in the En-1-/-, En-2-/- ventral midbrain culture a consistent number of TH positive cells had retracted their processes, rolled up, presented a chromatin condensed nucleus and were positive for activated caspase-3, one of the effectors enzymes in apoptosis (Fig 8.A-C´). Thus, En-1-/-, En-2-/- dopaminergic neurons showed morphological hallmark of apoptosis, also when placed into culture, providing evidence that a common mechanism of cell death accounts for the demise this neuronal population both in vivo and in vitro.

3. Engrailed is not directly involved in triggering apoptosis

At this point we wanted to understand if En was directly involved in the apoptotic cascade, by sensing for example intracellular signal that induce apoptosis. In such a case, according to our speculation, En silencing would derepress transcription of key molecules involved in the apoptotic cascade, causing eventually the demise of mDA neurons where it is normally expressed. Studies using neurotoxin as MPTP, 6-OHDA, or Rotenone (Ungerstedt et al., 1974; Langston et al., 1983; Betarbet et al., 2000) show a specific ablation of the mDA neurons in cultures, and recapitulate the death process normally occurring in Parkinson’s disease. Moreover neurotoxin treatment is associated with active transcriptional regulation driven by activation of the

Fig 8. En-1-/-, En-2-/- mDA neurons disappear by apoptosis after two days in culture. Cell culture at about 48 hours post dissociation stained against TH (A) and activated caspase-3 (B). Each sample was counterstained with DAPI (C) to identify cell nucleus. Mutant mDA neurons (arrows) retracted their processes, rounded up and are positive for activated caspase-3. An additional sign for apoptosis is the pyknotic nuclei (magnified C´). Scale bar= 64.
transcription factor NF-kB (Cassarino et al., 2000; Blum et al., 2001; Ryu et al., 2002) and c-Jun (Nishi, 1997). The time course of midbrain dopaminergic demise after MPP+, 6-OHDA, and Rotenone application resembles the one of the En-1-/-, En-2-/- mDA neurons, in fact the signs of active apoptotic process such as axon retraction, rounding up of the cell, activation signs of active apoptotic process such as axon retraction, rounding up of the cell, activation of the effector caspase-3 and chromatin condensation occur already within 24 hour after treatment. This homology in the timing and mode of programmed cell death lead us to the hypothesis that En could act as transducer and regulate transcription of a variety of genes involved in cell fate. So that ablation or silencing of these genes would lead to an apoptotic choice for the cell. We used all three neurotoxin in culture following the standard operating conditions. E12 wild-type ventral midbrain were dissected, dissociated and cultured for 3-5 days before neurointoxication. 24 hours after treatment 50% of the mDA neurons had undergone apoptosis process in line with what previously reported (Mochizuki et al., 1994), in addition all the phenotypical marker En, Nurr-1 (Backman et al., 1999), Pbx-1 (Sgado´ and Simon, unpublished data), Ptx-3 (Smidt et al., 1997) and Lmx-1b (Burbach et al., 2003) were downregulated in those cells after treatment with MPTP, 6-OHDA, or Rotenone. As an example we report here the effect of MPP+ intoxication on the expression of transcription factor such as En, Nurr-1, Pbx-1, which are constitutively expressed by the mDA neurons 24 hours after intoxication. mDA neurons showed typical signs of apoptosis and lost expression of all three phenotypical markers: En-1 (Fig 9.A-D´), Nurr-1 (Fig 9.E-H´), Pbx-1 (Fig 9.I-L´). Moreover by fixing the cells at time points before the 24 and 48 hours we were able to see a progressive loss of the marker in parallel with the processing of the PCD (data not shown). This suggest that En is not specifically sensing for the insult, but that neurointoxication profoundly impairs viability of the mDA neurons so that these cells generally switch off transcription factors expression as a consequence of cellular damage.
Fig 9. MPP+ treatment leads to general downregulation of the phenotypical markers *En*, *Nurr-1* and *Pbx-1* in mDA neurons and eventually cell death. Immunohistochemistry using Antibody against TH (A,A’,E,E’, I,I’), En (B,B’), Nurr-1(F,F’), Pbx-1(J,J’) and DAPI(C,C’,G,G’,M,M’). 48 hours after MPP+ intoxication TH positive cells (A’,E’,I’) indicated by the arrows have retracted their axons, downregulated all three phenotypical markers: En (B’), Nurr-1(F’), Pbx-1 (J’) and bare condensed nuclei (C’,G’,K’). TH positive cells from control culture (A,E,I) normally express En (B), Nurr-1 (F), Pbx-1 (J). Scale bar= 64µm
4. The midbrain DA neurons require *En-1* and *En-2* cell autonomously for their survival

The specific loss of midbrain dopaminergic neurons in the *En-1* and *En-2* double knockout (1-1) at a time when *En* expression sets in the mDA neurons (1-2) suggested that these transcription factors are essential for the survival of this neuronal population. However, since *En* is required for the normal development of the mesencephalic region (Wassef and Joyner, 1997), which is apparent in *En-1/2* double null mutant by the absence of the midbrain and anterior hindbrain (Liu and Joyner, 2001b), it remained open whether the midbrain environment would have any survival promoting activity on these neurons. In case of a non cell-autonomous requirement of the *En* genes, the midbrain depletion would deprive the dopaminergic neurons of trophic support committing them to cell death, observable in the *En-1/-, En2-/-* embryo. In turn, a cell autonomous requirement of the *En* genes implied that these transcription factors, themselves, support cell survival specifically in this neuronal population, notwithstanding the environment. The cell autonomous requirement is supported, in first line, by the evidence that *En-1* and *En-2* act in a gene dose effect so that the presence of one allele for *En-2* on a *En1/-* background leads to an intermediate phenotype in respect to the mDA neurons between *En1/-* and *En-1/-, En2-/-* (Simon et al., 2001).

In order to address this question we tried both a in vivo and `in vitro` approach.

4-1. *En-1/-, En-2-/-* chimeric mouse

We first designed a chimera mouse by injecting *En* double mutant stem cells into a wild-type blastula in order to achieve an `in vivo´ mixed population of *En-1/-, En-2-/-* and wild-type cell. The animals were viable, fertile, behaved normally, and lived up to adulthood. However at a closer sight the amount of midbrain DA neurons in the SN and VTA were reduced, consistently with the degree of chimerism (Thuret S. Ph.D Thesis, 2002). In order to expand the number of animal and confirm this first result obtained, we tried to derive stem cells from blastocysts of all phenotypes: *En-1/-, En-2-/-, En-1+/-, En-2-/-* and *En-2-/-*. The embryos were obtained from mating between *En-1+/En2-/-* and *En2-/-* mice. Thirty E3.5 blastocysts were placed onto
feeder layers of mouse embryonic fibroblast, and allowed to grow until an internal cell mass (ICM) had formed. More than 30% of the blastocysts gave rise to ICMs, which were then removed, carefully disaggregated to single cells and replated onto fresh feeder layers to allow formation of cell colonies. Unfortunately all the ICM-derived colonies after 4 days of culture were differentiated, which can be explained by the C57BL/6 background. Notoriously C57BL/6 ES cells are more difficult to maintain in culture since they tend to differentiate and easily loose their ability to colonize the germline (Auerbach et al., 2000; Schuster-Gossler et al., 2001). Thus, it is a standard procedure by deriving ES cells to superovulate the mother, which increases by 5 to 7 folds the number of blastocysts available. Moreover in order to restrict C57BL/6 ES cells differentiation it may have been appropriate in our case to use an inhibitor for ERK signaling, PD098059, which has previously been used to inhibit differentiation and enhance self renewal of stem cells (Burdon et al., 1999). ERKs are known to be involved in S/G1 transition and in part through the induction of cyclin D (Lavoie et al., 1996; Weber et al., 1997) and differentiation of ES cells is associated with G1 cyclin expression (Savatier et al., 1996), therefore inhibiting the transition to G1 would maintain the C57BL/6 ES cells in an undifferentiated stage, thereby increasing the yield of stem cell colonies.

In order to overcome all the evident disadvantages encountered in raising C57BL/6 stem cells, we are now bringing the En-1-/-En2-/- onto a 129 background. Having En-1-/-, En-2-/- 129 ES cells available would be a valuable tool for further genetic manipulation such as overexpression studies i.e. Bcl-2 (Martinou et al., 1994)(Martinou et al., 1994), or dopaminergic differentiation strategies (Hynes and Rosenthal, 2000; Kim et al., 2002).

4-2. Chimeric En-1-/-, En-2-/- ventral midbrain culture

In parallel to our stem cell derivation attempt we set up a “chimeric system” in cell culture by culturing dissociated En-2-/- and En-1-/-, En-2-/- mutant ventral midbrain together with wild-type ventral midbrain. To distinguish between the mutant and control wild-type midbrain dopaminergic neurons we injected the pregnant En-1+/-, En-2-/- mice with 5-bromodeoxyuridine (BrdU), and derived wild-type ventral midbrain tissue from unlabelled NMR2 embryos. BrdU is a thymidine analogue and is
incorporated into newly synthesized strand of DNA (Boccadoro et al., 1986). This compound is commonly used as kinetic marker for cell proliferation, and visualized by counterstaining using specific antibodies (Gratzner, 1982; Boccadoro et al., 1986). In order to obtain an homogenous BrdU labeling of the mesencephalic tissue we pulse injected En-1+-/-, En-2-/- pregnant females from E10-E12, at the time when the last mitotic event is occurring in the mDA neurons, until more than 90% of the cells were labeled (Fig 12. A-C). The ventral midbrain from the BrdU and untreated E12 embryos were dissected, En-1-/-, En-2-/- mutant, control littermate, and untreated control ventral midbrain were dissociated separately to single cell suspension, and counted. 150.000 cells were plated per coverslip as homogenetic culture, BrdU labeled En-1-/-, En-2-/- ventral midbrain alone, or heterogenetic culture, a mixture of BrdU labeled En-1-/-, En-2-/- and wild type untreated ventral midbrain (50: 50) (Fig 10).

Fig 10. Cartoon depicting co-culture experiment of En-1-/- En2-/- mutant cells (green) mixed with wild-type (pink): ventral midbrain containing the dopaminergic neurons (red) was dissected from En-1-/- En2-/- mutant and wild-type E12 embryos. The tissue pieces were trypsinated, the cells dissociated, their numbers counted, then plated on polyornithin/ laminin coated cover slips and cultured for up to 72 hour. The experiments were done with wild-type, mutant or a mixture of both. We determine the number of tyrosine hydroxylase positive cells at 1 hour, 24 hours and after 72 hours. Mutant mDA neurons fail to survive after three days in culture. Mutant cells were labeled by twice daily interperitoneal injections of BrdU from E9.5 –E12 to differentiate them from wild-type cells.

In both heterogenetic and isogenetic culture the En-1-/-, En-2-/- mutant DA neurons, expressing TH and BrdU, start to die with a delay of 24 hours of culture, since plated on PORN/laminin substrate, and entirely lost at 72 hours (Fig 11.D-F). The viability of the DA neurons in the BrdU labeled littermate controls remained, in contrast, almost unchanged, showing that the BrdU labeling had no unspecific toxic effect on mDA neurons (Fig 12).
The two chimeric experiments, "in vivo" and "in vitro", reveal that no environmental factor can rescue this cell population when ablated of En expression and strongly support that En-1 and En-2 are cell autonomously required in the midbrain DA neurons, and that the onset of En expression is essential for the proper maturation and maintenance of this neuronal population.

Fig 11. En1-/-, En2-/- mDA neurons are lost both in heterotypic, and homotypic cultures after 3 d.i.v. (A-C) 1 hour after plating, almost all cells, including TH-positive neurons (arrow) derived from En1/2-/- mutants were positive for BrdU (Red), after multiple intraperitoneal BrdU injections (C) Example of double labeled (TH=green, BrdU=red). (D-F) After three days in the mixture of En double mutant and control littermate ventral midbrain, only single labeled TH neurons and no TH-positive, BrdU-labeled cells were present in culture. Scale bars = 20µm

Fig 12. Quantification of En1-/-, En2-/- mDA neurons demise in heterotypic, and homotypic cultures. Numerical charts show the average number of TH positive cells after 24 hr hr and 72 hr in vitro: Control, mixture of heterozygote (En1+/--;En2-/-) and homozygote (En2-/-) BrdU labeled cells. In control case, number of mDA neurons decreased only slightly between 24 hours and 72 hours. Regardless, whether mutant En1-/-;En2-/- cells were mixed with wild-type or cultured on their own, the numbers of TH-positive cells derived from engrailed double mutants decreased almost to zero after 72h from a baseline of in average 45 TH-positive mutant cells at 24h. n≥12 for each bar, (Student’s t-Test, p<0.001)
5. RNA interference of En-1 leads to disappearance of the mDA neurons with En2/- background

Despite the initial differentiation of mDA neurons from En-1/- En2/- mutant E12 mice, when placed into culture, it remained open whether those cells were already committed to cell death before the tissue was dissociated, in that case the selective loss of the mutant mDA neurons in the mixture experiment would therefore reflect this early commitment.

To address this possibility, we took advantage of RNA interference (RNAi), which allows post-transcriptional gene silencing both in vivo and in vitro. This biological mechanism was first discovered in the nematode worm Caenorhabditis elegans (Fire et al., 1998) but is present in many other organisms such as Drosophila, plants and protozoans (Hammond et al., 2001). The proposed mechanism involves cleavage of dsRNAs by ribonuclease into 21-22 nucleotide RNA duplexes or small interfering RNAs (siRNA). These molecules trigger degradation of the cognate mRNA (Zamore et al., 2000; Bernstein et al., 2001). Recent experiment have shown that the application of small interfering RNA duplexes (siRNA), of 21 nucleotides in length, leads to specific mRNA degradation in mammalian cell lines (Elbashir et al., 2001) and primary cell culture (Krichevsky and Kosik, 2002). Applying the rules proposed by Elbashir S.M. (Elbashir et al., 2002) we constructed 21 nucleotides primers for En-1, and control oligos, such as lamin A/C, a nuclear protein often used as a standard control (Elbashir et al., 2001), scrambled oligos, generated by a random process, and Pbx-1, another homeobox transcription factor expressed by the mDA neurons (Sgado P. and Simon H.H. unpublished data). We then transfected the siEn-1, and the control oligos into ventral midbrain culture of homozygous null mutant mice for En-2, since the En-2/- do not exhibit any phenotype in respect to the mDA neurons (Simon et al., 2001). After isolation and dissociation of the E12 ventral midbrain, cells were plated onto PORN/laminin coated coverslips, let for 3 days into culture and then transfected using siRNA oligos duplexes at a concentration of 0.003-0.03µg/ml per well. All oligos were applied at this concentration range in order to avoid any off-target effect (Jackson et al., 2003). At the third day in culture, the mDA neurons show a complex network of neurites and all express En-1 and Pbx-1. Already 12 hours after transfection the first En-1 negative DA neurons were detected, but the number of TH positive cells remained unchanged (Fig 13.B).
The first signs of apoptosis and cell loss were detectable at 24 hours, reaching a peak at 48 hours, when the highest amount of En-1 negative mDA neurons was detected (13% of the control). During the following 48 hours the number of TH positive cells progressively declined to 75% and at 96 hours stabilized, when no more En-1 negative DA neurons were visible (Fig 13.B). In the control transfection no alteration in cell viability was detected (Fig 13.A), and the degree of silencing (12%) of the control marker Pbx-1 resembled the one of En-1 (Fig 15.D). Dieing En-1 negative mDA neurons, in the En-1 interfered En2-/ cultures, showed specific hallmarks of apoptosis, neurites retraction, rounded body, activated caspase-3 and chromatin condensation (Fig 14.A-D; E-H). In contrast all control cultures, mock transfected (Fig 14.A-D), Pbx-1, lamin A/C interfered cultures (Fig 15.A-C) were normal in all aspects we investigated. The experiment shows thereby that switching off En-1 expression in En-2-/ mDA neurons affects deeply the viability of these neurons, committing them to a suicide program.
Fig 14. En-1 siRNA applied to En2-/ ventral midbrain culture impair survival of the mDA neurons inducing them to undergo apoptosis (A-D) Immunohistochemistry on En1 transfected cultures after 48 hours. The transfected TH positive cell (arrow) was round, had no processes, was En1 negative (red) and possessed a fragmented, pyknotic nucleus (DAPI). (A’-D’) The mock-transfected control cells (double arrow head) maintained their neurites and were En1-positive. (D) Magnification of DAPI staining in C. (E-H) Confocal images of En1 transfected culture. Three TH-positive neurons are present in this field; one (arrow) had a rounded cell body, was En1-negative and stained positive for activated caspase-3 (blue). Two others (double arrowhead) had spindle shaped cell bodies with attached neurites, were En1-positive and caspase-3 negative. Scale bar A-H = 20µm, D = 10µm

Fig 15. RNA i using a siPbx ablates specific genes expression but is dispensable for survival of the mDA neurons. (A-B) 48 hours after transfection with Pbx1 specific RNA duplexes, Pbx1 expression is silenced in some mDA neurons (arrow) but is preserved in the majority (double arrow head). Regardless of Pbx1 expression, the cells maintained their neurites and the shapes of their cell bodies. (D) Data graph showing the degree of silencing 48 hours after transfection with Pbx1 and En1 specific RNA oligos. A comparable proportion of TH positive cells, 12%, are negative for Pbx1 and En1, respectively. The number of TH-positive cells is , although, only reduced after siEn1 transfection. Scale bar A-C = 20µm.
This resembles the cell fate of the En-1/- En2/- mDA neurons and indicates that the lack of any rescue effect of the wild-type environment on the En-1/2 mutant mDA neurons in the cell mixing experiment it is not an artifact of a previous commitment of this neuronal population.

The result from the RNAi experiment, thereby, provides further evidence of the cell autonomous requirement of the En transcription factor in the survival of midbrain DA neurons.

6. Rescue of double mutant DA neurons by caspase inhibition

As from our "in vitro" and "in vivo" studies it was evident that the mDA neurons demise is occurring via apoptosis, interference with this pathway could result in a rescue of the mDA neurons. Previously, it has been reported that synthetic caspase inhibitors, such as Boc-(Asp)-fluoromethylketone(BAF) under 6-OHDA intoxication (Lotharius et al., 1999), and N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk,) under MPP+ intoxication (Bilsland et al., 2002) had a protective effect on mDA neurons in culture by preventing cell death (50-70% recovery in both cases). Despite the significant recovery of mDA neurons viability, only partial restoration of neural projection, and [3H]DA uptake (Dodel et al., 1998; Bilsland et al., 2002) was observed. The rescue effect of the caspase inhibitors remains yet contradictory as Hartmann et al. (2001) report that addition of zVAD-fmk to MPP+ treated cultures potentiate the toxicity against mDA neurons by increasing necrosis.

We used the caspase inhibitor, zVAD-fmk, in the concentration where more than 50% recovery was observed, and applied it to the double mutant ventral midbrain culture 6 hr after plating, and let it for 72 hr in culture, at the point when cultures were fixed. From our experiment no recovery of mDA neurons in the En-1/-, En-2/- ventral midbrain culture could be detected (Fig 16), moreover we observed that in our treated control culture, the inhibitor zVAD-fmk exerted a toxic effect on the viability of the mDA neurons, confirming the toxic effect of the general caspase inhibitor observed by Hartmann et al, 2001.
In turn, the application of DMSO alone was able to slightly rescue the double mutant mDA neurons, whereas control culture remained unchanged. This can be explained alteration in the Ca\textsuperscript{2+} intracellular stores, as a result of membrane solubilization by DMSO, which can lead to temporary enhanced survival (Orrenius et al., 2003). The result has to be confirmed by applying depolarizing conditions (Yu and Chuang, 1997) that enhance the intracellular Ca\textsuperscript{2+} content that would reveal an active involvement of Ca\textsuperscript{2+} in the survival/death program in this system.

7. En-1 regulates p75 transcription

7-1. En-1 is transcriptionally repressing p75 expression

A tetracycline inducible cell line overexpressing En-1 has been established in the lab. Gene expression profiling was performed using the cell line upon induction with Doxycycline, and as a control the same non-induced cell clone. Microarray analysis from En-1 overexpression in a Neuro2A Tet-on cell lines, revealed that Engrailed is regulating p53 and p75. Interestingly enough both molecules are involved directly in neuronal cell death. p53 is a cell sensor component activated by the nuclear enzyme poly (ADP-ribose) polymerase (PARP) following DNA damage or ROS release. MPTP intoxicated mice show a strong poly ADP-ribosylation of p53 from PARP in the brain (Mandir et al., 2002), and there is evidence that p53 protein is necessary for
complete expression of MPTP neurotoxicity to dopamine neurons since p53-/ null mice result partially resistant to MPTP intoxication (Trimmer et al., 1996). p75NTR, on the other hand, is the low-affinity neurotrophin receptor. Recent evidence suggested a role for p75(NGFR) in developmentally regulated neuronal cell death of sympathetic neurons at the time when the ratio of p75 and TrkA is in favor of p75, and neurotrophin dependence is higher. Moreover Schwann cell isolated from p75-/- null mice (Syroid et al., 2000) or sensory neurons and hippocampal neurons treated with p75 blocking antibodies are resistant to NGF mediated apoptosis (Davey and Davies, 1998; Friedman, 2000). More recently it was reported, antibody directed against the extracellular domain of murine p75(NTR), activate the receptor and profoundly effects the survival of short-term cultures of sympathetic neurons (Freidin, 2001).

In light of the results emerging from the Microarray analysis and from the cell autonomous requirement for En in the mDA neurons, we wanted to know if in the En-1-/-, En-2-/- double null mice upregulation of these proteins occurs. For this purpose, we performed quantitative expression analysis by Real-Time PCR, using Assays on Demand (Applied Biosystem) for the murine p53 and p75, on embryonic ventral midbrain from En-1-/-, En-2-/- mice and control littermate, and as a control the cell line overexpressing En1 with or without doxicycline induction. Since no FACS sorter, to select mDA neurons, is available in our lab, and it appears that the total yield of neurons from embryonic tissue is rather poor (Barrett et al., 2001) we carefully dissected E12 ventral midbrain in a butterfly-like shape, where mDA neurons are located, and extracted RNA from the tissue pieces. We quantitatively analyzed the gene expression in the tissue at E12.5 at the time when the mDA neurons are present. From the quantitative analysis it appears that p53 expression remains unchanged either between the two engrailed phenotypes or the two cell lines (data not shown). p75(NTR) expression, in turn, results by three fold increased in the En-1-/-, En-2-/- mice is in respect to the control (mixed population of E12 En-1+/-, En-2-/- and En-2-/- ventral midbrain) On the other side, as it would be expected, p75 expression is consistent downregulated in the inducted cell line overexpressing En-1 in comparison to the non-induced control (Fig 17).

These results suggest that engrailed regulates the expression of p75 (NTR) in the midbrain, where the transcription factor is constitutively expressed, and that the
ablation of this transcription factor may lead to a receptor overexpression. Overexpression of p75 may, thus, override the balanced ratio between low affinity receptor to the high affinity receptor Trk, thereby rapidly triggering either neurotrophin-induced apoptosis, or stress-induced cell death (Barrett, 2000) in the neurons located in the midbrain including the dopaminergic neurons.

7-2. The p75(NTR) promoter contains consensus sequence for the homeodomain transcription factor engrailed

Another strong evidence for the regulation of p75 (NTR) by En-1 arise from the BLAST search and Mat-Inspector V2.2 analysis for high affinity binding sequence for the homeodomain transcription factor En, consensus sequence, in the mouse and human p75 promoter region. The analysis revealed that the p75 promoter contains two highly matching consensus sequences for the homeodomain transcription factor between −140-180bp and −250-340bp from the start codon for the Drosophila engrailed and the mammalian En, respectively (Fig 18), and that this region is strongly conserved between the two species (>98% homology). This suggests that En constitutively regulates the expression of p75(NTR) by transcriptionally repressing its expression. This is consistent with the classical role of Engrailed as a transcriptional repressor, as previously shown, for adhesion molecule connectin and neuroglian in spinal interneurons in Drosophila (Siegler and Jia, 1999) and diencephalic gene Pax-6 in mammals (Araki and Nakamura, 1999).
In addition, taking into account that the mode of and timing of cell death recapitulates the one observed in p75 mediated demise of sympathetic neurons (Freidin, 2001) and sensory neurons (Majdan et al., 1997). The present result further proposes that indeed in En-1-/-, En-2-/- over-expression of the p75 receptor may mediate apoptosis in the midbrain dopaminergic neurons.

8. En-1 overexpression in the chick

As previously demonstrated, the domain of the midbrain dopaminergic neurons in the En-1-/, En-2-/- E12 embryo is significantly smaller than the control littermate (Simon et al., 2001). This reduction could be explained by a regionalization deficit occurring in the En1/2-/- double null mutant embryo (Liu and Joyner, 2001b). Since one of the En function is to maintain midbrain identity, as shown, by negatively regulating repressors of mesencephalon-related genes (Wurst et al., 1994; Araki and Nakamura, 1999; Scholpp and Brand, 2001). Alternatively, it is possible that the number of midbrain dopaminergic precursors formed in the En-1-/-, En-2-/- embryo is comparable to the wild-type, but individual cells at different stages of maturity are observed, suggesting that the cell autonomous requirement for En may interest only a subset of the same population, differentiating progressively overtime. This is in line with the differentiation time course of mDA neurons in the mice, where the first postmitotic cells are detectable by E10 (Kawano et al., 1995), and fits with the progressive onset of En expression in the midbrain (Fig 8). Thus, when the first cell disappear in the midbrain of the En-1-/, En-2-/-, other cells are either just been born, or in an earlier differentiation stage when En is not yet required. To differentiate

Fig 18. The p75 promoter contains two highly matching consensus sequence for Engrailed. Schematic representation of the Mat-Inspector analysis of promoter region for the mouse and human p75 (98% homology within the two) shows that between −140-180 and −250-340 there are respectively two highly matching binding site for the Drosophila engrailed, and the mammalian En-1, respectively. Revealing a direct transcriptional regulation of the homeodomain transcription factor on p75 expression.
between these two possibilities a gain of function experiment on the chick embryo was performed. If the En genes determine the size of the competent mesencephalic tissue that can adopt a dopaminergic phenotype, by enlarging the expression domain of En-1 an increase in number of precursor cells and subsequently of mDA neurons should be observed. The En genes specify the midbrain and anterior hindbrain, from around stage 8 to 11 in the chick embryo (Araki and Nakamura, 1999). Chick embryos of this age were electroporated on one site using a non-competent retroviral vector RCASEn1 envA. This construct contains a mouse En-1 c-DNA (Joyner and Martin, 1987; Logan et al., 1992) flanked by two LTR sites of the RCASBP(A) (Hughes et al., 1987). To ensure that the infection of RCASBP does not induce, itself, any transformation in the chick brain, a mock electroporation was performed using RCANEn1 (Logan et al., 1996), this retroviral isoform lacks the splice acceptor site required for proper translation of En protein. The plasmid (RCASEn1) was electroporated in the developing neural tube at one side at Hamburger Hamilton (HH) stage 8-10 in ovo (Logan et al., 1996). This stage correspond to the mouse E8. At this time the progenitor cells are still uncommitted to a specific phenotype, and En is transiently overexpressed in a region where it is, normally, absent (Ye et al., 1998). En-1 overexpression was detected by whole mount RNA in situ hybridization, and distinguished from endogenous En-1 expression using a mouse specific probe. 2 RCASEn1 envA electroporated, HHS 8-10, chick embryos showed a strong staining for the mouse En-1 transcript, whereas the 2 mock electroporated controls showed no staining at all (data not shown).

8 RCASEn1 electroporated, E7, chick specimens, and 1 mock control, were analyzed. 7 of the RCASEn1 electroporated chicken showed no alteration in the amount of mDA neurons in respect to the mock control and unelectroporated side. Only in one single brain we see a decrease in the total amount of TH positive cells, accompanied by a tectal shrinkage at the electroporated side (Fig 19.A, A’). This latter example can be interpreted as a developmental impairment of the tissue due to a toxic effect of the plasmid.
Thus, from our results it appears that transient En-1 overexpression acts only as a regionalization factor of the midbrain and it has no influence on the specification of the competent tissue, that will give rise to dopaminergic neurons. It remains however open whether the specification of the dopaminergic phenotype does not require the cooperation of both transcription factors En-1 and En-2.
DISCUSSION
The molecular mechanisms responsible for the maintenance and survival of mDA neurons are just beginning to be unraveled. Here, I demonstrated that \( En \) genes are cell-autonomously required for the survival of mDA neurons and that the ablation of \( En \) expression induces apoptotic cell death. In addition I propose that the mechanism leading to neuronal cell death is due to overexpression of the low affinity neurotrophin receptor and death receptor p75.

**I. mDA neurons undergo apoptosis when ablated of \( En-1 \) and \( En-2 \) expression.**

\( En-1 \) and \( En-2 \) are expressed at the mid-and hindbrain boundary (Joyner, 1996), including the mDA from development onwards (Simon et al., 2001). Ablation of both transcription factor leads to a large deletion of the dorsal midbrain structure including the isthmus, the main source of Fgf-8 in the midbrain (Liu and Joyner, 2001b). Midbrain dopaminergic neurons are induced at the right position, express the postmitotic marker TH (Simon et al., 2001), but two days later, at E14, disappear. Here I demonstrate that \( En \) expression is switched on progressively in the mDA neurons, so that at E12 only few cells express it and by E14 all do. Since in the \( En-1-/- \) \( En-2-/- \) embryo mDA neurons are completely lost at E14, at the time, when \( En \) expression should set in, it is suggestive that \( En \) is essential for the survival and further differentiation of this neuronal population. There are several null mutations of transcription factors that lead to the prenatal loss of mDA neurons. In homologous recombinant mutant mice for \( Nurr-1 \) (Zetterström et al., 1997; Wang et al., 2003), mDA neurons fail to express their neurotransmitter phenotype and begin to disappear at E15 (Wallen et al., 1999). The \( aphakia \) mice, a spontaneous null mutation of Pitx3, (Semina et al., 2000) exhibit a specific loss of nigral DA neurons (Nunes et al., 2003; Van Den Munckhof et al., 2003). In null mutants for \( Lmx1b \), the entire population of mDA neurons is lost by E17 (Smidt et al., 2000). Furthermore, the null mutation for the trophic factor TGF\( \alpha \) (Blum, 1998) leads to a reduction of DA neurons in the SNpc. 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 unrelated. In this context, the \( En \) double mutant phenotype is of particular interest since the mDA neurons disappear as early as E14.5 and the entire dopaminergic population is
affected. En is, therefore, dispensable for the induction of the mDA neurons (Simon et al., 2001), however it seems to indirectly affect the size of the competent progenitor tissue. Indeed the shrinkage of the TH domain observable in the En-1/2 double mutant mice at E12 may result from the narrowing of the Fgf-8 domain to a small patch, located in the dorsal midbrain (Liu and Joyner, 2001b).

Since it has been previously demonstrated that the loss of the TH positive neuron in the En-1 -/- En-2-/- is not a consequence of a respecification of the neurotransmitter phenotype (Simon et al., 2001), the survival impairment of this neuronal population can be addressed to an essential requirement of En for the proper survival and development of these neurons. At this point it was essential to understand what mechanism underlies the dopaminergic cell demise and thereby unravel the molecular cause of the survival failure under En ablation. Three principle mechanisms for neuronal cell death have been described, namely apoptosis, necrosis, and autophagy (Pettmann and Henderson, 1998). Here we demonstrated that only few TH positive cells were left one day after induction (E13) in the midbrain of double null embryo, and that 30% of them bare specific hallmark of apoptosis, such as cell shrinkage, caspase-3 activation, and pyknotic nuclei. Those morphological signs of programmed cell death in mDA neurons of the En-1 -/- En-2-/- mice recapitulate the apoptotic profiles in the mDA neurons of other null mutant mice such as the Nurr1-/- (Saucedo-Cardenas et al., 1998; Wallen et al., 1999), the aphakia (Van Den Munckhof et al., 2003) and Lmx.-1b mice (Smidt et al., 2000). Evidence for programmed cell death involving the mDA neurons has been confirmed in many different experimental models for PD, following injury striatal excitotoxic injury (Macaya et al., 1994) or treatment with specific neurotoxins like MPTP (Tatton and Kish, 1997) and 6-OHDA (He et al., 2000). Apoptosis plays also a role in the regulation of the numbers of mDA neurons during the first 14 days after birth (Jackson-Lewis et al., 2000; Chun et al., 2002), likely reflecting a dependency of neurons on GDNF during this period (Burke et al., 1998; Granholm et al., 2000). Another line of evidence for apoptotic cell death of the mDA neurons comes from the postmortem analysis of brains from PD patients where signs of apoptosis were detected in dopaminergic neurons (Hartmann et al., 2000). Thus programmed cell death is triggered in many different experimental paradigms in this neuronal population, both during normal development and during the pathological degeneration. This speak for a preferential cell death mode for the
demise of the mDA neurons, suggesting that when these neurons are insulted and impaired in their normal functioning or ablated of essential components, they switch on a suicide program, which leads to their progressive demise.

II. Engrailed does not regulate axonal outgrowth of mDA neurons

Another remarkable feature of the mDA neurons in the En-1-/- En-2-/- mouse embryo is the lack of axonal outgrowth. This raised the possibility that En was regulating also the ability of these neurons to project an axon. During development of the central nervous system (CNS), neuron grow out neurites whose growing tips are guided by growth-permissive or inhibitory molecules synthesized by non-neuronal cell. Thus, under instruction of this guidance molecule the axons are directed in highly selective pathway towards their target where they eventually establish specific connection (Tessier-Lavigne and Goodman, 1996; Drescher et al., 1997b). This is also true for the dopaminergic neurons. As soon as these neurons become postmitotic, they start to express tyrosine hydroxylase (TH), and begin polymerization of tubulin in microtubules to grow out axons and dendrites (Perrone-Capano and Di Porzio, 2000). The orientation along which neurites grow is determined by ligand binding to receptors on growth cone, which controls actin polymerization in filopodia and lammellipodia (Tessier-Lavigne and Goodman, 1996). Midbrain dopaminergic neurons, thereby, elongate their axons, selectively, from the ventral midline towards the telencephalon, so that by E14.5 the nigral and ventral tegmental dopaminergic neurons, have reached their axonal target (Perrone-Capano and di Porzio, 1996): the dorsal striatum, nucleus accumbens and olfactory tubercle. The rostral pathfinding of the dopaminergic projections is the final result of non-permissive and permissive local cues distributed along the caudal- rostral path. An important group of local guiding cues are extracellular matrix protein (ECM) such as laminin, fibronectin, tenascin and proteoglycans (Fitch and Silver, 1997) produced by midbrain astroglia (Garcia-Abreu et al., 1995a). In addition differential expression of glycosaminoglicans (GAGs) confer non- permissive and permissive properties in the medial (M) and lateral (L) midbrain glia population respectively (Garcia-Abreu et al., 1995b; Garcia-Abreu et al., 2000; Mendes et al., 2003). The adhesion molecule L1, moreover, has multiple roles in axonal growth and guidance of the mDA neurons (Walsh and Doherty, 1997)
and acts also as a survival-promoting factor for this neuronal population (Hulley et al., 1998). In contrast netrins, which are expressed at the floor plate, mediate a repulsive effect preventing any axonal crossover at the midline (Lumsden and Davies, 1983; Tessier-Lavigne et al., 1988).

It has been previously reported that engrailed genes are involved in controlling axonal pathfinding by regulating expression of adhesion molecule such as connectin and neuroglian, homologous of the mammalian L1, in Drosophila interneurons (Siegler and Jia, 1999), and similar adhesion molecule in cockroach sensory axons (Marie et al., 2002). The evidence that in the En1-/-, En2-/- E12 embryo the mDA neurons were not projecting rostrally but bared only sparse, unidirectional, short extension, suggested that that En may control axonal outgrowth of the dopaminergic neurons either by regulating the expression of adhesive molecules, confirming the previous reports or by interfering with the cytoskeletal organization of these neurons. From the cell culture experiments using different permissive substrate such as laminin, 3D collagen matrix, and wild type ventral midbrain vesicles, we observed that 24 hr after plating the mutant dopaminergic neurons had extended projections of comparable length to the wild-type and had an a,b-tubulin pattern similar to control cultures. In addition, expression of the adhesion molecule L1 on En1-/-, En2-/- mutant mDA neurons resulted unchanged in comparison to the littermate control culture. These results, taken together, suggest that engrailed is not impairing the neurons in their intrinsic ability to extend an axon neither by controlling a-tubulin polymerization, nor by controlling the expression of the adhesion molecule, L1. In such a case it remains the possibility that the environment depleted of En expression is lacking glial derived permissive cues for the directional outgrowth of the neurites. This would recapitulate the role of en genes in the grasshopper where they specify neural precursor cell to a glial fate (Condron et al., 1994). Another component guiding the dopaminergic axons along the posterior-anterior path is represented by the repulsive cues such as Ephrin A2, A5 peaking at the isthmus (Cheng et al., 1995; Drescher et al., 1995; Nakamoto et al., 1996; Drescher et al., 1997a; Frisen et al., 1998). As previously described, the En1-/-, En2-/- mutant embryo has a large deletion of the MHB (Liu and Joyner, 2001b), in such case the repulsive-instructive activity arising from the isthmus would be missing thereby dis-orienteering the projection of the mDA neurons in rostral direction. In addition there is also evidence for diencephalic directional cues that
guide the dopaminergic axons rostrally similarly to other monoaminergic neurons, i.e. serotoninergic and noradrenergic. Those neurons reside in the hindbrain and extend their axons rostrally along the longitudinal axis (Nakamura et al., 2000). Ablation of engrailed, which is normally repressing diencephalic fate (Araki and Nakamura, 1999), could cause a caudal redistribution of diencephalic cues, which would impair the primary axonal elongation in the mDA neurons. To further investigate the causes of the projection deficit of the En-1 -/-, En-2/- mDA neurons in vivo, I am planning to analyze the glial profile in the En-1 -/-, En-2/- E12.5 ventral midbrain in comparison to control littermate by Microarray gene expression analysis. Beside, this will provide further information on the mesencephalic molecular cues, which confer permissive or non-permissive property to the tissue.

III. mDA neurons require En-1 and En-2 cell autonomously for their survival

As shown by this study, the developmental disappearance of the mDA neurons in the En-1 -/-, En-2/- occurs synchronous to the onset of En expression, and this speaks for a direct involvement of the En genes in the survival of the mDA neurons (Simon et al., 2001). Nevertheless, the large morphological defect at the midbrain and cerebellum in the En-1 -/-, En-2/- mice (Joyner, 1996) left open the question whether mDA neurons demise in the En double mutant was not caused by the loss of environmental factors, normally providing trophic support to developing neurons. In such a scenario the requirement of En would have been non cell-autonomous, whereas in case of a cell autonomous requirement of En, the En-1 -/-, En-2/- mDA neurons would be lacking fundamental components supporting cell survival, regardless of the environment. In order to address this question we performed two cell mixing experiment both “in vivo”, and “in vitro”, where En-1 -/- En-2/- mDA neurons were surrounded from a wild-type environment.

In both experimental paradigms, “in vivo” and “in vitro”, we observed the same result: either in the chimeric mouse made up of 50% En-1 -/- En-2 and wild-type cells, or the mixed primary mesencephalic culture, the mutant mDA neurons died in spite of the wild-type environment. This demonstrates that En genes are cell autonomous required for the survival of the midbrain dopaminergic neurons and
supports the essential role of the En genes in the maturation of the dopaminergic neurons. In addition, the cell death time course, and mode of cell death for the En-1 -/- En-2/- mDA neurons “in vivo” and “in vitro” resulted the same, which allowed us to adopt the “in vitro” paradigm to address further questions regarding the involvement of En genes in the survival/death pathway.

In order to understand whether En genes were directly involved in the apoptotic pathway by functioning as cell sensor and apoptotic repressor, as it is the case for other transcription factor such as p53 and NF-kB, we tested the effect of different neurotoxin specific for the mDA neurons. Already 24 hours after MPP+, 6-OHDA, and Rotenone intoxication we observed that the expression of En-1, Nurr-1, Pbx-1, Lmx-1b, Ptx-3, expressed specifically in this neuronal population, was ablated. This was indicating that En is not selectively silenced after cell damage, but the general sweeping out of the transcription factor expression is rather a consequence of the toxic insult, that leads eventually to cell death.

Since the apoptotic mode of cell death affecting the mDA neurons is extensively studied and new strategies to target programmed cell death are being developed, we tried to rescue En-1 -/-, En-2/- mDA neurons by using a general inhibitor of apoptosis, zVAD-fmk. This inhibitor has been reported to rescue mDA neurons after MPP+ intoxication up to 80%(Bilsland et al., 2002). In our En-1 -/-, En-2/- primary ventral midbrain culture, however, no rescue of the mDA neurons was observed after application of the inhibitor. Which is in line with what other authors observed, where zVAD-fmk exerted no rescue effect on MPP+ treated ventral midbrain culture (Hartmann et al., 2001b). Quite strikingly, in turn, the application of DMSO alone was able to partially rescue the double mutant mDA neurons. DMSO is an organic hydrophobic solvent, which solubilizes all type of cellular membrane, and can possibly release intracellular stores of ions such as Ca$^{2+}$. Changes in intracellular ion homeostasis has various effect for example under depolarizing condition (K$^{+}$ 20-50mM) a moderate increase in intracellular Ca$^{2+}$ is induced, thereby preventing spontaneous cell death of midbrain dopaminergic neurons (Douhou et al., 2001), however prolonged exposure to Ca$^{2+}$ leads to activation of calpains which produces a negative feedback loop, that blocks the rescue activity of calpains inducing plasma membrane disruption and secondary necrosis (Neumar et al., 2003). This may explain why some author report a shift to necrotic cell death 72 hours after application of the
caspase inhibitor zVAD-fmk (Hartmann et al., 2001b). A large increase in intracellular Ca$^{2+}$ promotes, in turn, excitotoxic cell death (Orrenius et al., 2003). It would be of interest to confirm the involvement of Ca$^{2+}$ in the death molecular pathway of the En-1 -/-, En-2/- mDA neurons upon K$^+$ depolarization in primary cell culture. One additional approach to investigate the dynamic of apoptosis, which is triggered by the ablation of the *engrailed* genes, would be inhibiting protein translation by application of cycloheximide in En-1 -/-, En-2/- ventral midbrain culture. If any rescue effect would be detectable that would tell us that *engrailed* is constitutively repressing key molecules involved in the apoptotic process, and that its absence would switch their production on, triggering the cell death program.

**IV. En-1 RNAi induces rapid apoptosis of En-2 -/- mDA neurons**

Despite all signs of viability that the *En1/2* double mutant mDA neurons showed during the first 24 hours in culture, it was still possible that the cells were already committed to cell death at the time of tissue dissociation. Thus, the selective loss of mutant mDA neurons during the cell mixing experiments might only reflect this commitment. To address this possibility, we silenced the *En* expression in mDA neurons by RNA interference (RNAi). RNAi was first discovered in the nematode worm, *Caenorhabditis elegans* (Fire et al., 1998), and it is a constitutive mechanism to regulate protein homeostasis, consisting in degradation of the cognate mRNA by 21-22 nucleotide RNA duplexes or small interfering RNAs (siRNA) (Zamore et al., 2000; Bernstein et al., 2001). Recent experiment have shown that the application of synthesized small interfering RNA duplexes (siRNA), 21-22 nucleotides in length, leads to sequence-specific mRNA degradation in cultures of mammalian cell lines (Elbashir et al., 2001) and mammalian neurons (Krichevsky and Kosik, 2002). Also when primary cell culture are rather resistant to transfection in comparison to cell line, as reported by other authors (Krichevsky and Kosik, 2002), we still chose this technique for our studies since we wanted to observe whether ablating *En-1* expression in *En2/-* ventral midbrain culture, which are viable in respect to the mDA neurons, would impair mDA neuron survival. Our RNAi experiments showed that the silencing effect occurred from 12 hour post-transfection (p.t.) until 4 days p.t. with a maximum silencing effect at 48 hours. This is in line with other RNAi studies where
half-life time of a protein is estimated around 40 hours (Jackson et al., 2003). The first morphological signs of apoptotic cell death and the primary cell loss were observed 24 hours after the application of the siEn-1 oligos, this time course resembles the disappearance rate of the midbrain dopaminergic neurons in the En-1 -/-, En-2/- embryos, and a similar time scale is also observed in 6-OHDA and MPTP induced degeneration of the nigrostriatal DA system (Sundstrom et al., 1988; Jeon et al., 1995; Zuch et al., 2000), and in hippocampal neurons after activation of the neurotrophin of the low affinity neurotrophin,“ death receptor”, receptor p75 (p75NTR) (Friedman, 2000). Each of these experimental models leads to the apoptosis activation within 24-48hr, leading to damage of mitochondria and to the release of cytochrome c, activation of the effector caspase-3 and eventually to cell death (Dobrowsky and Carter, 2000; Vila and Przedborski, 2003). This suggests that the lack of En triggers cell death following the common mitochondrial molecular pathway. The apoptotic 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, recapitulates the characteristic feature encountered in other PD models. It is feasible therefore to assume that neuronal degeneration observed in PD patients, and the dopaminergic neuronal loss in the En double mutants, may have a common molecular origin. In the En double mutant, large alterations in the level of gene expression downstream of En-1 and En-2 are probably the reason for the death of mDA neurons. The fact that in the En-1 -/- En-2/- [-synuclein is ablated (Simon et al., 2001) lead us to speculate that indeed this molecule among others, which are yet unraveled, is essential for the full maturation and viability of the mDA neurons. Differential Microarray analysis of E12 and E14 En-1 -/-, En-2/- and control littermate ventral midbrain is currently in progress aiming to unravel the target genes of En, and cast light on the essential molecular components for the maturation and survival of mDA neurons. Moreover, in order to identify the molecular pathway leading to cell death in the En-1 -/-, En-2/- it will be necessary to track the key components of the apoptotic cascade, according to present knowledge of the two principle apoptotic molecular pathway and its components, and define the molecules involved in the PCD following ablation of En expression. Once the molecules of the apoptotic pathway are identified, we could try to interfere with the cell death program by targeting the same molecules within the pathway. If the mDA neurons cell death occurs via the mitochondrial
pathway, as it is often the case for the dopaminergic neurons, we could then plan to rescue the En-1 -/-, En-2-/- mDA neurons by genetically targeting key molecules in this pathway. Since there is already evidence that Bax-/- (Vila et al., 2001), p53-/- (Trimmer et al., 1996) and Bcl-2 overexpressing mice (Yang et al., 1998) show a high resistance to MPTP intoxication. By knocking out pro-apoptotic key molecules, Bax, p53 or over-expressing antiapoptotic factors such as Bcl-2 first in 129 backcrossed En-1 -/-, En-2-/- stem cells and then differentiate them into dopaminergic neurons, we may rescue the En-1 -/-, En-2-/- phenotype.

V. En-1 acts as repressor for p75NTR expression.

Preliminary gene expression analysis of En1 over-expressing cell lines, obtained in my laboratory, suggested that two key molecules involved in the apoptotic pathway are regulated by the En genes, namely p53 and p75. p53 is an important pro-apoptotic protein, which rapidly senses cell damage and induce an apoptotic program as after MPTP intoxication (Mandir et al., 2002). Moreover p53 protein appears necessary for complete expression of MPTP neurotoxicity to dopamine neurons as p53-/- null mice result partially resistant to MPTP intoxication (Trimmer et al., 1996). MPTP treatment, on the other side, leads to a cell death which proceeds at a rate similar to the death of the mDA neurons in the En-1 -/-, En2-/- mice. Additionally in both systems, cell death occurs by apoptosis, suggesting that p53 may have been a potential target of engrailed regulation and its derepression in absence of En, could trigger apoptosis. The other molecule of interest was p75, the low affinity receptor for the neurotrophin NGF, BDNF, and NT-3. This receptor is member of the TNF receptor family as it shares with the other receptors an intracellular death domain and it is widely expressed during development (Yan and Johnson, 1988). Among its classical function in assisting the response of Trk proteins to their ligands (Barrett, 2000), it can promote an apoptotic signal depending on the different developmental stages of sensory neurons (Barrett and Bartlett, 1994). Upon neurotrophin treatment and in absence or low expression of Trk receptors, it can mediate an apoptotic signal, in different system such as glial cell (Casaccia-Bonnefil et al., 1996), a variety of neuronal cell lines (Rabizadeh et al., 1994) and hippocampal neurons (Friedman, 2000). Moreover, BDNF is an important neurotrophic factor for mDA neurons during
development (Hyman et al., 1991). This led us to speculate that the two neurotrophin receptors could play an essential role in the development of the midbrain dopaminergic neurons. To reinforce our hypothesis there is evidence that p75 can transduce constitutively and rather rapidly (8hr) an apoptotic signal in sympathetic neurons (Freidin, 2001), which is closely matching the death time course of the mDA neurons in the En double mutant and in the RNAi experiments. From our quantitative expression analysis of En-1 overexpressing cell line relatively to the non induced control and the ventral midbrain of En-1 -/-, En-2-/- developmental, stage E12.5 (when the mDA neurons are present) relatively to the control littermate, it appeared that p53 remained unchanged in all cases. In turn p75NTR was dramatically downregulated in the En-1 overexpressing lines in comparison to the control, whereas we measured a three fold expression increase in En-1 -/-, En-2-/- ventral midbrain in respect to the littermate control. This suggests that indeed p75 signaling may be involved in neuronal demise observed in the En-1 -/-, En-2-/-, by overcoming the survival effect mediated by the Trk proteins (Persson and Ibanez, 1993; Birling and Price, 1995). In addition the evidence that p75 juxtamembrane domain senses alteration to membrane components after oxidative stress and cell damage thereby triggering apoptosis (Hannun, 1996; Dobrowsky and Carter, 2000). Together with the result emerging from the comparative differential display carried out in our laboratory, which shows that one of the target gene of En is hydroxysteroid (17-[\(^\text{\textcopyright}17\)) dehydrogenase-12, an intermediate enzyme involved in sphingolipid synthesis, it is suggestive that in En double mutant alteration to the cellular membrane structure may activate p75 receptor, causing eventually cell death. In addition, analysis of the promoter region for p75 confirmed that there are two highly matching consensus sequences between –140-180bp and –250-340bp from the start codon for the Drosophila and mammalian En respectively. This speaks for a direct negative regulation by the transcription factor on p75 expression, which is in line with the “classical” role of engrailed as expression repressor (Araki and Nakamura, 1999; Siegler and Jia, 1999) and strongly suggested p75 as good candidate in the programmed cell death occurring in the En-1 -/-, En-2-/- mDA neurons.

To confirm our hypothesis regarding the involvement of p75 in the demise of the mDA neurons we need to restrict our analysis only to the mDA neurons. To do so, we are going to compare the pattern of expression of the En-1 -/-, En-2-/- ventral
midbrain at E12.5 with that at E14.5. Since the two stages differ only for the presence of the dopaminergic population, a subtractive analysis of the E12.5 on the E14.5 gene expression pattern may cast light on genes, which are differentially expressed in the midbrain dopaminergic neurons of the two phenotypes. Additionally, in order to confirm that in our system the apoptotic signal is indeed mediated by p75 signaling, we could try to block this signalling cascade by using antibodies against p75 that have been previously applied to culture of sensory neurons of the trigeminal mesencephalic nucleus and have resulted effective in preventing neurotrophin induced apoptosis (Davey and Davies, 1998).

VII. *En-1* has no direct influence on dopaminergic progenitors

In the *En-1* -/-, *En-2* -/- mutant mice, mDA neurons are induced in a smaller domain at E12.5 in comparison to the wild type this implies multiple scenarios:

I. *En* has a direct effect on the precursor cells, and the lack of these transcription factors restricts the competent tissue that can form dopaminergic neurons. II. The lack of *En* affects the survival of the mDA neurons, as it is demonstrated by our previous experiments, reducing the life span of these neurons to 24-48 hr. The first DA neurons are induced in the midbrain at E10.5 (Perrone-Capano and Di Porzio, 2000), according to short life span of these neurons, what we see at around E12.5 is a subpopulation of mDA neurons which has just completed the last cell cycle, whereas the older neurons have already been committed to death. III. The consistent loss of midbrain tissue in the *En-1* -/-, *En-2* -/- mouse affects the isthmic region where *Fgf-8* is expressed at the embryonic mid/hindbrain junction. This may impair the synergistic inductive effect of *Fgf-8* and Shh on the precursor cells of the mDA neurons. In order to answer these questions we planned a gain of function experiment by over-expressing the mouse *En-1* in chick. We wanted to investigate the effect of *En-1* misexpression on the induction of mDA neurons. Previously, Dittrich et al., 1997, have performed over-expression of *eagle* in *Drosophila* in order to find out whether the gene is required at the level of progenitors cells or at the level of the mature cells in the specification of the serotoninergic neurons. Similarly Araki and Nakamura, 1999, over-expressed chick *En-1* in the chick embryo at stage HH 10, at a time when
*En* is responsible for the patterning of the mesencephalon. The authors observed that misexpression of *En* in the diencephalon induced an enlargement of the mesencephalic tissue with rostral shift of the di-mesencephalic boundary and an ectopic tectum. The midbrain-patterning function of *En* gene has been explained by the direct repressing activity of this transcription factor on diencephalon-related genes, such as Pax-6, and indirect inducing activity mesencephalon-related genes such as Wnt-1, Fgf8, Pax-2, and Pax-8. In Previous studies a replication competent retroviral vector RCASBP(A)(Hughes et al., 1987) containing a mouse *En-1* cDNA was used to infect the chick neural tube at HH 8-10. Also in this case *En* misexpression produced a pronounced caudal variation of the cytoarchitecture of the rostral optic tectum, and rostral shift in the distribution of Eph-related receptor kinases, RAGS and ELF-1, which are normally expressed in a decreasing gradient P to A, reflecting the early *En* patterning (Logan et al., 1996). Those results indicate that *En* is responsible for the patterning and identity of the mesencephalon. Taking advantage of the regionalization function of *En-1* we wanted to understand whether an enlargement of the mesencephalic tissue, by *En-1* overexpression, could expand the mesencephalic competent tissue. In our over-expression experiments, where chick embryos were electroporated with non-competent retroviral vector encoding for the mouse *En-1*, we observed no effect on the number of mDA neurons at the midbrain. The present results offers different explanations i) overexpression of *En-1* alone does not have any effect on the mesencephalic dopaminergic precursors, since at this stage (HH8-10) it is responsible only for the regionalization of the mesencephalic tissue, and only later it is required for the survival of the mDA neurons. Nevertheless, it remains possible that *En-1* and *En-2* are both required for the specification of mDA neurons, since it has been demonstrated that just one *En-2* allele is able to maintain a subpopulation of mDA neurons alive (Simon et al., 2001). To investigate this possibility it would be necessary to perform simultaneous overexpression of both *En-1* and *En-2* ii) inductive additional signals to Fgf-8 and Shh, such as Fgf-17 (Liu and Joyner, 2001a), which are independent of *En* expression may be involved in commitment of the dopaminergic progenitors. Considering, also, that in the *En-1/-, En-2/-* the mDA neurons are still being induced (Simon et al., 2001), despite the consistent loss of the isthmic Fgf-8 domain (Liu and Joyner, 2001b). Thus enlargement of the Fgf-8 domain due to *En* overexpression would not be enough to
commit more progenitor cells to a dopaminergic fate. To further investigate this issue it may be interesting to analyze the pattern of expression of other Fgf family members in the En-1-/-, En-2-/-, and prove their inducing effect on neural stem cells (NSC) in culture. Finally iii) the developmental window when the overexpression is performed may be postum to an early specification of the midbrain dopaminergic precursor. Thereby the lack of inductive effect would be restricted to an experimental question and further electroporation experiment previous to HH 8 are planned in future to solve this issue.

Another interesting experiment would be to co-express En-1 with other intracellular mediator to observe any change in the induction of the mDA neurons. Lmx-1b is a good candidate since it shares features of the engrailed genes and it is also involved in MHB regionalization (Adams et al., 2000) and it is expressed before the mDA neurons are being induced (Smidt et al., 2000). In addition, similarly to the En-1 -/-, En-2-/- mouse mutant, in the Lmx-1b-/- mDA neurons are induced in a smaller domain (Smidt et al., 2000). This phenotypical homology between the two transcription factors raises the interesting question whether the two transcription factors could co-operate in the specification of the dopaminergic phenotype at progenitor level.

Conclusions

The outcomes of this study show that i) En genes are progressively expressed in the mDA neurons from E12 on ii) En genes are cell autonomous required for the maturation and survival of mDA neurons. Lack of En expression leads to apoptotic cell death. iii) En doesn’t regulate axonal outgrowth of the mDA neurons neither by regulating cytoskeletal organization, nor by modulating expression of the adhesion molecule L-1 on the mDA neurons. iv) En-1 function as transcriptional repressor of p75 expression and it is a good candidate for the death signaling which leads to the rapid demise of the mDA neurons. v) En-1 does not specify the midbrain precursors cells to their final dopaminergic phenotype

A major challenge will be to find out in more detail the target genes of En in the mDA neurons and understand how those molecules are involved in the full maturation of the mDA neurons. In addition unraveling components involved in the apoptotic cascade that mediates cell death in the En-1 -/-, En-2-/- mDA neurons will be useful
to perform gain of function experiment in order to rescue $En^{-1}$, $En^{-2}$ mDA neurons.
This work suggests that $En$ is a fundamental component of the mDA neurons, further studies also on human could help to understand if $En$ can be one of the gene involved in the etiology of PD.
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