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IDENTIFICATION AND CHARACTERIZATION OF GENES EXPRESSED BY THE MIDBRAIN DOPAMINERGIC NEURONS

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“A hundred times every day I remind myself that my inner and outer life depend on the labors of other men, living and dead, and that I must exert myself in order to give in the same measure as I have received and am still receiving.”

Albert Einstein

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ABBREVIATIONS

AADC	L-aromatic amino acid decarboxylase
AHD-2	Aldehyde dehydrogenase 2
ANR	Anterior neural ridge
K-ATP	ATP-sensitive potassium
BDNF	Brain-derived neurotrophic factor
cDNA	Complementary desoxyribonucleic acid
CNS	Central nervous system
DA	Dopaminergic
DAT	Dopamine transporter
dCTP	Desoxycytosine triphosphate
ddPCR	Differential display PCR
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide
DMEM	Dulbecco's modified eagle medium
E	Embryonic day
EDTA	Ethylene diamine tetra acetic acid
En	Engrailed
En-1	Engrailed-1
En-2	Engrailed-2
ES	Embryonic stem
FGF	Fibroblast growth factor
FBS	Fetal bovine serum
FP	Floorplate
GABA	γ-aminobutyric acid
GP	Globus pallidus
GDNF	Glial cell line-derived neurotrophic factor
GFP	Green fluorescent protein
HNF3	Hepatocyte nuclear factor 3
ICM	Inner cell mass
ISH	*In situ* hybridization
LIF	leukemia-inhibiting factor
MAP1B	Macrotubule associated protein 1B
MHB	Mid-hindbrain boundary
MPTP	1,2,3,6-methyl-phenyl-tetrahydropyridine
NPTII	Neomycin phosphotransferase II
NRG	Neuregulin
P	Posnatal day
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
PD	Parkinson's disease
PHHI	Persistent hyperinsulinemic hypoglycemia of infancy
PFA	Paraformaldehyde

RNA	Ribonucleic acid
RRF	Retrorubral field
Rt	Room temperature
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
SSC	Sodium chloride/ sodium citrate
Shh	Sonic Hedgehog
SN	Substantia nigra
SNC	Substantia nigra pars compacta
SNL	Substantia nigra pars lateralis
SNR	Substantia nigra pars reticulata
TH	Tyrosine hydroxylase
TGF	Transforming growth factor
TLZ	TauLacZ
UTP	Uracyl triphosphate
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area

-/-	Homozygous for a null mutation in the gene of interest
-/+	Heterozygous; only one allele of the gene of interest is mutated
+/+	Wild type for the gene of interest
%	Percent
˚C	Celsius degree
Ci	Curie
g	gram
hr	hour
l	liter
µ	micro
M	molar
ml	milliliter
mm	millimeter
mM	millimolar
mim	minute
msec	milliseconde
n	nano
sec	seconde
U	unit

SUMMARY

The dopaminergic (DA) neurons of the substantia nigra and ventral tegmentum are the main sources of dopamine in the central nervous system. They are associated with one of the most prominent human neurodegenerative disorders, Parkinson's Disease. Very little is known about the mechanisms operating during the intermediate period between induction and full differentiation of the DA neurons. The goal of this study was to identify and characterize genes effective during this phase and which mediate the midbrain DA neurons development. The two murine *Engrailed* (*En*) genes are expressed by the midbrain DA neurons from embryonic day (E) 11.5 until adulthood, and are required for the survival of this neuronal population. In the initial part of this study I further characterized the role of the *En* genes: the generation of *En* chimeric animals permitted to show that the midbrain DA neurons require these genes cell autonomously for their survival, and the over-expression experiment of *En-1* in the chick embryo suggested that the *En* genes are not required for the formation of midbrain DA precursor cells. In order to identify more genes that are specifically expressed by this neuronal population, a PCR-based differential display screen was used, comparing mRNA from several sources of the ventral midbrain and adult olfactory bulb. The midbrain tissue was dissected from wild-type mice at two developmental stages (E12 and E14) and also from E12 *En* double null mutant embryos that become deficient for the midbrain dopaminergic neurons at later stages of development. Seventy-one differentially expressed gene fragments were identified of which four are specifically expressed by this neuronal population. Three of these genes have been further characterized in this study: The hepatocyte nuclear factor 3α (*HNF3α*), the neuronal and B-cell differentiation factor, *Olf-1/Ebf*, and the neuregulin receptor, *ErbB4*. The *HNF3α* expression is highly specific, as it is only found in the midbrain DA neurons and another cell group in the diencephalon. It may be expressed in the precursors of the DA neurons as early as E9 and continues to be expressed in the postmitotic cells until the adult. Detailed analysis of the midbrain DA neurons from mice homozygous for a null mutation in *HNF3α* demonstrated that *HNF3α* is not

required for the early development of midbrain DA neurons, but a role in maintenance of this neural cell population is still possible. The *ErbB4* expression begins in the DA neurons at E11.5 and continues into adulthood, specifying the most rostral part of the substantia nigra compacta. Analysis of the expression pattern of ErbB4 ligands together with a detailed study of the midbrain DA neurons of adult mice with a nervous system specific deletion of *ErbB4*, suggested that the expression of ErbB4 after E11 is not essential for the midbrain DA survival, although its involvement into the early fate determination and neural differentiation of DA neurons is nevertheless a possibility. The *Olf-1/Ebf* gene is expressed transiently in mouse DA neurons from E10 to E13. Ectopic expression of *Olf-1/Ebf* in the chick midbrain by *in ovo* electroporation leads to an ectopic placement of DA neurons, suggesting that *Olf-1/Ebf* may be involved in midbrain DA neurons specification and differentiation. Finally, it was shown that neither HNF3α nor Olf-1/Ebf is regulated by *Nurr1* or the *En* genes. Thereby suggesting that multiple parallel pathways control the development of the midbrain DA neurons. Overall, this study took us a step further towards additional insights concerning the factors that determine the identity of such a particular cell population.

INTRODUCTION

1-General introduction

The cathecholamine dopamine is an essential regulator of many neural functions, including motor integration, cognition [Backman and Farde, 2001], emotive behavior and reward [Schultz, 2001] as well as sexual functions (for review [Giuliano and Allard, 2001]). Dopamine is synthesized from tyrosine by tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis.

The amount of dopaminergic (DA) neurons, in mammals, is very low in proportion to the total number of neurons in the brain. Most of them are found in three distinct nuclei located in the ventral midbrain: the substantia nigra (SN), the ventral tegmental area (VTA) and the retrorubral nucleus [Nelson et al., 1996]. DA neurons arising from the SN project to the striatum and receive input from various structures in the diencephalon and telencephalon. The ascending nigrostriatal pathway adjusts motor control and its degeneration is related to the motor disorder characteristic of Parkinson’s disease (PD) [Hirsch et al., 1988]. The limbic system and cortex receive input from the VTA. Neurons from the VTA are involved in emotional and reward behavior as well as in motivation [Schultz, 2001]. Changes in this system have been linked with schizophrenia, addictive behavioral disorders and attention-deficit hyperactivity disorder [Watanabe et al., 1998; Floresco et al., 2001].

The exact anatomical localization and functional differentiation of DA neurons in the mammalian brain is accomplished through actions and gradient displays of a variety of factors. Data from biochemical and genetic studies, from tissue transplantation and explant culture experiments have established that midbrain neural plate progenitors differentiate into DA neurons by the joint action of sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF8), and that these two extracellular inducers are necessary and sufficient for the early induction of DA neurons along the ventral neuraxis [Ye et al., 1998]. It is believed that the inductive molecules activate cascades of other signaling molecules and transcription factors, which lead to the final differentiation of DA neurons. Up to date, only little is known about what happens during the intermediate period between induction and full differentiation of DA neurons. My PhD thesis work was focused on the identification and characterization of genes that are required for the final differentiation of the midbrain DA neurons.

2-Anatomy and function of the midbrain DA neurons

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

The midbrain DA neurons are located in the ventral midbrain and are categorized into three major DA cell populations. The main midbrain DA neurons subpopulation is identified as the A9 DA cell group and is principally found in the substantia nigra pars compacta (SNC). The second subpopulation is found in the VTA and is identified as the A10 group [Paxinos, 1995]. The third midbrain DA neurons subpopulation is known as the A8 DA cell group and is located in the retrorubral field (RRF). Three-dimensional reconstructions of the SN/VTA DA neurons are presented in Figure I.1.

The A9 cell population is formed by a layer of DA neurons in the SNC, partially covering the substantia nigra pars reticulata (SNR) and extending laterally towards the substantia nigra pars lateralis (SNL). The SNC contains compactly grouped DA neurons. Often, ventral DA neurons give rise to tightly apposed groups of dendrites that invade the SNR [Bjoerklund and Lindvall, 1975]. These dendrites release dopamine (for review [Cheramy et al., 1981]), which can interact with dopamine autoreceptors found on adjacent DA dendrites or/and striatonigral neurons. More ‘basal’ dendrites, of SNC DA neurons spreading medio-laterally, invade the overlaying dorsal sheets of SNC and VTA neurons. Another kind of the SNC DA neurons is positioned in more dorsal aspects of the SNC. They give rise to dendrites radiating medio-laterally. This organization of split dentritic domains is the basis for the operative partition of separate input and output areas in the SNC [Fallon et al., 1978].

The A10 DA cell group is medial and dorsomedial to the SNC and SNR. The VTA contains a mix of non-DA neurons (20%) and DA neurons (80%) [Halliday and Tork, 1986]. At dorsally mid-anterior levels, the DA neurons are the continuity of the dorsal DA neurons of the SNC, and the two DA neurons populations are very similar. Other DA neurons have several radiating dendrites. At ventrally mid-anterior level the DA neurons are densely packed at the midline [Paxinos, 1995].

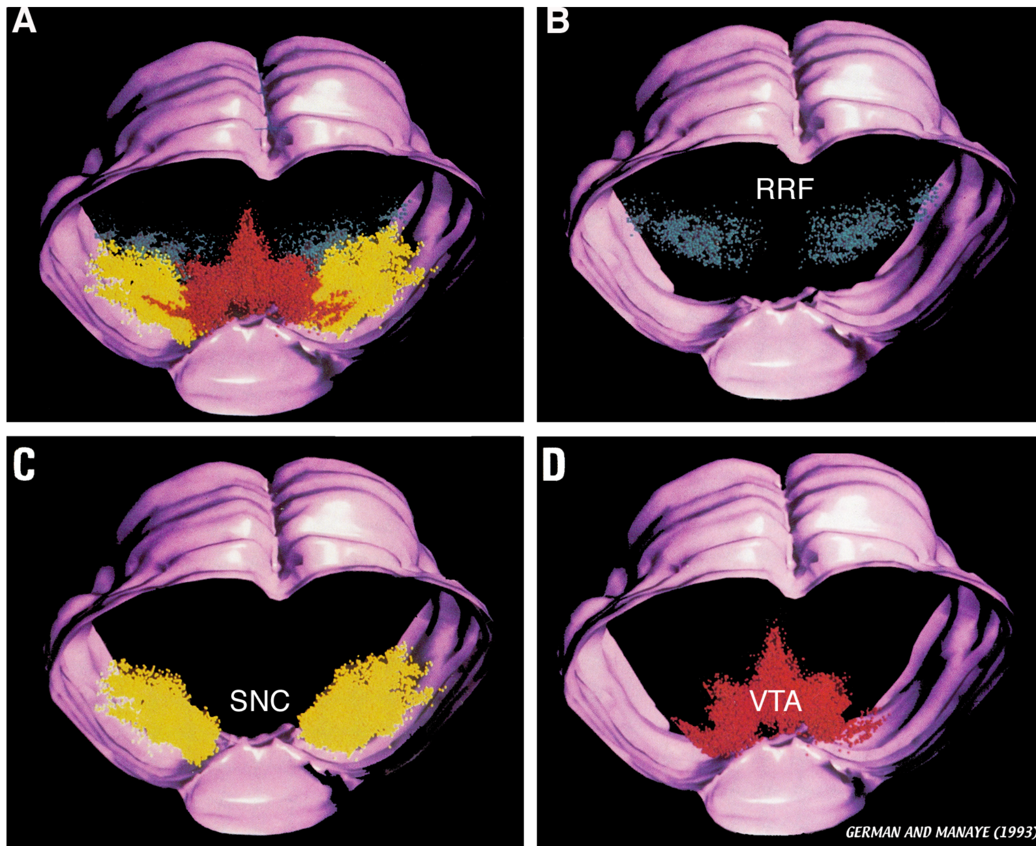

Figure I.1. Three-dimensional reconstructions of midbrain dopaminergic neurons. A8 neurons are illustrated in blue (RRF), A9 neurons are illustrated in yellow (SNC), and A10 neurons are illustrated in red (VTA). The rostral direction is in the foreground. (A) A8, A9, A10 viewed together. (B) A8. (C) A9. (D) A10. From German and Manaye, 1993.

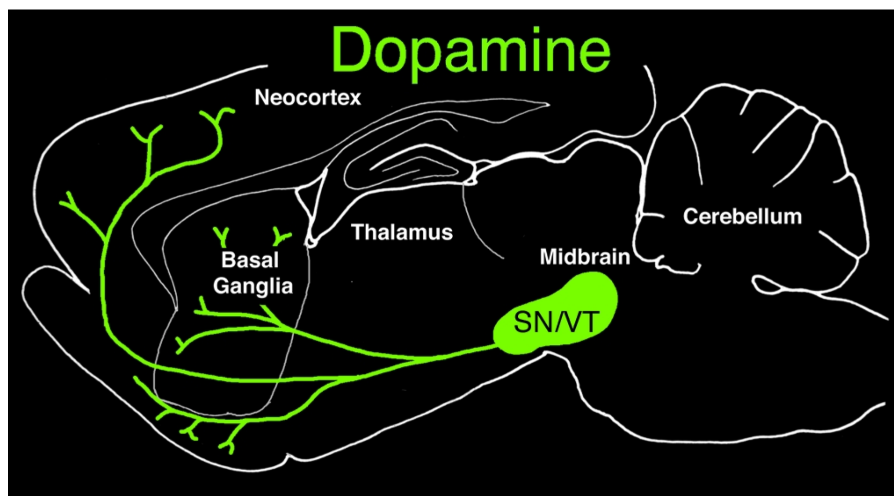

Figure I.2. Summary representation of dopaminergic projection pathways arising in the SN-VTA. Sagittal view.

Finally, the A8 DA cell population of the RRF seems to be a caudal extension of the SNC and SNL and have similar type of DA neurons [Paxinos, 1995].

2-2- The afferent projections from the midbrain DA neurons

The DA projections to the caudate-putamen (or striatum) arise from the ventral and intermediate sheets of the SNC and the ventro-lateral VTA. The dorsal and middorsal VTA and medial SNC innervate the nucleus accumbens and the olfactory tubercle. Projections to the amygdala arise from the VTA and the lateral SNC and SNL. The lateral septum is innervated by ventral VTA neurons and by adjacent medial SNC neurons. The neocortex is innervated by the dorsal-most sheet of SNC and VTA neurons. Minor DA projections reach the cerebellum, the hypothalamus, the raphe, the hippocampus, the ventral pallidum and the locus coeruleus [Ikai et al., 1992; MacRae-DeGuerce and Milon, 1983; Simon et al., 1979; Swanson, 1982; Kizer et al., 1976; Fallon and Moore, 1978; Gasbarri et al., 1991; Clavier et al., 1976; Faull and Mehler, 1978; Gerfen et al., 1982; Prensa and Parent, 2001]. A simplified overview of the major midbrain DA neurons projections is illustrated in figure I.2.

2-3- The inputs of the midbrain DA neurons

The γ-aminobutyric acid (GABA)-ergic neurons of the striatal patch innervate the dendrites of ventral SNC neurons in the SNR, and the ventral SNC (for review [Gerfen, 1992]). The GABA-ergic neurons of the globus pallidus and ventral pallidum project to the SNR and SNC (for review [Kalivas, 1993]). Some projections to the SNC and VTA arise from the amygdala, hypothalamus, preoptic area and cortex [Wallace et al., 1992, Wright et al., 1980; Hurley et al., 1991].

2-4- The functions of the midbrain DA neurons

The midbrain DA neurons have a wide network of connections in the entire brain and any changes in their amount, morphology or functionality will dramatically affect many neuronal functions. For example, the majority of the dense fibers of the A9 neurons project to the caudate-putamen to create the nigrostriatal pathway, this

pathway monitors voluntary movement by adjusting the responsiveness of striatal output neurons (for review [Gerfen, 1992]). The A10 neurons innervate extensively the limbic system as well as a subset of the cerebral cortex to create the mesocorticolimbic pathway. The mesocorticolimbic pathway, collaborating with the nigrostriatal pathway, adjusts locomotor activity and mediates emotion, motivation, and memory processing (for review [Le Moal and Simon, 1991]).
Depending on what midbrain DA sub-nucleus is altered and to which degree, the consecutive neuronal adjustments are different. When the DA neurons of the VTA are hyperactive, the mesocorticolimbic pathway is affected, and there is a hyperdopaminergic state in the brain causing abnormal behavior, as observed in schizophrenia [Watanabe et al., 1998; Floresco et al., 2001]. Degeneration of the DA neurons of the SN and VTA will results mainly in motor behavioral abnormalities because of the perturbation of the nigrostriatal pathway, but due to the multifaceted role and connection of the DA neurons, these symptoms will be associated as well with non-motor behavioral abnormalities. This neurodegenerative disorder is particular of PD [Hirsch et al., 88], and will be described in detail in the next chapter.

3-Parkinson's disease

3-1-Clinical traits and pathological findings

PD is one of the most common neurodegenerative disorders in humans, affecting 2% of the population over 65 years of age. This disease is caused mainly by a progressive degeneration of the DA neurons of the SN and VTA, the loss is estimated to be around 80% at the onset of symptoms [Fearnley and Lees, 1991]. Another pathological trait is the presence of degenerating ubiquitin-positive neuronal processes (Lewy bodies), which are found in all affected brain-stem regions [Gai et al., 1995]. The lost of DA neurons is not uniform through the SN/VTA. Neuronal loss tends to be the most in the ventro-lateral tier of the SNC, followed by the medial ventral tier and dorsal tier. This non-homogenous loss results in a regional loss in the DA projections. The striatal dopamine is lost, mainly in the dorsal and intermediate subdivisions of the putamen [Kish et al., 1988].

When dopamine is missing, inhibitory GABA-ergic output activity increases in the basal ganglia, in the internal segment of the globus pallidus (GP), and in the SNR. The increased output of the basal ganglia conducts to an excessive inhibition leading to a blackout of the thalamic and brainstem nuclei because they are over-inhibited by the internal GP and SNR through GABA (Figure I.3). Excess of inhibition on the thalamic nuclei leads to repression of the cortical motor system, very likely resulting in motor behavioral abnormalities, such as bradykinesia, hypokinesia, rigidity and tremor. The excessive inhibition on the brain stem nuclei may lead to abnormalities of gait and posture (For review [Lang and Lozano, 1998; Hornykiewicz, 2001]). Others main characteristic symptoms of PD consist also of autonomic failure with orthostatic hypotension, urinal incontinence and impotence. Other non-motor behavioral abnormalities observed in PD are: mental dysfunction with mood disorders, cognitive dysfunction and, sporadically, delusions and hallucinations [Gibb and Lees, 1991].

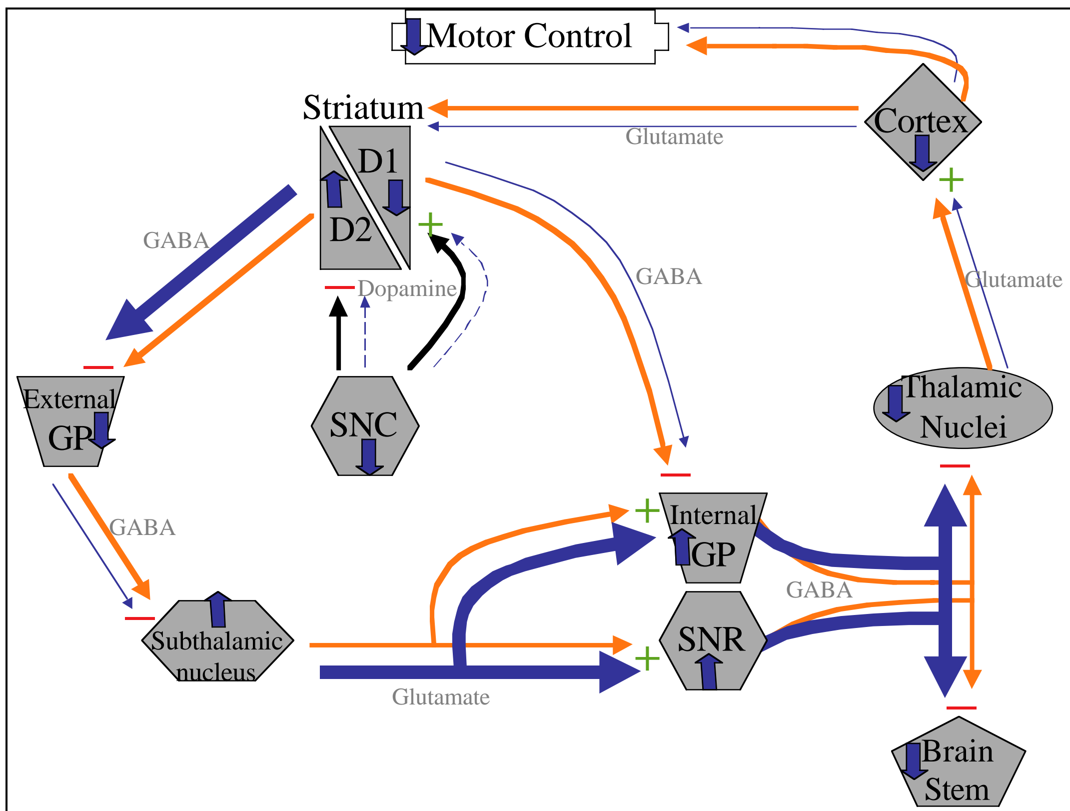

Figure I.3. Functional model of the nigrostriatal DA system and connected pathways.
For persons with normal motor control the pathway arrows are in orange. For persons with PD, the pathway arrows are in blue, the width of the arrows indicates the degree of functionality of each pathway compared with the normal state. The blue arrows inside each box indicate if there is a decrease or increase of activity of the brain region as compared with the normal level of activity. The neurotransmitters used by the different pathways are in gray. Plus green signs indicate excitation and minus red signs inhibition. Dashed lines indicate the reduction of dopamine level produce by the SNC in PD patients. PD patients have an increased inhibition of the motor thalamus -leading to suppression of the cortical motor system- and of the brain-stem locomotor areas resulting from over-activity of the internal GP and SNR. The high activity of these two areas is due to diminished inhibition from the striatum and to excess of stimulation from the overactive subthalamic nucleus.
D1, D1 dopamine receptors; D2, D2 dopamine receptors, SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; external GP, external portion of the globus pallidus; internal GP, internal portion of the globus pallidus; GABA, γ-aminobutyric acid. Adapted from Lang and Lozano, 1998.

3-2-Hypotheses of cell death mechanisms

The most relevant feature of PD neuronal degeneration is the distinct disappearance of the DA neurons. The mechanisms responsible for cell death in PD are not well understood. Some evidences suggest that the neuronal death in PD is abnormal apoptotic process [Hartmann and Hirsch, 2001], while others evoke a necrotic mechanism (for review [Andersen, 2001]). There are many causes and factors, which have been evocated for their implication in neuronal degeneration in PD. Among the most studied theories include, mitochondrial dysfunction (for review [Jenner, 2001]), oxidative stress (for review [Sayre et al., 2001]), deficient neurotrophic support [Kordower et al., 2000], and immune mechanisms (for review [Kuhn et al., 1997]). The vulnerability of the DA neurons in PD, is likely related to their singularity and may be due to their increased metabolic stress, high rates of protein oxidation, generation of toxins or failure to detoxify (maybe because of the presence of neuromelanin), and very specific needs for neurotrophic support or their ability to take up both endogenous and extrinsic toxic complexes through carrier mechanisms, such as the dopamine transporter.

3-3-Etiology: environmental and genetic factors

There is no real existing etiology of PD (for review [Olanow and Tatton, 1999]). The only evidence for an environmental factor in PD is associated to the toxin 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP). Drug addicts who took MPTP developed a syndrome that remarkably looks like PD [Langston et al. 1983]. Some epidemiological studies show that a couple of factors may augment the risk of developing PD as exposure to well water, pesticides, herbicides, industrial chemicals (for review [Tanner and Langston 1990; Jenner, 2001]). Some exogenous toxins, like trace metals, cyanide, organic solvents, carbon monoxide, and carbon disulfide have been related with the development of PD (for review [Montgomery, 1995]) [Bringmann et al., 1999; Hageman et al., 1999], but none of these toxins have been found in the brain of PD patients, nor a MPTP-like factor.

The participation of genetic factors in the risk of developing PD is strengthened by epidemiological studies. It was demonstrated that there is a higher frequency of PD cases in relatives of PD patients than in those of controls (for review [Bandmann et

al., 1998]), it was shown as well that there is a high concordance in monozygotic twins [Piccini et al., 1999]. Mutations in the *α-synuclein* gene have been found in some families with autosomal dominant PD [Polymeropoulos et al., 1997; Krueger et al., 1998]. Interestingly, α-synuclein is one of the main constituents of Lewy-bodies. Mice homozygous for a null mutation in α-synuclein exhibit a diminution in striatal dopamine and an attenuation of dopamine-dependent locomotor response to amphetamine [Abeliovich et al., 2000]. Another gene possibly involved in PD predisposition is the *parkin* gene, which encodes an ubiquitin ligase. Deletions of this gene have been identified in autosomal recessive juvenile forms of the disease in humans [Kitada et al., 1998; Shimura et al., 2000]. Among other gene candidates for a role into risk of developing PD are those involved in mitochondrial dysfunction, oxidative stress, deficient neurotrophic support and immune mechanisms.
The fact that either an environmental or a genetic factor could cause PD is suggested by the existence of the association of a PD syndrome with both MPTP and mutations in α-synuclein. Nevertheless, it is doubtful that the majority of PD cases will be elucidated by a unique cause. It is more likely that PD results from the co-action of genetic mutations and environmental toxins.

3-4-Treatment

Since the mid-1960s therapies which replace the lost neurotransmitter dopamine with its precursor L-dopa have been used for the treatment of PD. These therapies are very efficient in the early phase of the treatment and the patients experience an impressive restoration of neurological functions. Unfortunately, the efficiency of the L-dopa declines progressively and some side effects, like motor fluctuations including involuntary movements, start to occur after 6 months to 5 years of treatment depending on the patient (for review [Bezard et al., 2001]). These problems could be avoided by very recent surgical trials accomplished on PD patients. Embryonic mesencephalic tissues were grafted within the striatum of the patient to provide local delivery of dopamine. However ethical and safety issues, as well as an only partial functional recovery in PD patients necessitate additional development of the approach (for review [Dunnett et al. 2001]) [Freed at al., 2001].

Finally, outstanding results have been obtained with an alternative therapy called deep brain stimulation. Bilateral electrical stimulation of the subthalamic nucleus significantly improved akinesia, rigidity, tremor and reduced dyskinesis [Limousin et al., 1998].

4- Development of the midbrain DA neurons

The midbrain DA neurons are initially detected around embryonic day (E) 12.5 in the rat, near the mid–hindbrain boundary ([MHB] or isthmus) [Voorn et al., 1988]. The generation of DA neurons in this area depends on prior signals from organizers located nearby the ventral midbrain progenitor neurons and involves molecular differentiation cascades for the specification of neurotransmitter identity and the proper integration in the developing brain (Figure I.4).

4-1-The early organizers

Along the dorso-ventral and the antero-posterior axes of the neural tube, mature neurons develop in stereotypic positions (for review [Tanabe and Jessell, 1996]). The MHB, the floorplate (FP) and the anterior neural ridge (ANR) are three organizing centers, which operate along these two main axes and set up an epigenetic grid of Cartesian coordinates where neural progenitors start different cell fates consistent with their position on this grid [Rubenstein et al., 1994]. It is indeed the case for the midbrain DA neurons development (for review [Hynes and Rosenthal, 1999]).

4-1-1-Specification of the DA neurons along the dorso-ventral axis

Studies have shown that the midbrain and forebrain DA neurons differentiate near the FP. Moreover, they can be induced ectopically by the FP in dorsal midbrain in explants, or in transgenic mice [Hynes et al., 1995 a] and in dorsal forebrain explants [Ye et al., 1998], respectively. The molecule responsible for the FP activity is Shh. Indeed, it can imitate by itself the inductive effect of the FP [Hynes et al., 1995 b; Wang et al., 1995], and FP inductive effect is not possible in presence of Shh-blocking antibodies [Ye et al., 1998].
Therefore, it seems that DA neurons need Shh for their specification along the dorso-ventral axis. However, Shh cannot induce DA neurons in diencephalon or hindbrain (that do not normally contain endogenous DA neurons) [Ye et al.,1998] suggesting that it does not specify the DA neurons along the antero-posterior axis.

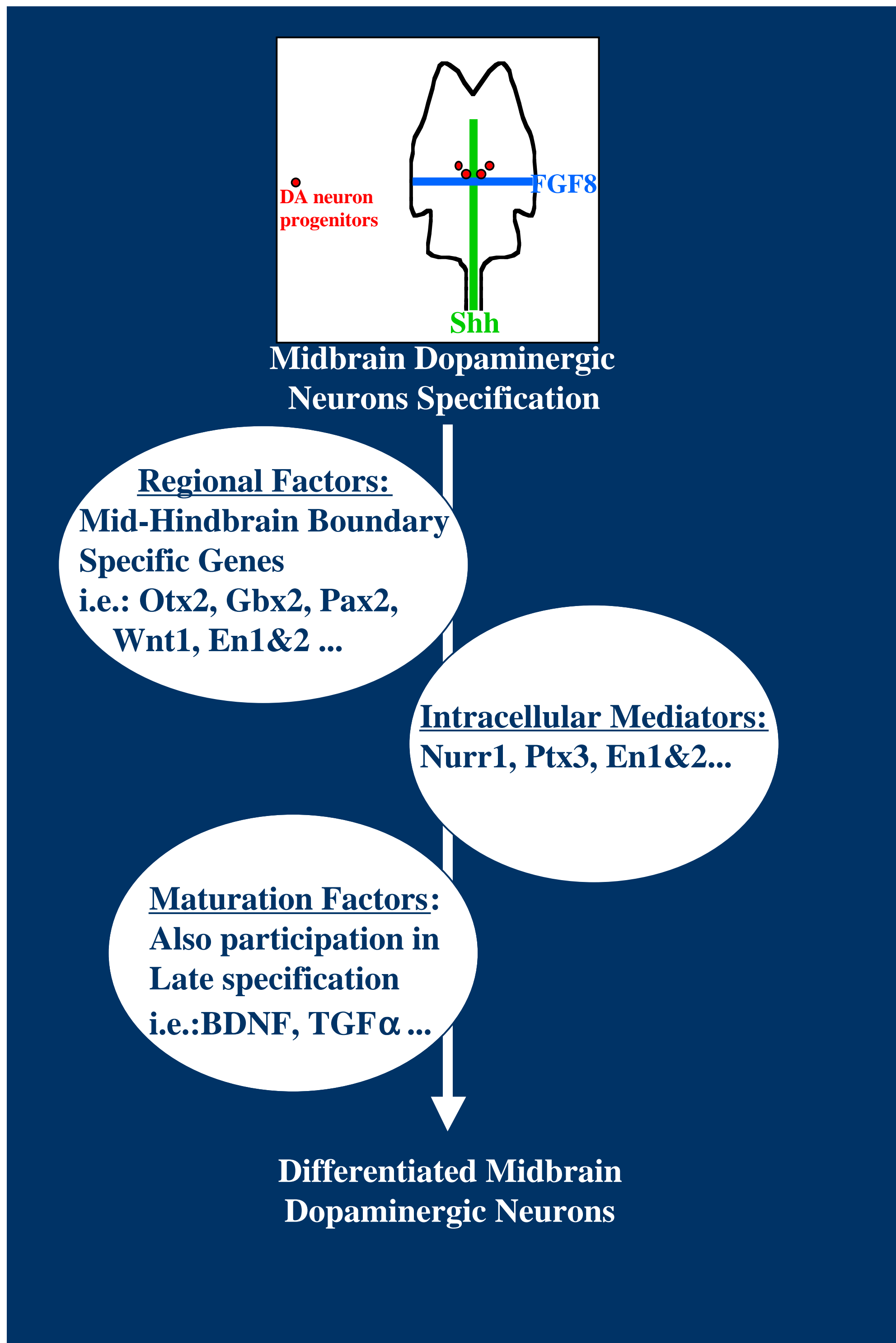

Figure I.4. Generation of the midbrain dopaminergic neurons.
Midbrain neural plate progenitors differentiate into DA neurons by the combined action of two extracellular inducers, Sonic Hedgehog (Shh) and fibroblast growth factor 8 (FGF8). The inductive molecules are thought to activate cascades of other signaling molecules and transcription factors, which lead to the final differentiation of DA neurons.

4-1-2-Specification of the DA neurons along the antero-posterior axis

Forebrain DA neurons have been found to be specified in the very anterior regions of the neural plate, in close proximity to the ANR [Ye et al., 1998]. The fact that the developing midbrain DA neurons are close to the MHB and that the forebrain DA neurons are close to the ANR, indicate that these two organizing centers may supply the DA neurons for location information along the antero-posterior axis. The molecule delimiting the position of the DA neurons along the antero-posterior axis is FGF8. Indeed, FGF8 is expressed at E9 in both the ANR and MHB, at very high levels in the transverse pieces of tissue that give rise to fore- and midbrain DA neurons [Ye et al., 1998]. At E14, the DA neurons are located nearby the remaining *Fgf8* signal. It was shown that blocking FGF8 in isolated mid- and forebrain explants prevented the development of both mid- and forebrain DA neurons [Ye et al., 1998]. The need of FGF8 for DA neurons existence is supported by experiments showing that mice with a mutation in the *Fgf8* gene lack TH-positive DA neurons [Ye et al., 1998]. Reverse experiments show that FGF8 is also capable of inducing DA neurons ectopically in the ventral diencephalon [Ye et al., 1998]. This demonstrates that FGF8 is very likely an endogenous factor that demarcates the location of DA neurons along the anterio-posterior axis of the neural tube.

4-1-3-Specification of the DA neurons by Shh and FGF8

FGF8 and Shh can induce DA neurons in the dorsal diencephalons only when applied together, but not alone. Therefore, a combined action is crucial for the specification of DA neurons, and the location of DA neurons along the anterio-posterior and dorso-ventral axes is delineated by the incorporation of these two molecules [Ye et al., 1998].

4-1-4-The MHB and its relationship to the midbrain DA neurons

The midbrain DA neuron progenitors are located in rat at E9 on the rostral side of (and within) the MHB, and they differentiate in this area between E9 and E14. It is

important to characterize the MHB to comprehend how it acquires a distinctive rostral-caudal polarity resulting in a specific rostral location to the midbrain DA neurons. The MHB is vaguely anatomically distinguish as the 'gap' in between the midbrain and hindbrain and molecularly depicted has having the ability to recreate particular patterns when transplanted to another area of the neural tube. Because of its ambiguous anatomical definition, a set of genes expressed within the MHB is used to define this organizing center. These genes cover a large domain that ends at the margin between the mid- and hindbrain (e.g. *Otx2* and *Gbx2*), are specifically expressed in midbrain (e.g. *Wnt1*), hindbrain (e.g. *Otp*), or are expressed within the isthmus (e.g. *Engrailed-1* and *Engrailed-2* (*En-1* and *En-2*), *Lmx1b*, *Pax2*, *Pax5, Pax8*, and *Fgf8*) (Figure I.5).

The MHB initially covers a wide area within the neural plate, including the region of both the future midbrain and hindbrain. Later this area progressively is reduced in size, between the headfold stage and mid-gestation, to finally occupy a region between the midbrain and hindbrain. *En-1* and *En-2, Fgf8*, *Wnt1*, *Otx2* are expressed at early stages (5 somites) in the areas of the neural plate, which give rise to midbrain DA neurons [Ye et al., 1998], *Lmx1b* is expressed throughout the caudal forebrain, midbrain and hindbrain [Adams et al., 2000]. *Fgf8*, *Wnt1*, *Otx2*, *Lmx1b*, *En-1* and *En-2* are still expressed by midgestation in close vicinity to DA neurons, where the latter three genes now even include expression within the DA neurons (details are given in the section 3-2-3). From E11.5 onwards, the DA neurons can be identified and express TH their mature marker. The MHB genes identified so far function to define, stabilize and maintain the boundaries of the MHB, implying a function in the midbrain DA neurons positioning and likely in their fate.

4-2-The factors that affect later events in midbrain DA neurons maturation

4-2-1-The maturation factors

The late development of midbrain DA neurons involves a lot of factors. These factors may not only play a role in the maturation of midbrain DA neurons, but might as well take part in their late specification. For instance, the receptor for

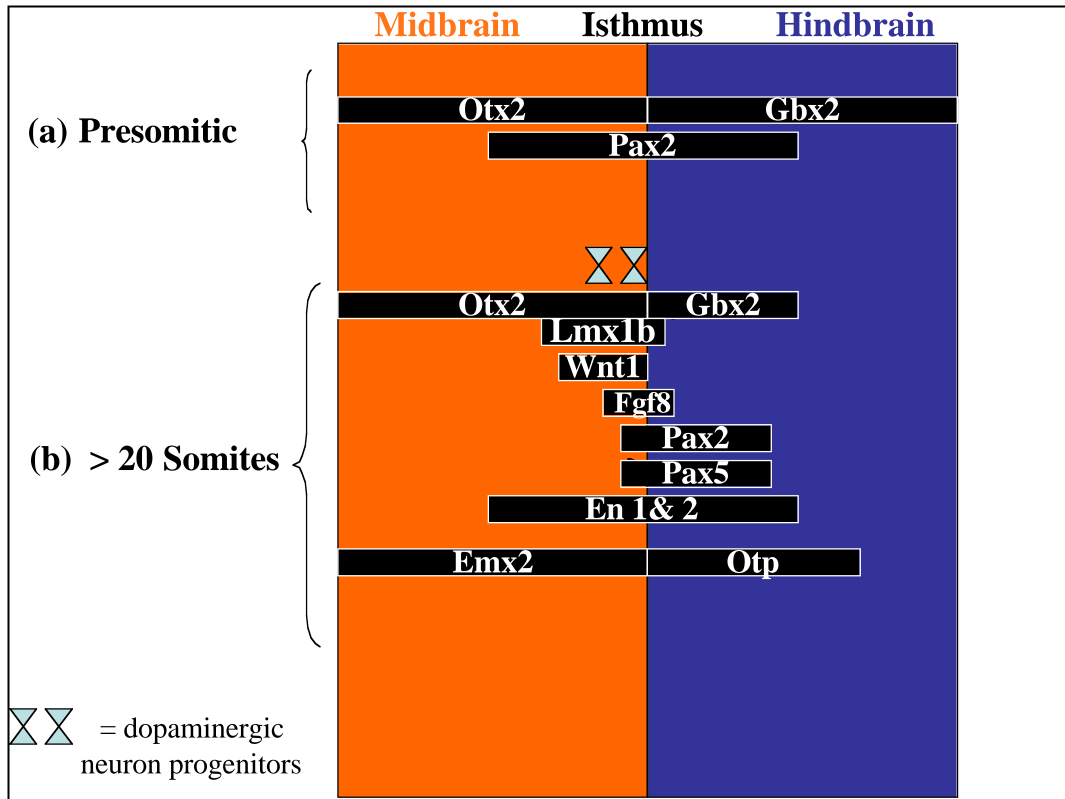

Figure I.5. Schematic of the relative position of mid-hindbrain boundary associated genes and of the dopaminergic neuron progenitors.
(a) Presomite stage: *Otx2*, *Gbx2* and *Pax2*. **(b)** > 20 somites: *Otx2*, *Gbx2*, *Lmx1b*, *Wnt1*, *Fgf8*, *Pax2*, *Pax5*, *En-1* and *En2*, *Emx2*, *Otp*. Adapted from Hynes and Rosenthal, 1999.

thrombin was shown to be expressed in the embryonic ventral midbrain, and DA neurons cultured in contact with thrombin change the length, branching pattern and number of neurites [Debeir et al., 1998]. Similar changes in DA neurons are generated by nerve growth factor proteins, such as brain-derived neurotrophic factor (BDNF) [Alonso-Vanegas et al., 1999], neurotrophin-3 and neurotrophin-4/5 [Studer et al., 1995; Hyman et al., 1994], by bFGF [Ferrari et al., 1989], and by members of the transforming growth factor (TGF) and GDNF protein families [Horger et al., 1998; Strelau et al., 2000]. Moreover, mice deficient in the TGFα gene showed a 50% reduction of DA neurons in the SN, but a normal amount in other DA nuclei [Blum, 1998], suggesting that TGFα may take part in the expansion or differentiation of this specific DA neuron.
There are more factors which play a role in the development, the survival, or the maturation of the midbrain DA neurons and/or whose receptors are expressed in the midbrain DA neurons. Although little is known about the function of these factors in the development of DA neurons *in vivo*, they could participate in late specification into particular subtypes or serve as mitogenic or survival factors for DA progenitors. These factors are for instance, the pituitary adenylate cyclase-activating polypeptide [Takei et al., 1998], calcitonin gene-related peptide [Burvenich et al., 1998], endothelin [Webber et al., 1998], neurotensin [Sotty et al., 1998], estrogen and progesterone [Kritzer and Kohama, 1998], substance P [Futami et al., 1998], retinoic acid [Samad et al., 1997].

4-2-2-The intracellular mediators

In contrast to the genes described above, very little is known about the intracellular proteins that mediate the development of DA neurons in reaction to inductive signals. Until now, five transcription factors have been identified to intervene in the development of the midbrain DA neurons: *Nurr1*, *Ptx3*, *Lmx1b* and *En-1* and *En-2*. The data described in this chapter suggest that none of these genes, by itself, is sufficient to induce DA neurons in the embryonic midbrain.
Midbrain DA progenitor cells, while still mitotically active, express the homeodomain transcription factor *Lmx1b* at E7.5 [Smidt et al., 2000] and *aldehyde dehydrogenase 2* (*AHD-2*) is detected from E9.5 [Wallen et al., 1999]. After

becoming postmitotic and beginning neuronal differentiation, the precursors of the midbrain DA neuron express the orphan nuclear receptor *Nurr1* at E10.5 [Zetterstrom et al., 1997], the homeodomain transcription factor *Ptx3* at E11 [Smidt et al., 1997] and the 2 homologues homeodomain transcription factors *En-1* and *En-2* at E11.5. At E11.5, these neurons begin terminal differentiation into DA neurons expressing TH, and L-aromatic amino acid decarboxylase (AADC), as well as other proteins necessary for dopamine function including vesicular monoamine transporter (VMAT) and dopamine transporter (DAT) [Lee et al., 1999; Foster et al., 1988; Fujita et al., 1993; Hansson et al., 1998].

4-2-2-1-*Nurr1*

Nurr1 is an orphan member of the steroid/thyroid hormone nuclear receptor superfamily and functions as a transcription factor (for review [Maruyama et al., 1998]) [Eells et al., 2001]. It plays a key role in the determination of the midbrain DA neurons identity. Mice homozygous for a null mutation in the *Nurr1* gene neither express the two enzymes of dopamine synthesis, TH and AADC, nor its transporter vehicles, VMAT and DAT [Zetterstrom et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998]. In the absence of *Nurr1*, the DA neuron precursors rapidly lose *ADH-2* expression after E10.5, while the expression of *Ptx3*, *En-1* and *-2*, and *Lmx1b* is maintained until at least E15.5 before it is gradually lost [Saucedo-Cardenas et al., 1998; Wallen et al., 1999; Smidt et al., 2000]. A subset of the DA neuron precursors undergoes apoptosis; however, a significant population remains at birth, principally in the VTA, as shown by the continued expression of exon 1 and 2 *Nurr1* mRNA (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Wallen et al., 1999; Witta et al., 2000).
Although Nurr1 expression can be induced by Shh [Ye et al., 1998], it may be responsible only for late steps in the differentiation of DA neurons. Furthermore, Nurr1 does not seem to be sufficient by itself to induce DA neurons, as no ectopic development of DA neurons is induce by Nurr1 ectopic expression in the dorsal midbrain of mutant mice (M. Hynes, A. Rosenthal, unpublished data, mentioned in the review [Hynes and Rosenthal, 1999]).

4-2-2-2-*Ptx3*

Ptx3, a homeodomain transcription factor, has been cloned from neuronal tissues and is exclusively expressed in midbrain DA neurons from E11 until adulthood [Smidt et al., 1997]. The consequence of ablating Ptx3 has yet been reported. Nevertheless, cell culture experiments with transient transfections showed that Ptx3 activates the *TH* gene through direct interaction with a single high-affinity binding site within the promoter. The activation of the TH promoter appears to be cell-dependent suggesting that Ptx3 action may be modulated by other regulatory mechanism(s) and factor(s) [Lebel et al., 2001].

4-2-2-3-*Lmx1b*

Additionally to its role in the patterning of mid/hindbrain region [Adams et al., 2000], the LIM homeobox gene *Lmx1b* is an essential regulator of dorsoventral patterning of the developing limbs [Johnson and Tabin, 1997]. Mutations in this gene evoke the nail patella syndrome [Dreyer et al., 1998; Chen et al., 1998] but no resulting patterns of expression of *Lmx1b* in brain or brain abnormalities are known to be connected to this syndrome. *Lmx1b* expression pattern is characterized by expression in the DA neurons of the SNC and of the VTA. Neural *Lmx1b* is first expressed at E7.5 and its expression is maintained in the SNC and VTA throughout life [Smidt et al., 2000]. In E12.5 *Lmx1b–/–* mouse embryos *TH*-positive cells are observed only in the VTA, and express *Nurr1*. These cells do not express Ptx3, indicating that Ptx3 is not necessary for *TH* expression. *TH*-positive cells are detected in the VTA up to E16 before they are lost. However, expression of TH is maintained in the DA and noradrenergic neural system in other brain regions. The absence of Ptx3 expression in the *TH*-positive neurons indicates that *Lmx1b* is essential for the proper specification of the midbrain DA neurons. *Lmx1b–/–* mice seem to lack the required molecular signals to maintain midbrain DA neurons, resulting in their lost during maturation of the organism [Smidt et al., 2000].
It appears that *Lmx1b*, together with *Ptx3*, sets up a molecular cascade unrelated to *Nurr1* and *TH*. This individual pathway seems vital for proper development of the system and may be related to neuronal specification of the midbrain DA neurons.

4-2-2-4-*Engrailed 1 and 2*

En-1 and *En-2*, are two homeodomain transcription factors. Their identification in mice [Joyner et al., 1985; Joyner and Martin, 1987] and later in others vertebrates, was based on their sequence similarity to the drosophila genes *Engrailed* and *Invected*. The drosophila genes have been shown to be required for embryonic segmentation [Kornberg, 1981; Lawrence and Struhl, 1982] and for later development of the nervous system [Lawrence and Johnston, 1984].

In the mouse and rat, *En-1* and *En-2* (and *En-3* in zebrafish*)* play an important role in the patterning of the mid/hindbrain region and all the En proteins have extensive functional overlaps [Hanks et al., 1995; Scholpp and Brand, 2001]. Mice depleted for *En-1* die shortly after birth and exhibit multiple developmental defects and lack the cerebellum and inferior colliculus [Wurst et al., 1994]. Mutants for *En-2* are viable and show no obvious defects in embryonic developement but exhibit an abnormal foliation in the adult cerebellum [Joyner et al., 1991; 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; Shigetani et al., 1997; Shamim et al., 1999] within the developing mesencephalon results in the disruption of the gradient of cytoarchitectonic differentiation of the optic tectum and further aberrant arborizations and perturbed targeting of nasal retinal ganglion cell (RGC) axons and complete degeneration of the temporal RGC axons.

En-1 and *En-2* have been recently described to be involved in the development of the SN and VTA. They are both expressed in the midbrain DA neurons from E11.5 of mouse development and up to adulthood (Figure I.6). Either of the two is sufficient for the generation and maintenance of this cell population. Their requirement is only apparent in the absence of both genes (Figure I.7). In *En* double null mutant, the DA neurons are induced in the ventral midbrain and express at least one of their typical differentiation markers, *TH*, but then fail to mature and disappear (Figure I.8). Interestingly, *α-synuclein* is not expressed in the midbrain DA neurons when no functional En-1 and En-2 protein is present [Simon et al., 2001]. This is particularly interesting since, as mentioned earlier, mutations in the gene *α-synuclein* have been found in some families with autosomal dominant PD.

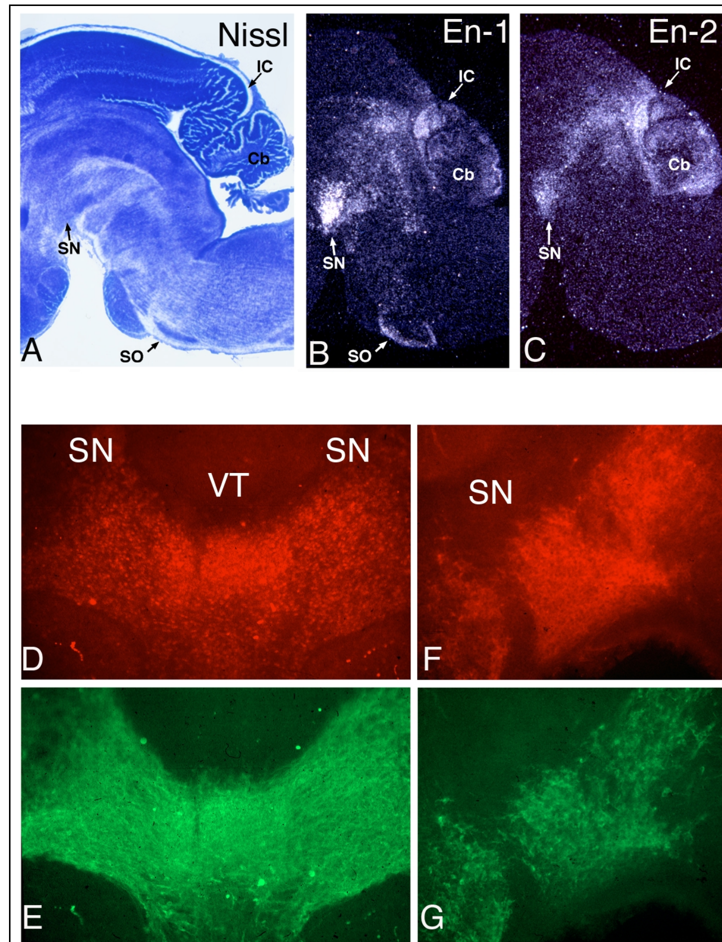

Figure I.6. DA neurons in the ventral midbrain of neonatal mice express *En-1* and *En-2*.

Nissl staining (A), *in situ* hybridization (B and C) and double immunolabeling (D–G) of sagittal (A–C, F, G) and coronal (D, E) sections of P0 mouse brains. A, B, C, adjacent sections of a P0 wild-type mouse hybridized with riboprobes against *En-1* (B) and *En-2* (C) reveal the distribution of En transcripts in the substantia nigra (SN), inferior colliculus (IC), and cerebellum (Cb). *En-1* is expressed at high levels throughout the ventral tegmentum (VT) and SN, whereas *En-2* is expressed at relatively high levels by only a small subset of cells and at much lower levels in most cells. *En-1* is also expressed in a subpopulation of cells in the superior olive (SO), a hindbrain nucleus. D, E, double immunohistochemistry on a coronal section of a P0 wild-type mouse brain using the αEnhb antibody, which recognizes both En-1 and En-2 proteins (D, red), and an antibody against TH (E, green). En and TH proteins are coexpressed in DA neurons of the SN and VT. En protein is located in the nuclei, whereas TH is located in the cell somata and their axonal processes. F, G, a lateral section of P0 mouse brain heterozygous for *En-1/Tau-LacZ*, double-labeled with antibodies against β-gal, the protein product of *LacZ* (F, red) and TH (G, green). TH and the β-gal reporter for *En-1Ttau-LacZ* are coexpressed in the somata and axons of the midbrain DA neurons. Adapted from Simon et al., 2001.

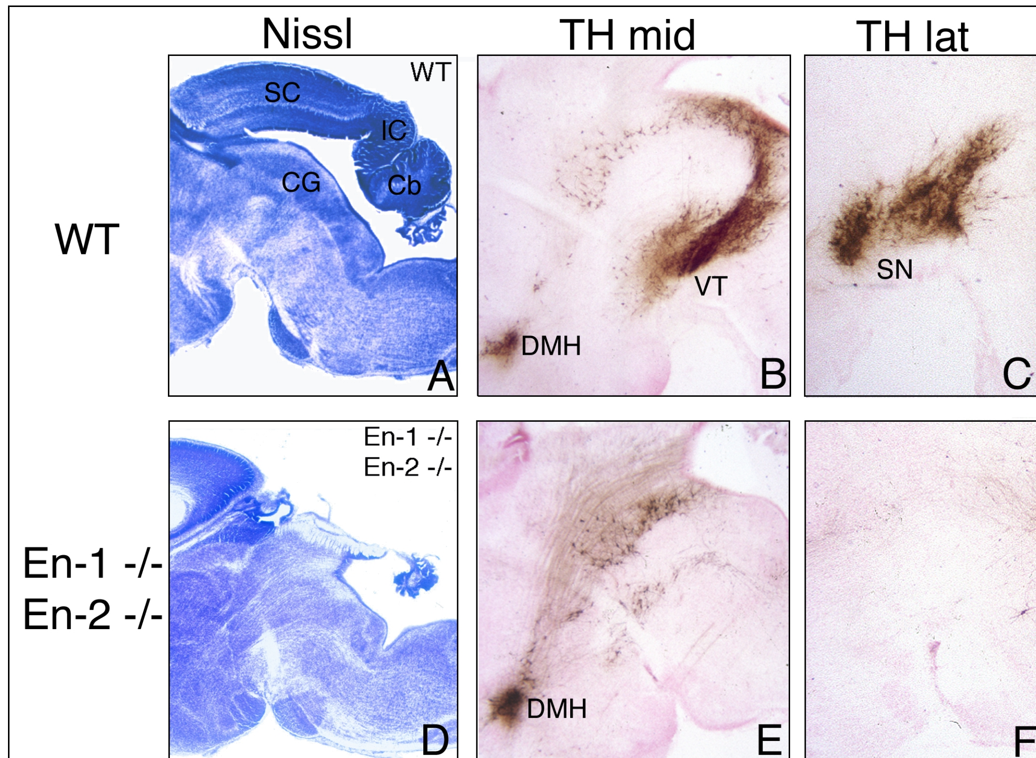

Figure I.7. Loss of midbrain DA neurons in *En* double mutant mice.
Nissl staining (A and D), immunohistochemistry on sagittal sections of P0 mouse brains using antibodies against TH to identify DA neurons (B, C, D, E) of wild-type (*WT*) mice (B and C), *En* double mutant (*En-1*-/-/*En-2*-/-) mouse (E and F). B and C, TH immunostaining reveals the normal distribution of the midbrain DA neurons. In the midline (mid) (B), the neurons of the ventral tegmentum (VT) are labeled, and in a more lateral (lat) section (C), those of the substantia nigra (SN) are labeled. E and F, DA neurons of the SN and VT are not detected by TH immunostaining, however, DA neurons of the dorso-medial hypothalamic nucleus (*DMH*) remain TH-positive (E), indicating that their DA phenotype does not depend on *En-1* or *En-2*. Adapted from Simon et al., 2001.

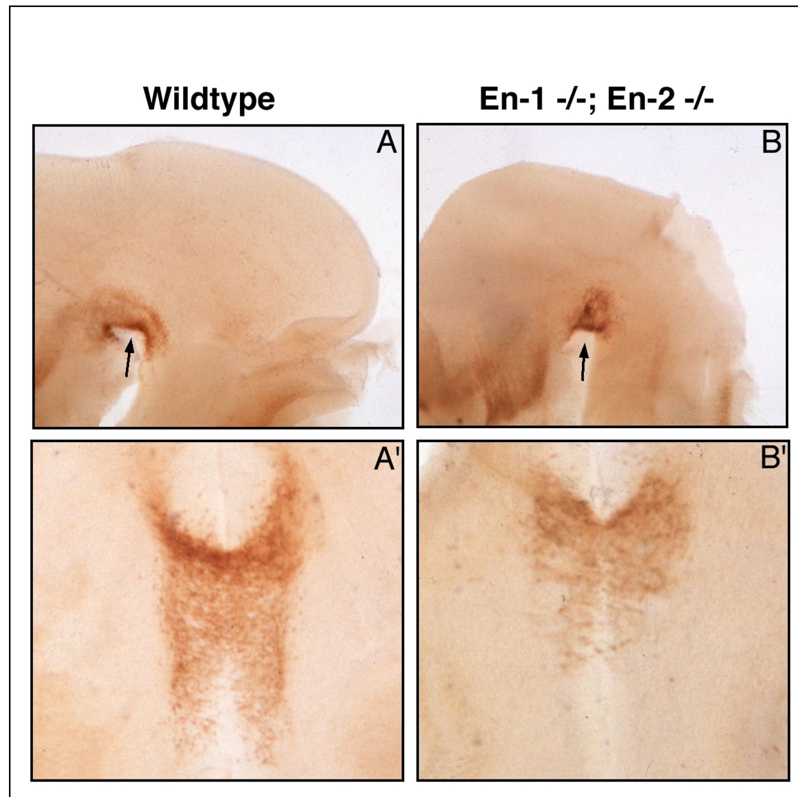

Figure I.8. DA neurons of the ventral midbrain are generated in *En* double mutants.

Whole-mount immunohistochemistry of E12.5 embryos using antibodies against TH. A, wild-type; B, *En* double mutants (*En-1*-/-/*En-2*-/-). Flat mount of ventral midbrain E12.5 embryos immunostained for TH. A', wild-type; B', *En* double mutants. A', the immunostaining reveals the bilateral distribution of DA neurons adjacent to the floor plate. B', a cluster of DA neurons is detected, although it is smaller than what is observed in wild-type or heterozygous mice. These neurons disappear in *En* double mutants over the next few days of development. Adapted from Simon et al., 2001

5-Aim of the study

The DA system is specified in the embryonic ventral midbrain around E12 in the mouse brain. As mentioned above, the function of this region to generate DA neurons depends on earlier signals from organizers neighboring the ventral midbrain progenitor neurons, and involves molecular differentiation cascades for specification of neurotransmitter identity and appropriate integration in the developing brain.

There are a number of genes identified that participate in the patterning of the ventral midbrain (see above); however factors involved in midbrain DA neurons-specific cascades and their cellular function in development of the midbrain DA neurons system are largely unknown. Only five transcription factors described above; *Nurr1*, *Ptx3*, *Lmx1b* and *En-1* and *En-2* are known to be implicated at this level in the midbrain DA neuron system, however the functional relationship between these genes is not clear.

The aim of this project was to identify and characterize genes, which mediate the development of the DA neurons during the intermediate period between induction and full differentiation of these neurons. Such genes would be of great value for further studies where specific drivers of midbrain DA neurons are needed, i.e. for in vitro experiments in tissue grafts used for therapeutic purposes.

6-Strategy

The continuous expression of *En-1* and *En-2* in the adult suggests that these genes are required for the entire life span of the animal. Indeed, in the *En* double null mutant mice, the DA neurons of the SN and VTA differentiate first, but then disappear soon after they have acquired their DA phenotype [Simon et al., 2001]. Since *En-1* and *En-2* are transcription factors, which bind to DNA and act as activators or repressors of gene expression, the altered expression of downstream genes in the double null mutants is likely to be responsible for the death of these neurons. A comparison of the gene expression pattern between wild type mice and *En* double null mutant mice should therefore reveal genes whose expression may be directly regulated by *En-1* and *En-2* and genes which are generally expressed by the midbrain DA neurons. A recently modified differential display method [Gesemann et al., 2001] was used in order to identify such genes. Subsequently, the characterization of the genes of interest was achieved by the study of their developmental expression patterns, by the analysis of mice depleted for these genes and by over-expressing them in the chick embryo.

MATERIALS & 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., 1991]. The *En1Tau-LacZ* mice were generated by a "knock-in" strategy in which the first 71 codons, including the start codon, were replaced by a *Tau-LacZ* sequence (TLZ) [Callahan and Thomas, 1994], and resulted in an *En-1* null allele. The construct and procedures are described in detail by Saueressig et al., 1999. Parental lines for producing the mutant mice deficient for both *En-1* and *En-2* were kept as *En2-/-*;*En1+/TLZ*. The initial genotyping of the mice for *En-2* wild-type and mutant alleles was done by PCR with three primers to detect in one reaction wild-type and mutant alleles. 'Common' primer: TTG AGA AGA GAG GCC CTG TA, 'wild-type' primer: CTG GAA CAA AAG GCC AGT GT, 'mutant primer' (located in the neomycin resistance gene): TCT CAT GCT GGA GTT CTT CG. PCR parameters: 5min-94°C, 36 cycles {45sec-94°C, 1min-54°C, 1min-72°C}, 5min-72°C, PCR conditions: as described in 3-4, with 1.5mM $MgCl_2$. The presence of the *En1TLZ* allele was detected by β-galactosidase staining of the toes. The toes were incubated at 37 °C for 3hr in the staining solution (4mM $K3[Fe[CN]_6]$; 4mM $K4[Fe[CN]_6]$; 2mM $MgCl_2$, and 0.4mg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside), then observed under the microscope. A blue staining had developed if the *En1TLZ* allele was present. The *En2-/-*;*En1TLZ/TLZ* mutant mice were recognizable by their typical head morphology: they lack a large part of the midbrain and the anterior hindbrain.

The *Nurr-1* deficient mice were generated by targeted deletion of exons 2, 3, and part of exon 4 [Zetterstrom et al., 1997]. The parental lines for producing the null mice were kept as heterozygotes. The genotyping of the mice for the *Nurr-1* wild-type or mutated alleles was done by PCR. Wild-type allele detection: sense primer: GTC GGT TTC AGA AGT GC, anti-sense primer: GTA AAC GAC CTC TCC GG. Mutated allele detection: sense primer: CCA ATG TCG AGC AAA CC, anti-sense primer: CGA TCC CCT CAG AAG AA. For both PCR the parameters were the same: 4min-94°C, 35 cycles {45sec-94°C, 1min-56°C, 1min-72°C}, 4min-72°C. PCR conditions: as described in 3-4, with 1.5mM $MgCl_2$.

The *ErbB4* conditional knock-out mice were generated by Rüdiger Klein and kindly provided by Martin Gassemann (unpublished data). The *nestin-cre*

transgenic mice [Tronche et al., 1999] were used to generate these mice in which the ErbB4 gene was mutated by Cre-mediated recombination of loxP-flanked *ErbB4* exon 2.
The *HNF3*α deficient mice were generated by targeted deletion of the entire winged helix DNA-binding domain and carboxy-terminal region of the protein and creation of an in-frame fusion with *E.coli lacZ* gene [Kaestner et al., 1999].
The C57/BL6 wildtype mice were bred at the animal facility at the University of Heidelberg. The day of vaginal plug detection is designated E0 and the day of birth, postnatal day (P) 0. The timed pregnant females were sacrificed by cervical dislocation.

2- Generation of the *En* chimeric mice

2-1- Preparation of the mouse embryo fibroblasts as feeder layer

2-1-1-Preparation of the mouse embryo fibroblasts

E12 to E14 mouse embryos were dissected in a dish with Dulbecco's modified eagle medium (DMEM) (high glucose, without pyruvate). The limbs, the internal organs and the head were removed. The carcasses were put into a tube containing DMEM and rinse 3 times with DMEM. The carcasses were then place on a dish and minced with a razor blade, and poured into a tube containing 10ml of 0.05% trypsin/EDTA in phosphate buffer saline (PBS) Ca^{++}/Mg^{++} free and incubated for 10min on a shaker at 37°C. 5ml from the incubated tube were removed and placed in a new 50ml tube with equal volume of DMEM plus 10% of newborn calf serum. Another 5ml of trypsin/EDTA was added to the first tube and incubated for an additional 10min. The second 5ml aliquot was then removed and added to the second 50ml tube with an equal amount of DMEM plus 10% of newborn calf serum. The latter step was repeated for five incubations. The content of the 50ml tube was then centrifuged and resuspended with DMEM plus newborn calf serum. The cells were then plated into eight 10-cm tissues culture dishes containing feeder medium (DMEM, 50µg/ml penicillin and streptomycin, 2mM glutamine, 10% fetal bovine serum (FBS) (Life Technologies, Inc., USA)). After one day in culture at

37°C and 5% CO_2 the medium was changed and then the cells were allowed to grow until the dishes were confluent. The cells were split 1:10 and grew until they reached confluence and then frozen into DMEM plus 20% FBS and 10% dimethyl sulfoxide (tissue grade culture, Sigma-Aldrich, Inc., USA).

2-1-2-Preparation of mouse embryo fibroblasts feeder dishes

It is necessary to mitotically inactive the mouse embryo fibroblasts before they can be used as feeder layer to grow ES cells. Mitomycin C (tissue grade culture, Sigma-Aldrich, Inc, USA) was used to cross-link DNA and block cell proliferation.
Confluent plates of mouse embryo fibroblasts on gelatin were treated with DMEM plus 10% newborn calf serum and 10μg/ml of mitomycin C for 3hr at 37°C and 5% CO_2. The dishes were washed three times with PBS. ES medium (DMEM (high glucose, without pyruvate), 15% fetal FBS ES cell tested, 2mM glutamine, 0.1mM non essential amino-acids, 50μg/ml penicillin and streptomycin, 0.1mM (Life Technologies, Inc., USA), β-mercaptoethanol (tissue grade culture, Sigma-Aldrich, Inc., USA), and leukemia-inhibiting factor (LIF) 200U/ml (Chemicon International, Inc., Temecula, CA, USA)) was then added 2hr before plating the ES cells.

2-2- *De novo* isolation of embryonic stem cells from blastocysts

The E3 blastocysts were flushed out from the uterine horns with M2 medium (Life Technologies, Inc., USA) and place individually into 10-mm-well tissue culture dishes containing a feeder layer and ES medium. After two days of culture at 37°C with 5% CO_2, the blastocysts attached to the culture dishes. The inner cell mass (ICM) became distinguishable and grew rapidly over the next two days. After 5 to 6 days, the ICM was disaggregated using a sealed end of a very fine Pasteur pipette. The clumps of cells were then washed twice with PBS Ca^{++}/Mg^{++} free (Life Technologies, Inc., USA), and were transfered with a very fine Pasteur pipette to a microdrop of 0.025% trypsin/EDTA (Life Technologies, Inc., USA) in PBS. The cells were incubated for 3min at 37°C, then the clumps were gently dissociated into smaller cellular aggregates of three to four cells. The content of the microdrop was then transferred into a fresh 10-mm feeder cells tissue culture well (see 2-3) with

ES medium. The individual cultures were inspected daily. Generally after 3 days, primary colonies of cells became visible and the stem-cells-like cells could be identified. They grow progressively but maintain the same ES-cell-like phenotype: the colonies remain composed of a homogenous population of stem cells and no cellular differentiation occurs. Stem cells are comparatively small, typically have a large nucleus containing one or more prominent nucleoli, and are tightly packed within the multi-layered colony. The colonies which fail to show any differentiation but retain cells of an exclusively ES phenotype were selectively removed between 7 to 8 days. These colonies were then dissociated in microdops of trypsin / EDTA (as described above) and passaged into fresh, small feeder layer wells to keep the cell density high. Depending on the relative rate of growth, these cultures were expanded 3-5 days later by trypsinizing the whole well and transferring its content into a larger feeder dish. This procedure is detailed in the laboratory manual 'Manipulation of the mouse embryo' by [Hogan et al., 1994]. The experimental stages of the techniques and the timings are summarized in figure II.I.

2-3- Establishment of the ES cell lines

To establish the ES cell lines it was necessary to subculture at 4-5 day intervals, splitting at 1:3, as their cell growth rate was relatively low (instead of usually 2-3 day intervals). To improve the viability of the cells, the media of semi-confluant cultures was changed 2-3hr before passage. The cell monolayer was washed twice with PBS and a small amount of 0.025% trypsin/EDTA in PBS was added to the tissue culture dish. After 3-4min of incubation at 37°C, the cells were detached from the dish and the cell clumps were broken up to give single-cell suspension by pipetting vigorously. The suspension was then washed with ES medium and replated onto fresh feeder wells. The ES cells were incubated at 37°C and 5% CO_2 and ES medium was changed every 1 to 2 days.

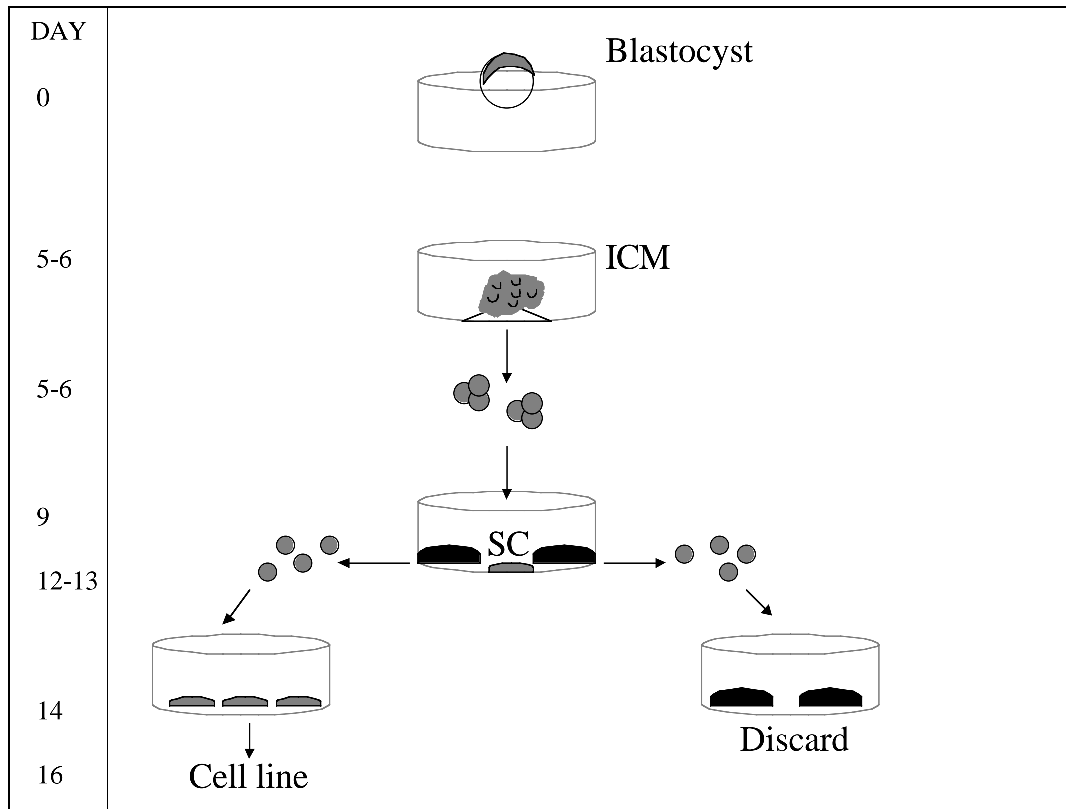

Figure II.1. Summary of the procedure for obtaining pluripotential stem cell lines. Blastocysts are recovered and placed into cell culture dishes with ES medium (day 0). By day 5-6, the inner cell mass (ICM) has proliferated to give a distinct cellular mass, which is dissociated, and the cells clumps are transferred to a fresh feeder layer well. On day 9, primary colonies are examined. Colonies that look like stem cells (SC) are individually removed, dissociated into single cells, and reseeded into fresh feeder wells on day 12-13. Two days later (day 14), the wells are inspected for the presence of ES cell colonies and wells containing stem cells are subcultured (day 16) to give permanent cell lines. Remaining wells are discarded. Adapted from Hogan et al., 1994.

2-4- Genotyping of the ES cell lines

The *En-1* gene was used to screen ES cells for *En2-/-;En1TLZ/TLZ* genotype as described by Saueressig et al., 1999. ES cells clones were screened for integration of *TLZ* in the *En-1* locus by Southern blot using an external 3' genomic DNA probe. The Southern blot analysis was carried out as described in the Basic Protocol section of 'Current protocols in molecular biology' [Ausubel et al., 1998]. Briefly, *Hind*III-digested genomic DNA samples from ES cells clones were run in an agarose gel and transferred over-night on a nylon membrane. The DNA samples were immobilized to the membrane by UV crosslinking. The membrane was then hybridized with a denatured 800bp *EcoR*I/*Hind*III DNA probe labeled with ^{32}P, overnight at 65°C. The membrane was washed at 65°C twice 15min with 2xSSC/0.1%SDS, then twice 15min with 0.2xSSC/0.1%SDS. Signals were detected by exposure overnight of the membrane to Applied Biosystems PhosphorImager cassettes, which were scanned on a PhosphorImager (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA, USA).

3- Differential display PCR experiment

3-1- RNA isolation

Embryos and adult brains were dissected in L15 medium (Life technologies GmbH, Germany), carefully removing mesodermal tissue and meningitis. The tissue pieces were briefly washed in medium and then put directly into lysis buffer (Qiagen GmbH, Germany). Each sample was passed through a Qiashredder (Qiagen GmbH, Germany) and applied on an RNeasy column (Qiagen GmbH, Germany). The RNA was then treated with RNase free DNase (Life Technologies GmbH, Germany) and repurified over an RNeasy column. The RNA was isolated from three independently collected samples of each of the tissue types. Small aliquots (250ng) were snap frozen in liquid nitrogen to avoid repeated thawing and freezing.

3-2- Reverse transcription

Each reverse transcription was done with 250ng of total RNA. One of the arbitrary 10mer primers (Operon Kit B (OPB) (Operon Technologies, Inc, Alameda, CA)) TGATCCCTGG, GGACTGGAGT, TGCTCTGCCC, GTCCACACGG, CTGCTGGGAC, CCTTGACGCA, TCCGCTCTGG, TTTGCCCGGA, CCACAGCAGT, GGACCCTTAC, GTTTCGCTCC, CATCCCCCTG, TGCGCCCTTC, GGTGACGCAG, TGGGGGACTC, GTAGACCCGT, TTCCCCCGCT, GGAGGGTGTT, AGGGAACGAG or ACCCCCGAAG), was added to the RNA to initiate first strand cDNA synthesis using the Superscript Preamplification System for First Strand cDNA Synthesis (Life Technologies GmbH, Germany). Reverse transcriptions were performed as suggested by the manufacturer using a thermal cycler. The obtained cDNA was applied to a Qiagen PCR purification column (Qiagen GmbH, Germany) and subsequently elute with 10mM TRIS pH 8.5.

3-3- Differential display PCRs

The differential displays PCRs experiment (ddPCR) was performed as described in detail by Gesemann et al. 2001. The cDNA of each sample was amplified using combinations of the OPB primers as upstream and downstream primers. ddPCR reactions were prepared on ice using 2µl of first strand cDNA and the following final buffer concentrations: 0.5µM of the primer used for reverse transcription, 0.5µM of secondary arbitrarily primer, 10mM Tris pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 25nM each dNTP, 0.075µCi/µl [^{33}P]dCTP and 0.2 U Taq polymerase (AmpliTaq, Perkin Elmer, Foster City, CA, USA). Following an initial 4min denaturing step the reaction mixture was subjected to three low stringency cycles (94°C for 45sec, 36°C for 60sec and 72°C for 90sec) and 39 high stringency cycles in which the annealing temperature was raised to 40°C. The PCR products were separated on a 6% denaturing polyacrylamide gel until the xylene cyanol dye reached the bottom of the gel (about 4h with 85 Watts). The sequencing gel was subsequently transferred to a piece of Whatman 3MM paper and dried without fixation. Gels were routinely exposed to x-ray films between 24hr and 36hr. Differences in the intensity of the bands were analyzed by visual inspection.

3-4- Reamplification and subcloning

Differentially expressed gene fragments were recovered from the gel by cutting through the overlaying film. Gel pieces were rehydrated in 100µl of deionized water and boiled for 10min. 2µl of the obtained supernatant was directly used for reamplification. Reamplification conditions were identical to the ddPCR conditions, except that the nucleotide concentration was raised to 200µM and no isotope was added. In addition, the original primers were replaced by 20mers containing the original OPB 10mers primers with added XbaI or XhoI restriction sites. The 4 bases preceding the cut site were chosen in a way that the annealing temperature of the new 20mer was around 62°C (XXXXTCTAGA-OPB-10mer #1-10; XXXXCTCGAG-OPB-10mer#11-20). Cycling parameters for reamplification were adjusted as follows: Two cycles with annealing temperature at 40°C, followed by 34 cycles in which the annealing temperature was raised to 60°C. After a final extension time of 5 minutes, the PCR reaction was loaded on a 3% agarose gel and the fragments were recovered using the Qiaex II gel isolation kit (Qiagen GmbH, Germany). Fragments were digested with the appropriate enzymes (Roche Diagnostics GmbH, Germany) and subcloned into pBluescript II SK (-) vector (Stratagene, La Jolla, CA, USA).

4- *In situ* hybridization

DNA fragments subcloned into pBluescript were used as template for making riboprobes. The linear templates used for *in vitro* transcription were generated either by restriction digest (Promega GmbH or from Roche Diagnostics GmbH (Germany)) of the plasmids or by amplifying the fragments, from the ddPCR experiment, using the following primer pairs: if the cDNA was in antisense direction to the T7 RNA Polymerase promoter site of the vector the primers AGCTCCACCGCGGTGGC and GGCCAGTGAATTGTAATACGA were used, if cDNA was in sense orientation the primer containing the T7 RNA polymerase promotor sequence AAAAATGTAATACGACTCACTATAGGGCCCACCG CGGTGGCGGCCGCTCTAGA and the primer GGGTACCGGGCCCCCCC TCGAG were used. This way, all templates contained a T7 site in antisense orientation. All templates were gel-purified and transcribed in the presence of ^{35}S-

UTP using T7 RNA Polymerase. The protocol for *in situ* hybridization was adapted from Goulding et al. (1993). Mouse embryos and postnatal mouse brains were fixed by either emersing in or perfusing with 4% paraformaldehyde (PFA). They were then dehydrated overnight in 70% ethanol, 2x1hr30 min in 80% ethanol, 2x30min in 96% ethanol, 2x45min in absolute ethanol, 1hr in ethanol/acetone (1:1), 2x45min in acetone (the dehydration times were shortened to half for embryos younger than E11, and extended to double duration for P8 brains), then embeded in paraffin for 2x1hr (Vogel, Germany). 8µm sections were cut and deparaffinated (2x7min in xylol, 5min each in a series of ethanol baths) then fixed in 4% PFA and then pretreated with acetic anhydride, and dehydrated in a series of ethanol baths. Hybridization occurred overnight at 55°C. Following hybridization, the sections were treated with ribonuclease A (20 µg/ml) at 37 ºC for 15min before being washed at high stringency in 0.2x SSC at 55ºC for 15min and 0.1 x SSC at 55ºC for 15min.

The slides were exposed on a Hyperfilm (Kodak) for 3 to 6 days, and developed for 4min in the D-19 developer (Kodak) and fixed in a non-rapid Kodak fixer for 10min. Selected sections were dipped into Amersham Hypercoat emulsion (Amersham Pharmacia Biotech Europe GmbH, Germany) and stored for 2-3 weeks in the dark at 4ºC. The developed sections were then counterstained with DAPI, again dehydrated and mounted in DPX (Fluka, Germany).

5- *In ovo* electroporation

The mouse *En-1*-coding region [Logan et al., 1992] was inserted into the pIRES2-EGFP vector (Clontech laboratories, Inc., CA, USA) with the CMV promoter replaced by the chicken β-actin promoter (kindly provided by C.Krull, University of Missouri, USA). The chicken Olf-1/Ebf mRNA (Ebf) is registered at the GenBank (AJ238322). *Olf1/Ebf*-coding region was cloned into the β-actin-IRES2-EGFP vector (Figure II.2). The single strand Ebf cDNA was generated from E7 chick brain mRNA (extracted with the RNAeasy kit from Qiagen GmbH, Germany) by reverse transcription, and amplified by PCR with primers containing both the EcoRV restriction site (sense primer: TCC GAT ATC ATG TTT GGG ATC CAG GAA AG, anti-sense primer: TCC GAT ATC TCA CAT GGG GGG AAC AAT

CA, PCR parameters: 4min-94°C, 35 cycles {45sec-94°C, 1min-60.5°C, 1min30sec-72°C}, 4min-72°C, PCR conditions: as described in 3-4). All the cloning procedures were done with primers synthesized by MWG-Biotech Sequencing GmbH (Germany), with the Superscript Preamplification System for First Strand cDNA Synthesis (Life Technologies GmbH, Germany) for reverse transcription reactions, with the Taq polymerase (AmpliTaq, Perkin Elmer, Foster City, CA, USA) for PCR, with restriction enzymes from Promega GmbH (Germany) for enzymatic restriction digestions and with the Takara kit (Bio Whittaker Europe, Germany) for the ligation reactions (100ng of insert and 100ng of plasmid incubated with an equal amount of Takara mix I, for 1hr at 16°C). All these procedures were done according to the manufacturer instructions.
The *in ovo* electroporation method [Muramatsu et al., 1997] was modified to obtain efficient transfection into brain vesicles [Funahashi et al., 1999, Nakamura and Funahashi, 2001]. Fertile chick eggs were incubated at 37°C for 1.5 days (stage 10-11). The windowed chick embryos were electroporated with *En1* or *Olf1* overexpression vector DNA at 3µg/µl. DNA mixed with a microdrop of 0.2% Fast green FCF dye (Sigma-Aldrich, Inc, USA) was pipetted into the midbrain vesicle using a picospriter until the vesicle was full. Pairs of electrodes (gold plated, 5mm distance between the electrodes, from Genetrodes by Genetronics Inc., CA, USA) were put beside brain vesicles as shown in figure II.3. A pulse of 25Volts, 50.7msec was charged five times by the electroporator (ECM 830 BTX, by Genetronics Inc., CA, USA). Eggs were sealed and embryos were allowed to develop to a specific stage, at which time they were dissected and fixed by immersion with 4% PFA for 6hr to 12hr.

6- Immunohistochemistry

Mouse embryos and postnatal mouse brains were fixed with 4% PFA, by perfusion for postnatal mouse brains, and/or by immersion for 12hr to 24hr for all the specimens. Brains, entire heads or embryos were cryoprotected in 30% sucrose, and cut at 10-12µm thickness on a cryostat. They were first blocked with 10% new born calf serum (Life Technologies GmbH, Germany), 0.1% Triton (Merck, Germany) in PBS for 1hr at room temperature (Rt) and then incubated overnight at 4°C with rabbit anti-Olf-1 (a kind gift from Randall Reed, Davis and Reed, 1996) 1:200 and

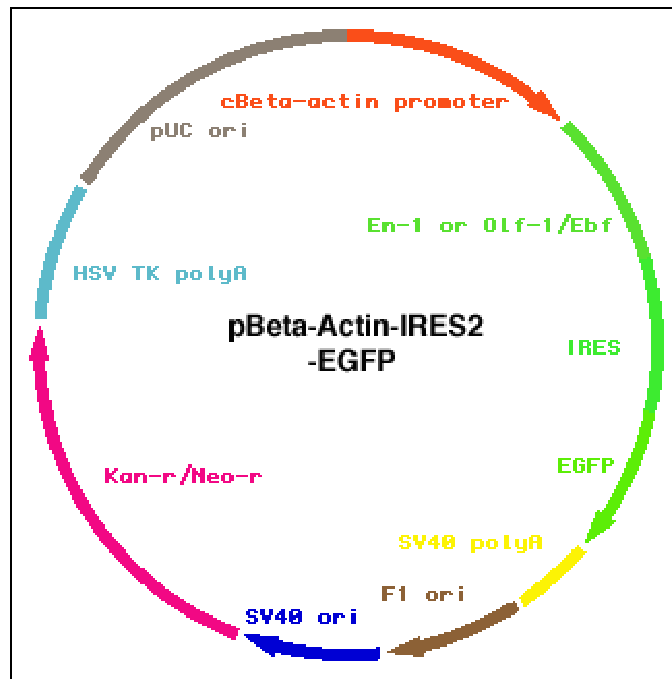

Figure II.2. Chick β-actin-IRES2-EGFP overexpression vector.
The mouse *En-1*-coding region or the chicken *Olf1/Ebf*-coding region was cloned into the pIRES2-EGFP vector (Clontech laboratories, Inc., CA, USA) with the CMV promoter replaced by the ubiquitous chick β-actin promoter. This vector contains the internal ribosome entry site (IRES) between the gene of interest (*En-1* or *Olf-1/Ebf*) and the enhanced green fluorescent protein (EGFP). This permits both the gene of interest and the EGFP gene to be translated from a single bicistronic mRNA. cbeta-actin, chicken β-actin; polyA, polyadenylation signals; ori, origin; Kan-r/Neo-r, neomycin/kanamycin resistance gene; HSV TK, herpes simplex virus thymidine kinase.

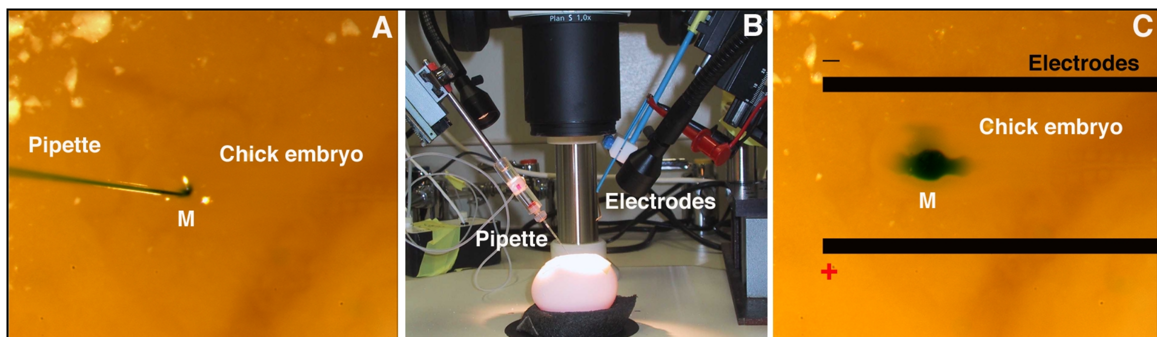

Figure II.3. *In ovo* electroporation into chick embryos.
A and C, dorsal view of the chick embryo brain vesicle. A, before injection, C, after injection of plasmid DNA labeled with Fast green in the midbrain vesicle (M) done with a pipette and a picospriter. DNA was electroporated by pulses charged between a pair of electrodes placed besides of the brain vesicle (C). The workstation is shown in B.

sheep anti-TH (Chemicon International, Inc., Temecula, CA) 1:400 diluted in blocking solution. Several washes with PBS were followed by incubation with Cy3™ conjugated donkey anti-rabbit, 1:500 and biotin conjugated donkey anti-sheep, 1:400 (Jackson, Inc, West Grove, PA) for 2hr at Rt. Sections were then washed again in PBS and incubated with Cy2™ conjugated streptavidin for 1hr at Rt. Finally, the sections were washed and mounted in Aqua Poly Mount (Polysciences, Inc, Warrington, PA, USA). A similar procedure was applied for chicken embryos, however the cryoprotection was done in 25% sucrose. Individual immuno-detections of Olf-1 or TH (rabbit anti-TH (Chemicon International, Inc., Temecula, CA) were done with the same antibody concentrations, using either Cy2™ or Cy3™ conjugated antibodies. For the detection of TH on whole mounts or thick floating sections the procedure was similar, but the preincubation was done with 1% Triton and 0.3% of H_2O_2 (Fluka, Germany), and the secondary antibody used was biotin conjugated donkey anti-rabbit 1:500 (Jackson, Inc, West Grove, PA), then the tissues were incubated in streptavidin conjugated horseradish peroxidase 1:2000 (Jackson, Inc, West Grove, PA) for 1hr. The signal was revealed by incubation with 1% Diaminobenzidine (Fluka, Germany) and 0.01% of H_2O_2 in PBS. The floating sections were placed on a slide, dehydrated and mounted in DPX.

7-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 6.0.

RESULTS

1-Further characterization of the role of the *En* genes in the midbrain DA neurons development

1-1-The midbrain DA neurons require *En-1* and *En-2* cell autonomously for their survival

Since the midbrain DA neurons express *En-1* and *En-2* throughout their postmitotic life and they disappear in the *En* double null mutant mice after they have been generated in the ventral midbrain [Simon et al., 2001], it is very likely that these cells require the *En* genes cell autonomously for their survival. Nevertheless, since the *En* genes are also required for the specification of the midbrain and anterior hindbrain which are in close vicinity to the developing SN/VTA [Millen et al., 1994; Wurst et al., 1994], it is possible that the embryonic environment of the DA neurons provides trophic support essential for their survival and the lack of this support causes them to disappear in the *En* double null mutant.

It was essential to address this question to determine the source of RNA for the differential display project; either ventral midbrain in the cell autonomous case, or the midbrain and the anterior hindbrain, which contain the entire *En* genes expression domain, in the non-autonomous case.

Since a transgenic approach would have been time and resource consuming, I generated chimeric mice made up of a mixture of wild-type and *En* double null mutant cells. A cell autonomous requirement should reveal itself by the loss of midbrain DA neurons, whereas in the case of a cell non-autonomous requirement, no changes in the distribution and numbers of the midbrain DA neurons are expected.

Blastocysts, obtained from matings between *En2-/-*; *En1+/TLZ* mice, were placed into culture dishes, containing embryonic fibroblasts as a feeder layer, and let grow until the ICM had formed. 50% of the blastocysts gave raise to an ICM, which was then removed, disaggregated and dissociated into smaller cellular aggregates and put back into culture until ES cells colonies could be identified. 30% of the ICMs gave raise to ES cells colonies, which were then dissociated and expanded. I obtained a total of 16 distinct ES cell lines. The genetic composition of the cell lines for *En-1* was tested by

Southern blot (Figure III.1). Three *En2-/-;En1TLZ/TLZ* ES cell lines, 7 *En2-/-;En1+/TLZ* ES cell lines and 5 *En2-/-;En1+/+* ES cell lines were obtained. Two *En2-/-; En1TLZ/TLZ* ES cell lines, and one *En2-/-;En1+/TLZ* ES cell line, were injected individually into wild-type blastocysts and implanted into pseudo pregnant female mice. A total of 13 chimeric animals generated from *En2-/-;En1TLZ/TLZ* ES cell lines and 6 chimeric animals generated from the *En2-/-;En1+/TLZ* ES cell line were born.
Chimeric animals were genotyped by PCR (from tail genomic DNA). All animals showed the amplification of a band positive for the presence of the wild-type *En-2* allele (from the wild-type blastocyst) and a band positive for the presence of the mutated *En-2* allele (from the *En2-/-* ES cells) indicating that the ES cell line participated in the generation of the tail tissue. The chimeric animals were then sacrificed at adult stages.
The original design of this experiment was supposed to take advantage of the fact that the mutant ES cells contained the *En-1TLZ* construct, making it possible to discriminate between wild-type and mutant cells wherever the *En-1* promoter is active. The initial plan intended to analyze the chimeric mice for En1TLZ positive dopaminergic neurons in the SN/VTA, and to compare it to a control region. Such a region is the trapezoid body, a brain nucleus, which expresses *En-1* but is unaffected in the *En* double null mutant. We expected the mutant cells to evenly contribute to this nucleus. It should have been then possible to find out the degree of chimerism by determining the proportion of the En1TLZ positive cells among the En-1 (wild-type protein) positive cells in the trapezoid body. Furthermore, if the requirement for *En-1* and *En-2* were cell autonomous, only an insignificant amount of En1TLZ positive cells should contribute to the SN/VTA, whereas if the requirement is non-autonomous, about the same proportion should be detected in the SN/VTA as in the trapezoid body. The first chimeric animals analyzed by this method did not show any En1TLZ positive cells in the SN/VTA or anywhere in the midbrain or hindbrain, including the trapezoid body. However, the finding alone that no En1TLZ positive cells were present in the SN and VTA did not permit to conclude that the *En* genes are cell autonomously required, indeed without En1TLZ positive cells in the trapezoid body there was no evidence for the participation of the mutant ES cells in the generation of the CNS. The absence of

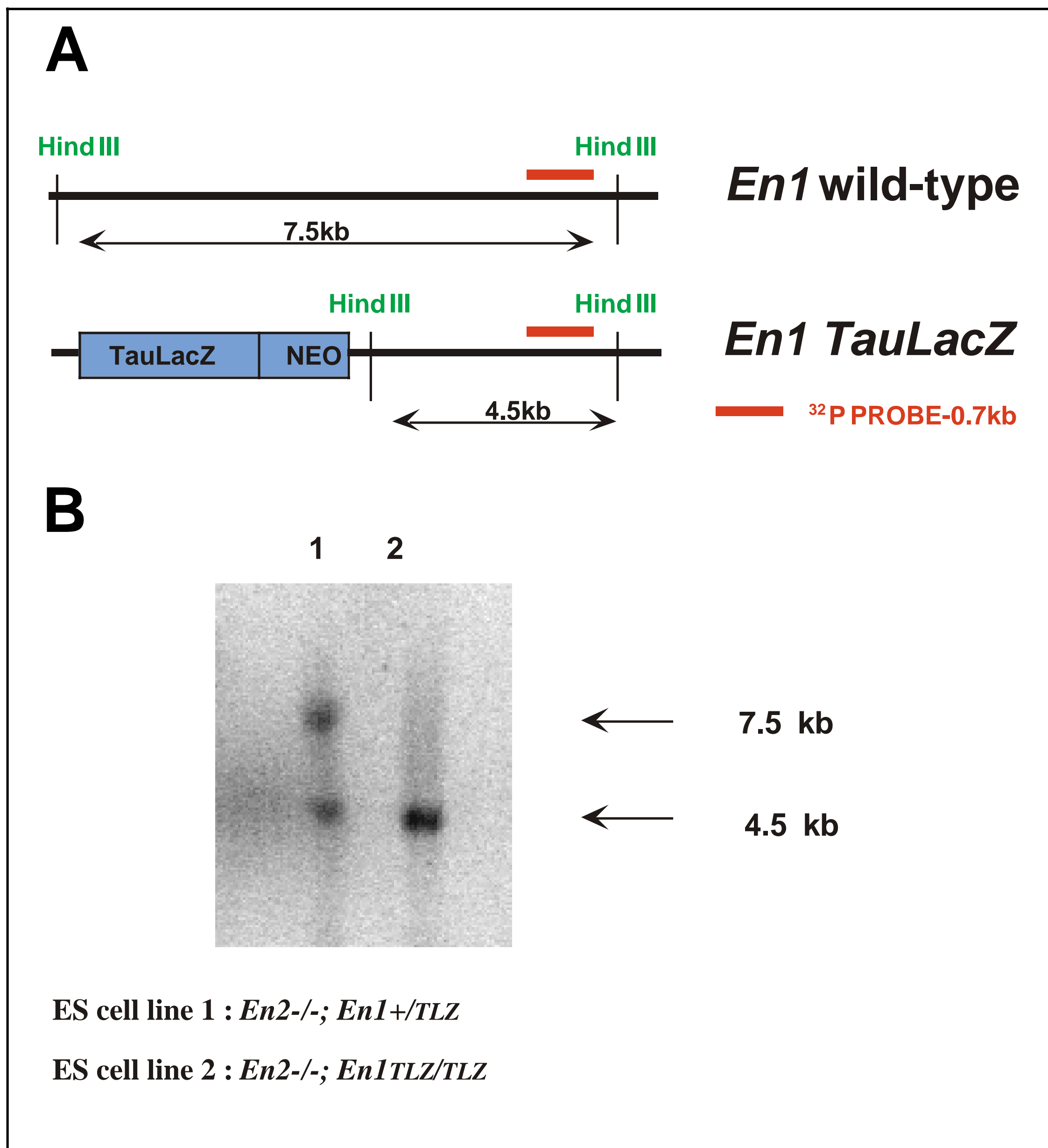

Figure III.1. Generation of ES cell lines mutant for *En-1* and *En-2*.
ES cell lines derived from blastocysts, obtained from *En2-/-;En1+/TLZ* mice matings, were analysed for the *En-1* allele.
(A) Targeting construct for inactivation of the mouse *En-1* gene [Saueressig et al., 1999]. (B) Southern blot of Hind III digested genomic DNA from heterozygous (1) and homozygous (2) ES cell clones probed with the 0.7kb probe, indicated in (A), reveal the wild-type (7.5kb) and the mutant (4.5kb) band.

En1TLZ positive cells in the trapezoid body is likely due to a postnatal requirement of the *En* genes in this nucleus.

Since it was not possible to use En-1 expression in the trapezoid body to determine the degree of chimerism, I immunohistochemically labeled brain sections with an antibody against the neomycin resistance gene: the *neomycin phosphotransferase II* (*NPTII*). *NPTII* was used for both *En-1* and *En-2* targeted deletion construct [Saueressig et al., 1999; Joyner et al., 1991]. Consequently, *NPTII* is expressed in all the mutant cells of the chimeric animals. Only one animal, derived from the *En2-/-*;*En1TLZ/TLZ* ES cell line, showed a significant NPTII signal when sacrificed at 16 months of age and interestingly this mouse looked older than its siblings (i.e. loss of hair and over-weight). A patchy NPTII expression pattern in the brain was observed (Figure III.2). The overall proportion of NPTII signal correlated in intensity and pattern with a 40% to 50% of chimerism in reference to a control chimeric animal (available in the laboratory from a different experiment, kindly provided by Robert J. Hindges) where the chimerism was estimated by the coat color. In other words, this brain contained a heterogeneous population made up of wild-type cells and of 40% to 50% cells depleted for *En-1* and *En-2*. None of the animals coming from the *En2-/-*;*En1+/TLZ* ES cell line showed a significant NPTII signal in the brain, even if a mutated *En-2* allele was detected in the initial screen by PCR. This suggests that the *En2-/-*;*En1+/TLZ* ES cell line was enable to contribute to the brain formation.

All the chimeric animals derived from heterozygous as well as *En* double null mutant ES cells, exhibited normal brain morphology. The midbrain DA neurons of these mice were analyzed by *in situ* hybridization, using a *TH* riboprobe on coronal sections. All analyzed chimeric animals derived from the *En2-/-*; *En1+/TLZ* ES cell line displayed a normal pattern and distribution of the midbrain DA neurons (data not shown). Among the chimeric animals derived from the *En2-/-*; *En1TLZ/TLZ* ES cell line, only the mouse with a significant NPTII signal in the brain exhibited an abnormal *TH* expression in the midbrain. The position of the *TH* expression domain is the same as in the wild-type, but the signal is very sparse (Figure III.3, compare A and B). The *in situ* hybridization results were confirmed by immunohistochemical labeling of the protein TH (data not shown). We observed a uniform reduction of the amount of midbrain DA

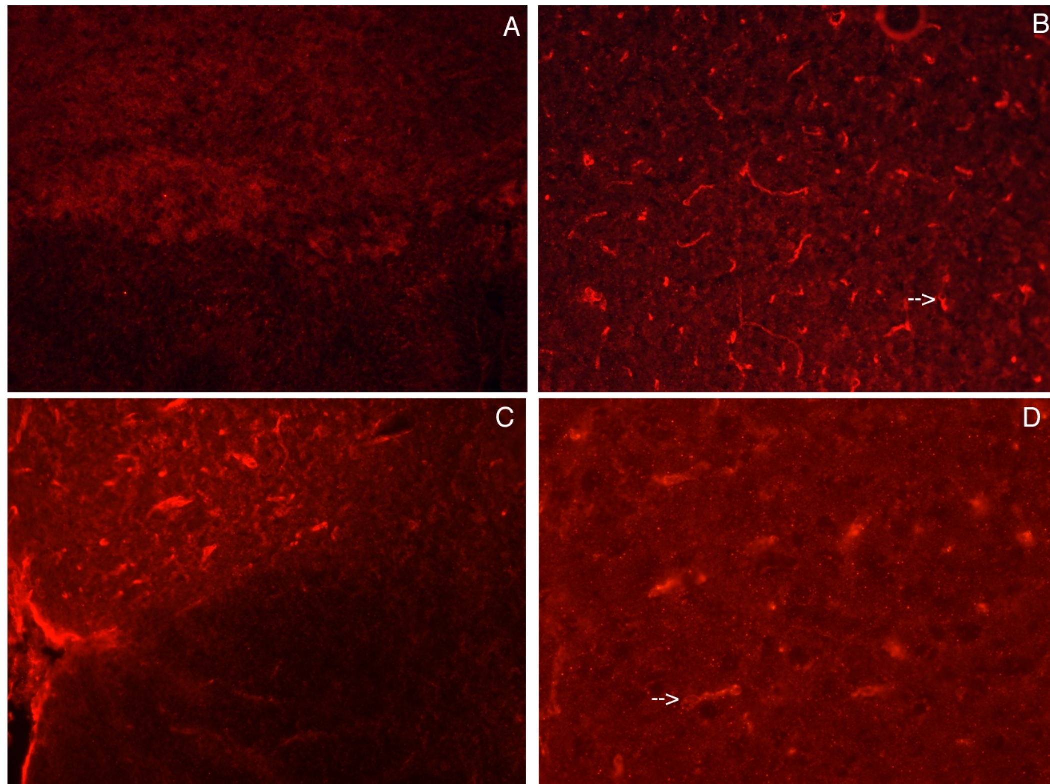

Figure III.2. Determination of the degree of chimerism in the brain of chimeric mice. Immunohistochemical labeling experiments were carried out on coronal brain sections with an antibody against NPTII to estimate the level of chimerism. A, no signal can be detected in brain sections from a wild-type mouse. B, a brain section from a control chimeric mouse, from which the chimerism was estimated with the coat color at 40 to 50%. The antibody detects various brain constituents as well as cell bodies (arrow), revealing a patchy NPTII expression pattern. C and D, brain sections from a chimeric mouse generated from the *En2-/-;En1*$^{TLZ/TLZ}$ ES cell line. The same patchy NPTII expression pattern can be observed, as described in (B). The overall proportion of NPTII signal in the brain is similar to the one observed in the control chimera, suggesting that the *En2-/-;En1*$^{TLZ/TLZ}$ ES cell line contributed about 40-50% to the cells in the brain of this chimeric mouse.

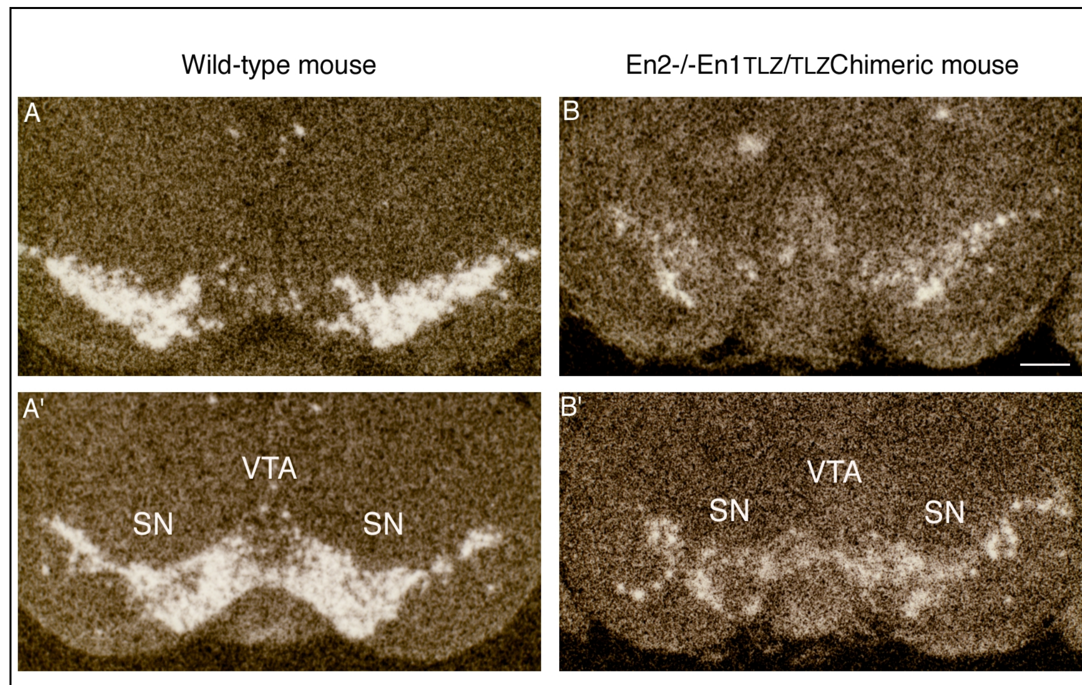

Figure III.3. TH mRNA expression in the midbrain DA neurons of *En2-/-;En1TLZ/TLZ* chimeric mice.

The DA neurons were detected by *in situ* hybridization using ^{35}S-labeled *TH* riboprobe on coronal sections of adult mouse brains. A and A' (A' a more caudal section), mRNA encoding TH shows an expression pattern in the ventral tegmentum area (VTA) and into the substantia nigra (SN) of wild-type mice. B and B' (B' a more caudal section), TH mRNA expression in the *En2-/-;En1TLZ/TLZ* chimeric mouse brain sections is weaker compared to wild-type. *TH* expression is still detected in the SN and VTA but the signal is very sparse, suggesting a reduction of the amount of DA neurons. Scale bar= 300μm.

neurons in the chimeric animal where the *En2-/-*;*En1TLZ/TLZ* ES cell line contributed significantly in the brain formation.

The loss of some midbrain DA neurons in the chimeric animal made of 40-50% of cells, which are not able to express functional En-1 and En-2, strongly suggests that the midbrain DA neurons require the En transcription factors cell autonomously for their survival. Whereas in the case of a non-cell autonomous requirement, we would have expected no loss of midbrain DA neurons. The wild-type phenotype of the cerebellum, colliculi and ventral midbrain, suggests that the wild-type cells can fully compensate in the entire brain for the loss of the mutant cells, with one exception; the midbrain DA neurons.

1-2-The *En* genes are not required for the formation of the midbrain DA precursor cells

The *En* double null mutant mice show TH positive neurons in ventral midbrain until E13, but the domain is always smaller than in the wild-type (i.e. at E12.5 see introduction, Figure I.9). A possible explanation is that the midbrain DA precursor cells are reduced in number due to the regionalisation deficit in the *En* double null mutant embryos [Simon et al., 2001]. Alternatively and more likely, the number of midbrain DA precursor cells formed in the *En* double null mutant is the same as in the wild-type, but we observe the individual cells at different stages of maturity. These cells are generated over two or more days. Since individual cells are at different time points of development, an En requirement would also reveals itself at different developmental stages. Thus, when the first cells disappear due to the lack of En, others are just born and/or at an earlier stage of differentiation when En is not yet required. Therefore, we would only be able to see the same amount of TH positive cells in the *En* double null mutant as in the wild-type at a very specific developmental stage.

To differentiate between these two possibilities, I performed gain of function experiments in chick embryos. If the *En* genes determine the number of precursor cells which give rise to midbrain DA neurons, increasing the size of En expression domain should increase the number of precursor cells which could be later detected as TH

positive cells. The *En* genes specify the midbrain and the anterior hindbrain from around stage 8 to 11 in the chick embryo [Araki and Nakamura, 1999]. Chick embryos of this age were electroporated with an En-1 over-expression construct. Full-length mouse *En-1* was clone into a pIRES2-EGFP expression vector bringing the gene under the control of an ubiquitous chick β-actin promoter. This vector contains the internal ribosome entry site (IRES) between the *En-1* gene and the enhanced green fluorescent protein gene (EGFP). This made possible to visualize the ectopic En-1 expression in these electroporated animals by fluorescence without the need for an antibody specific for the mouse En-1 protein. Stage 10 chick embryos were windowed, and overexpression vector DNA was injected into the midbrain vesicle, and electroporated. The electroporation method directs the DNA toward one side of the brain vesicle, having the advantage that later for the analysis, one side of the brain is normal and serve as an internal wild-type control as a comparison for the other manipulated side. After the electroporation process, the eggs were sealed and allowed to develop until stage 31 (the first chick midbrain DA neurons are detectable with a TH antibody at stage 29 (see 3-5-1-1)).

All the preliminary control experiments insuring the functionality of the *En-1* overexpression vector were done as described in paragraph 3-5-2-1. An obvious swelling of the tectum, which extends rostrally into the diencephalon, was observed on the electroporated side of these brains. This swelling indicates an increase of the number of neuroepithelial cells at this position or a re-specification of the diencephalon and anterior midbrain. Indeed, the tectal swelling was previously described by Araki and Nakamura, after a similar *En* mis-expression experiment to further demonstrate the role of En in defining the position of the dorsal di-mesencephalic boundary [Araki and Nakamura, 1999]. Next, the midbrain DA neurons were detected either on whole mount brains or on sections with a TH antibody. Interestingly, no significant changes were observed in the distribution and amount of the midbrain DA neurons on the electroporated side of the midbrain (Figure III.4).

By changing the *En-1* expression domain at this early stage when regionalization occurs, we observe an enlargement of the tectum, but we find no changes in the distribution or amount of the midbrain DA neurons. If the amount of DA precursor

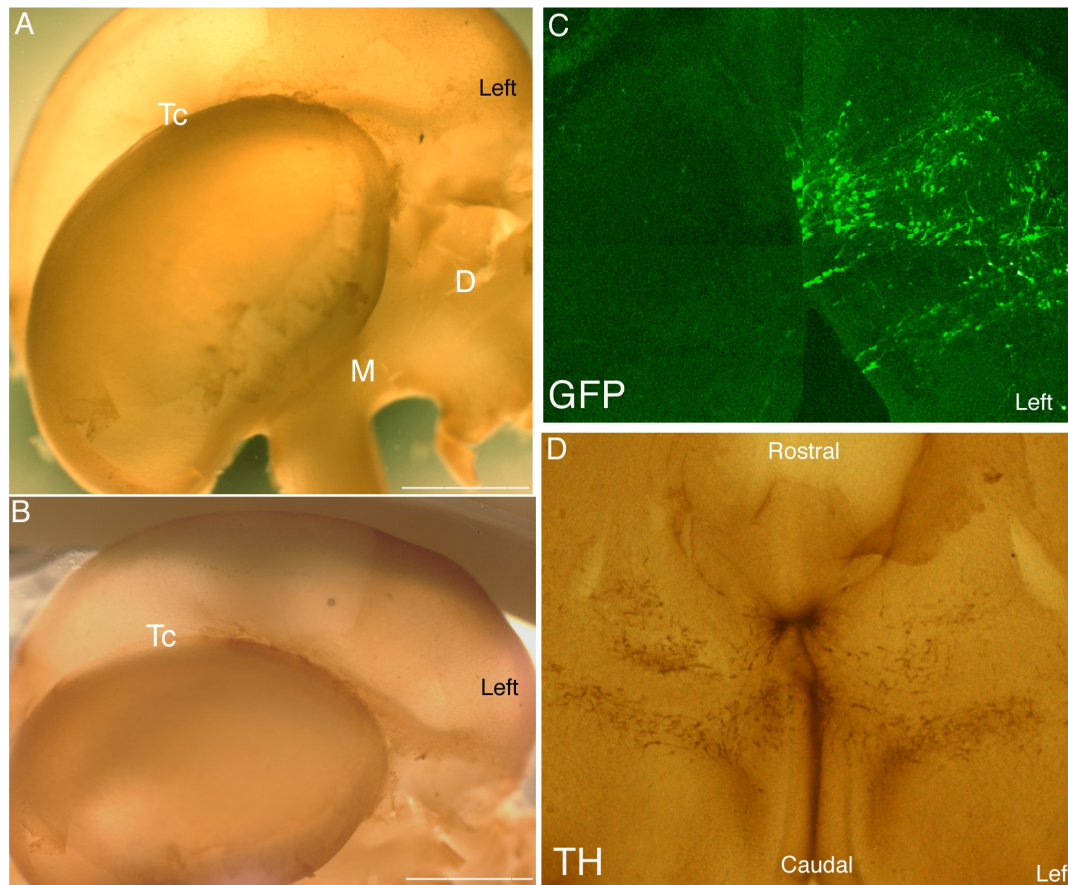

Figure III.4. Ectopic expression of En-1 in the left side of the midbrain in chick.
A and B, stage 31 entire brain vesicle, ectopic En-1 causes a tectal swelling extending to the diencephalon. C, horizontal section of the brain presented in B, showing GFP fluorescence restricted to the left electroporated side of the midbrain. D, whole mount TH immunostaining on the brain presented in A, the flat mount of the ventral midbrain shows a symmetrical TH expression pattern between the right and left side. No significant changes have been observed in the morphology and distribution of the midbrain DA neurons on the side of ectopic expression of En-1 as judged from the TH signal. Tc, tectum; D, diencephalon; M, midbrain. Scale bars=1mm.

cells were dependent on *En-1*, an expansion of the *En-1* expression domain should have increased the number of DA neurons. However, the lack of any additional TH positive cells demonstrates that *En-1* is not required for the generation of the midbrain DA progenitor cells.

All together, the generation of the *En2-/-;En1TLZ/TLZ* chimeric mice permitted to show that the midbrain DA neurons require the *En* genes cell autonomously for their survival, and the over-expression experiment of *En-1* in the chick embryo showed that the *En* genes are not required for the formation of midbrain DA precursor cells.

2- Identification of genes expressed by the midbrain DA neurons by differential display PCR.

Very few genes, which mediate the development of the midbrain DA neurons during the intermediate period between induction and full differentiation of these neurons, are known. In order to identify and characterize additional genes of this category, ddPCR was performed with four different types of tissues containing DA neurons. Approximately 500 μm^2 large pieces of ventral midbrain, as restricted as possible to the midbrain DA location, were dissected from E12 homozygous *En* double null mutant embryos (*En2*-/-;*En1TLZ/TLZ*), from E12 *En2*-/-;*En1+/TLZ* embryos, and E14 *En2*-/-;*En1+/TLZ* embryos (Figure III.5 A). At E12, the midbrain DA neurons express *En-1* and *En-2* in the wildtype and are still present in the *En* double null mutant [Simon et al., 2001]. In theory, a differential display experiment comparing wildtype tissue with *En* double null mutant tissue should reveal genes expressed by the midbrain DA neurons that are either under direct transcriptional control of the *En* genes or further downstream. Tissue from E14 embryos was also chosen since the DA neurons are lost at this age in the *En* double null mutants. Genes that are involved in the disappearance of the midbrain DA neurons in the mutants are likely to be expressed or downregulated at this age. Finally, adult olfactory bulb tissue was also added to the differential display experiment as an independent source of DA neurons. This tissue presumably expresses genes that are necessary for dopamine synthesis, transport, release and reuptake, which are commonly expressed by all DA neurons. Therefore the comparison of the ventral midbrain to the olfactory bulb most probably reveals some of the genes that are responsible for the unique characteristics of the midbrain DA neurons.

RNA was isolated from three independently collected samples of each of the four tissues. 15 pieces of E12 or E14 ventral midbrain yielded 7 to 10 µg of total RNA. The same amount was obtained from 3 adult olfactory bulbs. This RNA was used as a template to make single stranded DNA. Each reverse transcription was initiated with one of 20 arbitrary 10-mers primers (see materials & methods). 200 primer combinations (all 20 combined with the first 10, half of the possible combinations) were used to amplify cDNA fragments by PCR. A total of 2400 radioactive PCR

reactions were separated on a denaturing polyacrylamide gels. Gels were exposed to x-ray films and differences in the intensity of the bands were analyzed by visual inspection. Only fragments, which were differentially expressed in all three independent RNA samples, were isolated from the gels (for an example see Figure III.5 B, C). The differentially expressed gene fragments were reamplified by using 20-mers primers containing the original 10mers primers with added XbaI or XhoI restriction sites to facilitate the subcloning into the pBluescript II SK (-) vector. One hundred differentially expressed gene fragments were obtained and we were able to successfully reamplify 71 of them. Sequence comparison between each other and sequences at the Genbank database revealed 43 unique sequencing tags (see Table III.1). For verification of the differential expression patterns these 43 fragments were used for RNA *in situ* hybridizations on sections of E13 embryos and P0 brains. Some of the fragments (17) did not reveal any *in situ* signal at all. Thirteen genes were expressed specifically in the ventral midbrain of which 4 were expressed by the DA neurons. Seven fragments were found expressed in the double null mutant tissue but not in the three other tissue samples or *vice versa*. The only one of those, which was found to be expressed in the midbrain DA neurons, is the *Microtubule-Associated Protein 1B* (*MAP1B*). *MAP1B* was not expressed in the *En* double null mutant tissue, suggesting that the *En* genes may control its transcription. Indeed, a recent report demonstrated that *MAP1B* is transcriptionally regulated by the *En* genes [Montesinos et al., 2001]. However the abundant expression of *MAP1B* made the analysis focusing only on the midbrain DA neurons impossible. The three other genes expressed in the midbrain DA neurons and present in the *En* double null mutant tissue, which have been further characterized are the forkhead containing transcription factor *HNF3α* (hepatocyte nuclear factor 3), the neuronal and B-cell differentiation factor *Olf-1/Ebf* and the neuregulin (NRG) receptor *ErbB4*.

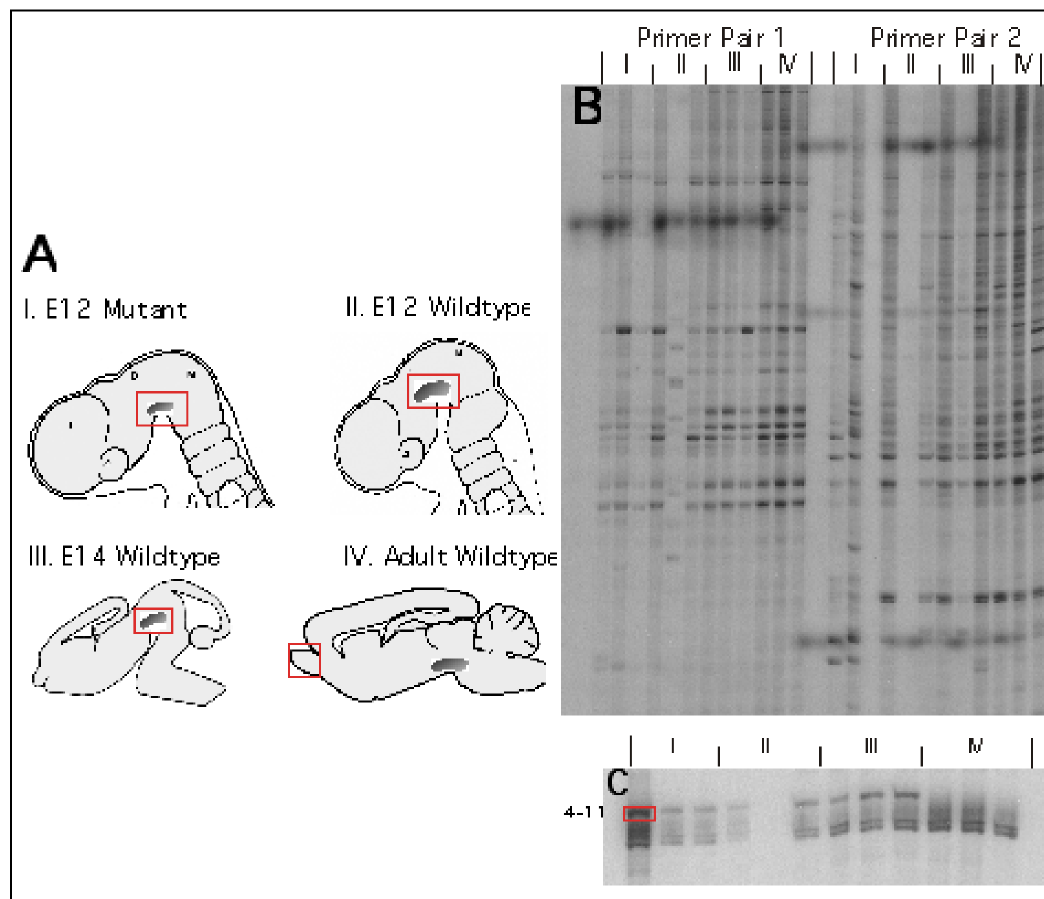

Figure III.5. Identification of genes specifically expressed by the DA neurons of the midbrain by differential display PCR (ddPCR).

A, Illustration on sketches showing the location of the different tissue pieces dissected for the ddPCR (red squares): E12 *En2*-/-;*En1TLZ*/TLZ embryo ventral midbrain (I), E12 *En2*-/-;*En1*+/TLZ embryo ventral midbrain (II), E14 *En2*-/-;*En1*+/TLZ embryo ventral midbrain(III) and adult olfactory bulb (IV). B and C, Example of a ddPCR experiment; PCR products were separated on a denaturing polyacrylamide gel (see materials and methods). B, two primer pair combinations are displayed, showing the individual amplification patterns. C, 4-11 message is amplified in all the tissue types (I to III) except in the olfactory bulb (IV). This gene fragment was later identified as *HNF3alpha*.

Total of amplified fragments	100	
Cloned fragments	71	
Unique sequencing tags	43	
In Situ Hybridization		
No signal	17	
Everywhere	2	
Outside of midbrain	11	
Ventral midbrain	13	
DA neurons	4	
Differential Display Criteria		
Present in:		
All tissues except E12 *En2-/-;En1TLZ/ TLZ*	5	MAP1b
All tissues except E12 *En2-/-;En1+/TLZ*	1	
All tissues except in olfactory bulb	13	HNF3a, Olf-1
E14 and olfactory bulb not E12	21	ErbB4
E12 *En2-/-;En1TLZ/ TLZ* only	2	

Table III.1. Summary of the outcome of the differential display PCR experiment. One hundred genes were differentially expressed and 71 of them were reamplified and cloned. The number of genes is given for each category. The different categories were made according to the gene expression location detected by *in situ* hybridizations, and according to the presence of the gene in the different tissues. The right most column indicates the candidate genes from the different categories, studied further in this work.

3- Characterization of the newly identified genes expressed in the midbrain DA neurons.

3-1- Developmental expression patterns in the midbrain DA neurons

In order to characterize further these genes, I carried out a detailed expression analysis by using *in situ* hybridization and immunohistochemistry on brain tissue from E9 to the adult. Their expression patterns were compared to the specific midbrain DA markers *TH*, *Nurr1* and *En-1*.

3-1-1- *HNF3α* expression in the DA neurons of the ventral midbrain

Previous studies found that *HNF3α* is expressed in the notochord and the floor plate during early embryogenesis [Monaghan et al., 1993; Sasaki and Hogan, 1993]. Here, I add to these data the *HNF3α* expression in the brain from E11 to the adult, focusing on the midbrain DA neurons. *HNF3α* was found to be expressed in the ventral embryonic midline at E9. Its anterior border of expression is approximately at the junction between tel- and diencephalon (Figure III.6 A,B). This region includes the most ventral part of the midbrain neuromere where the DA neurons are induced by an interaction between SHH and FGF8 [Ye et al., 1998]. In order to determine if its early expression is related to the DA neurons, we compared the *HNF3α* expression at E11 to *Nurr 1*, an early differentiation marker for this neuronal subtype [Zetterström et al., 1997], as well as to *TH*, the most commonly used marker to identify DA neurons. At this developmental stage, the *HNF3α* expression domain is still similar to the one found at E9, restricted to the ventral neural tube reaching into the diencephalon (Figure III.6 C). The smaller *Nurr1* expression domain is confined within the *HNF3α* domain, but *Nurr1* is not expressed on the ventricular site of the neuroepithelium (Figure III.6 E). Postmitotic DA neurons are revealed by the *TH* expression. The neurons are located within the *HNF3α* and *Nurr1* domains, but restricted to the pial surface, where postmitotic neurons are normally located (Figure III.6 D). The nested expression of the three genes and the early onset of the *HNF3α* expression suggest that it is expressed in

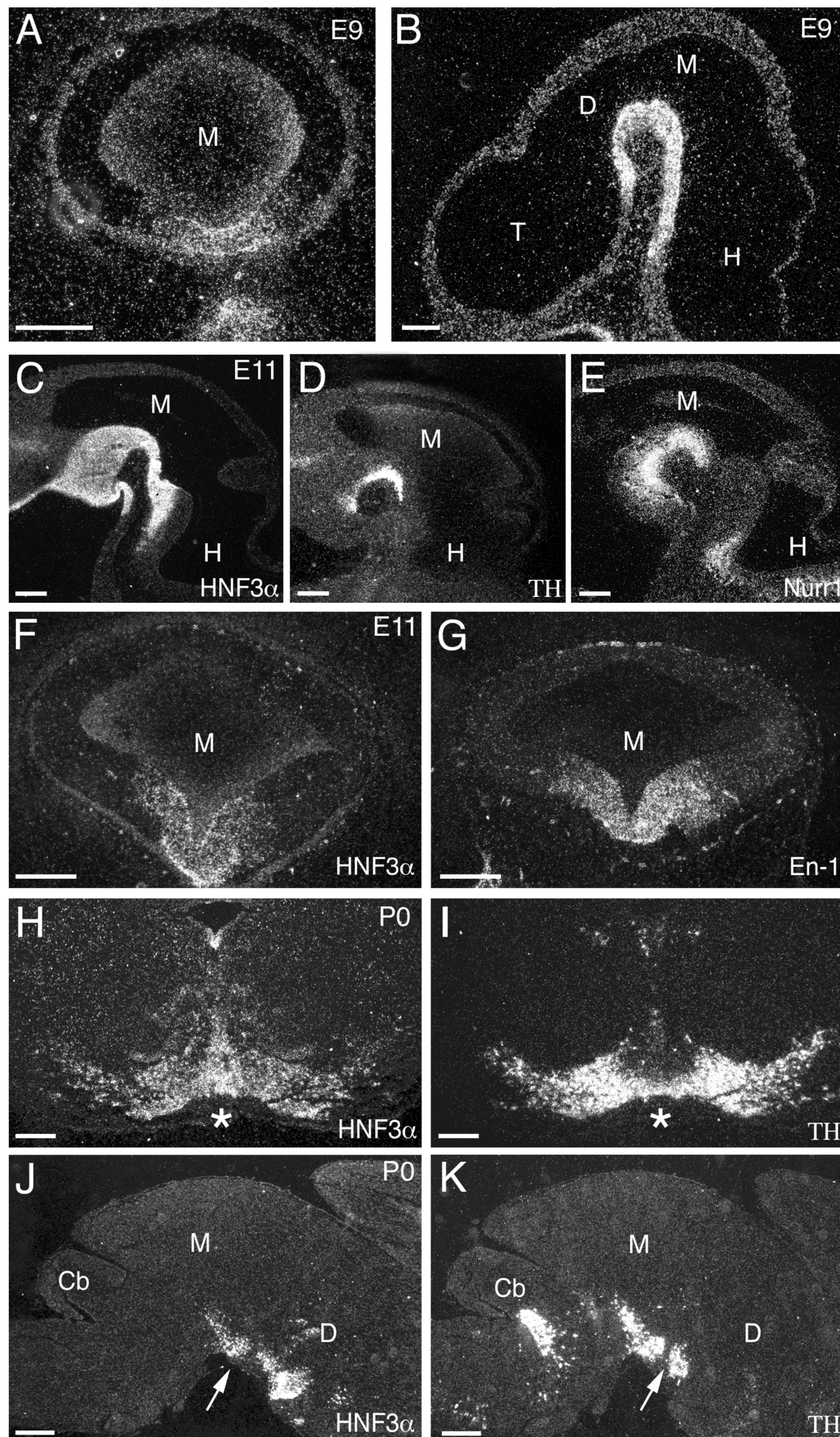

Figure III.6. *HNF3α* expression in the DA neurons of the ventral midbrain.
In situ hybridization using ^{35}S-labeled riboprobes on sagittal (B-E, J, K) and coronal /transverse (A, F-I) sections of mouse whole embryos (A-G) or P0 mouse brains (H-K).

A, E9, the transverse section reveals the early *HNF3α* expression in the ventral midbrain. B, in sagittal sections, the full extend of the *HNF3α* is apparent. Its rostral expression terminates at the border between telencephalon and diencephalon C, E11, *HNF3α* is expressed in the ventral midbrain from the ventricular to the pial surface. On parallel sections, two markers for dopaminergic neurons, tyrosine hydroxylase (*TH*) (D) and *Nurr1* (E) are found within the *HNF3α* expression domain, but neither of the two is expressed on the ventricular site of neurepithelium. F, G, two parallel transverse sections of an E11 embryo through the anterior midbrain: both *HNF3α* (F) and *En-1* (G) have the same distribution at this position. H, I, parallel coronal sections through anterior midbrain of a P0 brain show an almost identical distribution of *HNF3α* (H) and *TH* (I), identifying the cells expressing *HNF3α* as the dopaminergic neurons of the substantia nigra (SN) and the ventral tegmentum (VT). J, sagittal section through a P0 brain. *HNF3α* is expressed only at two locations in the CNS, the midbrain DA neurons (arrow) and another one in the hypothalamus. K, the *TH* labeling reveals that the expression is specific to midbrain DA neurons and not found in any other *TH* positive nucleus. T, telencephalon; D, diencephalon; M, midbrain; H, hindbrain; Cb, cerebellum. Scale bars, A-B=100 µm, C-I=200 µm, J,K=500 µm.

the precursor cells of the midbrain DA neurons before they become postmitotic. To support this idea, its expression was also compared to *En-1*, which is a selective marker for this neuronal subtype in the anterior midbrain [Simon et al., 2001]. The transverse sections show that the expressions of both, *HNF3α* and *En-1* overlap (Figure III.6 F, G). Finally, to determine whether the midbrain DA neurons or intermingled cells express *HNF3α*, *in situ* hybridization analyses were performed on coronal sections of P0 brain. The DA neurons of the SN and the VTA are well defined at this age by their position in the brain and the unique shape of the *TH* expression domain (Figure III.6 I, K). The *HNF3α* expression (Figure III.6 H, J) exactly coincides with TH in all sections where the SN and VTA are present. The sagittal sections show that *HNF3α* expression is highly specific for these cells. It is only found in the midbrain DA neurons and in several areas in the diencephalon, but nowhere else in the entire brain (Figure II.6 J). The identical expression pattern is found in adult animals.

3-1-2- *ErbB4* expression in the SN

The NRG receptor, *ErbB4*, was recently shown to be expressed in adult rats by the DA neurons of the SN [Steiner et al., 1999]. Since it was one of the genes isolated in the ddPCR, *in situ* hybridization on midbrain sections were performed in order to correlate its expression to the appearance of the DA neurons of the VTA and SN. At E9, transverse sections shows *ErbB4* in the entire midbrain with an elevated ventral signal (Figure III.7 A, B). The expression in the DA neurons is detectable from E11.5 onwards. The *ErbB4* signal is found in several patches in the ventral midbrain (Figure III.7 C) on the ventricular as well as the pial surface of the neurepithelium. The smaller *TH* (Figure III.7 D) and *Nurr1* (Figure III.7 E) expression domains are within the *ErbB4* expression domain, making it very likely that *ErbB4* and *TH* are co-expressed by the DA neurons at this age. The exclusive expression in the SN, but not in the VTA is only evident when the two populations of DA neurons are morphologically separated from each other at a later stage. In the rostral coronal section, the distinct shape of the SN can be detected with a probe against *TH*. The VTA is not present at this position (Figure III.7 G). In parallel sections, *ErbB4* is not only found in the SN but also at the

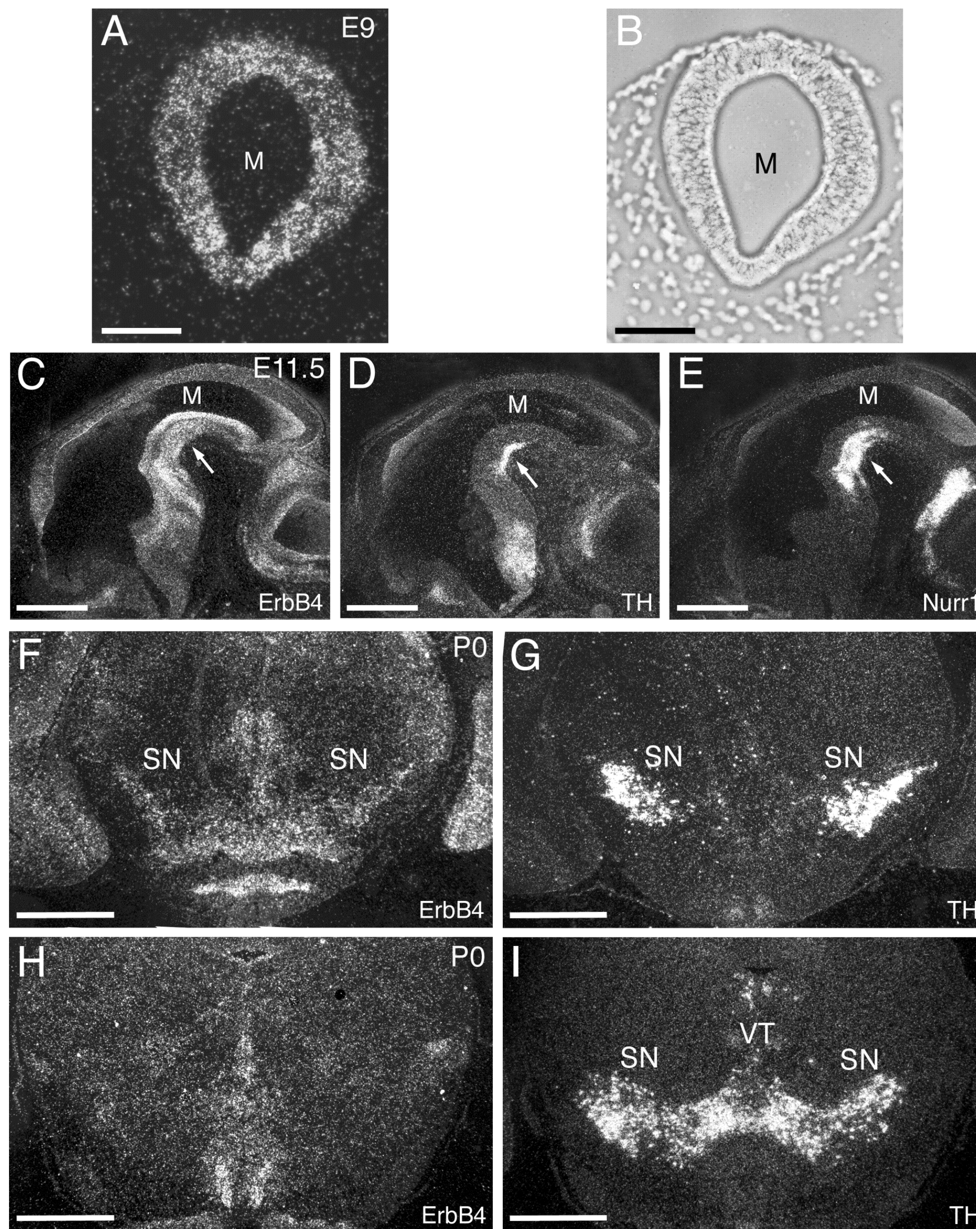

Figure III.7. *ErbB4* expression in the SN.
In situ hybridization using ^{35}S-labeled riboprobes on transverse sections of E9 mouse whole embryos (A,B), on sagittal sections of E11.5 mouse whole embryos (C-E) and on coronal sections of mouse P0 brain. (F-I). A, at E9, *ErbB4* is expressed in the entire midbrain neurepithelium. B, phase contrast image of A. At E11.5; C, *ErbB4* is expressed in the ventral midbrain on the ventricular and pial surface of the neurepithelium. On the parallel sections, *TH* (D) and the *Nurr1* (E) expression domain are smaller but within the *ErbB4* expression domain. At P0; F, rostral coronal section. *ErbB4* is expressed in the substantia nigra (SN), but several other domains of expression are also detectable. G, on the parallel section, the position of the substantia nigra is revealed by the *TH* labeling. H, I, sections caudal to F and G, *ErbB4* is only expressed in the midline, but not in the DA neurons of ventral tegmentum (VT) and the caudal substantia nigra. M, midbrain. Scale bars, A,B=100 μm, C-I=500 μm.

midline (Figure III.7 F). This midline signal is probably coming from the red nucleus. In more caudal sections both, the SN and the VTA are detected by the probe against *TH* (Figure III.7 I), however, no *ErbB4* expression can be detected in any of these structures (Figure III.7 H). This suggests that the population of DA neurons, which make up the SN can be divided in two subpopulations, a rostral ErbB4 positive population and a caudal ErbB4 negative population. ErbB4 expression was also detected in regions of the striatum (data not shown) as mentioned by Meyer et al., 1997.

3-1-3- Transient *Olf-1* expression in midbrain DA neurons

Olf-1/Ebf is a transcription factor that was recently identified as a differentiation factor for B-cells and striatal neurons [Lin and Grosschedl, 1995; Garel et al., 1999]. It acts during early phases of cell differentiation as a transcriptional regulator with an untypical zinc-finger binding domain. Previous reports have documentated its expression in mouse CNS from as young as E11. In contrast to these results, I found the onset of Olf-1 expression in the midbrain could be already detected at E9. Furthermore, it is transiently co-expressed with *TH* in the midbrain DA neurons at the embryonic stages briefly after they have become postmitotic. Co-expression of *Olf-1* and *TH* is detectable as soon as the DA neurons express TH. At E11, radioactive *in situ* hybridizations on sagittal sections show Olf-1 expression on the pial surface of the developing colliculus (Figure III.8 A), suggesting a role in differentiated neurons. In the ventral midbrain, there is an exact match of the expression domain with *TH* expressing cells (Figure III.8 B). The immunohistochemical double labeling on a coronal section (Figure III.8 E) reveals a co-localization of the two proteins. The Olf-1 antibody [Davis and Reed, 1996] detects the protein in the nucleus (Figure III.8 C) of all TH positive neurons (Figure III.8 D). However, the expression is lost in the next few days of development. The midbrain DA neurons begin to down regulate *Olf-1* at around E12.5. At E14, all TH positive neurons (Figure III.8 G) have lost the expression of *Olf-1* (Figure III.8 F). The Olf-1 protein is now only detectable at the midline of the coronal section, whereas the TH positive neurons are distributed more laterally. At P0,

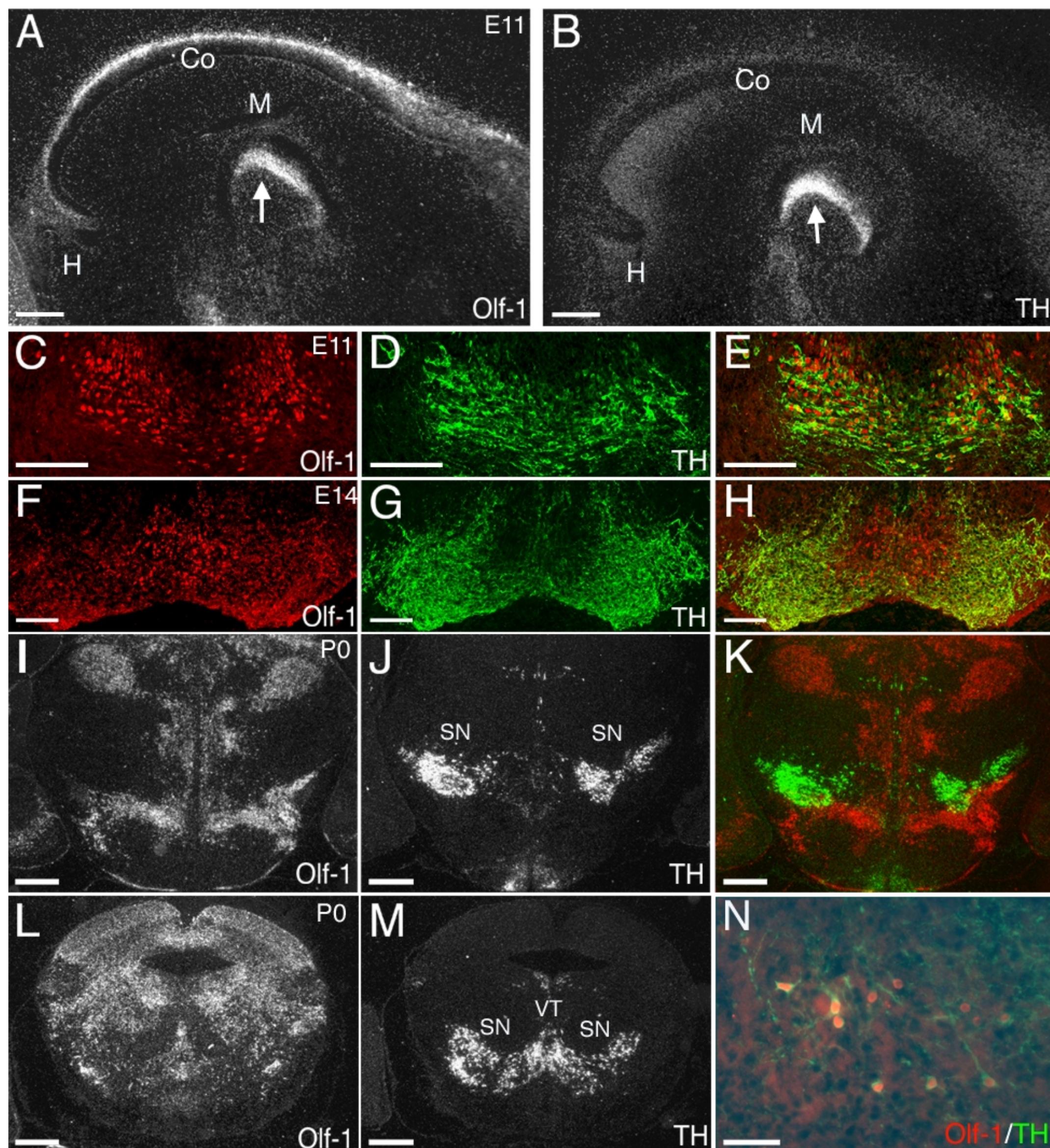

Figure III.8. Transient *Olf-1* expression in the midbrain DA neurons.
In situ hybridization using ^{35}S-labeled riboprobes on sagittal sections of E11 mouse whole embryos (A, B), coronal sections of P0 mouse brains (I-M) and immunohistochemical double labeling on transverse/coronal sections of E12 (C-E), E14 (F to H) mouse whole embryos and mouse P0 brains (N). A, *Olf-1* is expressed on the pial surface of the developing colliculus (Co) and in the DA neurons of the ventral midbrain (arrow). B, Parallel section stained against *TH*. The enzyme is expressed at the same location in the ventral midbrain as the *Olf-1* (arrow). C, D, E, at E11, the Olf-1 antibody detects the protein in the nucleus (C) of all TH (D) positive neurons; superimposed images (E). F, G, H, at E14, the Olf-1 antibody detects the protein in midline cells (F): The superimposed image with the TH immunolabeling (G) shows that the Olf-1 has been downregulated by the DA neurons (H). I, J, K, L, M, *in situ* hybridization on rostral sections against *TH* reveals the substantia nigra (J). On the

parallel section, there are several areas where the *Olf-1* is expressed (I). The false color superimposed image (K) of the two *in situ* hybridizations shows that the *Olf-1* and *TH* are expressed in vicinity to each other but they are not co-expressed. Only low *Olf-1*expression is detectable on a more caudal section (L), where the ventral tegmentum is located (M). N, high magnification of the double labeling experiment shows a few DA neurons co-expressing TH and Olf-1 at P0. M, midbrain; H, hindbrain; SN, substantia nigra; VT, ventral tegmentum; Scale bars A-B, F-H= 200 µm, C-E = 100 µm, I-M=500 µm, N=50 µm

Olf-1 and TH are both mainly expressed in vicinity to each other, but not in the same cell populations (Figure III.8 I, J, K, L, M). However, occasionally Olf-1 expression is found in a few DA neurons (Figure III.8 N).

3-2- *En* and *Nurr1* independent expression of *HNF3α* and *Olf-1* in the midbrain DA neurons

Since *HNF3α a*nd *Olf-1* were found expressed in all tissues derived from E12 and E14 embryos, including the *En* double null mutant, it was important to confirm whether the two genes are not under control of *En-1* and *En-2*. This verification is possible since the midbrain DA neurons are still present in the *En* double null mutant until E13. The *in situ* hybridizations on E12 sagittal sections show a small expression domain of *HNF3α* in the ventral midbrain of the deformed mutant brain (Figure III.9 A). The smaller expression domain of *HNF3α* is very likely due to the reduced number of DA neurons present in *En* double null mutants at this age [Simon et al., 2001]. The immunostaining of *En* double null mutants using the antibody against Olf-1 and TH show that all midbrain DA neurons co-express the two proteins in the mutant (Figure III.9 B), similar to the wild-type case (Figure III.8 E). These findings are in accordance with the results obtained from the ddPCR, where the expression of *HNF3α* and *Olf-1* is independent of the two *En* genes.

Nurr1 is one of the key regulators that determines the cell fate of the midbrain DA neurons [Sakurada et al., 1999] and moreover, is one of the earliest genes expressed by the postmitotic midbrain DA neurons. To determine whether *Nurr1* regulates the expression of *HNF3α* and *Olf-1* or whether these genes take part in other independent differentiation pathways, their expression patterns were analyzed in *Nurr1* mutant embryos. The unambiguous identification of midbrain DA neurons with an antibody against TH, as achieved for the *En* double null mutant, is not possible since the enzyme is not expressed in the midbrain in these mutant mice [Zetterstrom et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998]. However, the use of different markers for this cell group, like *Ptx3*, *En1* and *2*, and *Lmx1b*, demonstrated that the cells are present until at least embryonic day 15.5 [Saucedo-Cardenas et al., 1998; Wallen et al., 1999;

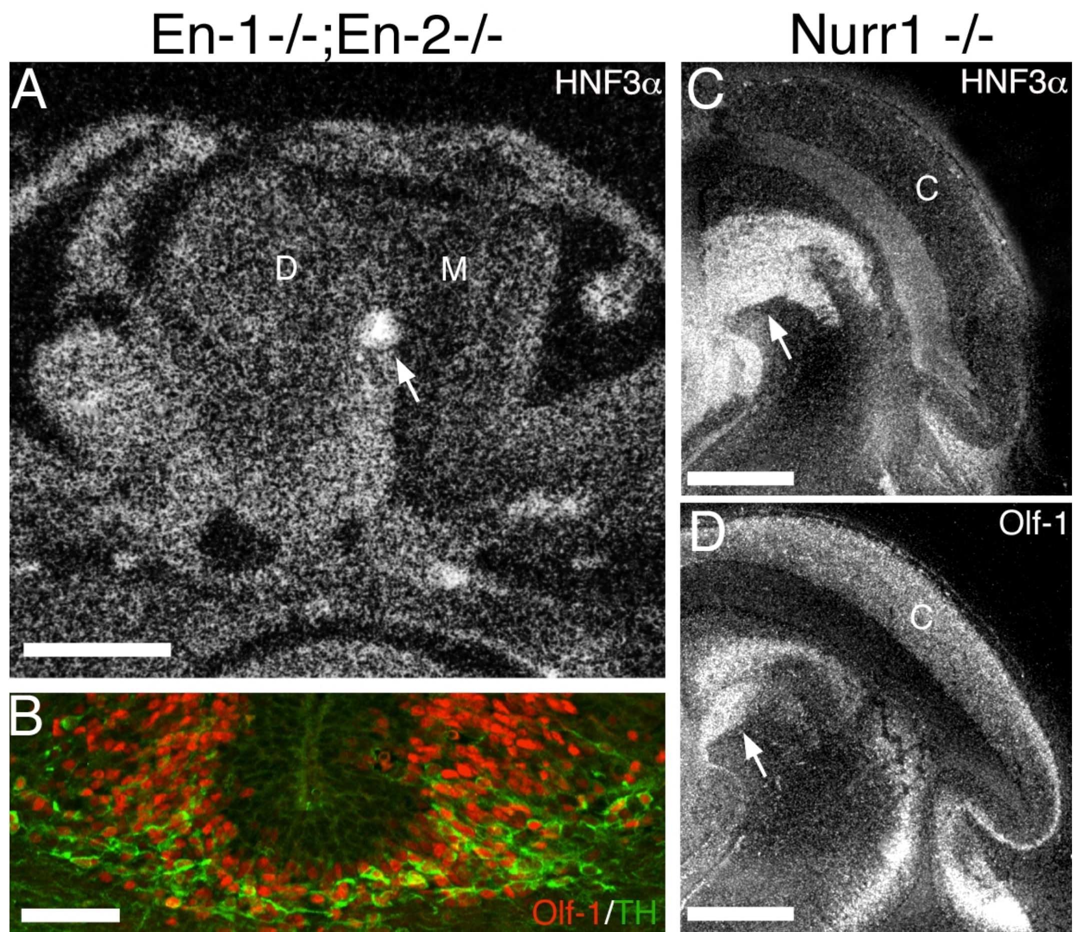

Figure III.9. Expression of *HNF3α* and *Olf-1* in the *Nurr1* mutant and *En* double mutant mice.
In situ hybridization using probes against *HNF3α* (A, C) *Olf-1* (D) and immunohistochemical double labeling with antibodies against Olf-1 (red) and TH (green) (B) on sections of E12 *En* double mutant (A, B) and *Nurr1* mutant (C, D). A, *HNF3α* shows a small expression domain (arrow) in the ventral midbrain on a sagittal section of *En* double mutant embryos in the same way as for *TH*. B, immunohistochemical double labeling on a coronal section of the *En* mutant embryo shows co-expression of Olf-1 and TH. Both genes are not regulated by *En-1* and *En-2*. C, D, parallel sagittal sections stained with probes against *HNF3α* and *Olf-1* show signals at the same location on the pial surface of the ventral midbrain (arrow). These coincidental signals are probably the DA neurons, which do not express *TH* in *Nurr1* mutant mice. Scale bars, A, C, D=500 µm, B=200 µm.

Smidt et al., 2000]. In E12 *Nurr1* mutant brains, an overlapping expression of *HNF3α* and *Olf-1* with the same size and shape as seen in wild-type sections was detected. The point of overlap in the ventral midbrain is very likely the location of the DA neurons (Figure III.9 C, D). These findings together with the early onset of expression of the two genes *HNF3α* and *Olf-1*, suggest that they both are regulated independently of *Nurr1*.

3-3-Investigation of the midbrain DA neurons in mice deficient for the *HNF3α* gene

To determine the function of *HNF3α*, I investigated homologous recombinant mice. These mice develop a complex phenotype that is characterized by abnormal feeding behavior, progressive starvation, persistent hypoglycemia and neonatal mortality between days 2 and 14 [Kaestner et al., 1999; Shih et al., 1999]. Dr. Kaestner provided us with some P8 brains of *HNF3α-/-* mutant mice and their wild-type littermates. The expression pattern of *TH* (Figure III.10), *Nurr-1*, *En*, *Ptx-3*, *Olf-1*, *VMAT*, *DAT*, *c-ret*, *GDNFRα*, *AHD2*, *AADC*, *DR2* and *ErbB4*, which are all genes expressed in the midbrain DA neurons, were studied by *in situ* hybridization using riboprobes on coronal sections. All of the genes that have been analyzed in this study are expressed in the midbrain DA neurons of mouse brains deficient for *HNF3α* and exhibit an expression pattern identical to the one observed in the wild-type (Table III.2). These data suggest that, in the context of the midbrain DA neurons, none of these genes are under the control of *HNF3α*.

3-4-Investigation of the midbrain DA system in nervous system specific *ErbB4* mutant mice

Here, I showed that the *ErbB4* expression initiates in the DA neurons at E11.5 and continues into adult stages, later specifying the most rostral part of the SNC. In order to get insight towards a possible role of *ErbB4* in the development of the midbrain DA neurons, I investigated the expression of molecular associates of ErbB4 in the midbrain

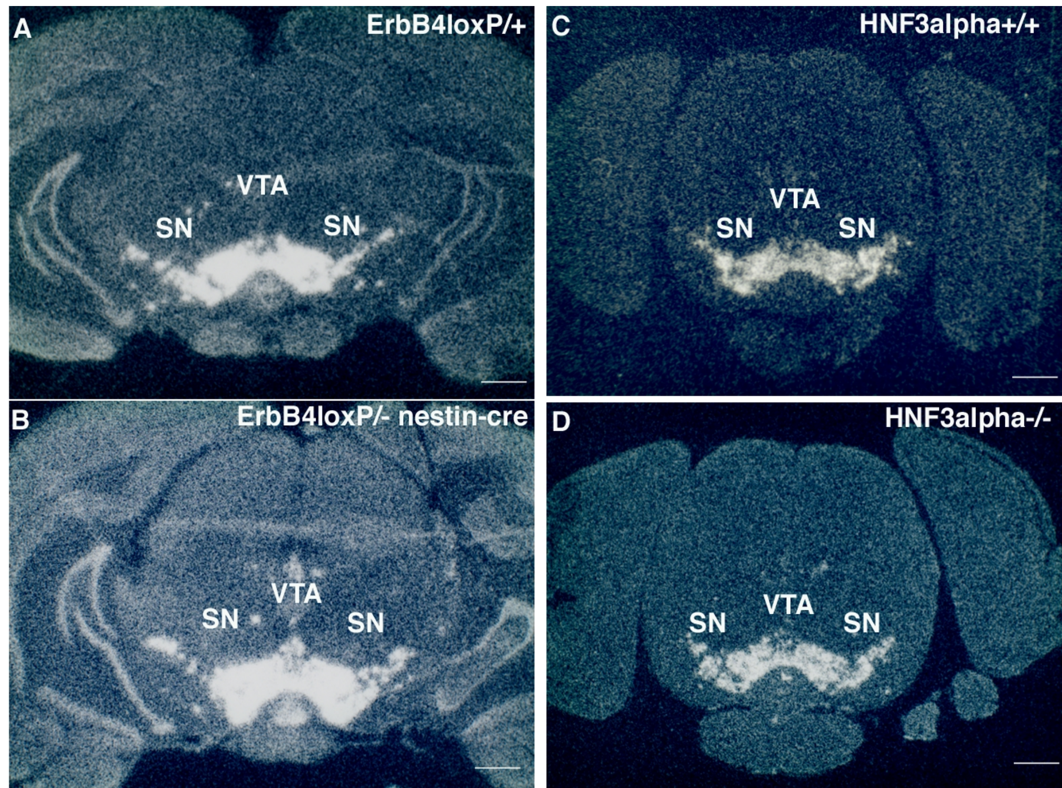

Figure III.10. Normal *TH* expression in midbrain DA neurons for mice lacking *ErbB4* in the nervous system and for mice deficient for *HNF3*α.
In situ hybridization using ^{35}S-labeled *TH* riboprobes on coronal sections of adult (A, B) and P8 (C, D) mouse brains. A, *ErbB4 loxP/+* mouse, one allele *ErbB4* flanked with LoxP, one wild-type allele, no *nestin-cre* (undisturbed expression of ErbB4), showing a wild-type expression of *TH*, in the substantia nigra (SN) and ventral tegmentum area (VTA). B, *ErbB4 loxP/- nestin-cre* mouse, nervous system specific *ErbB4* mutant. No changes in the *TH* expression pattern is observed. C, wild-type mouse for *HNF3*α. *TH* is detected in the SN and VTA. D, *HNF3*α *-/-* mutant mouse. No changes in the *TH* expression pattern in the SN and VTA is detected. Scale Bars= 500µm (A and B), 450µm (C and D).

Gene Expressed In midbrain DA neurons	Wild-type mice	*HNF3α -/-* mice	*ErbB4 loxP/- nestin-cre* mice
TH	+	+	+
En-1	+	+	+
Ptx-3	+	+	+
Olf-1	+	+	+
Pbx-1	+	+	+
VMAT	+	+	+
Nurr-1	+	+	+
DAT	+	+	+
c-ret	+	+	+
GDNFRα	+	+	+
AHD2	+	+	+
AADC	+	+	+
DR2	+	+	+
ErbB4	+	+	-
HNF3α	+	-	+

Table III.2. Summary of expression analysis in the midbrain DA of wild-type, *HNF3α -/-* mutant, and nervous system specific *ErbB4* mutant mice.

The analysis was carried out with *in situ* hybridization using ^{35}S-labeled riboprobes on coronal section of P8 brains and adult brains for *HNF3α-/-* mice and *ErbB4 loxP/- nestin-cre* mice, respectively. The (+) indicates the detection of the expression of the gene of interest in the midbrain DA neurons. The (-) indicates the absence of the gene.

DA neurons and the distribution of its ligands in the midbrain DA neurons and in the axonal projection targets of the SN. Furthermore, I analyzed nervous system specific *ErbB4* mutant mice for alterations in the midbrain DA neurons system.

3-4-1- Expression analyses of *ErbB2* and of the ligands of ErbB4 in the midbrain DA system

The receptor tyrosine kinase ErbB4, together with ErbB1, ErbB2 and ErbB3, is part of the very complex NRG signaling pathway. Many ligands have been described, and it has been shown that individual ligands can bind more than one receptor and induce different combinations of receptors, homo- or heterodimers, and transphophorylation (for review [Burden and Yarden, 1997]).

It is known that NRG ligands bind ErbB4 receptors and activates ErbB2 through heterodimerization (for review [Burden and Yarden, 1997]). Therefore, I initially investigated if the ErbB2 receptor is present in the midbrain DA neurons by *in situ* hybridization using a riboprobe on midbrain sections of wild-type P0 mice. No *ErbB2* expression was detected in the midbrain DA neurons.

As a next step, I determined if the ligands with the highest binding specificities for ErbB4 were expressed in vicinity of the midbrain DA neurons or within this cell population or in any of their projection targets. This information would provide insight to the co-existence and the possible interplay of the midbrain DA system and the NRG signaling pathway. The expression of the ligands was analyzed by *in situ* hybridization on brain sections of wild-type mice. Interestingly, two ligands, *Type III NRG-1* [Carraway et al., 1997] and *Betacellulin* (for review [Dunbar and Goddard, 2000]) are expressed in the striatum (or caudate putamen), from at least P0 onwards (Figure III.11). I also determined the expression of *Type I NRG 1*, *Type III NRG 2* [Carraway et al., 1997], and *NRG 3* [Zhang et al., 1997], but none of them was detected in the close vicinity of the midbrain DA neurons, or in their projection targets.

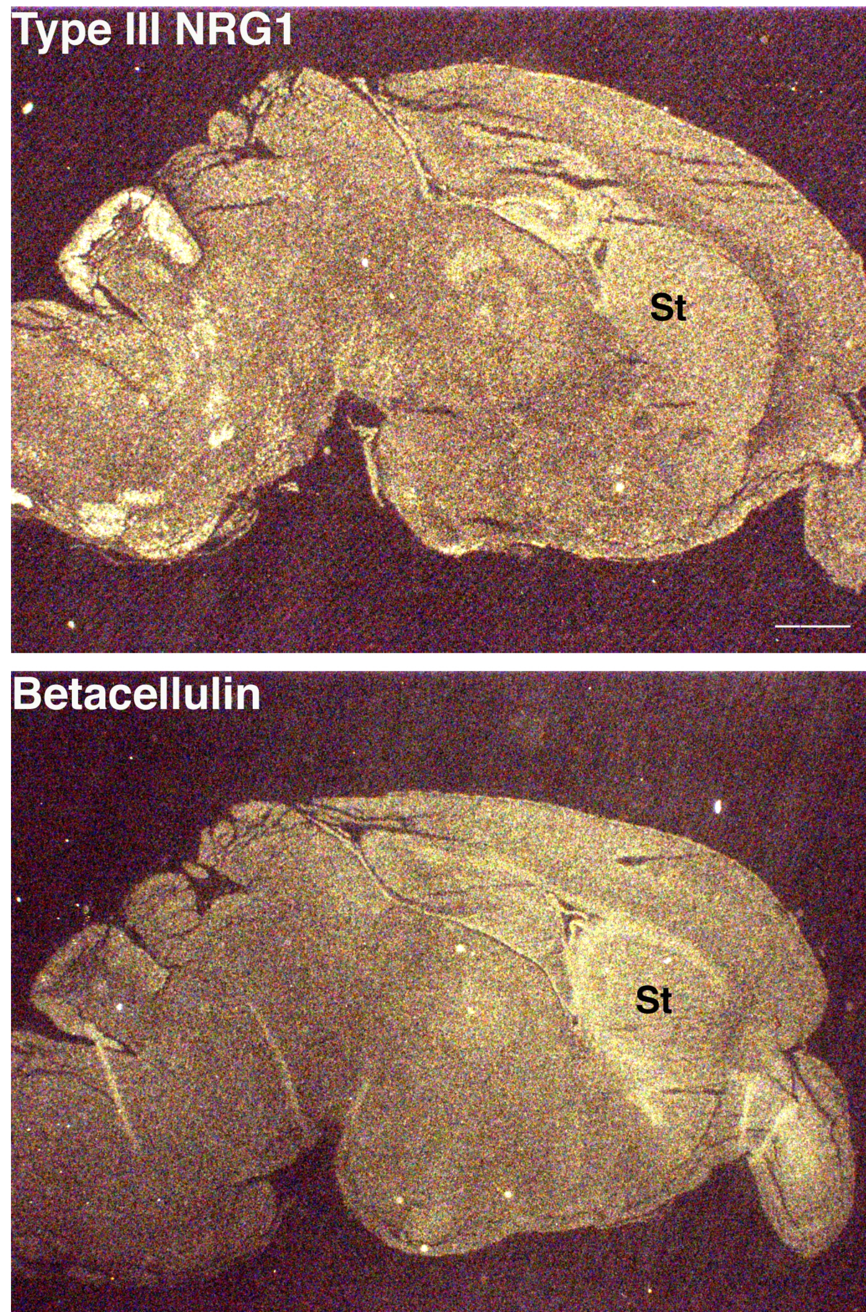

Figure III.11. *Type III NRG1* and *Betacellulin* are expressed in the striatum.
In situ hybridization using ^{35}S-labeled riboprobes on sagittal sections of wild-type P0 mouse brain. mRNA encoding *Type III NRG1* (upper panel) as well as *Betacellulin* (lower panel) are expressed in the striatum (St). Scale bar= 500µm.

3-4-2- Mice deficient for *ErbB4* in the nervous system

The mice with a loss-of-function mutation in *ErbB4* die around E10, from aborted development of the heart [Gassmann et al., 1995], an age when the DA neurons start to be generated in the ventral midbrain. Therefore, an investigation focusing on this neuronal population was not possible at this age.

As an alternative, Dr. Gassmann provided us with adult mouse mutant brains with a nervous system specific deletion of *ErbB4*. The initial embryonic lethality of the total *ErbB4* knock-out was bypassed: Mice with loxP-flanked *ErbB4* exon 2 were crossed with *nestin-cre* transgenic mice [Tronche et al., 1999]. The *nestin-cre* transgenic mouse line expressed the Cre recombinase under the control of the rat *nestin* promoter, which drive the expression specifically in the nervous system. In crosses to animals carrying a loxP-flanked *ErbB4* exon 2, an excision of the LoxP-flanked exon 2 occurs, leading to a specific deletion of *ErbB4* in the nervous system. The nervous system specific *ErB4* mutant mice used in this study (*ErbB4 loxP/- nestin-cre*) carried one *ErbB4* allele with its exon 2 flanked by LoxP, one *ErbB4* null allele [Gassmann et al., 1995], and the *nestin-cre*.

3-4-2-1-All the major genes expressed by the midbrain DA neurons are present in the mice deficient for *ErbB4* in the nervous system

I carried out a detailed expression analysis of 13 genes we know to be expressed in the midbrain DA neurons by *in situ* hybridization on brain sections of adult *ErbB4 loxP/- nestin-cre* mice. As a control, the analysis was done in parallel on adult brain tissue from *ErbB4 loxP/+* mice out of the same litter, which have one *ErbB4* allele with its exon 2 flanked by LoxP, and a wild-type allele, genotyped *nestin-cre* negative. I was unable to detect any alterations of expression in the *ErbB4 loxP/- nestin-cre* mice midbrain DA neurons for the following genes: *TH* (Figure III.10), *Nurr-1*, *En*, *Ptx-3*, *Olf-1*, *VMAT*, *DAT*, *c-ret*, *GDNFRα*, *AHD2*, *AADC*, *DR2* and *HNF3α* (Table III.2). This shows that the expression of these genes in the midbrain DA neurons are not affected by the loss of *ErbB4*.

3-4-2-2-The midbrain DA axonal projections are normal in the mice deficient for ErbB4 in the nervous system

As a next step, I examined whether the midbrain DA projections are affected in the mice lacking *ErbB4*, since two ligands of ErbB4, the Type III NRG1 and Betacellulin, as well as ErbB4 itself, are expressed in the striatum. This analysis was carried out by immunohistochemistry, using a TH antibody on sagittal sections of brain tissue from *ErbB4 loxP/- nestin-cre* adult mice in parallel with control specimens as described above. All *ErbB4 loxP/- nestin-cre* animals showed a wild-type-like axonal projection to the basal ganglia. I was also enable to detect any differences in TH staining intensity in the basal ganglia, suggesting a normal arborization in the axonal termini (Figure II.12). These results indicate that ErbB4 is not required for the proper establishment of SN/VTA DA projections.

Overall, the level of my analysis of the mice deficient for ErbB4 was insufficient to detect any function of the ErbB4/ligands signaling pathway for the development or the maintenance of the SN. However, it is possible that the mutant phenotype is of such subtle nature that it eluded us or that compensatory mechanisms take place.

3-5-Mis-expression of *Olf-1/Ebf* induces ectopic expression of TH in the chick midbrain

I showed that the *Olf-1/Ebf* gene is transiently expressed in the midbrain DA neurons from E10 to E13 in mouse. It is then down-regulated such that at E14, Olf-1/Ebf positive midbrain DA neurons were only detected occasionally. The timing of expression suggests a role for *Olf-1/Ebf* in the differentiation of the midbrain DA neurons. To further analyze the potential role of *Olf-1/Ebf* in generation of DA neurons, I applied *in ovo* electroporation procedures to misexpress *Olf-1/Ebf* within the chick midbrain.

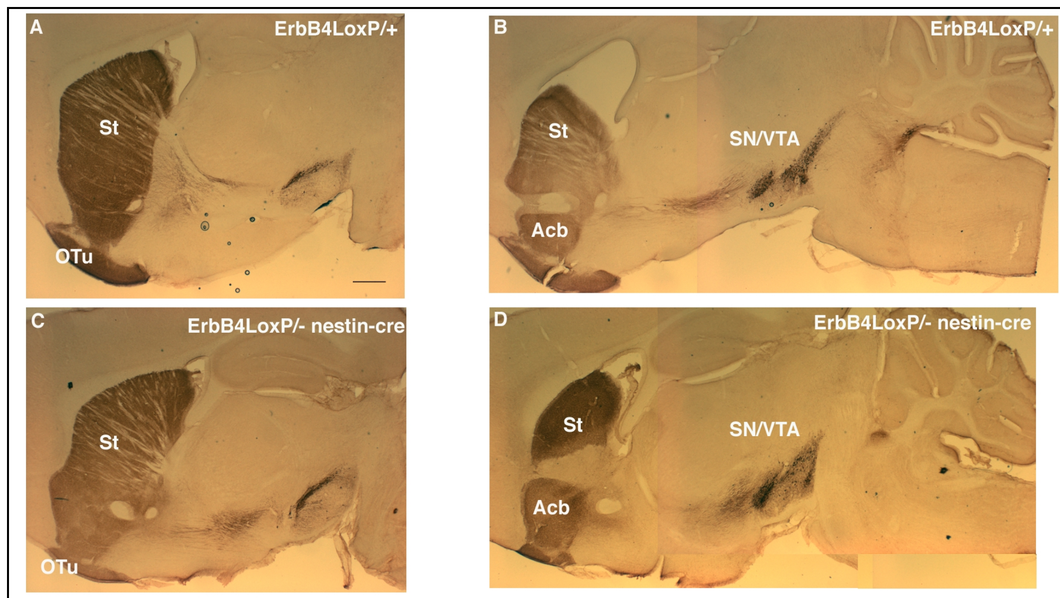

Figure III.12. The midbrain DA projections are normal in the nervous system specific *ErbB4* mutant mice.
Immunohistochemical labeling with TH antibody on sagittal sections of adult mouse brains. A and C are lateral sections, B and D are medial sections. A and B, *ErbB4 loxP/+* mouse, one allele *ErbB4* flanked with LoxP, one wild-type allele, no *nestin-cre* (undisturbed expression of ErbB4). The projections arising from the substantia nigra (SN) and ventral tegmentum area (VTA) are detectable along the way to their targets: the striatum (St), the nucleus accumbens (Acb) and the olfactory tubercle (OTu). C and D, *ErbB4 loxP/- nestin-cre* mouse, nervous system specific *ErbB4* mutant. The DA projections are identical to the ones observed in the wild-type. Scale bar= 500µm.

3-5-1-Groundwork

In the chick (*Gallus domesticus*), the midbrain DA neurons and their axonal projections are not as well characterized as in rodents. The innervations of the ventral forebrain [Metzger et al., 1996] arise from a single nucleus (*nucleus tegmenti pedunculopontinus pars compacta*) in the ventral midbrain [i.e. Wang et al., 1995; Kuenzel et al., 1997; Von Bartheld and Schober, 1997; Csillag, 1999; Watanabe and Nakamura, 2000], in contrast to mammals where these neurons can be separated in two distinct areas with unique properties, SN and VTA. Unfortunately, little is know about the development of the chick midbrain DA neurons. To pursue any *Olf-1/Ebf* over-expression experiments, it was first necessary to determine at what embryonic stage the chick midbrain DA neurons can be first detected, and whether they express *Olf-1/Ebf*.

Since the survival rate of the chick embryo after electroporation (stage 10) decreases over the time, it was important to determine at what embryonic stage the midbrain DA neurons can be first detected. For this purpose, chick embryos from stage 18 (E3) to stage 33 (E8) were analyzed for TH expression within the developing midbrain region. Depending on the age, I used either whole mount preparations or sectioned material. The first midbrain DA neurons, which are located in the ventral midbrain, are very few and sparse, and are first detected with an antibody against TH at stage 29 (E6.5). This is in contrast to rodents where the first expression of TH is detected around E11.5 (mouse), which corresponds to stage 18/19 (E3.5) in chick.

The presence of the protein Olf-1/Ebf (Ebf = chick homologue of the mouse Olf-1) in the embryonic chick midbrain was determined by immunohistochemical labeling on parallel sagittal sections of stage 31 chick (E7) using the same Olf-1 antibody as for the mouse study and a TH antibody. Due to the late appearance of TH in chick midbrain and in correspondence to the mouse data, I never detected Olf-1/Ebf co-expressed with TH. Nevertheless, the Olf-1/Ebf expression was always confined to the pial surface where postmitotic midbrain DA neurons are usually located (Figure III.13).

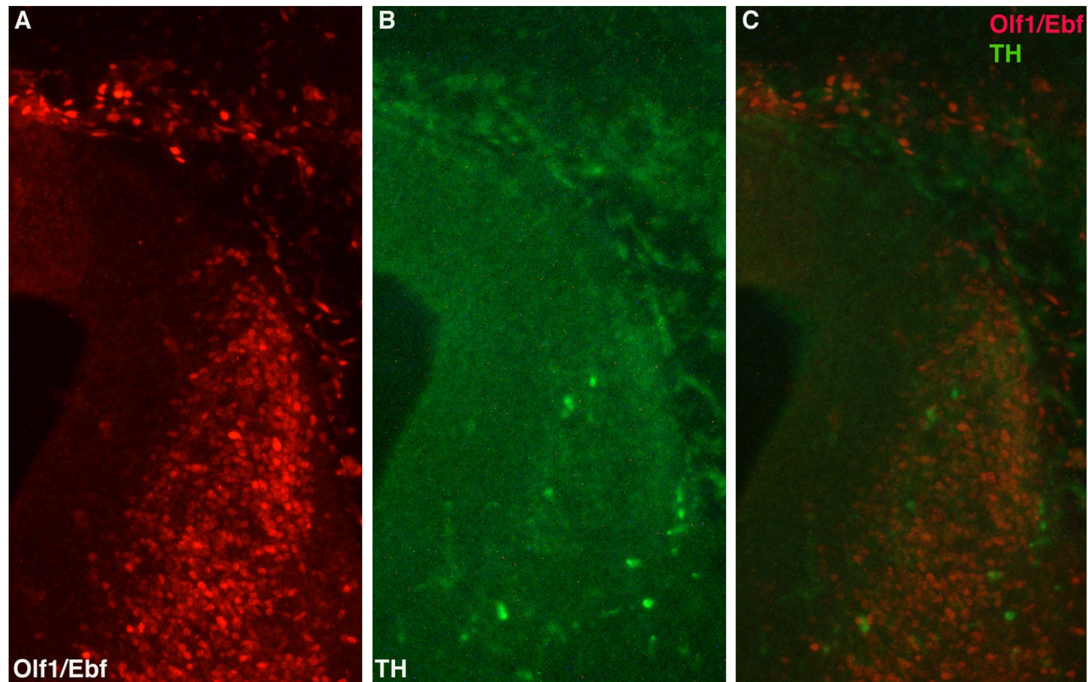

Figure III.13. Detection of Olf-1/Ebf in the embryonic chick midbrain. Immunohistochemical labeling on sagittal sections of stage 31 chick (E7). A, detection of Olf-1/Ebf in the midbrain with an Olf-1 antibody (red). B, detection of TH in the midbrain with a TH antibody on a parallel section (green). C, a superimposed image of A and B showing that the domain of expression of Olf-1/Ebf is broader and contains the TH expression domain. At this stage, both Olf-1/Ebf and TH are expressed in vicinity to each other, but they are not co-expressed in the same cells.

3-5-2-Induction of ectopic chick *Olf-1/Ebf* by *in ovo* electroporation

To examine whether *Olf-1/Ebf* plays a role in specifying the midbrain DA neurons, the chick *Olf-1/Ebf* was ectopically expressed in the midbrain of stage 10 chick embryos by electroporation of the Ebf-IRES2-EGFP expression vector, where the full-length chick *Olf-1/Ebf* cDNA (*Ebf*) was cloned, and then inserted in between the chick β-actin promoter and the IRES-EGFP.

3-5-2-1-Control experiments

To ensure that the electroporation of the pIRES2-EGFP expression vector by itself does not induce any transformation in the chick brain, I just injected the pIRES2-EGFP expression vector, without an insert, into the midbrain vesicle and electroporated it towards the left side. The chick embryos were dissected at E7, sectioned and studied under fluorescence microscope. The GFP signals were detected on the electroporated side of the midbrain but no induction of ectopic TH or any morphological abnormality was observed (data not shown). The average survival rate of the chick embryos until E7 was about 50%.

When the expression vector Ebf-IRES2-EGFP was electroporated at the same conditions as for the control experiment, the intensity of the GFP signals observed on the unilateral side of the midbrain was similar to the one detected when the control vector was injected. In order to verify that the bicistronic *Olf-1/Ebf-IRES-EGFP* construct is functional, electroporated embryos were isolated at E7, sectioned and inspected for double labeling of Olf-1/Ebf and GFP. Indeed, all the cells showing a GFP signal co-express Olf-1/Ebf. The Olf-1/Ebf antibody detects both endogenous (symmetrical expression on the left and the right side of the midbrain) and ectopic Olf-1/Ebf protein (expression only on the left side of the midbrain) (Figure II.14). There are no apparent morphological abnormalities found in the electroporated side of the brain. The average survival rate after electroporation of the Ebf-IRES2-EGFP vector was similar as in the control. However, if a larger amount of Ebf-IRES2-EGFP vector DNA was injected into the midbrain vesicle (by way of a higher DNA concentration),

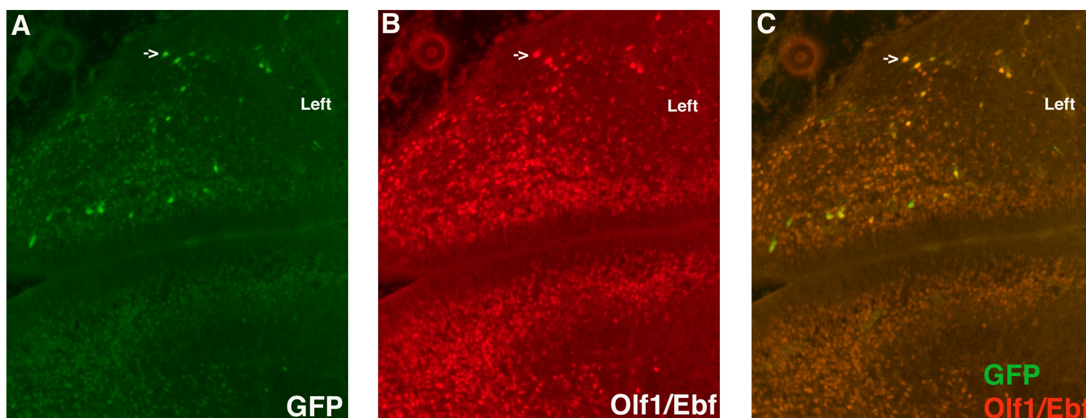

Figure III.14. Ectopic expression of Olf-1/Ebf in the left side of the midbrain in chick.
Immunohistochemical Olf-1/Ebf labeling on a horizontal section of E7 (stage 31) chick midbrain after *in ovo* electroporation at stage 10. A, GFP expression is detected in cells on the left side of the midbrain (green). B, same section as A, labeled by the Olf-1 antibody. The red signal shows both endogenous (symmetrical expression on the left and the right side of the midbrain) and ectopic Olf-1/Ebf protein (expression only on the left side of the midbrain). C, superimposed image of A and B, all the cells showing a GFP signal do express Olf-1/Ebf (arrows).

the survival rate would drop dramatically, whereas when a larger amount of the control pIRES2-EGFP vector DNA was injected, no change in the survival rate was found. This observation indicates that an over-expression of the Olf-1/Ebf protein in the midbrain may be toxic for the chick embryo to a certain extend.

3-5-2-2-Analyses of the TH expression in chick midbrains expressing ectopic Olf-1/Ebf protein.

In order to analyze the effect of the ectopic Olf-1/Ebf protein on the midbrain DA neurons, electroporated E7 midbrains were analyzed for TH expression. Fluorescent immunohistochemical TH labeling were carried out on horizontal sections of the brain vesicle. Ectopic TH expression was observed on the left electroporated side of the midbrain. TH positive cells were identified as ectopic when the 'symmetrical' equivalent of a group of TH cells was not present on the right control side of the midbrain. The ectopic expression of TH occurred in the ventral midbrain, only in the vicinity of the cells expressing Olf-1/Ebf. However, it is important to note that I did not find any cells which co-expressed TH and Olf-1/Ebf (Figure III.15 A, B, C, A', B', C').

In order to eliminate the possibility of an artifact due to a tilted cutting plane of the sections, and therefore showing an asymmetrical view of the midbrain, additional immunohistochemical TH labeling were carried out on E7 whole mount brain vesicles. For all the brains investigated, the presence of GFP expression first was confirmed in the unilateral side of the midbrain under a fluorescence microscope. The results from these whole mount stainings showed again an asymmetrical TH expression pattern. An ectopic TH expression on the left side of the ventral midbrain was observed in all brains with a strong unilateral GFP signal. The TH positive cells on the left side appear to be delocalized, in comparison to the right control side of the midbrain (Figure III.15 D, E). All together, these results show that a mis-expression of Olf-1/Ebf leads to ectopic TH expression in the chick midbrain, suggesting that Olf-1/Ebf can induce ectopic formation of DA neurons in the midbrain or that upon Olf-1/Ebf overexpression, the midbrain DA neurons do not migrate properly.

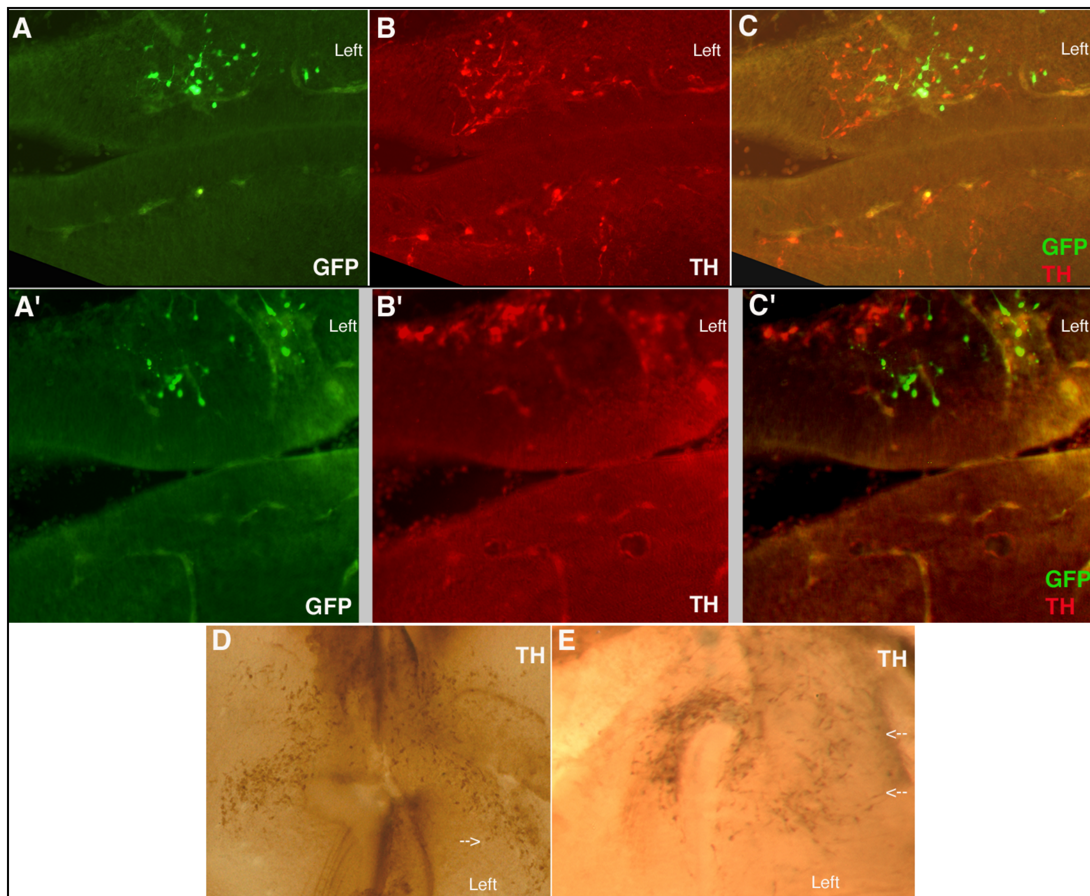

Figure III.15. Mis-expression of *Olf-1/Ebf* induces ectopic expression of TH in the chick midbrain.

Immunohistochemical TH labeling of E7 (stage 31) chick brains after *in ovo* electroporation of the Ebf-IRES2-EGFP expression vector in the left side of the midbrain. A and A' (two different cases) horizontal sections, GFP expression is detected in cells on the left side of the ventral midbrain (green). B, same section as A, TH positive cells (red) are detected in both left and right side of the ventral midbrain, with a higher amount of TH positive cells on the left side. Most of these TH positive cells on the left side are ectopic TH cells because their 'symmetrical' equivalents are not present on the right control side of the ventral midbrain. B', same section as A', TH is detected only in some cells on the left side of the ventral midbrain, all of them are considered ectopic as none are detected on the control right side of the ventral midbrain. C and C', superimposed images of A/B and A'/B' respectively. In both cases the ectopic TH positive cells are found in the vicinity of the GFP positive cells but there are no cells co-expressing TH and Olf-1/Ebf. D and E, entire brain vesicle. The flat mount of the midbrain (D) or whole mount (E) shows TH positive cells in brown. In both cases, an asymmetrical TH expression pattern is observed. The TH positive cells on the left side appear to be delocalized (arrows) when compare to the location of the TH positive cells in the right control side of the ventral midbrain.

DISCUSSION

Very little is known about the intracellular mediators that arbitrate the development of the midbrain DA neurons during induction and full differentiation of this cell population. The main focus of this study was to identify such mediators using a differential screening method to compare ventral midbrain tissue of wild-type versus *En* double null mutant mice. From this analysis, four genes were identified that showed specific spatial and temporal expressions in the midbrain DA neurons, suggesting a role of these genes in the determination of the midbrain DA neurons cell fate. I investigated their potential role in DA neurons development by analyzing mutant mice deficient in these genes, and by over-expressing them in the chick embryo.

1-Further characterization of the role of the *En* genes in the midbrain DA neurons development

1-1-The midbrain DA neurons require *En-1* and *En-2* cell autonomously for their survival

It has been previously shown that the *En* genes are required for the midbrain DA neurons survival [Simon et al., 2001]. We therefore decided to use tissue from *En* double null mutant mice for a differential screening method to identify other genes involved into the midbrain DA neurons fate. However, firstly we needed to determine whether the *En* genes worked in a cell autonomous fashion. The results obtained from the generation of the *En2-/-;En1-/-* chimeric mice demonstrated that the midbrain DA neurons require the *En* genes cell automomously for their survival.

The activity of *En* in the embryonic midbrain is down regulated as soon as the tissue is dissociated and cells are cultured (Prochiantz, unpublished data; Alberi and Simon, unpublished data). This finding made impossible to elaborate a simple culture experiment, involving dissociated cells of embryonic ventral midbrain of *En* double null mutants together with wildtype cells to determine whether or not the *En* genes are required cell automously by the midbrain DA neurons. An alternative way to address this question was the generation of chimeric mice where wild-type cells are intermingled with *En* double null mutant cells *in vivo*.

The blastocysts used for making the chimeric animals were isolated from female *En2-/-;En1+/TLZ* crossed with male of the same genotype. These females and males are derived from crossing of different mouse strains (129SV, C57BL/6 and ICR) making the blastocystes themselves originating from diverse mouse strains with various coat colors. The normal way to determine the degree of chimerism by coat color was therefore impossible. To detect the presence of the *En-2* mutant allele in the chimeric mice, a PCR approach was used. Positive signals were detected in DNA from the tail of all the animals generated, indicating that the ES cell lines participated in the generation of this tissue. Because such participation can be uneven in the generation of different organs, and the fact that the mutated allele is detectable by PCR, a very sensitive tool, does not mean necessary that the ES cell line contributed significantly to the generation of a particular organ. I therefore determined the level of chimerism in the brain by detection of NPTII.

The brain of the animal containing a heterogeneous population of wild-type cells and of 40-50% *En2-/-;En1TLZ/TLZ* cells had significantly less midbrain DA neurons. It is very likely that the DA neurons missing are the ones originally not expressing *En-1* and *En-2* corroborating that the DA neurons require *En-1* and *En-2* for their survival [Simon et al., 2001] and showing that no support from the wild-type environment expressing *En-1* and *En-2* was supplied to rescue them. These results support the main hypothesis: as the midbrain DA neurons express *En-1* and *En-2* and disappear in the *En* double null mutant [Simon et al., 2001], the cells require the genes cell autonomously.

Another significant outcome from this experiment is the fact that the brain morphology of the chimeric animal, composed of 40-50% of *En2-/-;En1TLZ/TLZ* cells, is normal and does not carry any defect in the brain regions derived from embryonic posterior midbrain and anterior hindbrain, while the *En* double null mutant mice lack the region around the isthmus [Liu and Joyner, 2001]. In this chimeric animal, it seems that the surrounding wild-type cells compensated for the loss of the mutant cells, or we would have observed morphological defects in these regions relative to the proportion of *En2-/-;En1TLZ/TLZ* cells. Moreover, it is very likely that the *En* genes are required in a cell autonomous fashion for the survival of the cells derived from embryonic posterior midbrain and anterior hindbrain, since no En-1TLZ positive cells were detected. The absence of any morphological defects

could be explained by the fact that the posterior midbrain and anterior hindbrain cell population requires the *En* genes while they are still proliferating. It is very likely that the wild-type cells increase their rate of proliferation (or less are dying) to compensate for the lost *En2-/-;En1TLZ/TLZ* cells in order to reach the normal mass of the posterior midbrain and anterior hindbrain. This is an often seen phenomenon, if the numbers of cells in the early embryo are reduced – either by chemical damage or physical removal – there is a general elevation in proliferation during later development to compensate for it (for review [Potter and Xu, 2001]).
Finally, it is important to mention that *En* double null mutant ES cell lines represent an excellent tool to demonstrate whether genes regulated by *En-1* and *En-2* are essential for the survival of midbrain DA neurons. Such genes like *MAP1B* (a gene pulled out from the differential display PCR experiment identified as not expressed in the *En* double null mutant tissue) or *α-synuclein* (a gene not expressed in the *En* double null mutant mice [Simon et al., 2001]) could be reintroduced into *En* double null mutant background (into the *En* double null mutant ES cell line). By studying the midbrain DA neurons of the mice derived from this experiment, we could determine whether these genes can rescue the *En* null mutant phenotype and if they are essential for the survival of midbrain DA neurons.

1-2- The *En* genes are not required for the formation of the midbrain DA precursor cells

The *En* double null mutant mice have midbrain DA neurons until E13, but the domain is always smaller than in the wild-type embryo. Two possible explanations exist for this observation. The precursor cells of the midbrain DA neurons are reduced in number due to the lack of *En-1* and *En-2*, or the analysis was not sufficiently detailed to find a stage where an equivalent amount of midbrain DA neurons are present in both genotypes. Indeed, a more detailed analysis would consist of looking at mouse embryos at different ages to find one age where the number of DA neurons in wild-type is the same as in the *En* double null mutant. However, this analysis requires a lot of mouse embryos and is not fully reliable because of the uncertainty of the equal staging of wild-type and mutant embryos. It

was therefore necessary to use a different approach to differentiate between these two possible explanations.

Dittrich et al., 1997, used in *Drosophila* an overexpression method to find out whether or not the gene *eagle* is required at the level of progenitor cells or at the level of the mature cells for the specification of serotonergic cells. A similar approach to this one was used and adapted to the chick animal model. *En-1* was overexpressed in the chick embryo at stage 10, inducing a change of *En-1* expression domain. The tectum was increased in size in such experiments, likely related to the role of *En-1* during regionalization. However, no changes in the distribution or amount of the midbrain DA neurons were detected. If the formation of midbrain DA neuron precursor cells was dependent on *En-1*, then an expansion of the *En-1* expression domain should increase the number and change the location of TH positive neurons. In contrast, these presented results revealed no changes, demonstrating that *En-1* is not required or maybe not sufficient by itself for the formation of DA neuron progenitor cells. It would be interesting to co-overexpress *En-1* with other intracellular mediators to see if changes in the midbrain DA neurons could be induced. *Lmx1b* would be a good candidate to co-express with *En-1*. Lmx1b has similar features when compared to the *En* genes, it is involved into MHB regionalization [Adams et al., 2000] and is therefore expressed during the DA neuron progenitors phase and later by the midbrain DA neurons [Smidt et al., 2000]. Furthermore, similar as the *En* double null mutant, midbrain DA neurons are first generated in the *Lmx1b* null mutant mice with a smaller domain than in the wild-type mice before they then disappear during further development [Smidt et al., 2000]. These common characteristics between *En* and *Lmx1b* in respect to the midbrain DA neurons makes it very interesting to further investigate whether the two genes co-operate together at the progenitor level to specify the midbrain DA neurons.

2-Identification of genes expressed by the midbrain DA neurons by ddPCR

This study was aimed to identify genes specifically expressed by the DA neurons of the ventral midbrain and/or regulated by the *En* transcription factors. Because the midbrain DA neurons require the *En* genes cell autonomously for their survival, the

source of the RNA for the screen was the ventral midbrain tissue, and was dissected as restricted as possible to the midbrain DA location, rather than the midbrain and the anterior hindbrain, which would have been taken in case of a non-autonomous requirement.

Four genes coming from this screen were identified to be expressed by the midbrain DA neurons. It appears that the most limiting factor to identify a greater number of genes was the sensitivity of the *in situ* hybridization. A signal was detected in 60% of the total number of probes. The size of the gene fragments might also play a role. Most of the fragments amplified with the arbitrary primers are smaller than 500 nucleotides, which limits their efficiency for *in situ* hybridizations. However, a more significant point is the part of the cDNA where the fragment is derived from. Several fragments isolated by the ddPCR belonged to previously identified genes, but showed only detectable signals when different probes of similar sizes than the original amplified fragments were used.

Initially, 43 individual sequence tags differentially expressed in wildtype and *En* mutant DA neurons were isolated. From these 43 fragments 7 were found to be expressed either exclusively in the double null mutant tissue or not expressed at all in it. It is very likely that only one of those, *MAP1B*, is regulated by En-1 and En-2 in midbrain DA neurons. This finding is supported by a recent article reporting that the *MAP1B* promoter is regulated by En. Montesinos et al. looked for some genes regulated by homeodomain transcription factors by internalizing the DNA-binding domain of En into rat cerebellum, and pulled out *MAP1B* by ddPCR. They did further *in vitro* and *in vivo* studies to confirm its regulation by En [Montesinos et al., 2001]. The general expression of *MAP1B* in all parts of the brain made it difficult to identify with a riboprobe its specific expression in the midbrain DA neurons. According to the recent insights concerning *MAP1B* [Montesinos et al., 2001], this gene should be seriously taken into consideration as a potential candidate for being able to rescue the *En* double null mutant phenotype and moreover being essential for the survival of midbrain DA neurons. Consequently, it would be necessary to characterize the MAP1B expression in the midbrain DA neurons in more details such as using double immunohistochemistry labeling with TH and MAP1B antibodies in order to see if some midbrain DA neurons are co-expressing the two proteins. Finally, the study of the midbrain DA neurons in the

MAP1B mutant mice [Edelmann et al., 1996] would be of great interest and very valuable.

3-*HNF3α* and its relation with the midbrain DA neurons

This study shows that the *HNF3α* expression is very specific in the brain, as it is only found in the midbrain DA neurons and in one other cell group in the diencephalon. It may be expressed in the precursors of the DA neurons as early as E9 and continues to be expressed in the postmitotic cells until adulthood.
Three genes belonging to the hepatocyte nuclear factor 3 (HNF3) family have been so far identified in the murine genome, *HNF3α, HNF3ß* and *HNF3γ* [Lai et al., 1993]. All of them are expressed during embryogenesis [Monaghan et al., 1993; Sasaki and Hogan, 1993], but only *HNF3α* and *HNF3ß* are expressed in the floor plate of the developing neural tube [Ang et al., 1993]. At E8 (4 to 10 somites), *HNF3ß* is found throughout the ventral neural tube, whereas *HNF3α* is expressed exclusively in the ventral midbrain. Only from E9 onwards, it is detectable in the hindbrain and in the spinal cord. The ventral midbrain is the region where the dopaminergic neurons are induced in the neuroepithelium by an interaction between Shh and FGF8 [Ye et al., 1998]. This developmental stage, when the tissue is susceptible for this signal and generates the DA neurons, coincides with the early expression of *HNF3α.* It is one of the earliest identified marker for midbrain DA neurons, preceding even *AHD2* and *Nurr1*, which start to be expressed at E9.5 and E10.5, respectively [Zetterström et al., 1997; Wallen et al., 1999]. From the developmental expression data, it is conceivable to think that *HNF3α* is one of the first genes upregulated in precursor cells in the midbrain DA neurons when they become committed. Judged from the size of the expression domain, the amount of *HNF3α* positive cells exceeds the number of later postmitotic DA neurons. It is plausible that a selection process reduces their cell number. An example for such an event is the expression change of the *Nurr1* gene: During early development, its expression domain is far larger than the area of future DA neurons marked by the *TH* expression. Nevertheless, later in development the cells surrounding the midbrain DA neurons do not express Nurr1 any longer [Zetterstrom et al., 1996]. A selection must have been taken place. A second scenario, where the early

expression at E8/9 is unrelated to the one found in the postmitotic DA neurons, requires a complicated mechanism where the *HNF3α* gene is upregulated twice in the same cells during development. The fact that neither *Nurr1* nor the *En* genes control the *HNF3α* expression supports the idea that it is an early marker for precursor of midbrain DA neurons and not a regional marker for the most ventral midbrain.

It can be speculated that an ectopic expression of *HNF3α* could induce DA neurons. However, overexpression of *HNF3α* in chick by *in ovo* electroporation resulted in the death of the embryos soon after and made it therefore impossible to analyze this in details (data not shown). Another way to assess such a hypothesis was to study the midbrain DA neurons in *HNF3α* null mutant mice. In these mutant mice, at P8 the midbrain DA neurons were present and seemed normally developed; no alteration of expression of the major markers could be detected. These results dismantle the statement that *HNF3α* could have a major role in midbrain DA neurons development. However, due to the limited amount of brains provided and the lack of live animals, it was not feasible to investigate all the anatomical failures possibly related to a defect in the midbrain DA neurons system (i.e. it would have been necessary to analyze if the DA neurons projections targets were well innervated). The discussion is still open towards a possible role of *HNF3α* into axonal path finding or organization of specific connectivity of the midbrain DA system. Because the mutant mice die early (P14) it is not possible either to see if the absence of the gene would affect the midbrain DA neurons in the long term, when the mice mature. To know if *HNF3α* was involved in the maintenance of the midbrain DA neurons, it would be necessary to create a conditional *HNF3α* knock-out where *HNF3α* is depleted only in the nervous system to bypass metabolism defects from which they die in the homologous recombinant mutant.

Another important issue concerning *HNF3α* is related to its remote correlation with PD. Mouse mutant studies [Kaestner et al., 1999; Shih et al., 1999] and the use of embroid bodies [Duncan et al., 1998] implicated *HNF3α* in the regulation of the blood glucose level. *HNF3α* null mutant mice die mainly because of severe hypoglycemia. In embroid bodies, *HNF3α* positively regulates *HNF1α* and *HNF4α*, two genes that have been genetically related to type 2 diabetes in humans

[Yamagata et al., 1996a; Yamagata et al., 1996b]. Interestingly, up to 80% of PD patients have an abnormal glucose tolerance (for review [Sandyk, 1993]) and some “slow“ environmental toxins have been implicated in both diabetes and PD [Eizirik et al., 1996]. This correlation may be caused by the *HNF3α* expression in pancreatic islet cells [Kaestner et al., 1999] and in the DA neurons of the SN/VTA. The link between diabetes and PD and the sensitivity to these toxins could be related to commonly expressed genes in these two cell types. For example, we can speculate a role of the ATP-sensitive potassium (K-ATP) channels in this scenario. Indeed, it was shown that the loss of functional K-ATP channels in pancreatic β-cells causes persistent hyperinsulinemic hypoglycemia of infancy (PHHI) where the K-ATP channels are permanently enabled to govern secretion of insulin [Kane et al., 1996]. The PHHI susceptibility locus encodes two particularly subunits of the K-ATP channel: the receptor with high affinity for sulfonylureas 1 and the inward rectifier potassium channel, $K_{IR}6.2$ [Aguilar-Bryan et al., 1995; Sakura et al., 1995]. Interestingly, functional somatodendritic K-ATP channels on DA SNC neurons have been identified and are composed of the same subunits combination [Liss et al., 1999]. Moreover, Roeper and Ashcroft, 1995, showed that the inhibition of mitochondrial complex I leads to a toxic activation of K-ATP channels and to a complete cessation of electrical activity in midbrain DA neurons consequently prohibiting dopamine release. This is relevant as complex I inhibition is one of the proposed trigger mechanisms of degeneration of midbrain DA neurons (for review [Hanna and Bhatia, 1997]). The dysfunction of the same K-ATP channel subunits in the pancreas and in the midbrain DA neurons, with *HNF3α* expressed in both tissues, leads to two PD features: abnormal glucose tolerance for review ([Sandyk, 1993]) and dopamine deficiency (for review [Lang AE, Lozano AM., 1998a,b)] respectively. It is conceivable that the transcription factor *HNF3α* could play a role as activator or repressor of genes expression for K-ATP channel subunits genes. Further studies are necessary to elucidate this issue.

4-*ErbB4* and its relation with the midbrain DA neurons

The adult expression of *ErbB4* in the SN of rats has recently been demonstrated by *in situ* hybridization in combination with dopamine depletion by 6-

hydroxydopamine treatment [Steiner et al., 1999]. I extended further this study by undergoing ontogenetic expression analyses of *ErbB4* in mouse. The *ErbB4* expression is initiated in the DA neurons at E11.5 and continues into adult stages, specifying the most rostral part of the SN.

Among the ErbB tyrosine kinase receptor family, ErbB4 is the only one to be expressed in the midbrain DA neurons (this study and [Steiner et al., 1999]). It is then very likely that, in the midbrain DA neurons system, the signaling specificity of the ErbB4/ligands network is going to be restricted to binding specificities and affinities characteristic to an ErbB4 homodimer. However, Sweeney et al., 2000, showed that ErbB4 receptor homodimers are capable of discriminating among ligands. Different ligands elicit similar amount of ErbB4 phosphorylation, but evoke different levels and patterns of tyrosine phosphorylation, recruit distinct adaptor proteins and activate different intracellular kinase cascades, thus providing a mechanism for expanding signal diversity without loss of specificity [Sweeney et al., 2000]. I showed that none of the typical ligands for ErbB4 are located in the midbrain DA neurons, but interestingly the *Type III NRG-1* and *Betacellulin* genes were found to be expressed in the striatum, one of the major projection targets of the midbrain DA neurons. Even more relevant is the fact that Type III NRG-1 is known to bind ErbB4 homodimers (with an intermediate affinity), and that the strongest binding affinity of Betacellulin is for ErbB4 homodimers [Dunbar and Goddard, 2000]. The binding of these two ligands could lead to two distinct effects upon ErbB4 phosphorylation. However, there is one issue; the two ligands and the receptors mRNA are expressed in two different cell populations of the nigrostriatal pathway, how can this work? Three different hypotheses that may explain this are; i) the ligands are picked up in the striatum by ErbB4 on the midbrain DA axons, ii) the ligands are expressed by the GABA-ergic neurons and transported to the axons termini in the SNC and are released, or iii) both routes could be used. Depending on what routes are used, it is possible to speculate about different roles for ErbB4/ligands signaling. This signaling could have a trophic effect on DA neurons. Such a signaling exists; the midbrain DA neurons contain receptors of GDNF (GDNFRα, c-ret) and this growth factor (expressed in the striatum) can promote survival of DA neurons [Lin et al., 1993; Glazner et al., 1998]. The ErbB4/ligands signaling could also have a neuronal differentiation effect as already shown in

different tissues by Zhao and Lemke. By using retroviruses carrying a *NRG-1* specific ribozyme in chicken, they demonstrated the function of NRG-1 in regulating the proliferation of neuroepithelia and differentiation of retinal ganglion neurons [Zhao and Lemke, 1998]. Moreover supporting this idea, it was shown in PC12 cells that ErbB4/Betacellulin signaling can induce neurite outgrowth [Vaskovsky et al., 2000].

All together, the results of this work jointly with previous studies suggest a possible role of the ErbB4 receptor and its ligands in the midbrain DA neurons system. In order to have an immediate idea regarding the importance of ErbB4 for the midbrain DA neurons system, this neuronal population was analyzed in adult mice deficient for *ErbB4* in the nervous system. The midbrain DA neurons in these mice are normal; all the major midbrain DA markers are present and show a normal expression pattern. This result could be explained by the up-regulation of another ErbB receptor, which would then complement the loss of ErbB4. But data from a gene chip screen searching for genes differentially expressed between *ErbB4* conditional knock-out and wild type brain tissues did not show any up-regulation of any other ErbB receptors (M. Schwab and C. Lai, personal communication).

Mice lacking completely *ErbB4* die at E10.5 due to a heart failure and moreover exhibit misprojections of cranial sensory ganglion afferent axons [Gassmann et al., 1995]. Because of this feature, it made sense to analyze the state of the midbrain DA projections in the conditional *ErbB4* knock-out mice. The results of this study showed that these animals elaborate normal DA projections and innervate all their targets, including the striatum.

A very important point to consider regarding the study of this conditional *ErbB4* knock-out mice is that the nestin-cre mice used to generate these animals, do not show a Cre recombinase activity in the nervous tissue before E11 (R. Klein, nestin-cre technical sheet from The Jackson Laboratory), which means that *ErbB4* is still expressed in the ventral midbrain from E9 until E11, exactly when midbrain DA neurons are being specified. We can only conclude from this conditional *ErbB4* knock-out mice study that ErbB4 does not seems to be essential after E11 for the midbrain DA neurons survival, and the establishment of their axonal projections. The fact that the ErbB4/ligands signaling does not have a particular role after E11 goes in favor to the hypothesis of an earlier role of this signaling in early fate

determination and neuronal differentiation of the midbrain DA neurons. This hypothesis could be investigated by studying the state of the midbrain DA neurons in the complete *ErbB4* null mutants, where *ErbB4* is rescued specifically in the heart (with a cre-mediated heart-specific repair). This would ensure the survival of the mice and the lack of ErbB4 expression in the brain at all stages.

5-*Olf-1/Ebf* and its relation with the midbrain DA neurons

This study shows that the *Olf-1/Ebf* gene is expressed transiently in mouse from E10 to E13, then the midbrain DA neurons begin to down-regulate *Olf-1/Ebf*, corroborating its possible role in the differentiation of the midbrain DA neurons. The Olf-1/Ebf (O/E) proteins O/E-1, O/E-2 and O/E-3 define a family of transcription factors that share structural similarities and biochemical activities [Wang et al., 1997]. They are expressed in the entire embryonic CNS [Garel et al., 1997]. Although, their expression is sometimes overlapping but always confined to postmitotic neurons. Their patterns of expression and inactivation of their genes in *C. elegans* [Prasad et al., 1998] suggest a role in neuronal differentiation. This concept has been supported by overexpression studies in *Xenopus* showing that their ectopic expression converts ectodermal cells into neurons [Dubois et al., 1998; Pozzoli et al., 2001].
Mouse mutant studies for *Olf-1/Ebf* revealed major defects in the immune system [Lin and Grosschedl, 1995] and the only significant CNS defect can be detected in the striatum, where the migration of striatal cells from the subventricular zone into the mantle layer does not occur in absence of *Olf-1/Ebf* [Garel et al., 1999]. The midbrain DA neurons have never been investigated directly, but indirect evidences strongly suggest that they are present in the *Olf-1/Ebf* mutant mice. The remaining striatum in the mutant mice is innervated by TH positive axons. Since the dopaminergic innervation of the striatum has its origin exclusively in the midbrain, it can be concluded that the DA neurons are present entirely or at least partially in the *Olf-1/Ebf* mutant mice. However, the largely overlapping expression of the members of the *O/E* genes family make it more than likely that compensatory mechanisms are taking place in the *Olf-1/Ebf* mutant mice and that only double or

triple mutants may reveal any function of the genes in respect to the midbrain DA neurons.

In order to determine a potential role of *Olf-1/Ebf* in the midbrain DA neurons specification and differentiation, in this study the chick *Olf-1/Ebf* was ectopically expressed in the midbrain of chick embryos. It appears that the mis-expression of *Olf-1/Ebf* induces ectopic TH expression, suggesting that Olf-1/Ebf can induce ectopic placement of DA neurons in the midbrain. However, the ectopic expression of TH occurred only in the vicinity of the cells expressing Olf-1/Ebf, and the two cell populations do not co-express the two proteins. First it is important to mention that at E7, more than 70 % of the GPF signal disappeared compared to the level at E5 (data not shown). This is probably due to the time limit of such a transient over-expression. This could mean that in most of the cases the initial *Olf-1/Ebf-GFP* expression domain was much broader after injection, and slowly decreased with time and appeared very reduced at E7. So it is possible that at the time of midbrain DA neurons specification/differentiation, the cells revealing an ectopic expression of TH did express an ectopic Olf-1/Ebf. Furthermore, as shown in the mouse, Olf-1/Ebf is co-expressed with TH during a very short period during differentiation of the midbrain DA neurons, and then is down-regulated. So it is very likely that E7 in the chick is already too late to see Olf-1/Ebf co-expressed with TH. It would be then necessary to look at earlier stages, and this would required the use of earlier markers than TH, i.e. AHD2 or Nurr1.

The detection of ectopic DA neurons in the midbrain signifies that the mis-expression of *Olf-1/Ebf* induces new midbrain DA neurons or alternatively, that the midbrain DA neurons are disturbed in their migratory behavior and are mis-located. The migratory behavior could be disturbed because the DA neurons express the ectopic Olf-1/Ebf by themselves (which we never saw, but maybe justified by the explanations described above), or the ectopic Olf-1/Ebf is only expressed in the midbrain DA neurons environment, which is enough to disturbe their migration route. To differentiate between the apparition of new DA neurons and mis-location of DA neurons, it would be necessary to count and compared the total number of DA neurons in the Olf-1/Ebf over-expressed side and in the control side of the midbrain.

Finally, the investigation of *Nurr1* -/- and *En* double null mutants mice demonstrated that *Olf-1/Ebf* is expressed independently of the two transcriptional regulators. It remains to be investigated the *Lmx1b* mutant mice whether there is any deficiency in the expression *Olf-1/Ebf*. The late onset of the *Olf-1/Ebf* expression in the TH neurons could be a sign that it is under the control of *Lmxb1*.
All together, the data from the mouse and the chick jointly suggest that Olf-1/Ebf is first very likely involved in midbrain DA neurons specification and differentiation, and second, it could be involved non-autonomously in the maintenance of these neurons as Olf-1/Ebf is still express in neighboring cells until adulthood and could signal to the midbrain DA neurons by the way of others molecules through transcriptional regulation.

6-Conclusion

The primary aim of this project was to identify and characterize genes involved in the development of the midbrain DA neurons, which are active during the intermediate period between induction and full differentiation of these neurons. The outcomes of this study were i) the further characterization of the *En* genes towards their cell-autonomous requirement by the midbrain DA neurons, and their non-requirement for the formation of the midbrain DA precursor cells ii) the identification of *MAP1B* as a gene regulated by En-1 and En-2 in the midbrain DA neurons, iii) the identification of *HNF3α, ErbB4* and *Olf-1/Ebf*, which are specifically expressed in the midbrain DA neurons between induction and full differentiation of these neurons, and finally iiii) the characterization of these three genes toward their involvement in the midbrain DA neurons fate, particularly with the finding that *Olf-1/Ebf* may be involved in the specification of these neurons.
It will be interesting to continue on the further analysis of this network of different genes. Other genes from this screen have yet to be followed up and will give important insights on the nature of the midbrain DA neurons.
Overall, this work took us a step further towards additional insights concerning the factors which determine the identity of such a particular cell population.

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