Dissertation

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The role of K_{ATP} channels in model systems of dopaminergic neuron loss in the ventral mesencephalon

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To my family and friends

To my love, who is both

(...)
There lies the port; the vessel puffs her sail;
There gloom the dark, broad seas. My
mariners,
Souls that have toil'd, and wrought, and thought with me,
That ever with a frolic welcome took
The thunder and the sunshine, and opposed
Free hearts, free foreheads,
you and I are old;
Old age hath yet his honor and his toil.
Death closes all; but something ere the end,
Some work of noble note, may yet be done,
Not unbecoming men that strove with Gods.
The lights begin to twinkle from the rocks;
The long day wanes; the slow moon climbs; the
Deep
Moans round with many voices.
(...)

Tho' much is taken, much abides; and tho'
We are not now that strength which in old days
Moved earth and heaven, that which we are, we are,
One equal temper of heroic hearts,
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield.

- Alfred Lord Tennyson, “Ulysses”

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Summary

The dopaminergic neurons of the ventral midbrain (mesDA neurons) form several distinct sub-populations which are involved in emotional control, reward behavior and motor control. In patients with the neurodegenerative disorder Parkinsons Disease (PD), one of these groups, the dopaminergic neurons of the substantia nigra, pars compacta (SNpc) gradually die. One focus of research has been to find an explanation why the SNpc neurons are more vulnerable to cellular stress than other neuronal populations. In engrailed mutant mice, the absence of the homebox transcription factors engrailed 1 and engrailed 2 (En1 and 2) causes a cell-autonomous and gene-dose dependent loss of mesDA neurons. Recently, my lab identified several genes differentially expressed between wild-type and engrailed mutant mice. Analysis of mouse mutants of one of the genes, the forkhead containing trancription factor 1, Foxa1 (formerly HNF3α) showed no phenotype in regard to the mesDA neurons. As it is well known that members of the Fox/HNF family of genes can compensate for each other, the focus of research shifted to one downstream target of Fox genes, the expression of K_{ATP} channels in mesDA neurons. K_{ATP} channels consist of Sur1 or 2 and Kir6.1 or 6.2 subunits and link the metabolic state of a cell to its membrane potential. The hypothesis was, that misregulation or impairment of these channels may lead to an increased electrical activity of the mesDA cells, putting them under heightened physiological stress which then may in turn cause cell death. Analysis of both Sur1 and Kir6.2 mouse mutants showed no change in TH+ cell number, cell density or density of axonal projections, thus a loss of functional K_{ATP} channels has no effect on the survival of mesDA neurons. After Liss and colleagues found a decreased cell loss when Kir6.2 mutant mesDA cells suffer a toxin insult, I revised my hypothesis and postulated that the normal open-closed state of K_{ATP} channels influences cellular survival when the cell is under oxidative stress. In this work, I show that blocking K_{ATP} channels in vitro by pharmaceutical means has a positive effect on cell survival when mesDA are under oxidative stress. Conversely, forced activation of K_{ATP} channels under these conditions leads to an increased rate of cell death. Furthermore, abolishment of K_{ATP} channel expression in En1-/+:En2-/- mice leads to a complete rescue of the mesDA cell of the SNpc. This highlights the importance of K_{ATP} channel function and may give a new direction in the development of drugs targeting Parkinsons disease.

Zusammenfassung

Die dopaminergen Neurone des ventralen Mittelhirn (mesDA) sind an der Kontrolle von Emotionen, Belohnungserwartung und Motorfunktionen beteiligt. In Patienten mit der neurodegenerativen Krankheit Morbus Parkinson sterben die dopaminergen Neurone der Substantia Nigra, pars compacta, ab. Es ist unklar, warum die SNpc Neurone gegenüber zellulärem Stress anfälliger sind als andere neuronale Zellgruppen. In engrailed Maus Mutanten, verursacht die Abwesenheit der Homeobox-Transkriptionsfaktoren engrailed-1 und engrailed-2 einen progressiven Verlust der mesDA Neurone. Kürzlich identifizierte unser Labor zahlreiche Gene, die zwischen engrailed Mutante und Wildtyp differentiell exprimiert werden. Die Analyse des Gens Foxa1, eines "forkhead-containing"-Transkriptionsfaktors, zeigte aber keinen Phänotyp im Bezug auf die mesDA Neurone. Da aber bekannt ist, dass die Mitglieder der Fox-Familie füreinander kompensieren können, fokussierten wir unser Interesse auf eines der down-stream Ziele der Fox Gene, die Regulation der Expression von K_{ATP} Kanälen in mesDA Neuronen. K_{ATP} Kanäle bestehen aus Sur1 oder 2 und Kir6.1 oder 6.2 Untereinheiten und stellen einen intrazellulären Energiesensor der Zelle dar. Unsere Hypothese war, dass eine Beeinträchtigung dieser Kanäle zu einer erhöhten elektrischen Aktivität der mesDA Neurone führt, die diese unter erhöhten physiologischen Stress setzt, der ultimativ den Tod der Zelle verursacht. Die Analyse von sowohl Sur1 als auch Kir6.2 Mausmutanten zeigte aber keine Veränderungen im Bezug auf das mesDA System, also hat ein Verlust von funktionalen K_{ATP} Kanälen keine Auswirkungen auf das Überleben der Neurone. Nachdem Liss und Kollegen in Kir6.2 Mutanten einen reduzierten Zelltod feststellten, wenn diese Tiere mit einem Toxin behandelt wurden, änderte ich meine Hypothese dahingehend, dass der normale Status der K_{ATP} Kanäle von Offen und Geschlossen das Überleben der Zelle beeinflusst, wenn diese unter physiologischem Stress steht. In dieser Arbeit zeige ich, dass die Blockade von K_{ATP} Kanälen einen positiven Effekt und die Öffnung einen negativen Effekt auf mesDA Neurone hat, wenn diese unter oxidativem Stress stehen. Des weiteren zeige ich, dass ein Fehlen von K_{ATP} Kanalexpression in En1-/+:En2-/- Mäusen zu der Wiederherstellung des mesDA Wildtyp-Phänotyps führt. Dies ist ein Hinweis für die Bedeutung der K_{ATP} Kanalfunktion und kann zu neuen Ansätzen in der Entwicklung von Parkinson-Medikamenten führen.

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Introduction

1 Introduction

The introduction consists of following topics:

- a short overview to describe the line of reasoning, the engrailed phenotype, the Fox genes and K_{ATP} channels
- important genes and how they influence mesDA development
- the mature mesDA system and its relation to Parkinsons Disease (PD)
- genetic and environmental risk factors of PD
- toxins as PD model systems
- evidence pointing to a role of K_{ATP} channels in mesDA neurons
- K_{ATP} channel structure and function
- goals of this work

1.1 Overview

The dopaminergic (DA) neurons of the ventral midbrain constitute several distinct sub-populations which are involved in emotional control, reward behavior and motor control (Fasano and Brambilla, 2002; Kawagoe *et al.*, 2004; Kitai *et al.*, 1999; Spanagel and Weiss, 1999). In patients with Parkinsons Disease (PD), cells of one of these groups, the DA neurons in the substantia nigra, pars compacta (SNpc) gradually die. The main innervation target of these cells, the striatum, is depleted of dopamine, leading to resting tremor, bradykinesia, postural instability and rigidity of muscles as the classical symptoms (Duvoisin, 1992; Fahn, 2003).

Investigation of familial cases of PD led to the discovery of several mutated genes, implicating a deficiency in protein degradation, impairment of safe-guards against exitotoxicity and oxidative stress. Additionally, several mutations in mouse and a number of toxins all lead to a more or less distinct degradation of the nigral DA neurons and leave other dopaminergic populations unaffected. Whereas some of these events specifically target mesDA neurons (either because the mutated gene is expressed solely in these neurons or the toxin is only taken up by them) the cause of the death in other cases is less clear and points to a specific vulnerability of the nigral DA neurons to cellular stressors.

1.1.1 The engrailed phenotype

In engrailed mutant mice, the absence of the homebox transcription factors engrailed 1 and engrailed 2 (En1 and 2) causes a cell-autonomous and gene-dose dependent loss of mesDA neurons. The two proteins can compensate for each other, so that both En1-/- and En2-/- mutations alone do not affect the mesDA neurons, aside from cell density in the case of En1 (Simon *et al.*, 2001). In the En2-/- background, however, a single deletion of En1 (hereafter referred to as En^{HT}) causes a progressive loss of SNpc neurons starting around postnatal day 15 (P15) and peaking at P90 (Sgado *et al.*, 2006). Mice with a double homozygous deletion of both isoforms lose the complete set of mesDA neurons between embryonic day 12 (E12) and E14. The cells do not undergo a change in cell fate (Simon *et al.*, 2001). The progressive loss in the En^{HT} mice mirrors the loss of SNpc neurons in PD patients, where those are the most affected while the neuronal population of the neighbouring ventral tegmental area is not or only slightly impaired. Recently, our lab performed a differential display analysis, in which ventral mesencephalic tissue from three sources was used to search for genes which are specific to mesencephalic neurons and/or under the control of the engrailed genes (Thuret *et al.*, 2004).

1.1.2 Possible roles of Foxa1 and Foxa2

Among the differentially expressed genes identified, the fork head containing transcription factor 1, Foxa1 (formerly HNF3α) (Costa *et al.*, 1989; Lai *et al.*, 1990), was of particular interest, as it is expressed in mesDA neurons from E9 onwards. However, analysis of the mouse Foxa1 mutant (Kaestner *et al.*, 1999) showed no change in the level of expression of the gold-marker for dopaminergic neurons, the rate-limiting enzyme of dopamine synthesis, tyrosine hydroxylase (TH), as well as the expression of several other genes known to be expressed by mesDA neurons. As it is well known that members of the Fox/HNF family of genes can compensate for each other (Lee *et al.*, 2005; Sund *et al.*, 2000; Wan *et al.*, 2005), I focused my research on two downstream genes of the Fox family, the K_{ATP} channel forming proteins Sur1 and Kir6.2.

1.1.3 K_{ATP} channels in systems of cellular stress

K_{ATP} channels in neurons act as control elements by coupling the metabolic state of the cells to their membrane potential, therefore I thought it possible that misregulation or impairment of these channels may lead to an increased electrical activity of the mesDA cells, putting them under heightened physiological stress that then may in turn cause cell death when other factors are added (Liss *et al.*, 2005). K_{ATP} channels are octaheteromeres of 4 sulfonyl receptor (SUR) sub units and 4 inwardly rectifying potassium channel elements (KIR6.x). In a given channel, both SUR and KIR6.x sub units are of the same type and this combination is specific to a certain type of cell. In mesDA neurons, only Sur1, Sur2 and Kir6.2 isoforms are expressed (Liss *et al.*, 1999a).

Our analysis of both Sur1 and Kir6.2 mutant mice showed no change in TH+ cell number, cell density or density of axonal projections, thus a loss of functional K_{ATP} channels has no effect on the survival of mesDA neurons. When Liss and colleagues found that a decreased cell loss occurs when Kir6.2 mutant mesDA cells suffer a toxin insult (Liss *et al.*, 2005), I revised my hypothesis: Not a loss of function of K_{ATP} channels is causing mesDA cell loss, but the normal opening may be a contributing factor.

In order to adress this hypothesis, I investigated the effect of open and closed K_{ATP} channels on the cell survival of mesDA neurons. I used both pharmacological reagents in vivo and vitro and homozygous recombinant mutant mice deficient of the channels. Furthermore, I studied the effect of a non-functional K_{ATP}-channel on the En^{HT} mice, which normally show a progressive mesDA cell loss.

1.2 The Development of Midbrain Dopaminergic Neurons

1.2.1 Early Development

MesDA neurons are some of the first neuronal populations in the mammalian central nervous system (CNS) which become postmitotic (Altman and Bayer, 1981). The region in which these cells are generated is laid down after the fore-, mid- and hindbrain has been specified (Joyner, 1996). Their progenitors receive positional information from several organizing centers during their development. In mammals, two of these centers, the anterior visceral endoderm (AVE) and the node, are both induced by extraembryonic tissue. In mice, this happens around E7-E8.

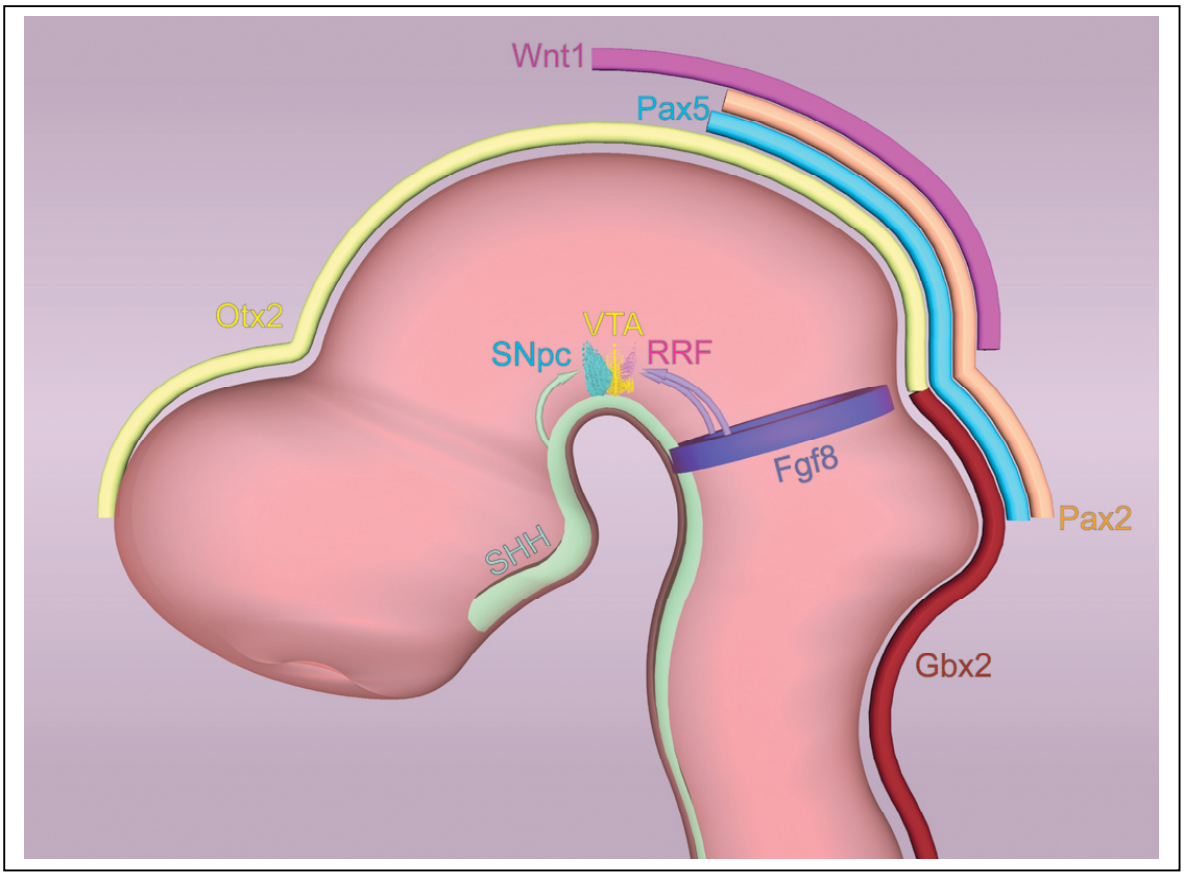


Figure 1.1: Gene expression domains in the developing midbrain
Overview of genes which are important for the development of mesDA neurons. These genes provide positional information and change the fate of progenitor cells to enter a mesDA developmental pathway (Alavian *et al.*, 2007).

For the AVE, signals from the extraembryonic ectoderm induce Nodal expression in the underlying cells of the epiblast which leads to the development of this centre (Brennan *et al.*, 2001). The trophoblast induces node formation in the epiblast via the

expression of Arkadia (Episkopou *et al.*, 2001). The AVE then in turn induces head formation and the first factors sub-dividing the brain start to be expressed, among them the drosophila orthodenticle homologue 2, Otx2.

1.2.2 Regionalisation of the Brain

1.2.2.1 Otx2 and Gbx2

The expression of Otx2 and the gastrulation brain homeobox gene Gbx2 defines the position of another of the organizing centres in the brain, the mid/hindbrain boundary (MHB), also called isthmus or mid/hindbrain organizer (MHO). By repressing each other as well as defining/limiting the expression of genes along the rostro-caudal axis of the brain they form two areas bordering on each other. At this border, the MHB is formed.

Before gastrulation, Otx2 starts to be expressed in the epiblast and anterior visceral endoderm (Matsuo *et al.*, 1995; Simeone *et al.*, 2002). Later in development, the expression domain of Otx2 narrows to a region comprising the fore- and midbrain regions (Broccoli *et al.*, 1999). In the Otx2 mutant mice, the forebrain and midbrain regions are deleted (Acampora *et al.*, 1995), whereas the expression domain of Otx2 is enlarged caudally in Gbx2 mutant mice (Wassarman *et al.*, 1997) (Millet *et al.*, 1999). Similarly, Gbx2 expression, initially present in all three germ layers from the posterior embryo up to the hindbrain, is limited to the anterior hindbrain by E8.5 (Wassarman *et al.*, 1997).

Mice ectopically expressing Gbx2 in the midbrain lose all mesDA neurons normally generated in that region by a shift of the hindbrain rostrally (Millet *et al.*, 1999). In a similar experiment, ectopic expression of Otx2 in the rostral hindbrain expands the area where mesDA neurons are forming (Puelles *et al.*, 2004).

In Otx2/Gbx2 double mutant mice, the expression of MHB genes like the fibroblast growth factor 8 (Fgf8) is unchanged, although they have been shifted to a more anterior position, suggesting that the expression of these genes is necessary for proper placement of the MHB but not for the initiation of expression of genes (Li and Joyner, 2001).

1.2.2.2 Pax2, Pax5, Pax6

The position of the MHB is further maintained by the paired box genes, Pax2, Pax5 and Pax6. Pax2 is expressed from E7.5 on in the anterior mouse embryo before neural tube closure. Later, its expression in the CNS is restricted to the neural plate of the prospective midbrain/hindbrain (Rowitch and McMahon, 1995).

Pax5 is expressed later (E9.5/3-somite stage) and in contrast to Pax2, which is also expressed in the developing eye, ear and urogenital tract (Bouchard *et al.*, 2000), it is specific to the developing brain (Rowitch and McMahon, 1995) (Asano and Gruss, 1992). Both Pax2 and Pax5 are expressed in the mes/metencephalon (Mastick *et al.*, 1997).

In Pax2/5 double mutant mice, the posterior midbrain and the cerebellum are lost (Figure 1.1). However, one wild type Pax2 allele is necessary and sufficient to rescue the mouse to a normal patterning of midbrain and cerebellum (although there are still severe malformations in other parts of the body) (Urbanek *et al.*, 1997).

Pax2 expression is unchanged in Pax5 mutants and vice versa, suggesting that both genes do not regulate each other (Schwarz *et al.*, 1997). However, Pax2 and Pax5 are structurally so similar that cloning of the Pax5 gene into the Pax2 locus completely rescues the embryo from the Pax2 -/- phenotype in respect to cerebellum and midbrain effects, as well as in the developing eye, ear and urogenital system, where Pax5 is not expressed (Bouchard *et al.*, 2000).

Pax6, on the other hand, is expressed in the forebrain and hindbrain vesicles (Walther and Gruss, 1991). The caudal limit of Pax6 expression in the forebrain marks also the posterior commissure separating fore- from midbrain. In mice lacking Pax6, the pre-tectum normally developing at the posterior commissure is lost and the boundary between fore- and midbrain is shifted rostrally (Mastick *et al.*, 1997).

In summary, the Pax genes are expressed at an early point in brain development and delineate the major brain regions, reinforcing the MHB, which forms a signaling centre at the rostrocaudal axis and positions the region where mesDA neurons will form.

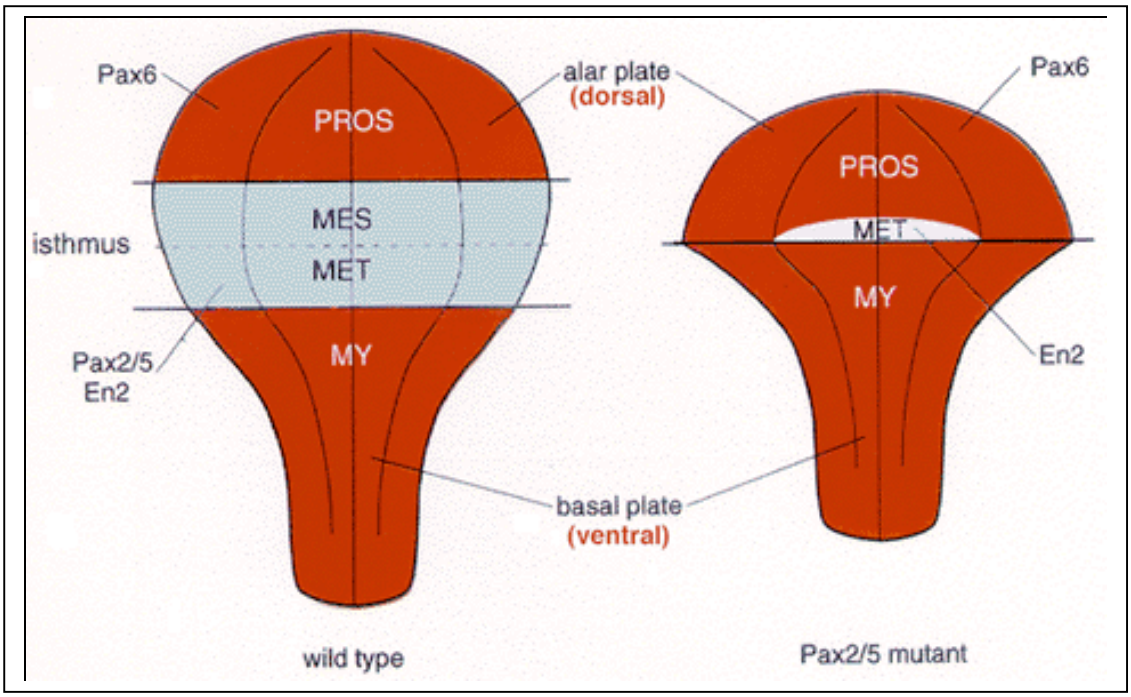


Figure 1.2: Schematic representation of a wild type and Pax2/5 double mutant mouse brain.
The posterior midbrain and the cerebellum, the areas where Pax2 and Pax5 are normally expressed, are severly reduced in the double mutant (Schwarz *et al.*, 1997).

1.2.3 Defining the Ventral Midbrain

The early dorso-ventral axis of the CNS is defined by both a morphological border and by the secretion of diffusible factors. The sulcus limitans provides a physical barrier between the ventral floor and basal plates and the dorsal alar and roof plates. The diffusible factors, for example the bone morphogenic proteins (BMPs) (De Robertis and Kuroda, 2004; Rhinn *et al.*, 2006) are secreted by the roof plate in a dorsal to ventral gradient, whereas sonic hedgehog (Shh), a drosophila hedgehog homologue, is secreted from floor plate and notochord (Ye *et al.*, 1998). Shh and the fibroblast growth factor 8 (Fgf8) act together to induce mesDA development. While Shh provides a ventral to dorsal gradient, the Fgf8 is secreted along the rostral-caudal axis of the developing embryo. Together with Fgf4, they provide responsive cells with positional information for the induction of dopaminergic and serotonergic cell fates (Ye *et al.*, 1998). Fgf8 is part of group of genes expressed at or around the MHB which form a feedback loop of expression. This loop maintains gene expression and provides a reinforcement of positional identity along the rostro-caudal axis.

1.2.3.1 Fgf8 and Shh

The narrow expression of Shh at the level of the floor plate of the posterior part of the neural tube (at the level of spinal cord and hindbrain) widens at the MHB to cover almost the entire basal plate of the midbrain and caudal forebrain (Echelard *et al.*, 1993; Ye *et al.*, 1998). Cells bind Shh at Patched receptor complexes and transduce the signal via Smoothened transmembrane proteins and Gli family transcription factors (Fuccillo *et al.*, 2006) (Blaess *et al.*, 2006). While early experiments suggested that Shh is sufficient and necessary for induction of mesDA neurons (Hynes *et al.*, 1995), more recent work provides evidence that Shh functions more like a survival and proliferation factor than a true patterning molecule (in respect to midbrain development), as the gene expression and morphology of the developing midbrain does not change when Shh is ablated (Britto *et al.*, 2002; Ishibashi and McMahon, 2002). Nevertheless, Shh critically maintains expression of Fgf8 (Blaess *et al.*, 2006) at the MHB and of Pax6 (Ericson *et al.*, 1997).

When Fgf8 soaked beads were implanted into the diencephalon (prosomere 2) of chicken embryos, an ectopic En2, Fgf8 and Wnt-1 expression could be detected (Crossley *et al.*, 1996). Implantation into a more caudal part of the diencephalon (prosomere 1) or directly into the midbrain led to the same result, but signal spread across the prosomere boundaries was restricted (Martinez *et al.*, 1999). The 5' promotor region of Fgf8 has intronic binding sites for both the engrailed and Pbx1 proteins (Gemel *et al.*, 1999). Fgf8 is also able to induce Pax2 and Pax5, Wnt1, and Gbx2 and to repress Otx2 (Liu and Joyner, 2001b; Liu *et al.*, 1999). Ectopic expression of either En1/2, Pax2 and 5 and Fgf8 in the diencephalon leads to the expression of the other genes from that group and pushes the diencephalic cells into a tectal path of development (Araki and Nakamura, 1999; Funahashi *et al.*, 1999; Okafuji *et al.*, 1999). This demonstrates the ability of Fgf8 to establish a feedback loop which strengthens the MHB in the wild type.

Two other members of the fibroblast growth factor family, Fgf17 and Fgf18 are also expressed at the isthmus and are maintained by Fgf8 (Liu *et al.*, 2003; Maruoka *et al.*, 1998; Xu *et al.*, 1999). When beads containing Fgf8 where implanted into embryos, an ectopic expression of both Fgf17 and Fgf18 was observed (Liu *et al*., 2003). In mesencephalon/rhombomere-1-specific conditional Fgf8 knock-out mice, expression of both genes is initiated but lost rapidly (Chi *et al*., 2003). Mis-expression of

Fgf17/18 in chicken expands the midbrain and leads to the expression of genes similar to those seen in a similar experiment with Fgf8a (Liu *et al.*, 2003).

1.2.3.2 Wnt1, Wnt3a and Wnt5a

Wnt1 and Wnt5a (and to a lesser extend Wnt3a), homologues of the drosophila wingless segmentation gene, are expressed in the developing midbrain of the mouse from around E8.5. Wnt1 is also expressed in the dorsal roof plate. Wnt1 expression is located anterior of the MHB and in two stripes on both sides of the medial floor plate within the cephalic flexure (Bally-Cuif *et al.*, 1995). Mice lacking Wnt1 expression are completely lacking the midbrain, cerebellum and the anterior hindbrain, as well as the initially present expression of other genes important for mesDA development like En1, En2 and Fgf8 (McMahon and Bradley, 1990) (McMahon *et al.*, 1992). Wnt1 regulates the expression of homeobox transcription factor engrailed 1 (En1) (Bally-Cuif *et al.*, 1995). Expressing En1 under control of the Wnt1 enhancer in Wnt1-/- mice leads to an almost complete rescue of the Wnt1 phenotype (Danielian and McMahon, 1996). In vitro, Wnt1 predominantly increases the proliferation of Nurr1 positive precursors and Wnt-5a increases the fraction of these Nurr1 positive cells expressing Pitx-3 and c-Ret. This suggests that both Wnt1 and Wnt5a, which is specific to the ventral midbrain, are necessary for proper proliferation and differentiation of mesDA neuron precursors (Castelo-Branco *et al.*, 2003). A newer report by the same group suggests that Wnt function is mediated through secretion by local glia cells which maintain their regional identity by expressing Pax2, En1 and Otx2 (Castelo-Branco *et al.*, 2006) and, on the molecular level, by activating the canonical (Wnt3a) and non-canonical (Wnt5a) pathways of Wnt-signaling (Schulte *et al.*, 2005).

1.2.3.3 Engrailed 1 and engrailed 2 (early function)

The homeobox transcription factors engrailed 1 and 2 are expressed around the forming MHB from the 1-somite stage (En1) and 3-5-somite stage (En2) onwards (Joyner *et al.*, 1985) (Davis and Joyner, 1988; Joyner and Martin, 1987). Despite a low sequence homology of 30% outside the homeobox, En2 cloned into the En1 locus is able to compensate for En1 and rescue the otherwise lethal En1-/- mouse mutant (Hanks *et al.*, 1995). In En1/2 double knock-out mice, large portions of mid- and hindbrain are deleted, as well as the cerebellum. These reductions are already

noticeable in En1-/- mice which also have cerebellar defects (Wurst *et al.*, 1994) but not in En2-/- mice, where only a slightly erroneous cerebellar foliation can be observed (Joyner *et al.*, 1991; Millen *et al.*, 1994). The large deletion in En1/2 mutants is similar to the phenotype of the Wnt1 mutation (McMahon and Bradley, 1990; McMahon *et al.*, 1992; Thomas and Capecchi, 1990). While the early induction of Fgf8 at the isthmus is independent of engrailed, the later maintenance is under the regulatory control of En1 and 2 (Liu and Joyner, 2001a). Additional data comes from experiments done in chicken. Ectopic expression of high levels of En1 via a retrovirus leads to expression of Fgf8 in ectopic positions in the mesen- and diencephalons (Shamim *et al.*, 1999). Injection of En2 into 1-2 cell stage embryos of Oryzas or Xenopus also lead to expression of Fgf8 as well as Pax2, Wnt1 and the gene itself (Ristoratore *et al.*, 1999).

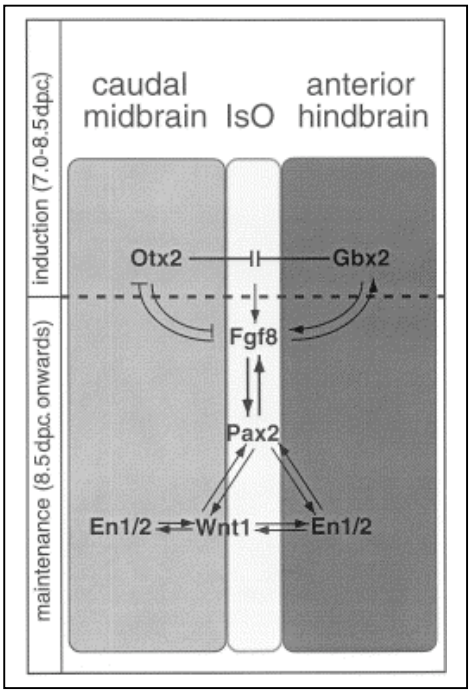


Figure 1.3: Establishment and maintenance of the MHB in the mouse.
The mutual repression of Gbx2 and Otx2 at 7-8.5 dpc establishes the MHB, Fgf8 expression maintains it and is part of a feedback loop of Fgf8, En1, En2 and Wnt1 from 8.5 dpc onwards (Acampora *et al*., 2001).

1.2.3.4 Tgfβ

Another gene of interest, the transforming growth factor beta (Tgfβ), is also expressed in the midbrain floor plate from E3 onwards in chicken (Unsicker *et al.*, 1996). It is not sufficient for the early induction of the mesDA neurons. Neutralizing either Shh in the presence of Tgfβ or Tgfβ in the presence of Shh impairs the development of mesDA neurons in E12 rat embryonic ventral midbrain cells but does not affect the noradrenergic neurons of the locus coeruleus or the dopaminergic neurons of the diencephalon (Farkas *et al.*, 2003). Incubation of chicken embryos with a Tgfβ

antibody before the mesDA neurons have been generated leads to a 60% cell loss five days later. There is always a loss of mesDA neurons, no matter when the antibody is added to the chicken embryos. This also happens, even if the cells have finished maturation, suggesting that Tgfβ not only acts as an inducing factor in generation of these neurons but also as an survival factor later in development and adulthood (Roussa and Krieglstein, 2004).

1.2.4 Differentiation of the mesDA precursors

After the establishment of the MHB and the induction of mesDA development, the precursor cells start to express a number of genes specific for dopaminergic neurons (like the rate-limiting enzyme of dopamine synthesis, tyrosine hydroxylase (TH)). Some of these genes are furthermore only expressed in the DA cells of the ventral midbrain and not found in other dopaminergic populations in the brain, making them targets for investigation in order to understand the distinct vulnerability of the dopaminergic neurons of the nigra.

1.2.4.1 Lmx1a and Lmx1b

Among the genes necessary for proper mesDA differentiation are two structurally related proteins, Lmx1a and Lmx1b. They encode LIM homeodomain-containing transcription factors. Despite having roles in early development, both are still expressed after E9.5. Lmx1a is an early activator of differentiation, e.g. maintaining expression of *Msx1*, both of which then activate Ngn2 expression (Kruger et al., 2002; Millonig et al., 2000). Ngn2 is necessary for an early shift of progenitor cells from glial to pro-neural fate (Bertrand *et al.*, 2002), while Msx1 expression prevents mesDA progenitor cells from entering alternative cell fates by repressing other genes like Nkx6.1, which is required for the generation of motor neurons in the spinal cord (Vallstedt *et al.*, 2001). Lmx1a is also necessary and sufficient to induce ectopic mesDA neurons in chicken embryos; these neurons express Nurr1, En1, Pitx3, Lmx1b and DAT like wild-type mesDA neurons. When Lmx1a expression is knocked down with RNAi, a reduction of mesDA cell number can be seen (Andersson *et al.*, 2006).
At E7.5, Lmx1b is widely expressed in the caudal forebrain, midbrain, and hindbrain but eventually its expression becomes restricted to the SNpc and VTA (Burbach *et al.*,

2003). Within the cephalic flexure, mesDA neurons of Lmx1b-/- mutants show a lack of Pitx3 expression but not of TH or Nurr1 (Burbach *et al.*, 2003). At E12.5, neurons are present which express Pitx3 but not TH or vice versa, suggesting that these neurons have not matured properly. Lmx1b is also required for the induction of Wnt1 expression in the chicken mid/hindbrain boundary region as well as being maintained by Fgf8 (Adams *et al.*, 2000). It is possible that the effect of Lmx1b on Pitx3 is in fact via its control over Wnt1 expression (Riddle and Pollock, 2003; Smidt *et al.*, 2000).

1.2.4.2 Engrailed 1 and engrailed 2 (late function)

The two homeobox transcription factors En1 and En2 are not only important for the early establishment and maintenance of the MHB, they start to be expressed on both sides of the isthmus around E11.5 and E14 in mice and are expressed in mesDA neurons during adulthood (Joyner *et al.*, 1985) (Davis and Joyner, 1988; Joyner and Martin, 1987).

Via cell-mixing and RNAi experiments as well as through the generation of chimeric mice, our lab was able to show that engrailed is necessary for the survival of mesDA neurons in a cell-autonomous manner (Alberi *et al.*, 2004) and that the neurons are not lost because the surrounding cells lack this gene. The engrailed-deficient mesDA neurons start to disappear at exactly the time when engrailed starts to be expressed. There is also a gene-dose dependency of engrailed by the mesDA neurons, as the loss of these cells becomes more pronounced, the more alleles of the two engrailed genes are deleted. For instance, mice with an En1-/+:En2-/- mutation initially have a normal number and distribution of mesDA neurons, however, as time progresses, these cells are lost, starting at P15 and peaking at 3 months of age with an 80% loss (Sgado *et al.*, 2006). In the full double mutant, the cells are completly lost. While this loss can be observed as early as E9, the mesDA neurons are first generated and even start to express their transmitter phenotype, but die between E12 and E14 (Simon *et al.*, 2001).

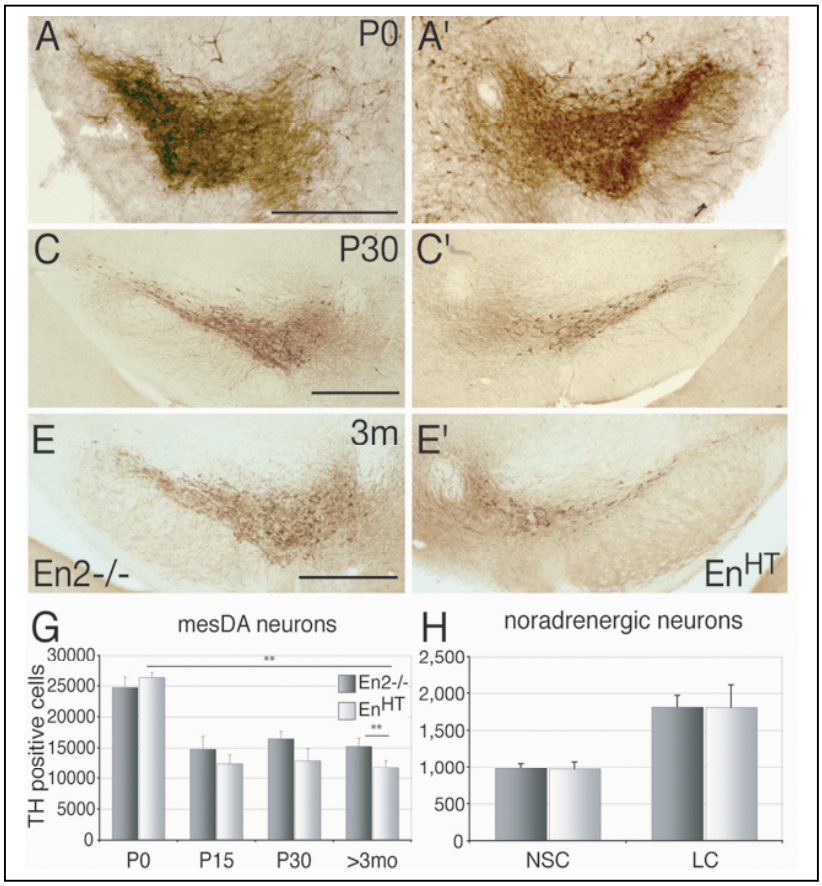
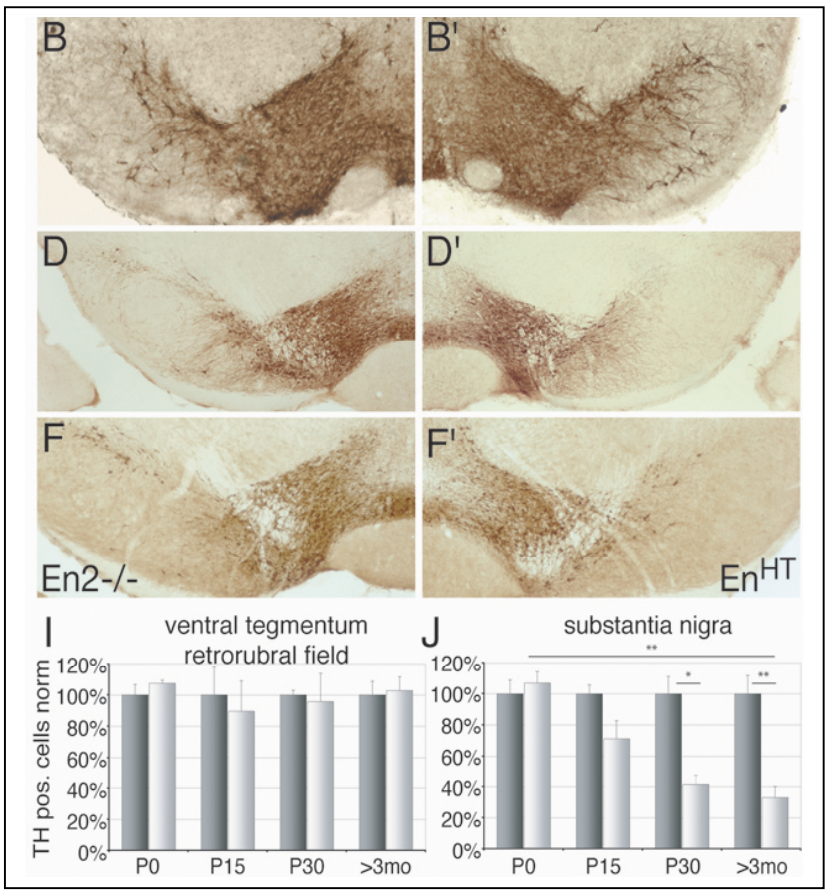


Figure 1.4: The En^{HT} (En1-/+:En2-/-) midbrain phenotype.
(picture divided in two parts)
Dopaminergic neurons of the substantia nigra are progressively lost (compare wild type A, C, E with A', C', E': cell count in J on the next page). The dopaminergic neurons in the neighbouring VTA and retrorubal field are not affected, nor are noradrenergic neurons in the locus coruleus and locus subcoruleus (NSC).



The En^{HT} (En1-/+:En2-/-) midbrain phenotype. Dopaminergic neurons of the substantia nigra are progressively lost (compare wild type A, C, E with A', C', E': cell count in J). The dopaminergic neurons in the neighbouring VTA and retrorubal field are not affected, nor are noradrenergic neurons in the locus coruleus and locus subcoruleus (NSC). Adapted from (Sgado *et al.*, 2006).

1.2.4.3 Pitx3

The paired-like homeodomain transcription factor 3 (Pitx3) is, with the exception of the eye, only expressed in the mesDA neurons of the ventral midbrain. The expression starts between E11.5 and E12.5 (Smidt *et al.*, 1997). In *aphakia* mice, which are Pitx3-/-, the neurons of the SNpc but not of the VTA are lost. It has been speculated that this might be due to differences in the molecular setup between the neuronal populations of the SNpc and VTA areas (Nunes *et al.*, 2003). The mesDA loss lowers the level of dopamine in the striatum by ca. –90% (Smidt *et al.*, 2004), the mice, however, fail to develop strong motor deficits. A lower motor activity can be observed which is treatable with L-DOPA, the same medication Parkinson patients receive. On the cellular level, the mesDA neurons in *aphakia* mice fail to migrate early in development. However, it is likely that Pitx3 has other functions in the terminal differentiation and/or early maintenance of these cells, as they have already finished proliferation by the time Pitx3 starts to be expressed (Smidt *et al.*, 2004).

Pitx3, as well as Nurr1, controls the expression of TH in nigral DA neurons by activating the promotor through a high affinity binding site (Lebel *et al.*, 2001; Maxwell and Li, 2005). In a number of in vitro experiments in which cells were artificially differentiated, Pitx3 was shown to induce the expression of mesDA specific genes (Sasai, 2002) (Zigova *et al.*, 2000).

1.2.4.4 Nurr-1

Nurr-1 (also known as Nr4a2, NOT, RNR-1 and HZF3) is a nuclear receptor belonging to a conserved superfamily of ligand-activated transcription factors (Aranda and Pascual, 2001; Giguere, 1999). Its called an orphan receptor because its ligand binding site is blocked by hydrophobic amino acid side chains (Volakakis *et al.*, 2006). It may, however, bind to RXR nuclear receptors and these heterodimers may in turn be activated by cognate ligands like docosaheaenoic acid (Wang *et al.*, 2003).

Nurr1 starts to be expressed in the ventral midbrain, as well as in other DA nuclei of fore- and hindbrain, at around E10.5 in mice, the time when mesDA neurons start to express TH and leave the cell cycle (Zetterstrom *et al.*, 1996). In Nurr1 mutant mice, the cells are generated and can be detected by the expression of mesDA specific genes like Pitx3 and engrailed but they are lost soon after (Perlmann and Wallen-Mackenzie, 2004). The cells fail to express vesicle monoamine transporter 2

(VMAT2), dopamine transporter (DAT) and TH (Castillo *et al.*, 1998) (Smits *et al.*, 2003). Not surprisingly, Nurr1 is able to bind directly to the TH promotor (Kim *et al.*, 2003) and a similar binding sequence was found in the DAT promotor (Sacchetti *et al.*, 2001; Sakurada *et al.*, 1999).

TH expression is not impaired in the DA nuclei of the olfactory bulb, diencephalons, as well as in the hindbrain and brain stem, where Nurr1 is not expressed. There is conflicting evidence if the normal target innervation of mesDA neurons in Nurr1 mutants is present or not ((Wallen *et al.*, 1999) and (Zetterström *et al.*, 1997) vs. (Witta *et al.*, 2000)). In Nurr1 mutant mice, the tyrosine kinase Ret, which is an important co-receptor for tropic factors like GDNF, is not expressed either (Baloh *et al.*, 1997; Robertson and Mason, 1997) (Wallen *et al.*, 2001). GDNF is one of the pivotal tropic factors required for the postnatal survival of mesDA neurons, as well as being protective against neurotoxin induced cell death (Burke *et al.*, 1998; Clarkson *et al.*, 1995; Tomac *et al.*, 1995).

Given its relatively late expression in mesDA neurons, Nurr1 has been considered a purely post-mitotic survival factor which is also important for terminal differentiation. In neuronal stem cell culture, Nurr1 together with Pitx can induce terminal maturation of ES cell cultures into midbrain dopaminergic phenotype (Martinat *et al.*, 2006).

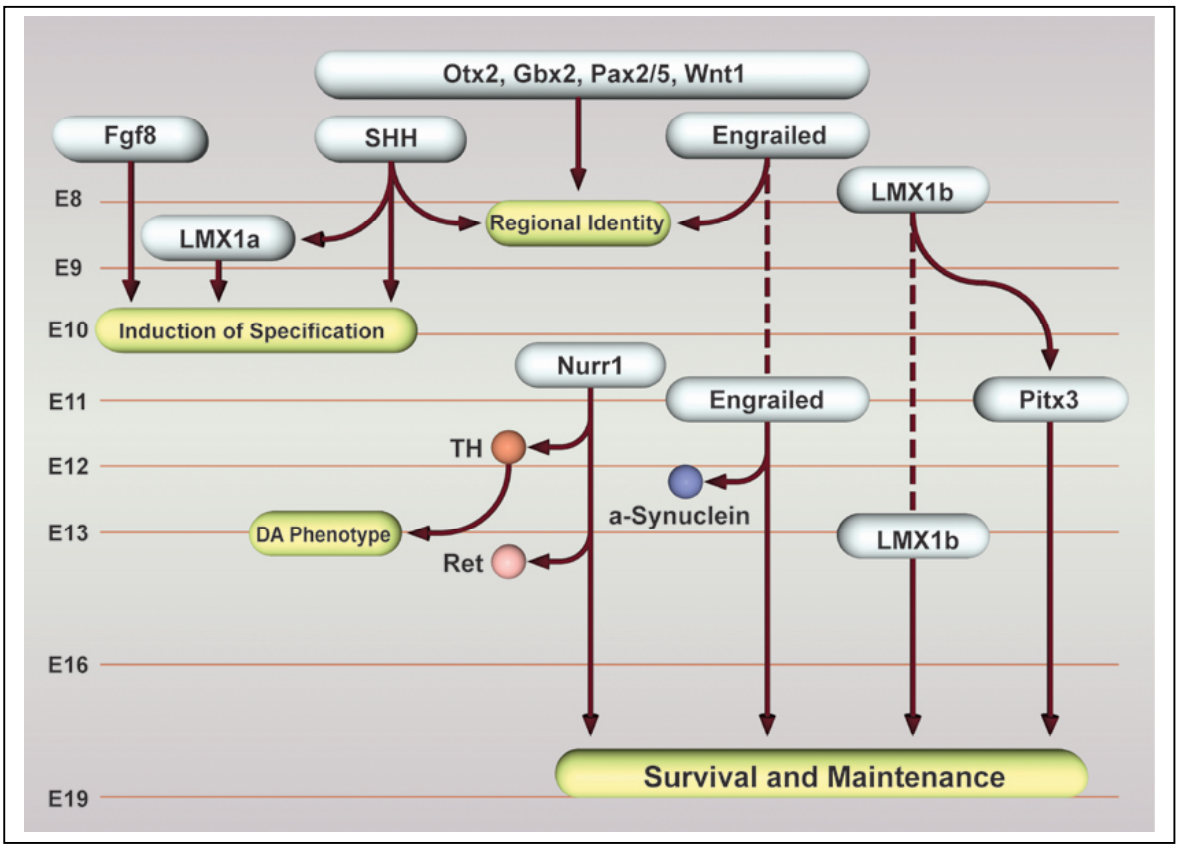


Figure 1.5: Timeline of gene expression and their function in mesDA neurons.
This picture shows the chronological order of genes important for mesDA neuron development. Dashed lines represent expression in the region and not necessarily in the mesDA neurons (Alavian *et al.*, 2007).

1.3 The mature mesDA system

The mesDA neurons of the adult human are divided into three subpopulations which form distinct pathways and innervate different target areas in the brain. The neurons of the retrorubral field (RRF, also called A8), the ventral tegmental area (VTA, also called A10) which forms the meso-limbic and meso-cortical pathways, innervating areas in the neocortex and the ventral striatum (Bayer *et al*., 1995; German *et al*., 1989). Functionally, these neurons are linked to emotion and reward and involved in schizophrenia and drug addiction (Tzschentke and Schmidt, 2000). The last subpopulation, the neurons of the substantia nigra pars compacta (SNpc, also called A9), targets the dorsal striatum. Proper function of this population is necessary for the induction and coordination of movement (Kish *et al*., 1988).

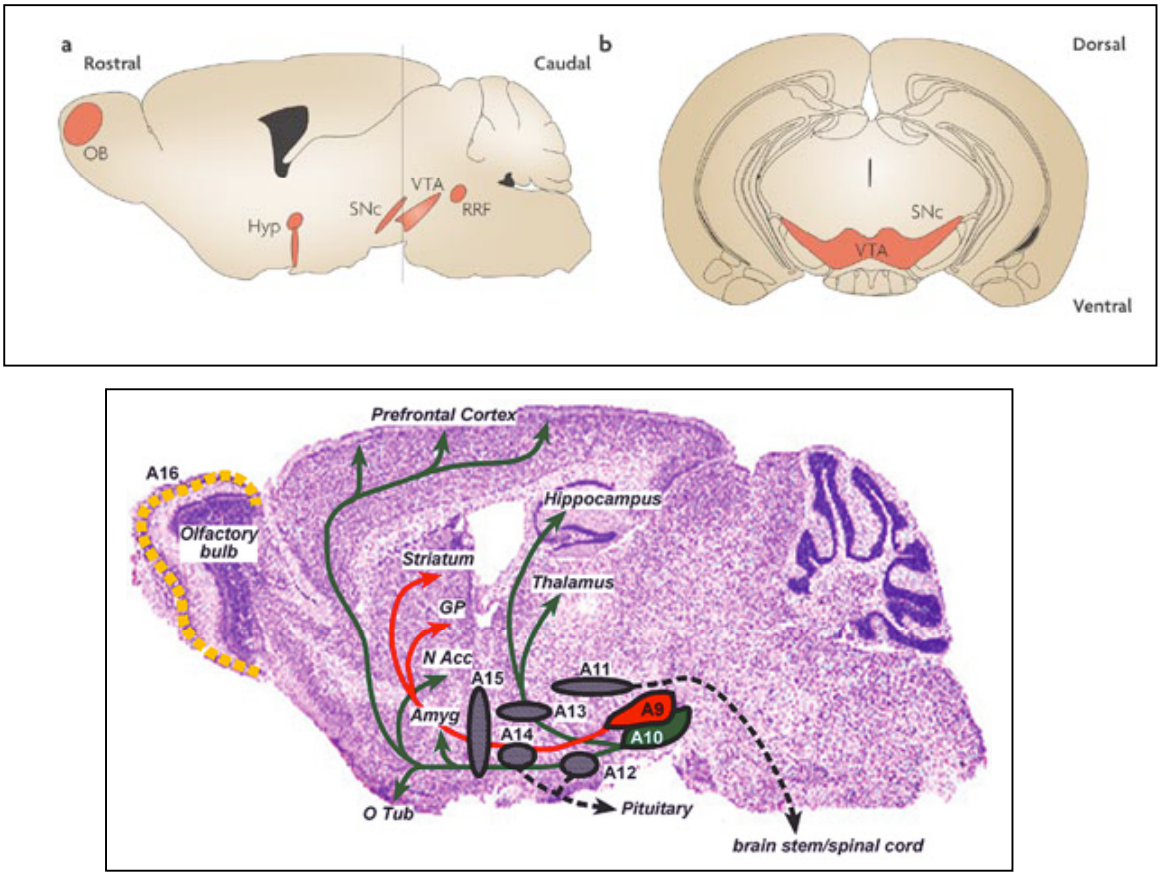


Figure 1.6: The dopaminergic nuclei of the murine brain and the innervation targets of SNpc and VTA. MesDA neurons coming from the SNpc innervate the dorsal striatum/globus pallidus, VTA neurons innervate predominantly areas in the prefrontal cortex and olfactory tubercle. (a and b adapted from (Smidt and Burbach, 2007), lower picture (Prakash and Wurst, 2006))

1.4 Parkinson's Disease (PD)

In 1817, the english doctor James Parkinson described in his publication "An Essay on the Shaking Palsy" (reprinted in (Parkinson, 2002)) a disorder causing "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace; the senses and intellects being uninjured." This disorder, now known as Parkinson's Disease was subsequently linked to a loss of nigral DA neurons (Tretiakoff, 1919). In order to be diagnosed with Parkinsonism, patients have to show at least two of the six symptoms of resting tremor, flexed posture, the on-and-off inability to move the feet, postural instability, rigidity and bradykinesia. One of the two symptoms has to be either tremor or bradykinesia. Furthermore, to be diagnosed with PD, theses symptoms have to have an asymmetric onset, include resting tremor and show a good response to levodopa (Fahn, 2003; Gibb and Lees, 1988; Hughes *et al.*, 1992). The motor impairments are linked to a 70-80% loss of the mesDA neurons in the substantia nigra (Bernheimer *et al.*, 1973). PD affects roughly 1-3% of the human population over the age of 60-65 (de Rijk *et al.*, 2000), while familiar forms occur rarely but with an onset as early as 30 years age. The prevalence of PD is similar among different ethnic groups, but different between the same ethnicities in different locations (Schoenberg *et al.*, 1988).

The loss of the SNpc neurons is distinct, no other neuronal populations are similarly affected, though degeneration can be detected in other areas (locus coeruleus, raphe nuclei, nucleus basalis of Meynert, cingulate and entorhinal cortex, olfactory bulb and autonomic nervous system) (Schulz and Falkenburger, 2004). Cellular inclusions containing predominantly α-synuclein and ubiquitin (Spillantini *et al*., 1997), the so-called Lewy-bodies, are present throughout the brain (Ohama and Ikuta, 1976) (Braak and Braak, 2000), altough their possible role in the progress of the disease is still unclear (Gibb and Lees, 1988) (Lang and Lozano, 1998) (Fahn, 2003). The DA neurons are lost progressively up to the point where the remaining neurons can not longer compensate for the falling level of dopamine in the striatum and the motor symptoms start to appear (German *et al.*, 1989). The effect on other parts of the brain can be seen by the development of secondary symptoms, e.g. 25 to 40% of PD patients develop dementia as well as depression and panic attacks. The range of secondary symptoms differs from patient to patient and may include fatigue, sleep

disorders, problems with digestion (e.g. constipation, megacolon) and sensory symptoms including pain, numbness, tingling and burning sensations in up to 40% of patients (Fahn, 2003).

Despite biochemical changes and reduced number of axon terminals, the mesDA neurons of aged healthy humans and other primates (De Keyser *et al.*, 1990) (Kubis *et al.*, 2000; McCormack *et al.*, 2004; Rinne *et al.*, 1990) are not reduced in number. PD is therefore not the result of the naturally occuring aging process, although age is a risk factor.

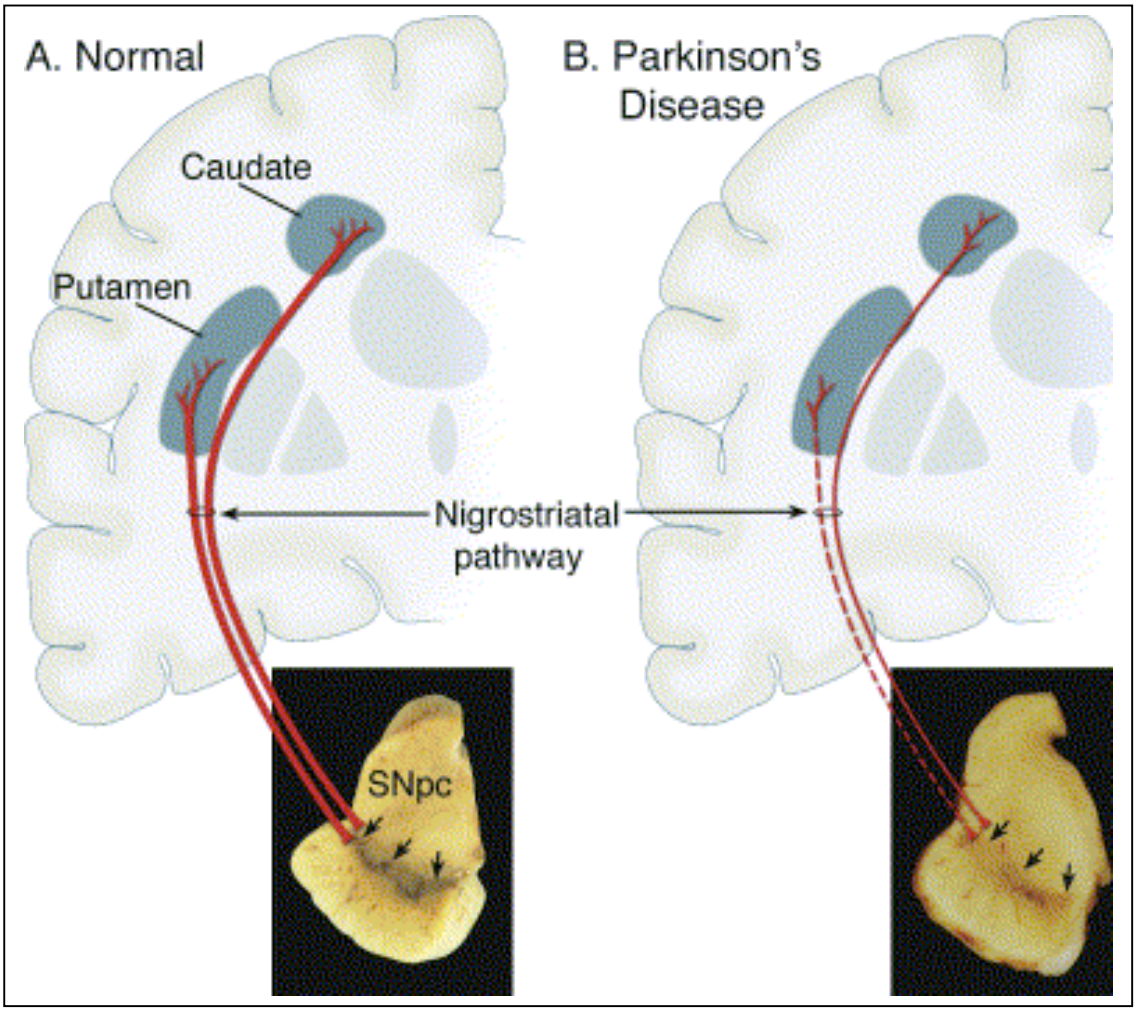


Figure 1.7: Coronal sections of human brains showing SNpc of a healthy and a PD human subject
The graphic depicts normal and affected striatal innervation. Note the reduced color in the nigra of the PD brain. (picture adapted from (Dauer and Przedborski, 2003))

1.4.1 Genetic and environmental risk factors of PD

No singular event causes Parkinsons Disease, nor is there a clear picture which of a various number of factors has to be present for an individual to develop the so-called

sporadic PD. However, the disease is most likely caused by a convergence of genetic pre-disposition and environmental factors affecting a person throughout his or her life. Over the years, screenings for risk factors in the populace of various countries were undertaken.

1-3% of all PD cases develop the symptoms at an earlier age than the majority of patients. Often under 50 years of age, in some cases as young as 20, these PD patients have frequently a familiar history of PD at similar ages. The identification of carrier families and genetic screening lead to the identification of, up to date, thirteen genomic loci, dubbed PARK genes. In seven of these loci the mutated gene could be identified. This offered an insight into the molecular workings of the disease. The known mutations with identified underlying protein can be grouped according to their penetrance, the autosomal recessive mutations of Parkin, PINK1 and DJ1 and the autosomal dominant mutations of alpha synuclein, LRRK2 and UCH-L1. These will be discussed on the next pages.

1.4.1.1 The recessive mutations (Parkin, DJ-1 and Pink1)

The first identified gene was Parkin, a member of the RING family of proteins. It has a ubiquitin-ligase domain at the amino terminus and there is evidence that it acts in a complex with a range of proteins to tag specific proteins for degradation (Staropoli *et al.*, 2003). There is no direct evidence how a mutation in these genes might cause PD; the most common hypothesis speculate that dysfunction of Parkin causes abnormal protein aggregation, leading either to a not clearly defined mass of proteins or to a distinct aggregation of PD-relevant proteins e.g. α-synuclein, one of the proteins found in Lewy bodies. A third hypothesis puts parkin in a cell-survival context, arguing that over-expression of parkin is protective in several cell-apoptosis models, maybe indirect via its various substrates, some of which act in cell survival. However, mice deficient for parkin show little or no alteration in cell numbers (aside from the locus coruleus in one case) and evidence for changes is dependent on the genetic background of the mice. (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Perez and Palmiter, 2005).

DJ-1 belongs to the Thi domain family and is associated with chaperone, protease and other functions. Its chaperone function is activated by oxidative stress and supressed in a reducing environment. When oxidized, this chaperone function appears to have a

wide range of targets, including binding to and preventing the fibrilization of α-synuclein (Shendelman *et al.*, 2004; Zhou *et al.*, 2006).

DJ-1 deficiency makes dopaminergic neurons sensitive to oxidative stress (Shendelman *et al.*, 2004), whereas over-expression is protective against oxidative toxic insults (Canet-Aviles *et al.*, 2004; Martinat *et al.*, 2006; Taira *et al.*, 2004).

Pink1 (PTEN-induced kinase 1, PARK6) is a protein with a serine/threonine kinase domain and a mitchondrial targeting sequence. It normally accumulates in the intermembrane space of mitochondria. All known mutations of this gene in humans lead to a reduced or total loss of function of the protein, either by a reduction of transported protein to the intermembrane space or by an impairment of the kinase activity (Palacino *et al.*, 2004; Periquet *et al.*, 2005; Valente *et al.*, 2004).

The small amount of data on gene function in humans is somewhat offset by research in drosophila. There, inactivation of a Parkin orthologue leads to mitochondrial dysfunction in flight muscle and sperm, causing progressively sterility, an inability to fly and a reduced survival rate. However, since human Parkin cannot compensate for a loss of the orthologue in drosophila, it is still unclear if the proteins are interchangeable.

As with Parkin, the mouse mutant of DJ-1 shows little alteration on the cellular level while there is evidence for increased sensitivity to oxidative stress in drosophila (Menzies *et al.*, 2005; Meulener *et al.*, 2005).

The Pink1 mutation effectively mirrors the Parkin phenotype as the flight muscle function is impaired, as well as the sperm motility. Furthermore, Parkin over expression in drosophila null for Pink1 largely rescues the animals, but not vice versa (Clark *et al.*, 2006; Park *et al.*, 2006). The effect is specific as Parkin over expression is unable to protect against other, e.g. toxin insults, strongly suggesting that, at least in drosophila, Parkin is acting downstream of Pink1. Human Parkin protein has the same protective effect, strengthening the argument that the mechanism is conserved (Clark *et al.*, 2006; Park *et al.*, 2006). Based on this data, a mechanism by which the three genes may interact has been proposed (as shown in Figure 1.8).

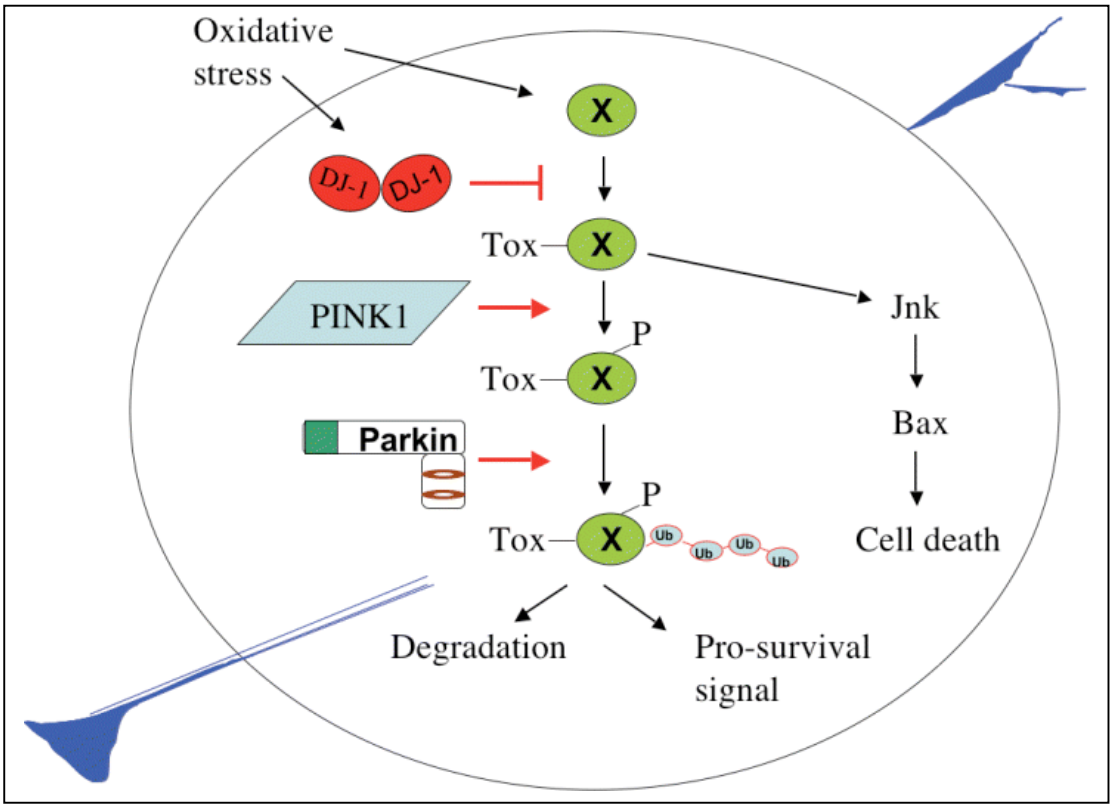


Figure 1.8: Unknown substrate proteins (X) are caused to accumulate in a toxic state (Tox-X) by oxidative stress.
This is thought to activate the JNK/Bax apoptotic pathway which ultimately leads to cell death. DJ-1 may inhibit the formation of Tox-X upstream of Pink1 and Parkin, which are part of a degradation pathway (Abeliovich and Flint Beal, 2006).

1.4.1.2 The dominant mutations (α-synuclein, LRRK2, UCH-L1)

α-Synuclein is the most promiment member of the PARK genes as its mutations are not only linked to PD, but also the protein itself can be isolated from the fibrillary mass of Lewy bodies (same as the more recently identified UCH-L1, see below), providing a link between familiar and idiopathic PD (Polymeropoulos *et al.*, 1997; Spillantini *et al.*, 1997). The neuronal function of α-synuclein is connected to neurotransmitter release; in its absence vesicle trafficking is altered in knock-out mice and in vitro (Abeliovich *et al.*, 2000; Cabin *et al.*, 2002; Fortin *et al.*, 2005). It has also been implicated in defective protein degradation (Tanaka *et al.*, 2001) altered macroautophagy (Stefanis *et al.*, 2001) and defective chaperone-mediated autophagy (Cuervo *et al.*, 2004). Over expression of a-synuclein is cytotoxic, both in naturally occurring human cases as well as in various animal models (Miller *et al.*, 2004; Singleton *et al.*, 2003).

The 286kDa ROCO family kinase LRRK2 was first described by (Funayama *et al.*, 2002) in a Japanese family. The protein has low expression levels in the whole brain,

slightly higher in striatum and parts of the cortex, but is not expressed in the mesDA neurons (Galter *et al.*, 2006)), putamen and lung (Zimprich *et al.*, 2004). To date, 19 mutations of the gene are known (Taylor *et al.*, 2006). The mutations G2019S and R1441G are the most well-studied. In Ashkenazi Jews and North African Arabs, the mutations can be found in over 10% of all PD cases (Lesage *et al.*, 2006); (Ozelius *et al.*, 2006): outside these groups it is far less common (0.5-1.5%) (Gilks *et al.*, 2005; Nichols *et al.*, 2005). The mutations are incompletly penetrant (not following a clear mendelian inheritance pattern) and enhance the chance to develop the disease (Mata *et al.*, 2005) (Gilks *et al.*, 2005) (Lesage *et al.*, 2006) (Ross *et al.*, 2006). The G2019S mutation appears to alter the 'activation loop' of the kinase domain, based on structural homology to other protein kinases. The resulting increased kinase-activity could be the cause of neurodegeneration (Greggio *et al.*, 2006; West *et al.*, 2007).
The expression of UCH-L1 (Ubiquitin carboxy-terminal hydroxylase L1) is neuron-specific and widespread throughout the brain. Its function is to recycle chains of ubiquitin to monomers by hydroxylation (Larsen *et al.*, 1998; Solano *et al.*, 2000). It is also known to have ligase function and one known substrate of UCH-L1 is α-synuclein. It has been speculated that an alteration preventing the proper function of this protein may lead to α-synuclein clogging of a cell, disrupting its function and causing cell death (Liu *et al.*, 2002).

1.4.2 Environmental risc factors

Next to genetic factors, environmental factors have been a major focus of study. The fact that different populations of the same ethnic group have unequal chances to develop PD, depending on where they live, points to an influence of environmental agents. A range of factors has been investigated over the years, for instance exposure to pesticides (Brown *et al.*, 2006), wellwater drinking, the impact of living in a rural environment (Seidler *et al.*, 1996), the influence of smoking and coffee drinking and many more. However, the influence of these factors is not clear yet., as studies performed on e.g. caucasian and asian populations often show different results (Schoenberg *et al.*, 1988) (de Lau and Breteler, 2006). However, there is a number of substances causing a more or less specific SNpc DA cell loss, which has been used in PD research as a means to induce rapid mesDA degeneration and which where in some cases identified as humans were exposed and developed PD-like symptoms.

1.5 Major Toxic Insult systems of PD research

1.5.1 MPTP

1-Methyl-4-phenyl-1,2,3,6- tetrahydropyridine is a potent heroin-like by-product of a meperidine analogue. The appearance of PD-like symptoms in a number of drug-addicts who injected MPTP contaminated heroin in the early 1980s (Langston *et al.*, 1983) led to its discovery. It is very lipophilic and easily crosses the blood-brain barrier. Non-dopaminergic cells take it up and convert it via monoamine oxidase B (MAO-B) to MPP+. Released into the extra-cellular space, it is actively taken up by mesDA neurons with dopamine transporter (DAT) (Javitch *et al.*, 1985; Mayer *et al.*, 1986). In the cells, MPP+ binds to complex I at the same residue that another mesDA toxin, rotenone, binds and inhibits the respiratory chain (Nicklas *et al.*, 1985). This leads to a drop in ATP levels in the cell which in turn causes an intracellular calcium overload (Chan *et al.*, 1991; Fabre *et al.*, 1999). The increased calcium level activates degrading phosphatases and proteases which disrupt cell function and lead to apoptosis. High MPP+ concentrations may also promote excito-toxicity via a glutamate release, leading to a massive influx of calcium by the activation of NMDA receptors and production of reactive oxygen species (ROS) (Hasegawa *et al.*, 1990). In mice over-expressing Cu/Zn and Mn superoxide dismutases, two enzymes deactivating free radicals, the toxicity of MPTP is decreased (Przedborski *et al.*, 1992).

Furthermore, oxidative damage also depletes intra-cellular ATP levels by activating poly-ADP-ribose polymerase. In mice lacking that enzyme, the toxicity of MPTP is significantly reduced (Mandir *et al.*, 1999).

MPTP in primates is able to induce most of the PD symptoms, like tremor, rigidity, slowness of movement postural instability and even freezing. The group of drug abusers who suffered from these symptoms reacted positive to treatment with either L-DOPA or dopamine agonists. Like PD, MPTP causes a greater loss of mesDA neurons in the SNpc than the VTA (shown for primates (Sirinathsinghji *et al.*, 1992; Varastet *et al.*, 1994) and mice (Muthane *et al.*, 1994; Seniuk *et al.*, 1990). Unlike PD, however, there is neither any degeneration in the neurons of the locus coruleus nor

convincing evidence of Lewy bodies in MPTP-treated primates (Forno *et al.*, 1993). Additionally, there are signs of reactive microglia and inflammation years after MPTP insult in human as well as in other primates (Langston *et al.*, 1999; McGeer *et al.*, 2003).

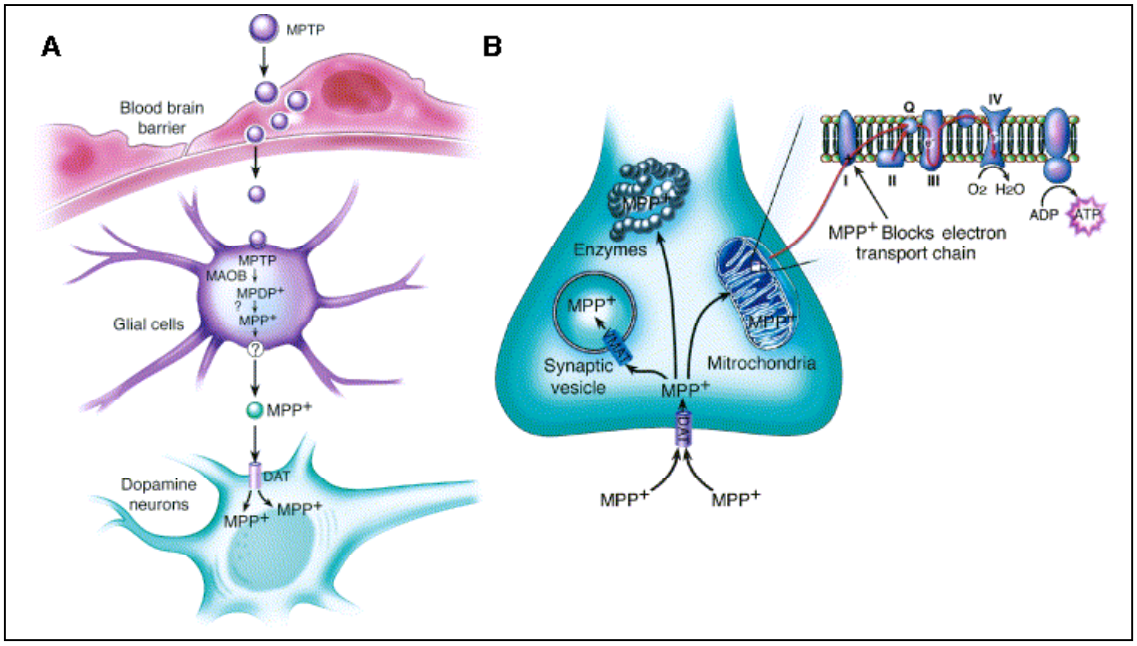


Figure 1.9: Overview of MPTP action.
(A) MPTP passes the blood-brain barrier and is taken up and converted to MPP+ by non-neuronal cells via MAO-B. MPP+ is then released into the extra-cellular space and specifically taken up via DAT by mesDA neurons. (B) Inside the cell MPP+ binds to complex I of the respiratory chain and inhibits ATP production (Dauer and Przedborski, 2003).

1.5.2 Rotenone

Rotenone is a naturally occurring compound found in the root of many plant species and has been used as an insecticide and fish toxin. Like MPTP, is a well-studied inhibitor of complex I of the respiratory chain. Since its very hydrophobic, it passes the blood-brain barrier with ease and leads to a systemic inhibition of complex I, unlike MPP+ which specifically taken up only by mesDA neurons (Talpade *et al.*, 2000). Nevertheless, systemic application in rats led to a distinct neurodegeneration of mesDA neurons, postural abnormalities and slowness of movement. They also have, as the only toxin model system, cytoplasmic inclusions reminiscent of Lewy-bodies, which are immuno-positive for alpha-synuclein and ubiquitine, two proteins commonly found in Lewy-bodies. The effect of rotenone on rodents, however, is not

easily reproducible and might vary both within strains as well as between strains (Betarbet *et al.*, 2000).

1.5.3 6OHDA

6-Hydroxydopamine (6OHDA) was the earliest used compound to induce a Parkinson-like phenotype in rodents. It cannot pass the blood-brain barrier and is therefore usually injected unilaterally directly into the brain to produce a more (if the location of injection is the SNpc) or less (if it is injected into the Striatum) rapid loss of mesDA neurons. The toxic effect of 6OHDA is thought to involve the generation of free radicals and oxidative stress (Werner *et al.*, 1994). 6OHDA has the disadvantage that it does not lead to Lewy bodies and that damage to other neurons is possible (Beal, 2001). Nevertheless the unilateral loss of mesDA neurons can be used for testing gene therapies and cell transplantation systems (Bjorklund *et al.*, 2002).

1.6 Oxidative Stress and K_{ATP} channels

Taking both the genetic and toxin based data into account, it appears that both impairment of protein degradation processes and oxidative stress is involved in mesDA degenration during PD. In a search for possible downstream targets in the event of oxidative stress, Liss and Roeper investigated mesDA K_{ATP} channels and their function during a mitochondrial complex I inhibition via MPTP or rotenone. My own research led to a possible function of the engrailed genes upstream of K_{ATP} channel regulation via the Fox family of genes.

1.6.1 K_{ATP} channels

1.6.1.1 K_{ATP} channel function

ATP-sensitive potassium channels are non-voltage dependent, selective channels expressed widely in multiple organs of the mammalian body, including pancreatic islet cells, heart, skeletal muscle, vascular smooth muscle and brain (Ashcroft, 1988). They couple cellular metabolism to electrical activity by opening when the intracellular ATP/ADP ratio decreases and by closing as the ratio increases. Closure of K_{ATP} channels leads to Ca2+ influx. In beta-islet cells, this mechanism leads to the secretion of insulin whenever the glucose level in the blood (and therefore the intracellular ATP/ADP ratio) is high.

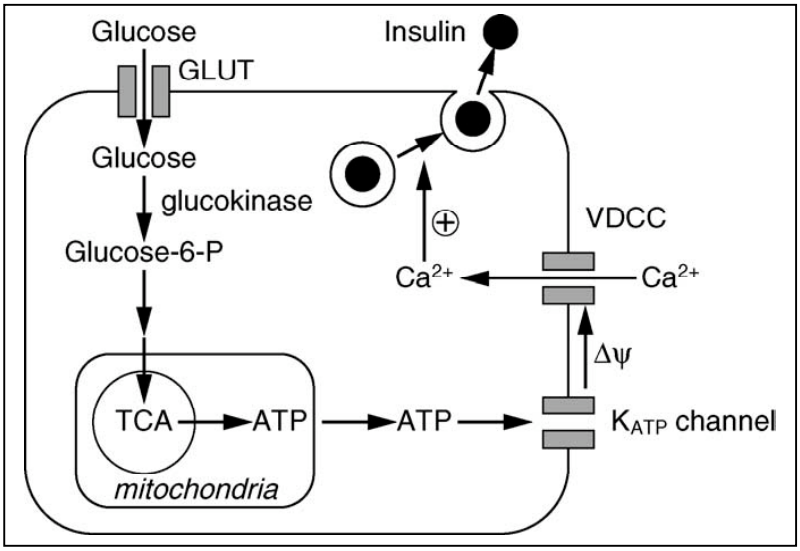


Figure 1.10: Glucose-stimulated insulin secretion.
Glucose is transported into ß-cells via the GLUT transporter. Rising intracellular ATP closes the K_{ATP} channels, which depolarizes the cell membrane. This opens voltage-dependent calcium channels, leads to an influx of calcium; the high intracellular calcium concentration in the ß-cell triggers insulin secretion (Miki and Seino, 2005).

In contrast, events causing the ratio to drop, like ischemia, lead to a K+ efflux and hyperpolarization of the cell membrane, therefore to a shortening of action potentials

and conservation of energy, which is thought to protect the cell for a time against cytotoxic processes (Miki and Seino, 2005).

1.6.1.2 K_{ATP} channel structure

K_{ATP} channels are formed by the joining of eight subunits, four ca. 40kDa Kir6.x pore-forming proteins (either Kir6.1 or 6.2), belonging to the inward rectifier K+ channel family and four ca. 160kDa sulfonylurea receptor proteins (Sur1 and 2), which are part of the ATP binding cassette (ABC) transporter family. Sur2 exists in two region-specific isoforms, called Sur2A and B (Inagaki *et al.*, 1996). In each channel, only one type of Kir6 and one type of Sur is present. The combinations of Kir6.x and Sur subunits specify their distinct pharmacological and electrophysiological properties. In mesDA neurons, Sur1 and Kir6.2 isoforms are expressed almost exclusively (Liss *et al.*, 1999a).

1.6.1.3 Involvement of K_{ATP} channel function in mesDA neuron activity

Using patch clamping on both wild type and Kir6.2 mutant mice, Liss et al. identified Kir6.2 as the sole pore forming part of DA neurons of the SNpc and VTA. RT-PCR showed Sur1 expressing neurons in similar frequencies in both populations as well as Sur2 in under 5% of all cases. Expression levels for Sur1 were twofold higher in the SNpc. Treatment with either 10µM MPTP or 100nM rotenone and subsequent patch clamping revealed that neurons of the SNpc but not VTA cease spontaneous action potential firing.

Kir6.2 mutant mice treated chronically with MPTP were resistant to the usually occurring SNpc cell loss, while the low cell death occurring in the VTA was similar compared to wild type levels. Furthermore, in a drastic genetic model of mesDA degeneration, the *weaver* mouse, inactivation of Kir6.2 by crossing both mutant strains led to slight reduction of postnatal cell death in the SNpc. Taken together, activation of plasma membrane K_{ATP} channels in the mesDA neurons of the SNpc leads to cessation of electrical activity, whereas inactivation enables the spontaneous firing of the neurons again and rescues the cells in both an acute chemical and genetic model of mesDA cell loss (Liss *et al.*, 2005).

1.6.1.4 Genetic control of K_{ATP} channel expression in the midbrain

A second line of evidence putting K_{ATP} channels in a mesDA cell survival context came from our own research. As stated earlier, in En1-/+:En2-/- mice, the loss of wild type gene expression leads to a cell-autonomous and gene-dose dependent degradation of the mesDA neurons (Alberi *et al.*, 2004). In the En2-/- background, a single deletion of En1 (En^{HT}) causes a progressive loss of SNpc neurons starting at around postnatal (P) day 15 and peaking at around 90 days of age (Sgado *et al.*, 2006).

Our lab recently performed a differential display experiment, in which ventral mesencephalic tissue from three sources was used to search for genes which are specific to mesencephalic neurons and/or under the control of the engrailed genes (for a detailed scheme, see Figure on next page) (Thuret *et al.*, 2004).

1.6.1.5 Foxa1 expression is controlled by engrailed in the midbrain

Among the genes identified in the differential display, the forkhead containing transcription factor 1, Foxa1 (formerly HNF3a) (Lai *et al.*, 1990), was of particular interest. It is expressed in mesDA neurons from E9 onwards. However, analysis of the mouse Foxa1 mutant showed no change in the level of expression of TH, as well as the expression of several other genes known to be expressed by mesDA neurons. However, this investigation was limited to young mice, as the full mutant dies between P2 and P12 (Kaestner *et al.*, 1999)**.** In one of my projects, I tried to generate a conditional knock-out of Foxa1. However, I was not able to complete this and focused on the work presented here.

1.6.1.6 Foxa2

As it is well known that members of the Fox/HNF family of genes can compensate for each other (Lee *et al.*, 2005; Sund *et al.*, 2000; Wan *et al.*, 2005), we focused our research on the close homologue Foxa2 (formerly HNF3b) (Lai *et al.*, 1991), which is also expressed by mesDA neurons (Besnard *et al.*, 2004). Interestingly, both Foxa1 and Foxa2 regulate the expression of several mesDA genes during development, for instance Nurr1, En1 and TH (Ferri *et al.*, 2007). Furthermore, Foxa2 is known to regulate the expression of the K_{ATP} channel forming subunits Sur1 and Kir6.2 in beta-islet cells (Sund *et al.*, 2001).

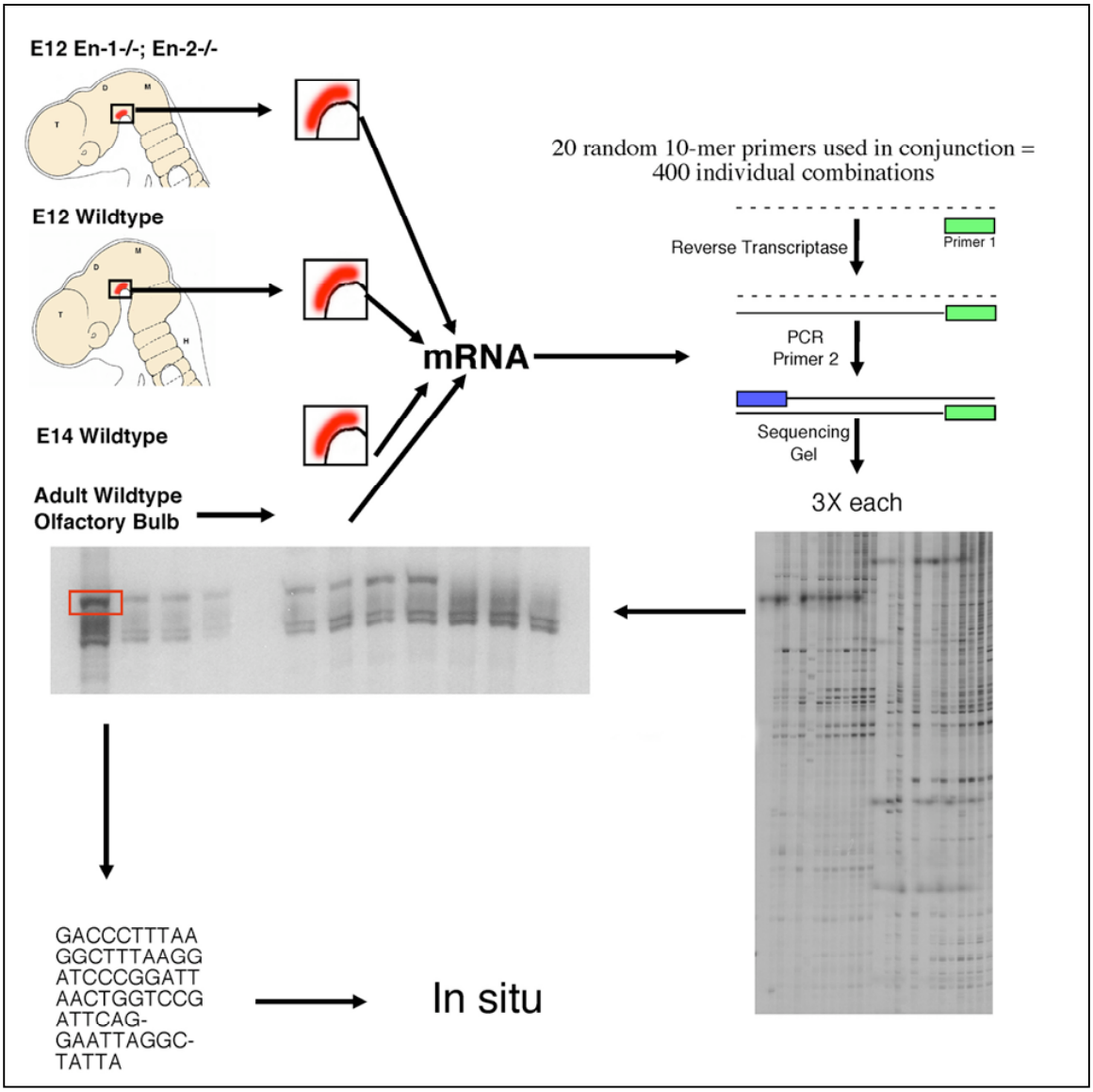


Figure 1.11: Overview of the differential display.
Ventral midbrains of E12 En1/2 mutant mice, wild type, E14 wild type and adult olfactory bulb were dissected. The mRNA was isolated and used in a RT PCR with random primers to generate DNA fragments. These were separated on a sequencing gel and the patterns of the lines were compared. Fragments appearing differentially were excised from the gel and sequenced (Thuret *et al*., 2004).

However, analysis of both Sur1 and Kir6.2 mouse mutants showed no change in TH+ cell number, cell density or density of axonal projections, thus a loss of functional K_{ATP} channels has no effect on the survival of mesDA neurons. Therefore, I revised my hypothesis, that not a lack or impairment of K_{ATP} channels is causing cell death, but a functional K_{ATP} channel, which opens under physiological stress and leads to cell death.

1.7 Goals of this work

In this work, I investigated the effect of a forced open or closed state of the K_{ATP} channel in mesDA neurons. I show that pharmaceutical K_{ATP} channel activation mediates enhanced cell death of TH+ neurons in primary cultures of E12 ventral midbrain under toxic conditions. I show that, in the reversal of these experiments, blocking the channel leads to an enhanced survival rate of DA cells compared to control conditions. Furthermore, I demonstrate that the slow progressive cell loss occurring in En^{HT} mice is rescued by crossing the strain with Sur1-/- mice. These findings point to the K_{ATP} channels of the mesDA system as potential targets for pharmaceutical treatment in Parkinson's Disease.

Materials and Methods

2 Materials and Methods

2.1 Generation of mutant mouse strains and genotyping

The En1/tau-lacZ mice were generated by a knock in strategy, replacing the first 71 codons plus start codon with the Tau/LacZ sequence (Callahan and Thomas, 1994) (Saueressig *et al.*, 1999).
Generation of the En2-deficient mice has been described elsewhere (Joyner *et al.*, 1991). The two lines were crossbred and maintained in an En2-/- background, so that three possible genotypes occurred (En1 +/+, En1 -/-, En1 +/-). The En1/En2 homozygous mutant dies at P0. At E12 it is easily detectable by a missing cerebellum (see (Simon *et al.*, 2001; Wurst *et al.*, 1994) for description).
Sur1 mutant mice were generated using the 5' end of the gene to construct a targeting vector replacing exon 2 of the gene with a puromycin resistance cassette (Seghers *et al.*, 2000).

2.1.1 Genomic DNA extraction

Tail biopsies from 2-3 weeks old mice were obtained and stored at –20°C. The tissue extraction was digested overnight at 55°C in proteinase K buffer [50 mM Tris pH 8, 100 mM EDTA, 100 mM NaCl, 1% SDS supplemented with 5 mg/ml proteinase K (Applichem, Germany)]. Supernatant was added to equal volume of 1:1 Phenol/Chloroform, mixed and shortly centrifuged. The upper phase was used for DNA precipitation, which was performed in high salt solution. 1/10th volume 5M NaCl and 1/2 volume 100% ethanol were added, the mixture was shaken vigorously and incubated for a minimum of 20 minutes at –80°C. The solution was then centrifuged for 20 minutes at room temperature. Precipitated DNA was rinsed with 70% Ethanol and then resuspended in 100-500µl TE (10 mM Tris pH 8, 1mM EDTA). The solution was heated at 65°C from two hours to overnight to further resuspension of the DNA and then used for PCR genotyping. Alternatively, the GenElute Kit (Sigma, Germany, Cat.: G1N70) was used according to manufacturers instructions. In brief, 200µl 100% Ethanol was added to 500µl Proteinase K digested lysate and transferred to pre-washed GenElute columns. After a short centrifugation,

the columns were washed twice and the DNA was eluted with 200µl of the appropriate Elution buffer.

2.1.2 Primers and PCR settings for genotyping

The genotyping for En2 wild type and mutant alleles was performed by PCR containing three primers, the common primer, TTGAGAAGAGAGGCCCTGTA, the wild type primer, specific for the En2 homeobox, CTGGAACAAAAGGCCAGTGT, and the mutant primer, TCTCATGCTGGAGTTCTTCG in the neomycin gene (which only occurs in the mutated allele).

For En1, the primers for detecting the knock-in Tau-LacZ allele were GTGTCCGGCAGCTTGGTCTT on the Tau-LacZgene, and TTCGCTGAGGCTTCGCTTTG, starting at position 224 of the exon 1.

For Sur1, the primers for detecting wild-type alleles were AGGTTGTTGGTGGAGGTCAG and CCAACACGAGCCTTGAACTT. For detecting knock-out alleles, AGGTTGTTGGTGGAGGTCAG and GCTACTTCCATTTGTCACG were used.

The PCR settings for En2 and Sur1 were 5 min at 94°C, 45 sec at 94°C, 1 min at 54°C, 1 min at 72°C, 35 cycles, 5 min at 72°C.

For En1 they were 5 min at 94°C , 45 sec at 94°C, 1min at 54°C, 1 min at 72°C, 30 cycles, 5 min at 72°C.

The PCR mix in all cases consisted of 2.5 mM $MgCl_2$, 10x buffer, 1 mM dNTP and 2% primer mix and 1% Taq polymerase (all from Fermentas, Germany) plus 1-3 µl DNA (2% of the total volume).

En2 common primer	TTGAGAAGAGAGGCCCTGTA
En2 wild type primer	CTGGAACAAAAGGCCAGTGT
En2 mutant primer	TCTCATGCTGGAGTTCTTCG
En1 mutant primer tLacZ	GTGTCCGGCAGCTTGGTCTT
En1 mutant primer exon1	TTCGCTGAGGCTTCGCTTTG,
Sur1 wild type primer A	AGGTTGTTGGTGGAGGTCAG
Sur1 wild type primer B	CCAACACGAGCCTTGAACTT
Sur1 mutant primer A	AGGTTGTTGGTGGAGGTCAG
Sur1 mutant primer A	GCTACTTCCATTTGTCACG

Figure 2.1: Primers used for genotyping

2.2 Primary cell culture

2.2.1 Coating of coverslips

Cover slips were dipped into 96% EtOH, ignited and then put into 24 well plates. 500µl of 0.01mg/ml Poly-ornithin (Sigma, Germany) was added per well and incubated for one hour. The poly-ornithine was removed, the cover slips were washed three times with sterile H2O and 1mg/ml Laminin was added in F12:DMEM (Cambrex, Belgium) at a 1:1000 ratio. The cover slips were incubated for at least two hours.

2.2.2 Culture

Pregnant mice twelve days post coitum (day of vaginal plug = E0) were sacrificed using CO2, the body was dabbed in 70% ethanol to prevent fur from contaminating the uterus. The uterus was taken out in toto and placed in a plate containing ice-cold Leibovitz's F15 medium (Cambrex, Belgium). The embryos were collected and, in the case of engrailed 1 mutants, genotyped. Detecting the En1 mutation was accomplished by incubating toes from sacrificed mice in Xgal Solution containing 5 mM $K_3Fe(CN)_6$ (Sigma, Germany), 5 mM $K_4Fe(CN)_6$_$3H_2O$ (Sigma, Germany), 2 mM $MgCl_2$ and with 1 mg/ml X-Gal (AppliChemGmbH, Germany) for 30 min at 37ºC.

The midbrain was taken out, the meningis was removed and the ventral part of the mesencephalon was placed into a tube containing ice-cold Leibovitz's F15. Brains were pooled according to genotype. After all samples were collected, the medium was removed and a 0.25% Trypsine/EDTA solution (Invitrogen/Gibco, Germany) was added. The tubes were incubated for ten minutes at 37°C, after which the trypsine was removed and the tissues were washed with 1:1 medium/FCS. The solution was removed and 500µl fresh medium was added. A fire-narrowed glass pipette was used to separate the cells from each other by triturating the cell solution gently up and down. The cells were centrifuged down (3200 rpm) 10 minutes (Beckmann TL, Rotor TLS 55), the medium was removed and fresh medium was added. The cells were pipetted up and down to distribute the cells equally in the solution and then were plated out. For each brain in the pool, five 10mm cover slips were used (amounting

for roughly 150000 cells per slip). The cells were diluted so that 100 µl per slip were used.
Each cover slip was placed on a separate holder and incubated for one hour at 37°C, to let the cells settle and attach to the surface. After this hour, the cover slips were placed in 24 well plates, 500 µl medium was added and after 24h, another 500 µl.

2.2.3 Neurotoxins used on primary cell culture

Ventral mesencephalic culture was first cultured for two to five days. One day before toxin insult, the medium was changed to serum-free medium. One hour before toxin insult, cells were pre-incubated with 1 mM tolbutamide, 10 mM TEA or 100 µM pinacidil. Cells were exposed to 10 µM MPP+, 40 nM rotenone (both Sigma, Germany) or 200 µM 6-OHDA (Invitrogene, Germany) for 24 h, after which the medium was removed, cells were fixed with 4% PFA and stained (see Immunohistochemistry)

2.2.4 Medium used for primary cell culture

DMEM/F12 with 1 % glutamine (Invitrogen, Germany)
1 % PenStrep (50 U/ml Penicilin, 50 µg/ml Streptamycin)
5 % FCS (10% for E18 cultures)
0,36 % Glucose (=33 mM, Sigma, Germany)
0.25 % BSA (Sigma, Germany)
1% N2-supplement (500 µl N1 supplement + 200 µl Insulin, Invitrogen, Germany)

In case of serum-free medium, the medium was the same as above, but lacking FCS.

2.3 Implantation of osmotic minipumps

All operations were done according to the guidelines of German animal handling law. Postnatal day 15 (P15) mice were anesthetized with isofluran (2-5 vol% in O_2). The area of the lower head to upper back was shaved. The osmotic mini-pump (1002D, Alzet, USA) was filled with 100 µl of either tolbutamide, glibenclamide (both diluted in DMSO) or DMSO alone. The PE tubing of the Brain Infusion Kit 3 (Alzet, USA)

was filled with the respective solution to prevent air bubbles and attached to the pump. The length of the tubing was determined with the growth of the mouse in mind (ca. 2 cm).

An incision was made along the midline of the head, starting right behind ear level down to the neck. The skin was pulled away from the incision and the bone of the skull was dried with a cotton tip; a pocket under the skin on the back of the mouse was formed using the blunt plastic cap of a syringe. A needle was used to drill a hole in the skull roughly 1 mm from both the line of Bregma and the midline. The pump was put into the pocket and the cannula was placed into the drilled hole and held until the Loctite glue had dried. The skin was closed with a suture, occasionally, a wound clip or tissue glue was added as an additional precaution. After a subcutaneous injection of 100 µl (5 mg/ml) of the analgesic Carprofen (Pfizer, Germany), the mice were allowed to regain full consciousness in a separate cage. After normal behaviour (grooming, exploration etc.) was seen, the mice were put together in the cage with the mother and the rest of the litter. On the next morning all operated mice received another injection of the analgesic. The mice were screened for abnormal behaviour, pain, open sutures, weight loss etc. from then on for two to four weeks until sacrifice. In the case of open sutures, mice were anesthezised again and the suture was closed. The mice were weaned from the mother one week after the operations but kept together with littermates. Animals which were exposed for four instead of two weeks were operated again to change the pump. Those animals were anesthesized and a small incision was made on the back were the pump was situated. The pump was detached from the tubing, a new pump was attached and the wound was closed with wound clips. The animals were killed after two or four weeks after the operation and treated as described in the next section.

Adult wild-type mice were treated in a similar fashion, the only difference being the model of the pump (1007D, Alzet, USA), which has an operating range of one week. After the operation the mice were allowed to recover for two days, after which they were injected with MPTP (20 mg/kg) once every day.

2.4 Preparation of postnatal mouse brain sections

Postnatal day 0 (P0) animals were anesthetized on ice for 3 minutes, the breast was opened and the heart was perfused with 4% PFA. Older mice were killed using CO2 and treated the same. The skull was opened and partly removed, the brain was taken out in toto. Following this, the tissue was fixed in 4% PFA over night and cryoprotected by incubation with 30% sucrose solution over night.
The brains were embedded in OCT Neg-50 Cry-medium (Microm) and sectioned on a cryostat (Microm). Sections of 50µm or thicker were taken up in 24 well plates containing PB and all subsequent steps were done with floating sections. Sections of 14 to 25µm were taken up on Superfrost/Plus Adhesion Microscope slides (NeoLab, Germany).

2.5 Immunohistochemistry

Sections were blocked using a PB solution containing 10% heat-inactivated FCS and 1% Triton. After one hour, the blocking solution was removed and the primary antibody with blocking solution was added. Incubation was carried out at 4°C over night. Sections were washed three times with PB and secondary antibody in blocking solution was added 1:500 and incubated either for four hours at room temperature or over night a 4°C. Sections were washed three times in PB and incubated with either 1:500 streptavidin-horseradish peroxidase for DAB colour reaction or streptavidin-Cy2 for fluorescent stainings (both Dianova, Germany). 0.5 ml DAB (Sigma, Germany) solution was diluted in 10 ml PB (=0.05%), 3.3 µl H_2O_2 per 10 ml was added. Solution was put in each well (in case of 24 well plates) or on cover slips (in case of sections). Cells/Sections were developed to satisfaction and then washed twice with PB. Cover slips with cells were put cell-side down on a cover slip on a drop of aqua-polymount (Polyscience, Germany) and counted. Sections were dehydrated in an ethanol series (30%, 60%, 90% and absolute) and 2x in Xylol for five minutes. Sections were then overlaid with DPX (Sigma, Germany) and counted.

Primary antibodies used:
1:1000 Rabbit α-TH (AB152, Chemicon, Germany)

1:250 Rabbit α-Pbx1/2/3 (Santa Cruz, USA)

Secondary antibodies used (all Dianova, Germany):

1:1000 Goat α-rabbit-biotin

1:1000 Streptavidin-horse radish peroxidase

2.6 Cell counting procedures

Stained cells were counted under a common light microscope at x20. Cover slips were slowly moved from side to side and the resulting lanes at the widest expansion through the objective were counted to make sure that all cells were only counted once. Tiny irregularities of the edge of the cover slips were used as landmarks when the cover slip was moved up to the next lane, so that no cell was counted twice.

2.7 Data management and statistics of primary cell cultures

Cell numbers obtained from cover slip countings were pooled per condition tested. Percentages of cell survival were first calculated by dividing a cell count by the respective average control cell number for that specific experiment. From these percentages, an average effect for the condition was calculated. The effect of tolbutamide, TEA and pinacidil on toxins was normalized against the average effect of the drug alone. Standard error of mean was calculated from normalized data, where applicable. P-values were calculated by using the total data from normalized conditions and toxin alone, using a two-tailed Student's t-test. P-values below 0.001 were considered significant.

2.8 Image processing

All images were captured with a CoolSnap camera (Photometrics, Germany) through a Zeiss Axiophot, a Leica Macrophot, or a Leica TMS MP Multi Photon Confocal microscope and processed using Phylum 2.6.1 and Adobe Photoshop 7.

2.9 Additional methods applied in chapter 4: Miscellaneous Results

2.9.1 Cloning of a HNF3a/Foxa1 flox plasmid

The method to obtain specific genomic DNA fragments to generate a viable knock-in plasmid has been described recently, a one-step cloning process (Scholz *et al.*, 2006).

2.9.1.1 Database search for Foxa1 cloning

Mouse Genome Blast at http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html, default settings – Database = genome, Program = blastn, use MegaBLAST, Expect = 0.01, Filter = default, Descriptions = 100, Alignments = 100. In some cases, the MegaBLAST option was removed and the Filter option set to "none" to determine the location of BAC end sequences. The accession numbers for the mouse genes used for the database searches: *Deleted in Colorectal Carcinoma* (DCC) NM_007831, *Dopamine Receptor 2* (Drd2) NM_010077, *engrailed-1* (En1) NM_010133, *Foxa1* NM_008259, Nerve Growth Factor Receptor 1 (Ngfr or p75) NM_033217 and *Pbx1a* NM_183355.

2.9.1.2 Programs used for in silico cloning

Gene Construction Kit 2.5 (SciQuest, USA) for generation of the restriction maps and the virtual assembly of vectors and inserts before designing primers. Oligo 6.8 from (Molecular Biology Insights, Inc., USA) to search for appropriate primers. The search for primer pairs was always performed at highest stringency. All of the chosen primer pairs had a suggested annealing temperature of 55°C or higher.

2.9.1.3 PCR conditions and restriction digests for BAC cloning

The BAC clones were all purchased for a nominal fee from the Children's Hospital Oakland Research Institute, USA, (http://bacpac.chori.org). All used restriction enzymes are commercially available. The ligations were performed with the TaKaRa Ligation Kit Version 2 (BioWhittaker, Germany) at 16°C for at least 30 min. The PCR reactions were all done with standard solutions (PCR buffer with NH_4SO_4 (Fermentas); 12.5 μM dNTPs (Roche Diagnostics); 1.5 mM $MgCl_2$ (Fermentas)); PCR settings were one cycle of 5 min at 94 °C, followed by 20-25 cycles with 30' at

94°C, 60’ at 55°C or higher (determined by primer set used), 45’ at 72°C and a final step of 7 min at 72°C.

2.9.1.4 Cell culture of ES cells

One 6 cm plate and sixteen 9 cm plates of growth-inhibited MitoC treated feeder cells were prepared for growing ES cells until electroporation and for G418 selection after electroporation. 24 h before ES cells were plated on MitoC feeder plates, the medium was changed from feeder medium to ES cell medium. One vial containing TBV-2 ES cells was quickly thawed at 37°C and then transferred to ES cell medium. The cells were pooled by a centrifugation step at 1000 rpm for five minutes, then were resuspended in 5 ml of fresh medium and plated out on a 6 cm MitoC feeder plate. The medium was changed at exact intervals of 24 hours to keep the cells in synchronized division. The cells were grown to confluency and then splitted with 0.03% trypsine in a 1:6 ratio to the 9 cm plates. The medium was changed two hours prior to every trypsination. Before electroporation, eight 9 cm plates were coated with a 0.1% gelatine solution for 20 minutes. The plates were then air-dried. The medium of three of the ES cell plates was aspirated and the plates were washed twice with PBS before they were trypsinized. A Pasteur pipette was used to break up cell clumps with gentle pipetting. Cells were pooled, spun down and resuspended in 20 ml ES medium, of which 10 ml was transferred to each of two gelatine plates. The cells were incubated at 37°C for twenty minutes. The supernatant containing the ES cells (which don’t attach themselves as readily as the feeder cells) was transferred to a second set of gelatine plates. After that the process was repeated with the other 3 ES cell plates and all ES cells were finally pooled and counted.

At least $20x10^6$ cells were used per electroporation. ES cells and NotI-linearized DNA construct were taken up in 0.7 ml ice-cold PBS and put into an electroporation cuvette. Electroporation took place with a 240 V pulse at 500 μF. The cuvette was kept on ice for twenty minutes afterwards. The cell slurry was re-suspended in 100 ml ES cell medium and spread evenly over ten 9 cm Mitoc treated feeder plates.

After 24 hours, the medium was changed to Neomycin-containing ES medium (350ug/ml Neomycin) and changed every 24 hours afterwards. After ca. four days, the selection started, visible by ES cell colonies dieing. After selection in G418 (Neomycin) containing medium for ten days, cells on electroporation plates had

colonies of surviving ES cells. Twelve hours before picking clones, MitoC treated feeder cells were placed into flat-bottomed 96-well plates. Two hours before picking, the medium on the ES cell plates was changed and a number of 24 well plates was gelatine-coated. The plates were washed twice with PBS and then put under PBS with Pen/Strep. Colonies were picked with the help of sterile 200 µl tips and a binocular light microscope. Only colonies containing undifferentiated cells were picked. Cells were put into the 96 well plates (propagation) as well as into the 24 well plates (DNA for analysis). Medium on both types of plates was changed every 24 hours. Special effort was made to freeze cells of the 96 well plates before they started to differentiate. Plates were frozen for one hours at –20°C, and stored at -70°C in 12.5% DMSO, 25% FCS in ES medium.

2.9.1.5 Genomic Southern Blots

About 10 µg of DNA was digested over night. The samples were run on a 0.7% agarose gel overnight at 25-28 V, 70-80 mA or six hours at 35 V. Gel was equilibrated and blotted with 0.4 M NaOH on Zeta-Probe GT Genomic Tested (BioRad, Germany) over night. The membrane was washed twice in 5X SSC and then dried.

The rediprime II random prime labelling system (GE Healthcare, Germany) was used according to manufacturers instructions. 2.5 to 25 ng of Probe DNA in 45 µl 10 mM Tris HCL pH 8.0, 1 mM EDTA (TE-buffer) was used. Probes were heated at 95°C for five minutes, snap-cooled on ice for five minutes and centrifuged briefly. Afterwards, 5 µl of Redivue ^{32}P dCTP (= 50 µCi) (GE Healthcare, Germany) was added and mixed until the solution was completely purple. The tube was incubated at 37°C for at least ten minutes. The membrane was pre-incubated with QuikHyb mix (Stratagene) for twenty minutes. 14-15 ml QuikHyb were preheated to 68°C. The probe was denatured as above and added to preheated mix. Membranes were incubated on a rotator for at least one and half hours to a maximum of three hours

Post-Hybridization washes were 2x SSC/0.1x SDS RT five minutes, 0.1x SSC/0.1x SDS at 68°C for 30 minutes and 0.1x SSC/0.1x SDS at 68°C for 30 min. A final wash with VE water was done to reduce salt residues and background. Membranes were wrapped with saran wrap and exposed on film for 2-4 hours or overnight, depending on strength of signal.

2.9.2 Pet-1 DIG in situ hybridization on whole mount

E12 mouse embryos where immersion-fixed in 4% para-formaldehyde and stored in 100% methanol at -20°C. The digoxigenin (DIG)-labeled Pet1-specific antisense RNA probe was prepared using a 1 kb fragment of the mouse Pet-1 cDNA encoding an unconserved region of the Pet1 protein downstream of the ETS domain (Hendricks *et al*., 1999). DIG-Pet1 probes were synthesized according to manufacturer's instructions (Roche, Germany). Embryos were re-hydrated, treated with 10 mg/ml Proteinase K for ten minutes, and post-fixed in 4% paraformaldehyde/0.1% glutaraldehyde for twenty minutes. Hybridization was carried out over night at 70°C. Embryos were washed, blocked with 10% sheep serum for two hours, and incubated over night at 68°C with anti-DIG antibody (Roche, Germany) 1:10.000 in 10% sheep serum. Color reaction was carried out using NBT/BCIP (Roche, Germany).

2.9.3 En1/Pbx1a Tetracycline inducible expression vectors

The generation of En1/Pbx1 TetON N2A cells has been described elsewhere (Paola Sgado, Univ. Diss., Heidelberg, 2006, IX, 118, XLII Bl). In brief, inducible expression was achieved with the use of the RevTet-On Expression System (Clontech, Germany). In order to obtain stable transduced cell lines a retroviral vector based strategy was chosen. The Tet Systems are based on two elements from the tet operon of the E. coli Tn10 transposon: The Tet repressor protein (TetR) and the tet operator DNA sequence (tetO). The gene of interest is cloned into the pRevTRE response vector downstream of the tetracycline-responsive element (TRE), which contains seven direct repeats of the 42-bp tetO sequence and the minimal immediate early promoter of cytomegalovirus (PminCMV). G418 resistant Neuro2a neuroblastoma cells expressing the reverse tetracycline-controlled transactivator (rtTA) were kindly provided by M. Wegner, Nuernberg (Peirano and Wegner, 2000). Stable transfection of retroviral TRE vector carrying En1-IRES-Pbx1a genes was performed using calcium phosphate precipitates and hygromycin selection. The stable transfected Neuro2a population was splitted up into single cell clones. Single clones were then analyzed by PCR and immunocytochemistry and selected based on the correlation between expression of the gene of interest in presence and absence

(background expression) of tetracycline (induction/background ratio). The resulting clones were maintained in DMEM containing 10% serum, G418 (300 µg/ml; Gibco BRL) and hygromycin (300 µg/ml; Gibco BRL).

2.9.4 ChIP assay

En1/Pbx1a Tet-induceable cells were grown to near confluency in 5% FCS containing OptiMEM (Gibco, Germany). Cells were induced with 5 mg/ml doxycyclin solution for two days. Cells were fixed with fresh 1% formaldehyde at 37°C for ten minutes. Cells were washed with PBS containing protease inhibitors and then incubated with lysis buffer. The solution was sonificated to achieve DNA lengths between 200 and 1000 bp. Solution was transferred to ChIP Dilution buffer (Upstate, USA) and preincuabted for 30 minutes with 60 µl Salmon Sperm DNA/Protein A agarose slurry (Upstate, USA). Solution was incubated over night with 1:250 rabbit α-Pbx1a antibody (Santa Cruz, USA). Solution was then incubated with 60 µl Salmon Sperm DNA/Protein A agarose slurry and centrifuged. The slurry was washed with Low Salt, High Salt and LiCl Immune Complex Wash Buffers and twice with TE (all Upstate, USA). 250 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$) (Upstate, USA) was added to slurry and incubated for 15 minutes. Supernatant was pooled with second elution. 20 µl NaCl was added to supernatant and incubated for four hours at 65°C to reverse histone-DNA binding. 2 µl of 10 mg/ml Proteinase K was added in 30 µl TE to solution and incubated for one hour at 45°C. DNA was then precipitated by column (GenElute, Sigma, Germany). Samples were run at a nested PCR under standard conditions (see e.g. engrailed 2 PCR conditions, above).

Outer primers	AAGTGTCCAGGGATCTAATTC +
	TCTGCCACTCCTAACTCACA
= fragment size 567bp	
Inner primers	GTTTTCCTCTCTTGGG +
	CTACTTGCCTATGAGGTACAT
= fragment size 209bp	

Results

3 Results

The results are composed of five parts. First, I established non-toxic conditions for the drugs tolbutamide, tetraethylammonium (TEA) and pinacidil on primary ventral midbrain cell cultures of E12 mice. Then, the effect of these agents on mesDA neurons in primary cultures treated with the toxins MPTP, rotenone and 6OHDA is shown. Second, evidence is presented of a rescue of the progressive En^{HT} nigral phenotype by the ablation of Sur1 expression. Preliminary results in the third section deal with an implantation of osmotic minipumps containing tolbutamide and glibenclamide, another K_{ATP} channel blocker into P15 En^{HT} mice, to stop the progressive nigral DA cell loss these animals develop. The fourth part shows results of similar implantations into adult wild-type mice, followed by sub-acute MPTP treatment.

In the last part, I discuss miscellaneous results obtained and published during my work, first, a novel one-step cloning technique for isolating defined DNA fragments of various genes from a larger DNA source, which in my case were bacterial artificial chromosomes (BACs). In a direct application of this technique, a loxP knock-in construct of exon 2 of the Foxa1 gene was designed in silico, the necessary DNA sequences from three BACs were isolated and used to generate ES cells carrying the knock-in gene. Furthermore, I investigated the requirement of the engrailed genes in the development of the noradrenergic neurons of the locus coruleus and locus subcoruleus and the serotonergic neurons of the median and dorsal raphe nuclei.

At the end, a ChIP (ChromatinImmunoPrecipitation) assay to investigate the possible binding of Pbx protein to the promotor region of AADC was performed and an analysis of Lmx1b heterozygous mutant mice to study possible gross changes in mesDA cell number and distribution.

3.1 Pharmacological treatment of primary mouse ventral midbrain cultures

In recent experiments performed in my lab, primary cultures of E12 mouse ventral midbrain cells were treated with sub-acute doses of the toxins MPTP, rotenone and 6-OHDA to induce partial neuron loss. Pre-incubated with 1 mM of the K_{ATP} channel blocker tolbutamide, the primary cultures treated with rotenone were partially rescued.

To further investigate the effect of the state of K_{ATP} channels on the survival of mesDA neurons under conditions of oxidative stress, I expanded these experiments and used tolbutamide, the K_{ATP} channel opener pinacidil and an additional potassium channel blocker, TEA (Tetraethylammonium). TEA is binding to a wide range of K-channel subtypes on the plasma membrane and directly blocks the pore of the channel, whereas pinacidil binds to the Sur part of the channel and activates channels on the plasma membrane and channels present on the inner mitochondrial membrane.

In addition to rotenone, two other toxins were used. MPTP and 6OHDA are taken up specifically by dopaminergic neurons and like rotenone, cause DA cell death predominantly by inhibition of the respiratory chain.

3.1.1 Culture system and concentrations for drugs and toxins

The system used for study of the effect of toxins and K_{ATP} channel drugs were primary cultures of E12 wild-type embryos. In order to prepare these cultures, in brief, E12 embryonic brains were dissected, the ventral mesencephalon was pooled from all animals of one litter, trypsinized and plated homogenously on poly-ornithine/laminine coated cover slips under DMEM:F12 medium. The cultures can be used in a time window between one day and two weeks. During the second week, several still dividing cell types will start to grow into colonies and cover the plated cells. In order to rule out secondary effects of these clusters depriving underlying mesDA cells and causing unspecific cell death, only cultures of less than one week of age were used for experiments. After one day, the non-dividing mesDA neurons had formed neurites and were easily detectable after TH-immunostaining, as single cells or small groups of three to five cells. The number on each cover slip per prepared pool of cells ranged from 150 to 600 cells between experiments. The cells were counted under a light microscope (as described in Materials and Methods). Every

condition was tested on at least three, on average on five cover slips per experiment. Per condition, at least 13 cover slips were counted in a minimum of three separate experiments.

As a first step, different concentrations of each drug were tested for their toxicity by using gradients. For the K_{ATP} channel blocker tolbutamide, the concentrations used were 500 μM, 1 mM and 2 mM. For the K_{ATP} channel opener pinacidil concentrations from 1 μM, 100 μM and 500 μM were tested and for the unspecific plasma membrane potassium channel blocker TEA 1 mM, 10 mM and 25 mM. A small amount of cell death caused by pinacidil, tolbutamide or TEA was tolerated in order to guarantee a maximal effect of the drug.

These gradients established the concentrations for each subsequent experiment of tolbutamide at 1 mM, of pinacidil at 100 μM and of TEA at 10 mM.

Sub-acute doses of MPTP (10 μM), rotenone (40 nM) and 6OHDA (200 μM) were used to generate an incomplete rate of cell death, in which the cells could still react to outside cues. Cell cultures in serum-free medium were pre-incubated with the respective drug for one hour, then the toxin was added and the cultures were incubated for 24 hours. 6OHDA was added for only 15 minutes and the medium was then exchanged with fresh medium containing the drug. After incubation, cultures were PFA-fixed and immunohistochemically labelled for TH. The stained cells were counted under a microscope. The counted number of surviving cells for (toxin + drug) effect were corrected against drug alone (see data management & statistics in Material and Methods).

3.1.2 Tolbutamide alleviates rotenone-induced cell death

Tolbutamide is not readily soluble in water and was dissolved in DMSO before use. 2.5 μl of a 185 mM stock solution were used to pre-incubate each of the respective cover slips. Upon addition of tolbutamide, the plates were stirred gently to facilitate an even distribution of the drug.

After pre-incubation with 1 mM of this K_{ATP} channel blocker cell survival on rotenone treated cells of E12 mesDA primary cultures was increased significantly by 21% ($p=0.00047$) (the survival rate increased to 56%±4; (rotenone control (35%±3)).

1 mM tolbutamide had no effect on MPTP-treated cells (survival rate 40%±4; (MPTP control (41%±5))).

It had a slightly negative effect on 6OHDA treated cells (survival rate 71%±4; (6OHDA control (82%±5))), however this was not statistically significant (p=0.71602).

The differential effect of tolbutamide on the cultures treated with the three toxins suggests that the molecular mechanism leading to cell death is different between these models.

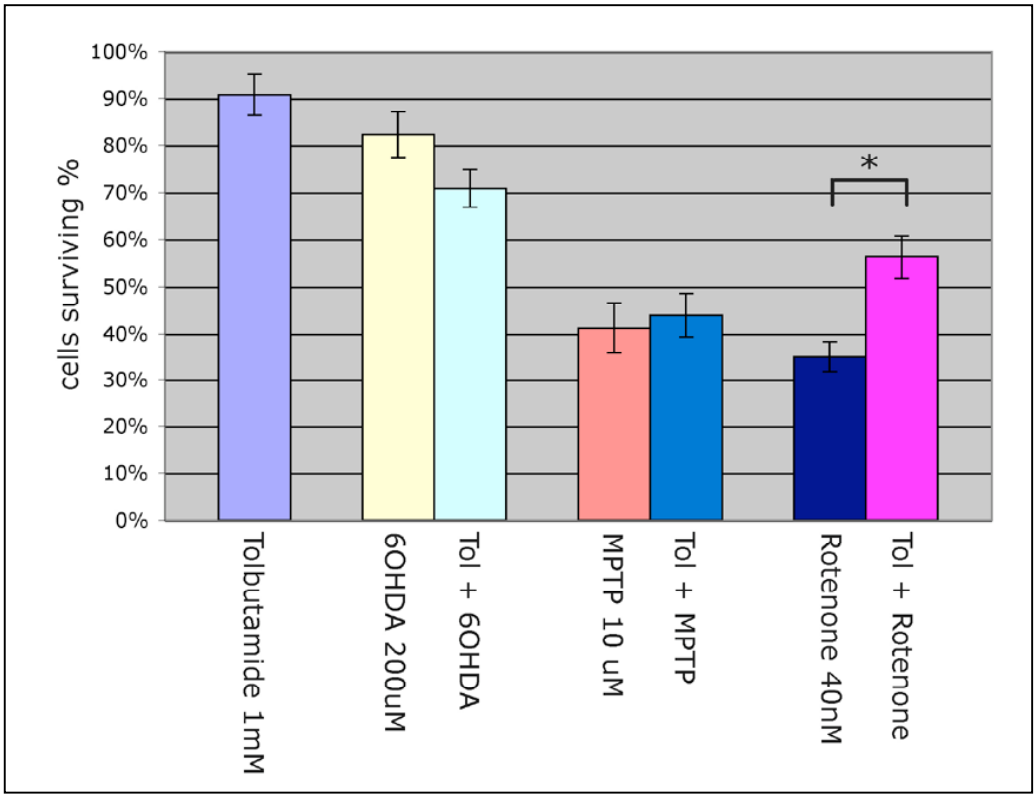


Figure 3.1: Effect of tolbutamide treatment on toxin insults

Percentage of cells surviving in primary cultures of E12 mouse ventral mesencephalic dopaminergic neurons after toxin and tolbutamide treatment. 1 mM tolbutamide had a slight but insignificant effect on 6OHDA treated cultures. Tolbutamide had no effect on MPTP treated cultures, but it increased the amount of surviving cells after rotenone treatment by 11% (p=0.00047). n = 13 to 21, asterisk marks significance

3.1.3 TEA has a positive effect on both rotenone and MPTP treated cells

In order to elucidate a possible specific effect of K_{ATP} channel blockade on cell death from a possible unspecific effect of impairing K^+ flux across the cell membrane or the effect of other potassium channel types, 10 mM of the unspecific potassium channel blocker TEA was used on primary E12 ventral midbrain cultures treated with all three toxins. TEA is soluble in water and was here dissolved in serum-free medium.

TEA enhanced cell survival after rotenone treatment significantly by 16% (p=0.00128) (the survival rate increased to 51%±3; (Rotenone control (35%±3))).
TEA had an even stronger effect on MPTP treated cells, the rate of cellular survival was raised significantly by 33% (p=0.00013) (the survival rate was increased to 72%±5; (MPTP control (41%±5))).

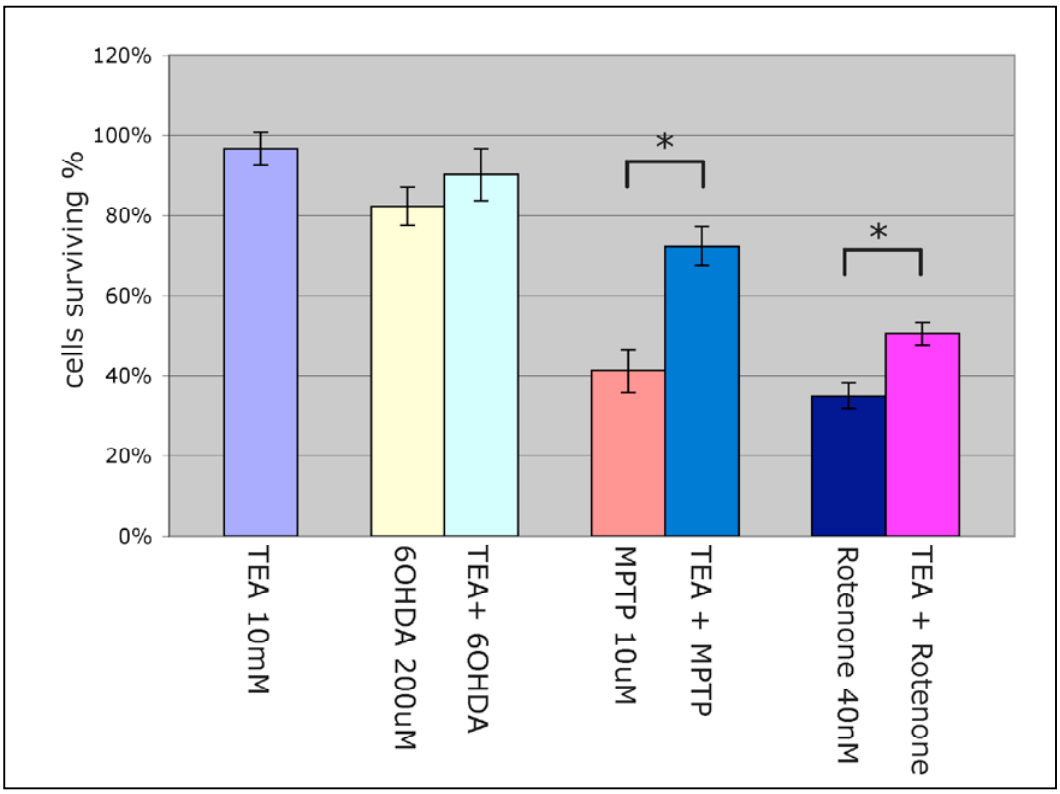


Figure 3.2: Effect of TEA treatment on toxin insults
Percentage of cells surviving in primary cultures of E12 mouse ventral mesencephalic dopaminergic neurons after toxin and TEA treatment. 10 mM TEA increased cellular survival very slightly by 8% on 6OHDA treated cells. TEA had a significantly increased cell survival on MPTP treated cells by 33%. Similarly, TEA enhanced cell survival after rotenone treatment by 16% to 51%±3. n=16 to 21, asterisk marks significance

TEA had only a very slight effect on 6OHDA treated cells and increased cellular survival by 8%, however, this effect was not significant (p=0.32580)
(survival rate 90%±6; (6OHDA control (82%±5))).
Thus, TEA enhances cell survival in the MPTP and rotenone assays, possibly by either affecting other potassium channels aside from K_{ATP} channels, by providing a more thorough blockade of K_{ATP} channels than tolbutamide or by being more resistant to degradation processes than tolbutamide.

3.1.4 Pinacidil enhances cell death in all toxin assays tested

Since blocking of K_{ATP} channels enhanced cell survival of mesDA primary cultures, I wanted to know whether the K_{ATP} channel specific opener pinacidil would decrease cell survival in these assays. This question is even more interesting considering the well established role of a positive effect of K_{ATP} channel activation in conditions of cellular stress like hypoxia. Primary cultures of E12 ventral midbrain cells were treated with 100 µM pinacidil under the established toxic conditions of 200 µM 6OHDA, 10 µM MPTP or 40 nM rotenone, to investigate whether this would exacerbate the rate of cell death.

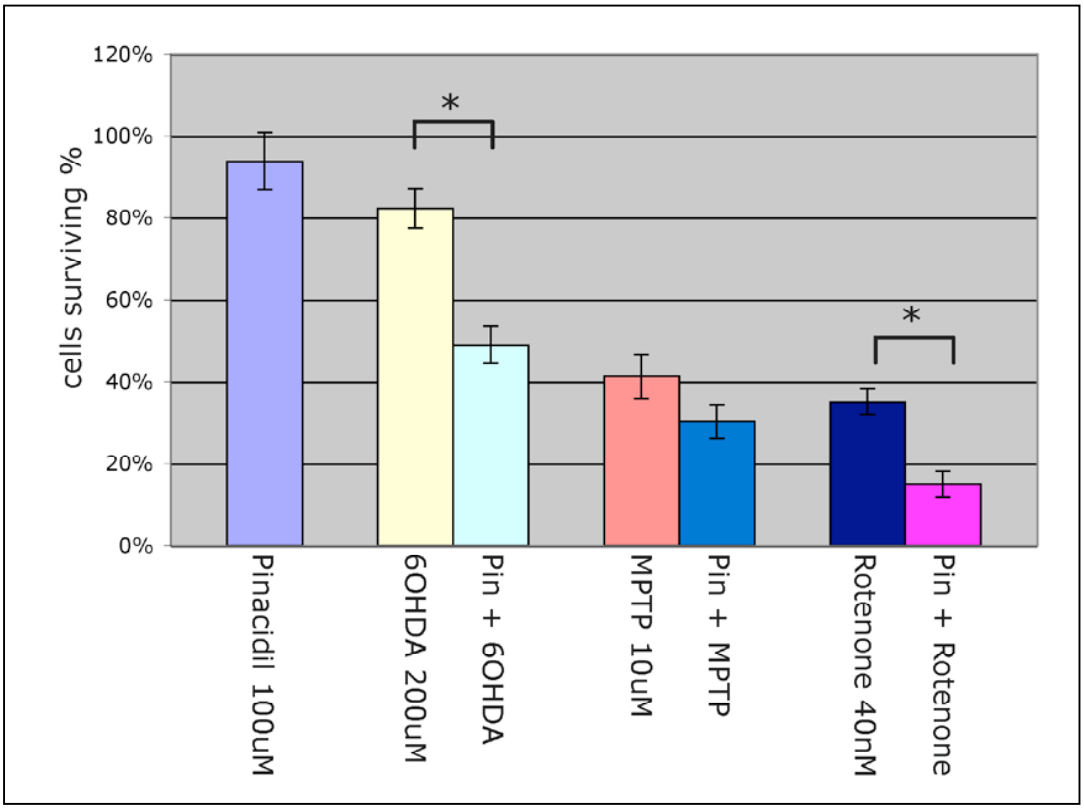


Figure 3.3: Effect of pinacidil treatment on toxin insult
Percentage of cells surviving in primary cultures of E12 mouse ventral mesencephalic dopaminergic neurons after toxin and pinacidil treatment. After pre-incubation with 100 µM pinacidil, the survival rate on 6OHDA treated cultures dropped by 33%, on MPTP treated by 11% and on rotenone treated cultures by 20%. n= 15 to 21, asterisk marks significance

This was indeed the case, pinacidil enhanced the amount of cell death under all three toxic conditions.

The effect was the most severe on 6OHDA primary cultures. Here the survival rate dropped by 33% (p=0.00001). (Survival rate was 49%±5%; (6OHDA control (82%±5))

Pinacidil caused a decrease in cell survival on rotenone treated cultures as well. Here the rate dropped by 20% (p=0.00012). (Survival rate was 15%±3%; (rotenone control (35%±3)))

The effect of pinacidil on MPTP treated cultures was the least severe and not significant (p=0.11112). Nevertheless, cellular survival of the mesDA neurons dropped by 11%.

(Survival rate was 30%±4%; MPTP control (41%±5)))

These results challenge the view of K_{ATP} activation as a means to prevent cell death. Not only is the rate of cell death increased, in comparison to tolbutamide and TEA, pinacidil has a stronger effect on the amount of cell death/survival and affects cells treated with all three toxins and not just rotenone (as tolbutamide) or rotenone and MPTP (as TEA).

3.1.5 Overall influence of drug treatment in toxin models

Given that the three toxins used are all predominantly causing cell death via oxidative stress, the different effects of tolbutamide, pinacidil and TEA are interesting and suggest an induction of cell death by distinctively different molecular mechanisms. The different effects become even more apparent when cell survival rates are compared to the baseline effect of each toxin. Tolbutamide had a significant effect on rotenone treated cultures, improving cell survival by 60%. The slight reduction in cell survival tolbutamide caused on 6OHDA treated cultures is difficult to interpret. 6OHDA is known to have rather broad array of ways to cause cell death, e.g. the generation of quinones, and its toxicity might not be related to K_{ATP} function. TEA enhanced cell survival on rotenone treated cultures by 44% and by 75% on MPTP treated cultures. As a rather unspecific blocker of potassium channels, the strong effect TEA has on MPTP treated cultures might be a sign of a different mode of apoptosis triggered, which is influenced by blocking other types of potassium channels. This is further strengthened by the effect of the K_{ATP} channel opener pinacidil. This drug had a significant negative effect on 6OHDA and rotenone treated cultures, while MPTP treated cultures were relatively unchanged. Cell survival rates dropped by 41% on 6OHDA treated cultures and by 57% on rotenone treated cultures when compared to the baseline toxin effect. This is no simple reversal of the effect of

tolbutamide and channel blocking but suggests that e.g. overall K+ efflux from the cell due to pm K_{ATP} opening or high levels of ROS due to m K_{ATP} activation might lead to a more severe rate of cell death.

Taken together, the negative effect of tolbutamide on 6OHDA cultures, blocking of K_{ATP} channels had a positive effect in the context of oxidative stress of mesDA neurons, while the activation of K_{ATP} channels increased cell death. Other mechanisms than the production of ROS contribute to cell death and the positive effect of TEA suggests these mechanisms either do not to involve K_{ATP} channels, but possibly other types of potassium channels or might provide evidence for K+ efflux from the cell.

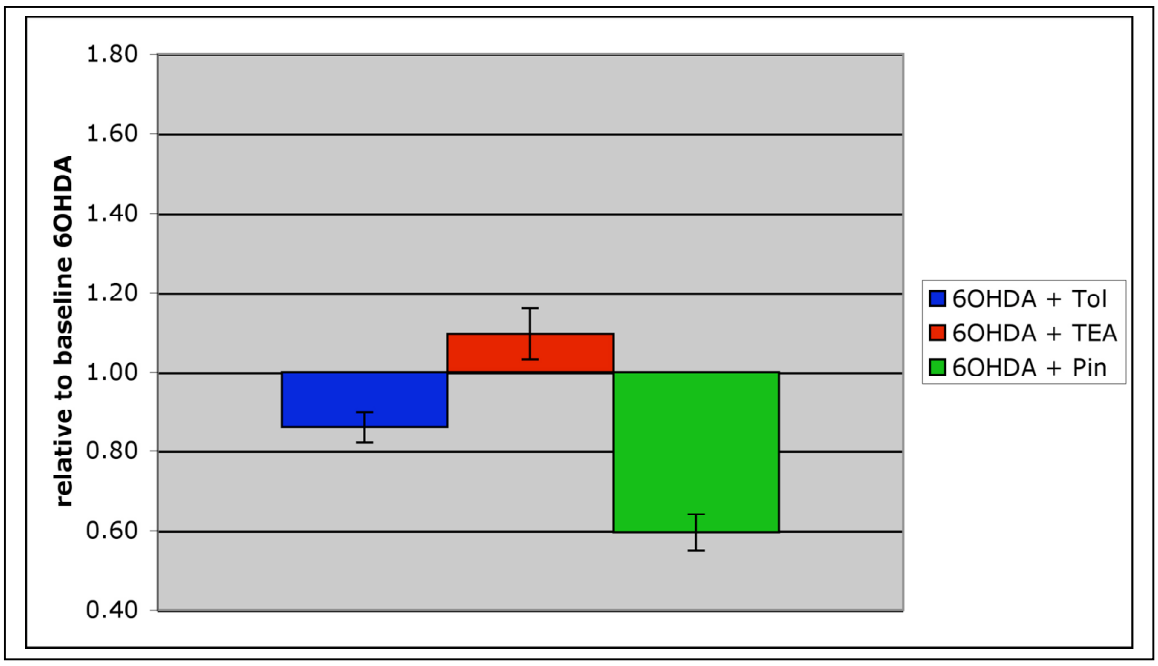


Figure 3.4: Baseline change of cell survival 6OHDA
Compared to baseline 6OHDA effect, cell survival decreased by 14% for tolbutamide, increased by 10% for TEA and decreased by 41% for Pinacidil, the only significant effect.

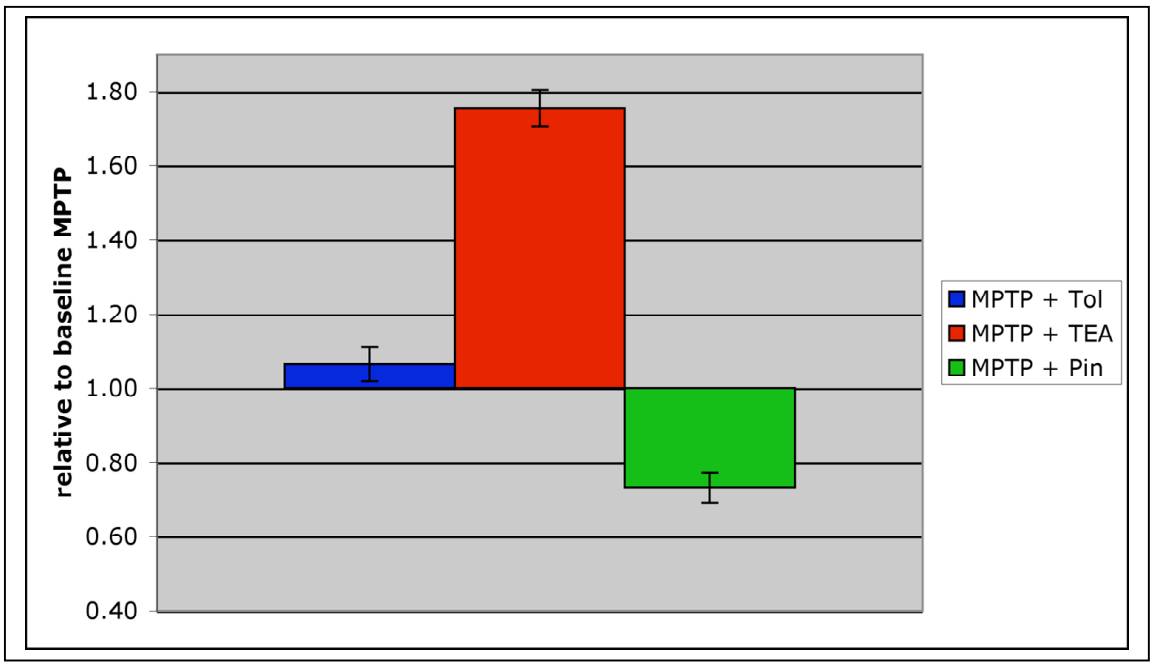


Figure 3.5: Baseline change of cell survival MPTP
Compared to baseline MPTP effect, cell survival increased by 7% for tolbutamide, increased by 75% for TEA and decreased by 27% for Pinacidil. Both the effect of TEA and pinacidil were statistically significant.

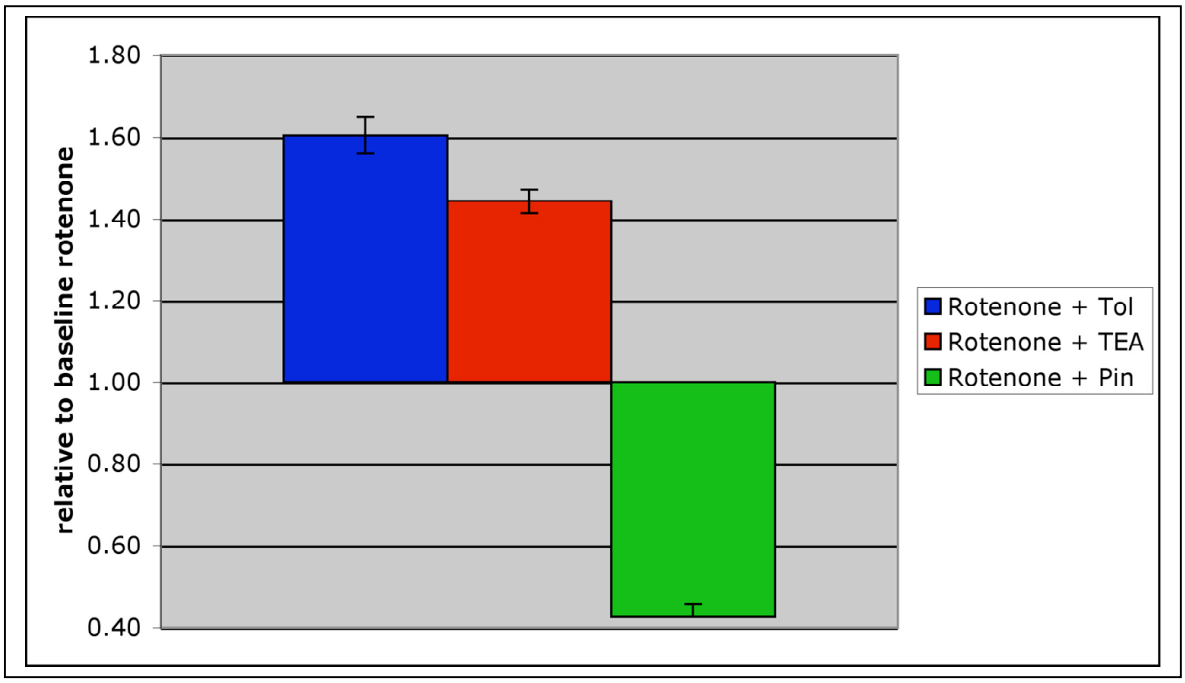


Figure 3.6: Baseline change of cell survival Rotenone
Compared to baseline rotenone effect, cell survival increased by 60% for tolbutamide, increased by 44% for TEA and decreased by 57% for Pinacidil. All effects were statistically significant.

3.2 Targeted deletion of Sur1 rescues En^{HT} mesDA phenotype

Mice carrying a single deletion of engrailed 1 in the background of the full En2 knockout (En^{HT}), loose the SNpc part of the mesDA neurons gradually, starting at around P15 and peaking at three months of age. Mice carrying a homozygous deletion of the Sur1 gene do not express a K_{ATP} channel. Previous results from my lab showed that this viable and fertile mutant has no deficiencies, with regard to mesDA neuron number, distribution and axonal fibre density. In an attempt to transfer the in vitro blocking of K_{ATP} channels to an in vivo situation, En^{HT} mice were crossed with Sur1 mice, left to age for at least 90 days, after which animals were sacrificed and stained immunohistochemically against TH. Sections of mice 19 months and older were compared on a gross morphological level to En^{HT} and wild type mice of similar ages.
The additional homozygous deletion of Sur1 in the background of En^{HT} completely rescues the loss of the SNpc DA neurons that the En^{HT} mice develop till P90. A normal copy of Sur1 (Sur1-/+) is unable to prevent the cell loss. Taken together, this suggests that the mesDA neurons of En^{HT} mice undergo apoptosis following a mechanism which is dependent on K_{ATP} activation. As a single copy of Sur1 is not able to prevent nigral cell loss, the decision is not dose-dependent but probably a choice point for triggering apoptosis. It is also possible that the deletion of a single Sur1 allele is able to slow the SNpc neuronal degeneration. However, in order to elucidate this, animals of a younger age need to be compared to matched En^{HT} controls.

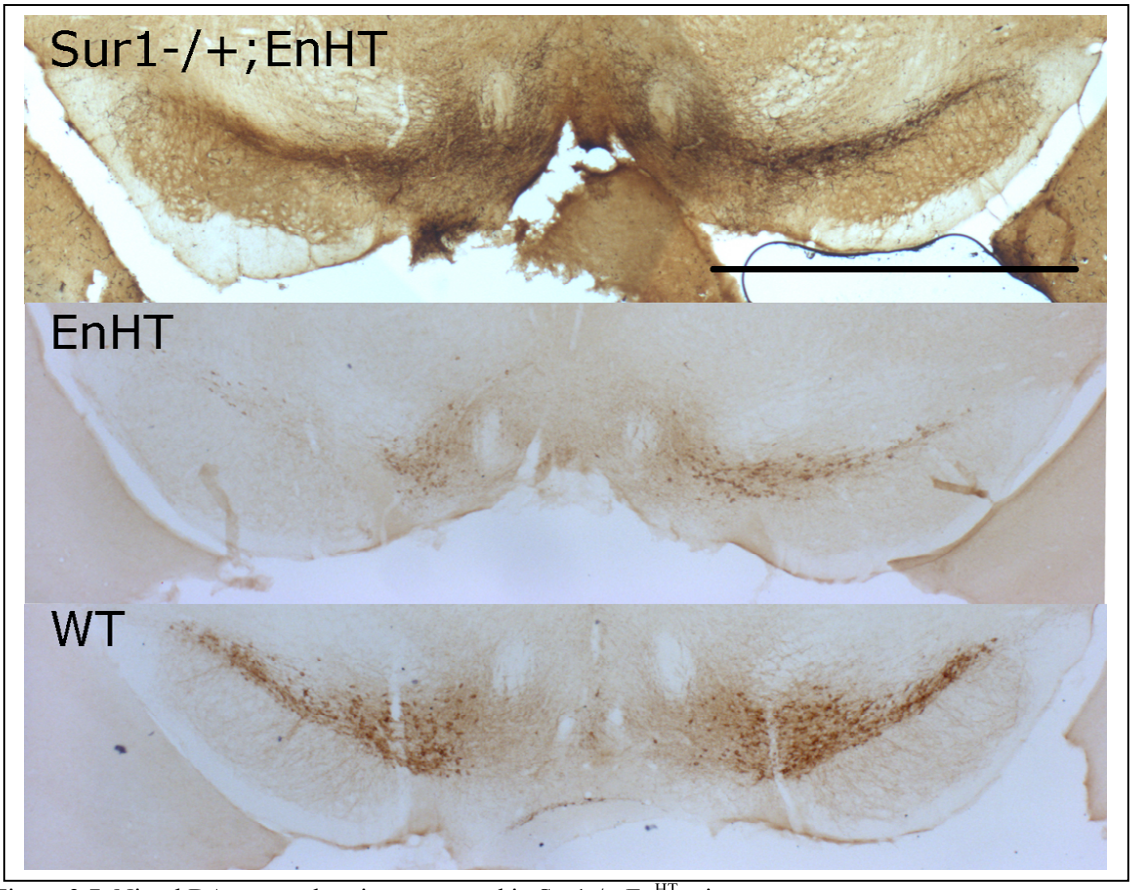


Figure 3.7: Nigral DA neuron loss is not rescued in Sur1-/+;En^{HT} mice
Coronal view adult mouse brain on the level of the SNpc. MesDA neurons are visualized via TH immunostaining. (Sur1-/+; En^{HT}) While the TH staining shows fibers in the shape of the SNpc, there are only few cell bodies. (En^{HT}) As with Sur1-/+; En^{HT}, the neurons in the SNpc are reduced in number. (Wild-type). Normal number and distribution of SNpc neurons. Scale bar is 1mm, dorsal is up.

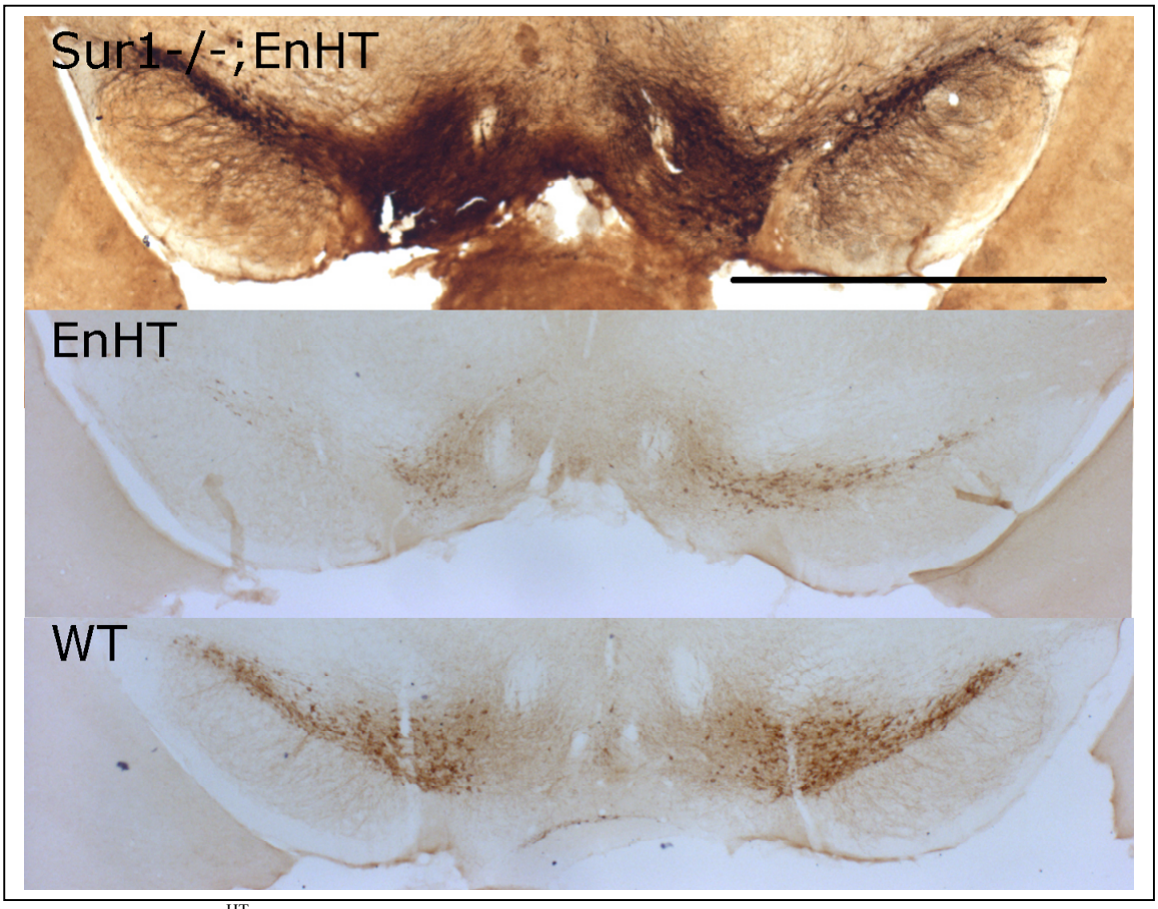


Figure 3.8: Sur1-/-;En^{HT} mice have wild-type like SNpc
Coronal view adult mouse brain on the level of the VTA. MesDA neurons are visualized via TH immuno-staining. Sur1-/-;En^{HT} mice show a normal number and distribution of DA neurons ins the SNpc. En^{HT} mice show an almost complete lack of SNpc DA neurons. Scale bar is 1mm, dorsal is up

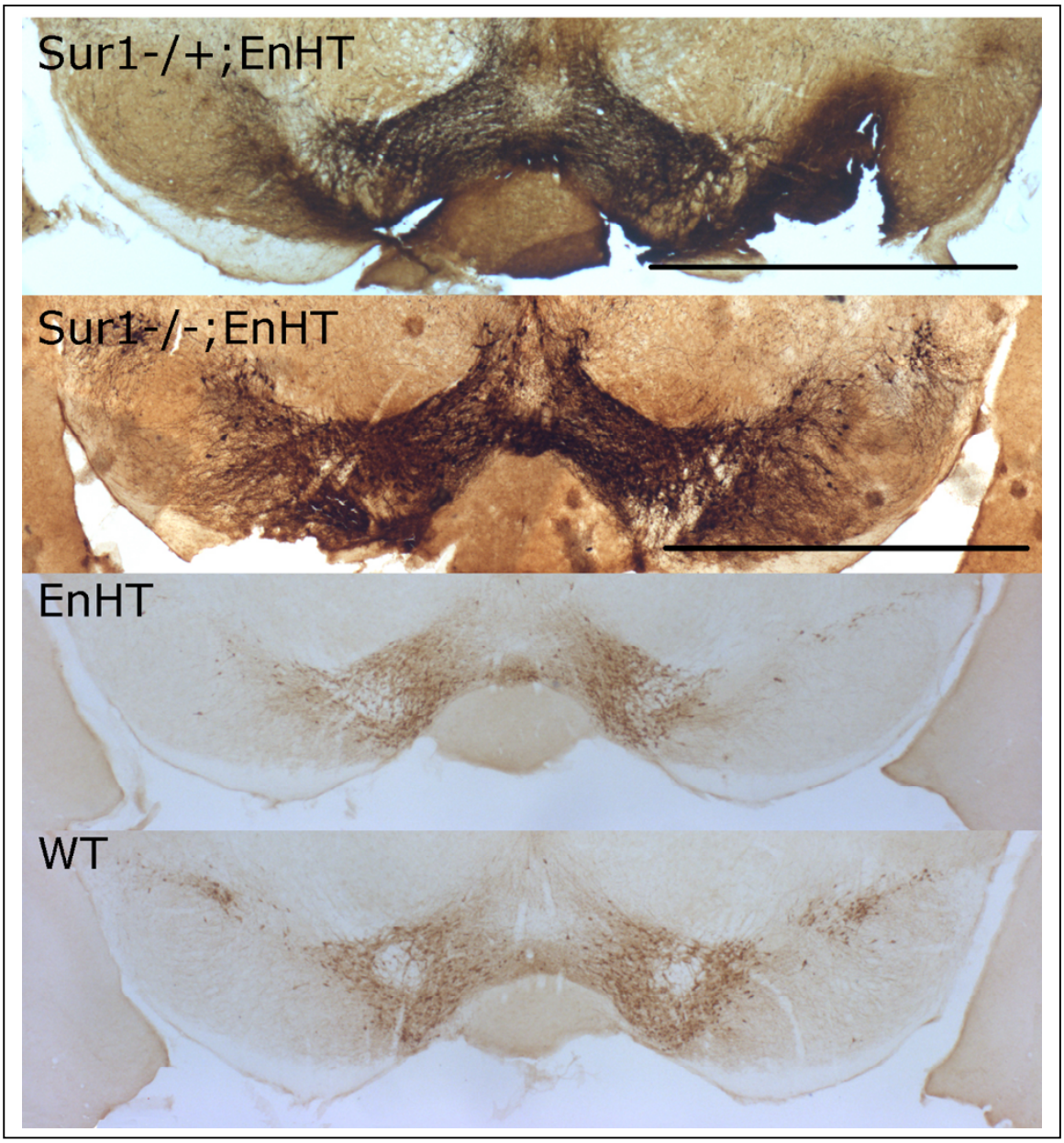


Figure 3.9: Sur1-/+;EnHT and Sur1-/-;EnHT mice have a normal VTA
Coronal view adult mouse brain on the level of the VTA. MesDA neurons are visualized via TH immuno-staining. All animals show a normal number and distribution of VTA DA neurons. Scale bar is 1mm, dorsal is up

3.3 Preliminary results on engrailed mice and K_{ATP} channel action

As a second in vivo model to validate the data from both the cell culture and the Sur1-/-; En^{HT} mice, I investigated the effect of tolbutamide and another K_{ATP} channel blocker, glibenclamide on young En^{HT} mice. In order to compare the amount of cell death occurring in the SNpc of P15 En^{HT} mice to En^{HT} mice in which a K_{ATP} channel blocker had been pumped into the brain for two or four weeks (starting at P15) and because both blockers are not able to pass the blood-brain barrier, it was necessary to use osmotic mini-pumps (Alzet, USA). The pumps were connected via PE tubing to a cannula puncturing the skull and reaching into the lateral ventricle of the pup. The concentrations of tolbutamide (250 µg/day) and glibenclamide (1µg/day) in this experiment was calculated using known clearance rates of both drugs in human (Spraul *et al*., 1989; Veronese *et al*., 1990)**.** We hypothesized that blocking K_{ATP} channels in En^{HT} mice before they start to loose their mesDA neurons in the SNpc will have a positive effect on cell survival. P15 litters were genotyped, the En^{HT} mice were selected for operation and separated into groups which were implanted with pumps containing glibenclamide or tolbutamide or into control groups with pumps containing only the solvent DMSO as a control. Animals were sedated with isofluran and kept on a heated pad to maintain normal body temperature. The pump was implanted into a pocket under the skin on the back of the animals, the connected BrainInfusionKit was then implanted 1mm rostro-lateral of the point of Bregma. Operational procedures had to be modified for mice of this young age. In brief, the concentration of isofluran had initially to be higher (5vol%) to successfully put the mice into deep sedation. Wound clips were not used because the tool for applying the clips proved to be too crude to guarantee successful wound closure without doing harm to the animals. Initial concerns that the mother would not accept the pubs back into the litter after operation were unfounded. Of 33 animals operated, only a single case of separation was registered and in this case it was likely that the animal was suffering from a side effect of the operation and was too weak to join the litter.

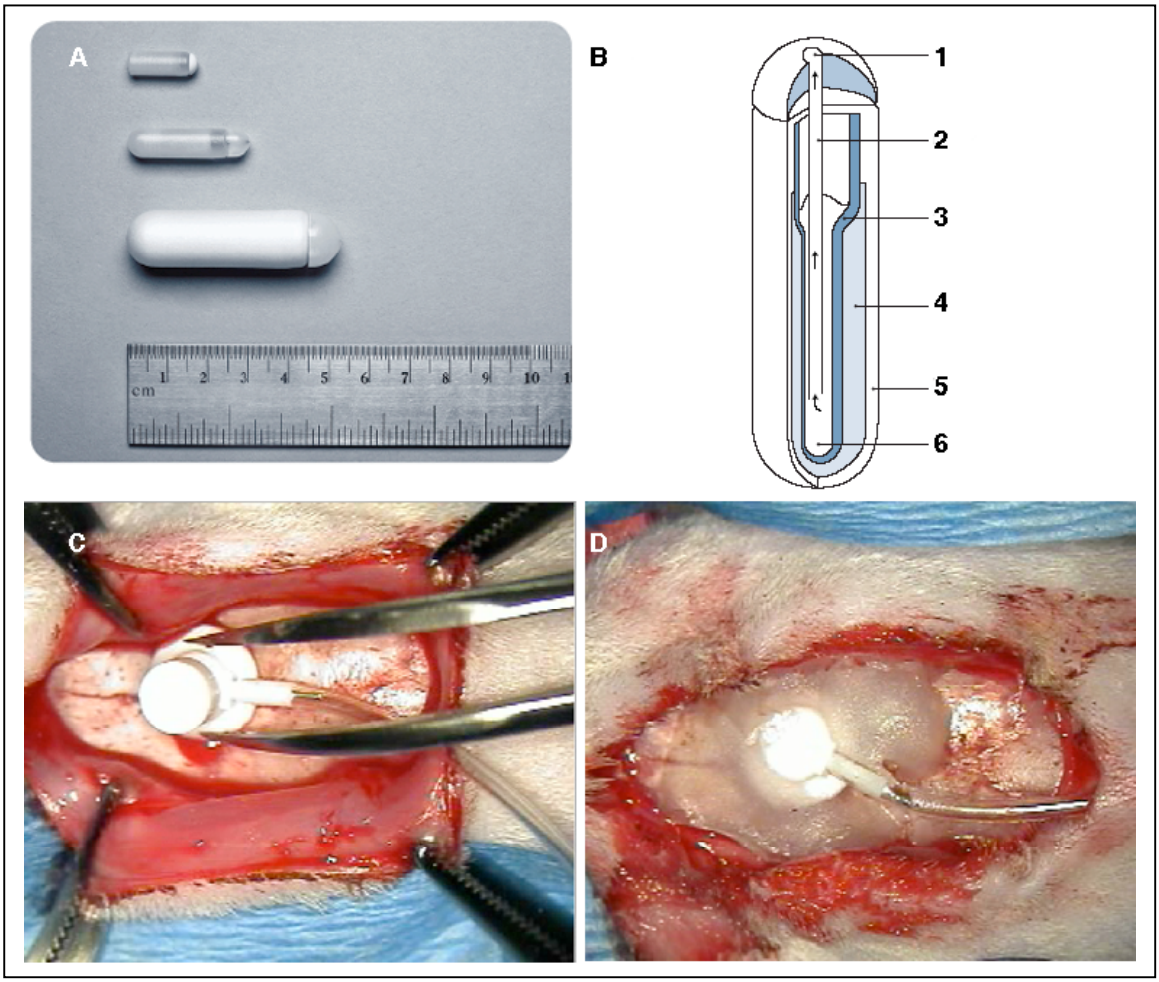


Figure 3.10: Implantation of osmotic mini-pumps
(A) Various models of the Alzet pump. In the experiments the top-most model (1002D) was used. (B) The structure of a pump. 1 = delivery portal; 2 = flow moderator; 3 = impermeable reservoir wall; 4 = osmotic agent; 5 = semi-permeable membrane; 6 = reservoir (C and D) Intraventricular implantation of the cannula, here performed on a rat. (C) After a hole is drilled through the skull, the cannula of the BrainInfusionKit is placed in it. Connected to the cannula is the PE tube leading to the pump. (D) The cannula is fixed on the skull, here with dental cement; on mice, super-glue can be used. The tubing disappears under the skin in the pocket where the pump was placed. The upper part of the cannula will be removed before the skin is closed with wound clips or a suture. (A and B are adapted from documentation available from Alzet, USA. C and D are stills taken from training videos available from Alzet, USA)

The pumps were running for two weeks after which the mice were either sacrificed and dissected or re-implanted with a new pump and left to survive for another 14 days. Unfortunately, the fixation of the cannula (BrainInfusion Kit III, Alzet**,** USA) to the skull

was not optimal for mice of this age. In roughly 80% of the operated mice, the cannula was not attached to the skull anymore after the experimental period. Furthermore, there was excessive necrotic tissue in the bone around the hole in the

skull and in the underlying brain tissue. Most likely, the postnatal growth of brain and skull caused friction between the fixed-in-place cannula and the expanding tissue around it. In consultations with Alzet representatives, there was no knowledge of any trials with osmotic pumps on mice of this age, nor any suggestions on how the problem could be solved. Therefore, this project was modified to use adult mice with osmotic pumps and cause progressive mesDA cell loss via a sub-acute injection of several doses of MPTP (see below). Interestingly, adult mice did not loose their cannulas, nor did any excessive necrosis occur, strengthening my theory about friction being the problem in P15 mice.

3.4 Pharmacological treatment of wild-type mice with osmotic mini-pumps

Similarly to the rationale and technique described in the previous section, adult mice were implanted with an osmotic mini pump containing tolbutamide but then injected with MPTP. Instead of the slow progressive loss of nigral DA neurons in P15 En^{HT} mice, here I wanted to cause mesDA degeneration over a short period of time with MPTP and study the effect of blocking the K_{ATP} channels in the brain with tolbutamide. MPTP can be applied in many concentrations and over various time frames to cause a faster or more progressive cell loss. I used a sub-acute dose of MPTP over the course of three days to generate an incomplete cell death tolbutamide should be able to influence (similar to the primary culture system). The MPTP model was chosen because of the relative ease it can be applied to rodents (Przedborski *et al.*, 2001).

The pump and drug were given two days time to establish a stable flux of solution into the brain. Furthermore, as the animals were given 100 µl (5 mg/ml) of the analgesic carprofen (Rimadyl, Pfizer, Germany) after the operation, the time was necessary to provide a wash-out of the system to make sure that no side-effects from a presence of the analgesic occurred.

On day three, a dose of MPTP (20 mg/kg) was injected intra-peritoneally into the mice once per day for three successive days. Over the next four days, the mice were monitored for changes in health and/or weight. On the fourth day after the last injection, the mice were sacrificed and the striatum was dissected from the brains and stored for HPLC measurement of the dopamine content. The remaining midbrain was

immersion fixed in 4% PFA and stored for later use. Of the first set of mice, five out of six died for unknown reasons. There are common risk factors for adverse effects known in MPTP treatment that include e.g. the use of certain mouse strains, female animals and animals younger than four weeks. All these factors could be excluded in my case as only male animals of three to six months of age were used. However, injecting animals subcutaneously rather than peritoneally had a positive effect on the survival rate of mice, most likely due to the delayed uptake of the toxin through the skin (Przedborski *et al.*, 2001). I was able to operate a second set of mice and achieved about 60% survival this time. Unfortunately, all animals surviving, except one, were part of the control group, carrying pumps with the solvent DMSO. The single animal showed no alteration in the number of mesDA neurons. While it is known that MPTP treatment may have huge variances in outcome, a single animal is not enough to say whether the lack of phenotype comes from this variance or an effect of tolbutamide. Concerning the small number of tolbutamide treated animals which survived, it can not be ruled out that the general blocking of K_{ATP} channels in the whole brain via tolbutamide generates side effects which might enhance the chance of death. However, before I could address this issue, the project had to be put on hold due to safety concerns regarding the housing and injection of the animals.

4 Miscellaneous Results

4.1 Engrailed genes are required for the development of dorsal raphe nucleus/locus coruleus (Simon HH, Scholz C et al., 2005)

Aside from their role in the regionalization of the midbrain and being a survival factor for mesDA neurons, the two engrailed genes were likely candidates for the early specification of four midbrain/anterior hindbrain cell clusters. The precursor and mature cells of the noradrenergic locus coruleus (LC) and L. subcoruleus (NSC) and the serotonergic median and dorsal raphe nuclei (MRN and DRN) lie within the expression domains of both En1 and En2 (Davis and Joyner, 1988).

4.1.1 Serotonergic and noradrenergic cell loss in En^{HT} mice

We could demonstrate that the two engrailed transcription factors are necessary for the proper specification of two of those nuclei, the serotonergic dorsal raphe nucleus (DRN) and the noradrenergic locus oruleus (LC) in a gene-dose dependent manner. Brain sections taken from P0 En1-/-, En1-/-;En2-/- and wild type mice were stained with an antibody against TH to detect noradrenergic neurons and against serotonin (5-HT) to visualize serotonergic neurons.

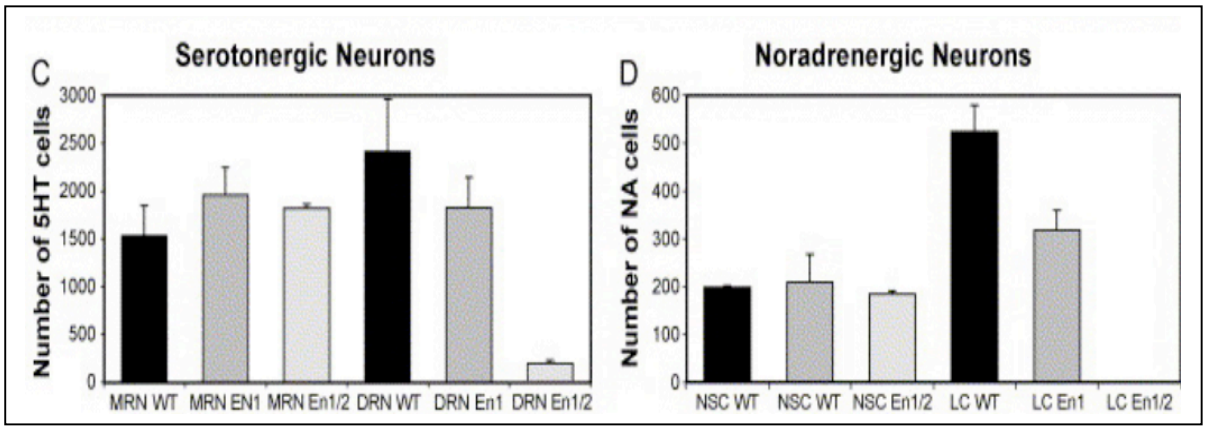


Figure 4.1: Comparison of cell numbers between wild type, En1 and En1/2 mutants.
While the number of cells in MRN and NSC are remain unchanged, there is a gene-dose dependent reduction of cells in the engrailed mutants. (n=2) (second "NSC WT" in D should read "NSC En1"; adapted from (Simon *et al.*, 2005))

To measure the amount of cell loss between neighbouring MRN and DRN and of the LC and NSC the cells were counted under the microscope. While the cell populations of DRN and LC decrease with the number of deleted engrailed alleles, the

respectively adjacent MRN and NSC are unaffected. This suggests that the both DRN and LC are generated from a different subset of cells than their neighbouring nuclei. Furthermore, the gene-dose dependent loss shows that the engrailed genes can compensate for each other, as had been shown for the development of the mesDA neurons (Sgado *et al.*, 2006).

4.1.2 Engrailed is required at an early stage of DRN and LC development

To investigate whether engrailed is present in DRN and LC at early stages of embryonic development, anti-TH and anti-5-HT antibodies in fluorescent immunostainings were combined with an immunostaining against tauLacZ. tauLacZ is part of the construct deleting the start codon of the En1 locus in the mutants and therefore encompasses its wild type expression domain. In fluorescent overlays of the three stainings, I was able to show that cells of the DRN, located in the periaqueductal central gray, do not express engrailed at E12. Even at P0, which was the second time point investigated, they do not express engrailed, though En- and 5-HT-positive cells are co-localized at these ages.

The noradrenergic neurons of the LC are outside the expression domain of the engrailed genes at both ages investigated. This suggests that engrailed is necessary for proper LC and DRN development at a precursor stage rather than for the post-mitotic, differentiated cells of both nuclei, as has shown to be the case for serotonergic midline cells in drosophila (Lundell *et al*., 1996) and mesDA neurons in mammals (Simon *et al.*, 2001).

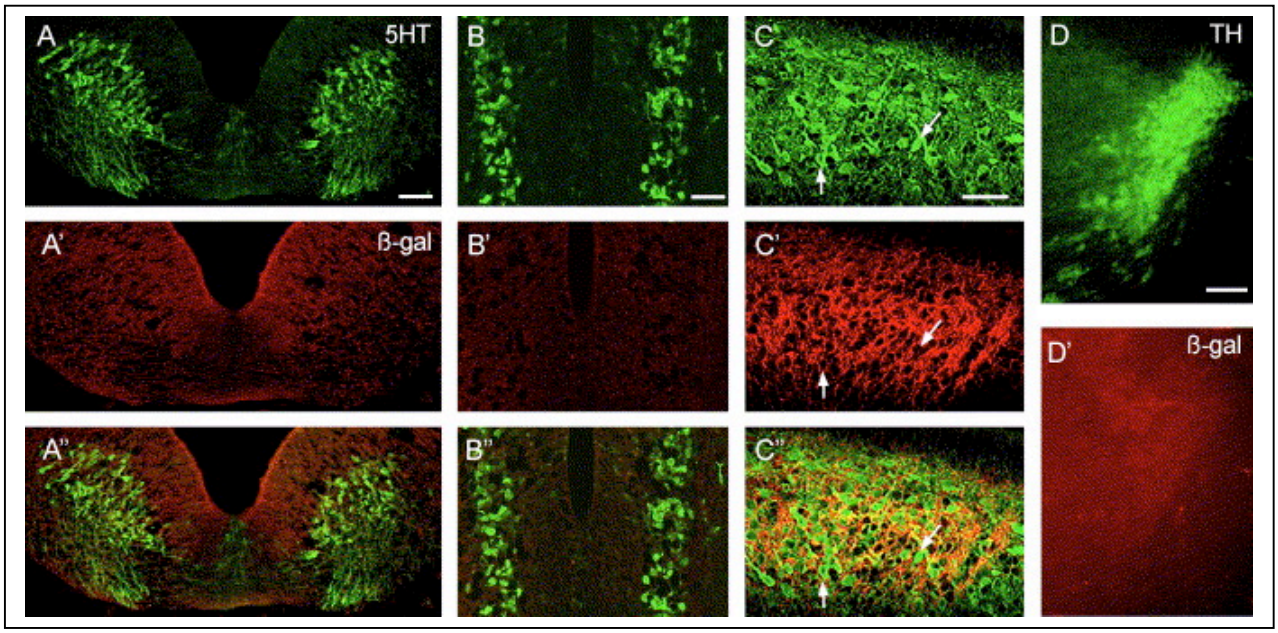


Figure 4.2: Serotonergic neurons of the DRN and noradrenergic neurons of the LC do not express the engrailed genes at E12 and P0.
Confocal images of sections from E12 (A and B) and P0 En1+/tLZ mice (C–C'', D, and D') immunostained with antibodies against 5-HT (A–C), tyrosine hydroxylase (TH) (D), and β-galactosidase (β-gal) (A', B', C', and D'). Merged images (A'', B'', and C''). (A–A'') Coronal section of E12 embryo at the level of rhombomere 1. 5-HT and β-gal are co-localized but are not co-expressed by the same cells. (B–B'') Posterior section of the embryo. 5-HT-positive neurons located outside of the En1 expression domain. (C–C'') Sagittal section through a P0 En1+/tLZ brain. DRN neurons are located in the periaqueductal central gray containing the processes of En-expressing cells. White arrows in A–C mark examples of two serotonergic DRN neurons that are En1 negative. The yellow signals in C'' are processes of En1/tau-LacZ-+ cells and serotonergic neurons overlapping in the Z-plane of the confocal images. (D and D') Double labeling of a sagittal section through the locus coruleus of an En1+/tLZ brain using antibodies against TH (green) (D) and h-gal (red) (E). The TH-positive neurons in the locus coruleus do not express En1. Dorsal to the top, rostral to the left, scale bars = 100 μm (Simon *et al.*, 2005).

4.1.3 Deficits of the DRN stem from a loss of precursor cells

Finally, we showed that the deficits in the DRN of P0 engrailed mutant mice are due to a loss of precursors and not the result of a re-specification event. In-situ hybridizations with a probe detecting transcripts of the ETS domain transcription factor Pet1 revealed, on a gross morphological level, a reduced number and distribution of prospective serotonergic cells in the DRN at E12. This suggests that the cells are lost in the mutant and have not entered another cell fate. Pet1 expression precedes the appearance of serotonin for about twelve hours and is essential for the development of serotonergic neurons in the CNS (Hendricks *et al.*, 2003).

In wild type mice, it marks the entire extent of the serotonergic hindbrain raphe nuclei (Hendricks *et al.*, 1999). The noradrenergic neurons of the LC could not be detected at E12 in En1/2 mutants even though they appear as early as E11 in the posterior midbrain of the wild type (not shown).

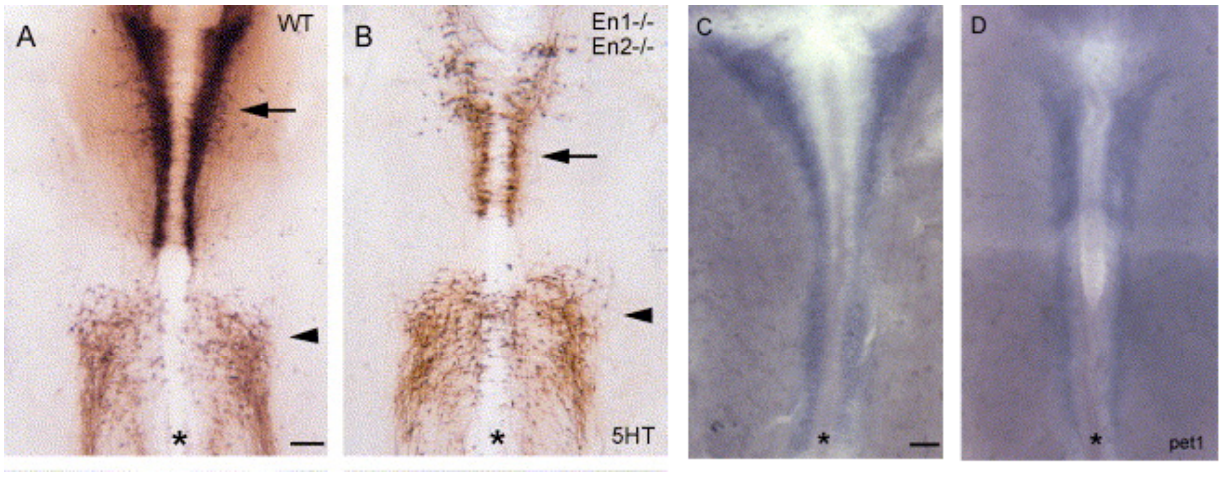


Figure 4.3: Population of serotonergic neurons is diminished as early as E12 in the engrailed double mutants.
Immunostaining using antibodies against 5-HT (A and B) and in situ hybridizations using riboprobes against Pet1 (C and D) on flattened hindbrain whole mounts dissected from wild type (C) and En1/2 mutant embryos (D). (A and B) In E12 wild type, bilateral stripes of densely packed serotonergic neurons (arrow) are located adjacent to the floor plate. These cells will give rise to the DRN and MRN. In the E12 engrailed double mutant, the domain of 5-HT-positive neurons in the rostral hindbrain is significantly reduced (arrow), whereas a more caudally located group of serotonergic neurons (arrowhead) is unaffected. (C and D) Pet1 in situ hybridization reveals the same pattern. A smaller rostral domain and a normal distribution of Pet1-positive cells caudally indicating that the serotonergic neurons, which give rise to the DRN are not generated in the En1/2 mutant embryos. The asterisk marks the midline. Anterior is to the top. Scale bar = 200 μm. Sections in A and B were processed by HH Simon. Adapted from (Simon *et al.*, 2005).

Taken together, the loss of LC and DRN points to a requirement of the engrailed genes for the proper development of the two nuclei. Furthermore, since the adjacent nuclei, the locus subcoruleus and the median raphe nucleus, are unaffected by the deletion, these nuclei must arise from different pools of precursor cells than locus coruleus and dorsal raphe nucleus.

4.2 In silico design and generation of Foxa1 knock-in targeting construct (technique described in (Scholz *et al.*, 2006))

Foxa1 became focus of our research after the initial differential display experiment done in our lab. Between ventral midbrains of E12 En1/2 mutant mice, wild type, E14 wild type and adult olfactory bulb, the gene was found to be expressed in both wild type and engrailed mutant tissue but not in wild-type olfactory bulb, which comprises an unrelated population of DA neurons (Thuret *et al.*, 2004). In order to determine if Foxa1 is necessary for proper mesDA development, the number of TH+ cells in the ventral midbrain of P0 Foxa1 mutant mice (Kaestner *et al.*, 1999) and their axonal density in the striatum was determined. Furthermore, it was investigated if the expression of other mesDA specific genes was changed. In both cases, no difference between Foxa1 and wild-type mice could be detected (unpublished results). The inactivation of the Foxa1 gene leads to an early death of the affected mice between P2 and P12, caused by hypoglycemia due to down-regulated glucagon levels (Kaestner *et al.*, 1999). Therefore, a requirement of the adult animal for Foxa1 in the mesDA neurons might not be visible until later in life, similar to what can be seen in engrailed mutant mice (Sgado *et al.*, 2006).

To circumvent a Foxa1 requirement in other organs, I generated a construct for gene targeting, to produce a mouse with loxP (locus-of-X-over-P1) sites flanking exon 2 of the Foxa1 gene, the same exon targeted in the original full mutant (Kaestner *et al.*, 1999). LoxP sequences are recoginition sites for the Cre (cyclization recombination) recombinase. This recombinase recognizes the loxP sites and cuts out the DNA the loxP sites flank (Orban *et al.*, 1992). By breeding the Foxa1 loxP knock-in mice with mice expressing Cre under the control of a mesDA specific gene it would have been possible to obtain mesDA specific Foxa1 mouse mutants.

4.2.1 One step cloning of DNA framents of defined size

The underlying technique to generate the necessary DNA fragments of the Foxa1 gene to clone the knock-out construct makes use of the fact that the mouse genome is fully sequenced and the sequence published (Gregory *et al.*, 2002). BAC libraries of human, mouse and rat, each covering the respective genomes more than 10 fold

(Osoegawa *et al.*, 2000), are stored as individual clones in the Children's Hospital Oakland Research Institute (CHORI). The BAC clones comprising the sequences of interest can be identified by their end sequences (Zhao, 2001; Zhao *et al.*, 2001) via BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1990), and bought from the CHORI (http://bacpac.chori.org/) for a nominal fee. With the DNA sequence known, I could map restriction sites to it and plan digests so that the resulting fragments could directly be used in the cloning of e.g. knock-out constructs. To obtain said fragments, after restriction digests the whole pool of fragments was cloned into a standard Bluescript II SK (-) vector (Stratagene).

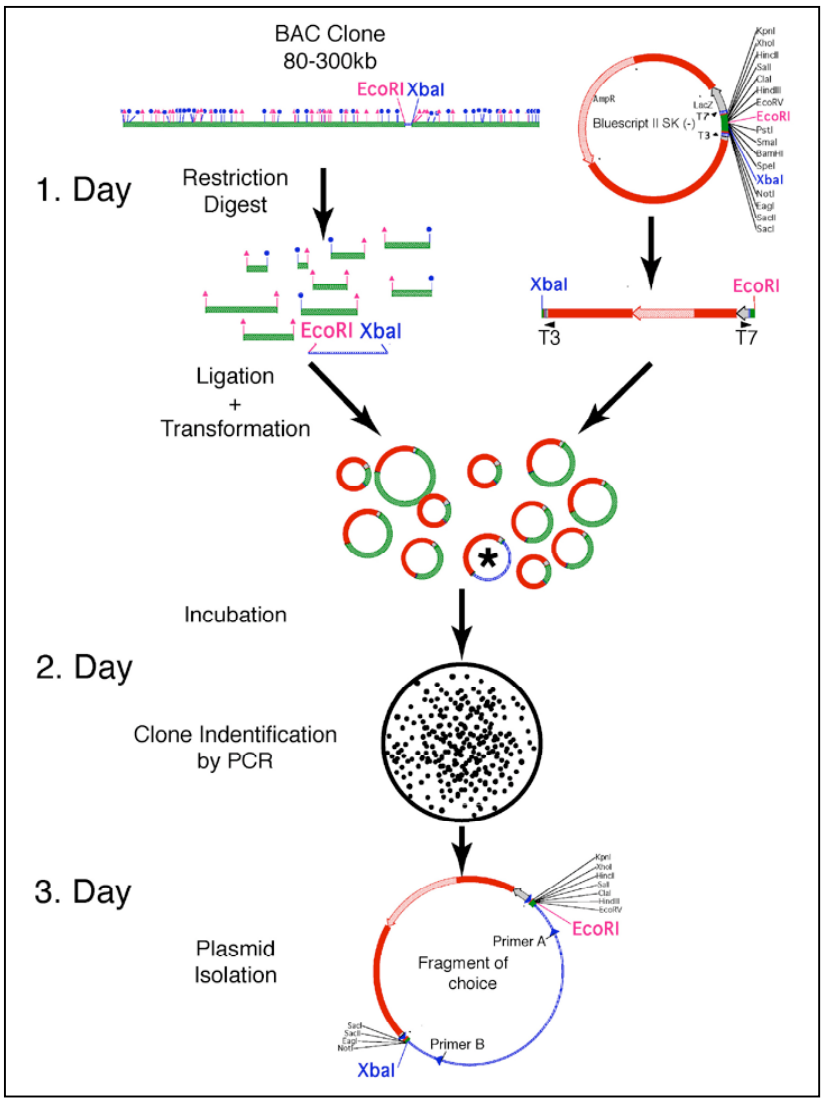


Figure 4.4: One-step cloning of BAC fragments
Example of the strategy to obtain a defined genomic fragment from a BAC clone. Day One: The BAC clone is cut with restriction enzymes flanking the fragment of interest (XbaI and EcoRI). All resulting fragments are subcloned into a vector (Bluescript II SK(-)) which was cut with the same enzymes. After ligation and transformation, the bacteria are plated out and grown overnight. Second Day: The individual clones are picked and subjected to PCR with one primer specific for the targeted DNA fragment, the other for the vector (T7 + Primer A and T3 + Primer B). The positive clones are grown overnight and the plasmid DNA is isolated at the third day (Scholz *et al.*, 2006).

One day later, I picked colonies and the one carrying the fragment was identified by a standard PCR using fragment-specific primers plus a primer specific for the T3 promotor on the vector. After the PCR reaction, the positive clones were placed in 1-2 ml of LB medium and incubated over night. On the next day, the plasmids were purified by cell lysis and precipitation and analyzed by restriction digests.

Following this procedure, it required the picking of 25 to 50 individual clones to identify a positive plasmid, no matter if one or two enzymes were used for the digestion of the BAC clones. The number of clones I had to analyze by PCR amplification to obtain the targeted DNA was lower than expected, if one assumes that all fragments generated by the restriction digests are equally likely to be inserted into the vector. There may be a bias for insertion of fragments of 1kb and larger, however, I did not test this further. In 2 out of 24 experiments, even extensive screening of more than 300 colonies yielded no result, however, a repetition employing different restriction enzymes was in both cases successful. It is likely that the sequence information was inaccurate, but other reason cannot be excluded. In total, 17 fragments of six different genes were isolated following this technique.

Gene	BAC Clone ID (TIGR)	Enzymes	#	Positions	Size kb	Primer	pBs
Drd2	RP23-416F20	EcoRI	38	119219 to 124483	5,2	TGGGAAAGGGCTACAGCAT	T7
		EcoRI/XbaI	52	115249 to 118123	2,9	GAGGATCATGGGAGGGGAC	T7
		XbaI/AvrII	52	118123 to 119796	1,7	GCCTGAAACTCCTGTTGCT	T3
Foxa1	RP23-91E17	EagI/AscI	3	177512 to 180868	3,3	ATTGATCTTGGGGAAAGGTT	T3
		ClaI/XhoI	4	180678 to 183368	2,7	CACACCCGAGGGCTCA	T3
		HindIII	67	173869 to 181355	7,5	CGCCTCAGTCCACTCCA	T3
		BamHI	36	185555 to 188790	3,2	GGATCTAGCCTGCCGAATC	T3
		EcoRI/EagI	58	107638 to 112173	4,5	CCCGTGTTGGCGTAGGACA	T7
Pbx1	RP24-293G19	XhoI/EcoRI	16	372141 to 380000	7,9	TTTAAATTTGGGTAAGATTC	T7
Dcc	RP23-4L9	BamHI	56	80066 to 87352	7,2	AATACCTGGGCTTGGAAC	T7
		BamHI/NheI	81	80066 to 87352	7,2	AATACCTGGGCTTGGAAC	T7
		XbaI	58	56496 to 62426	5,7	TGGTTAAAGAAGGTGGCT	T7
p75	RP24-347P1	XhoI	10	69790-73585	3,6	GCTAAGTGGGCTGCTCAG	T7
		SmaI	19	18896-24615	5,7	GTTATGGTTGGCTAGGGA	T7
En1	RP23-2751222	EcoRI/XhoI	18	6468 to 10215	3,9	TGGGCAAGGGAATCAGC	T7
		XhoI/XbaI	23	10215 to 14529	4,3	GCGCATCCTCCAAGAGACT	T7
		XbaI/NarI	15	14529 to 19904	5,4	GCTGACCTCTTACGCTTCTT	T7

Figure 4.5: Examples of isolated mouse genomic DNA fragments

The table contains the gene names, the identifier for each TIGR BAC clone, the restrictions enzymes which were used for the digests, the number of fragments produced by each digestion (#), the exact numeric position of the isolated fragment in the BAC clone, their sizes in kilobases the primers used for the PCR reactions together with the T7 or T3 primers. En1 = engrailed-1 Foxa1 = fork head box A1 transcription factor DDC = deleted in colorectal carcinoma DRD2 = dopamine receptor 2 Pbx1 = pre-B-cell leukemia transcription factor 1 pBs = Bluescript II SK (-). T3 primer sequence: GTAATACGACTCACTATAGGGC, T7 primer sequence: AATTAACCCTCACTAAAGGG (Scholz *et al.*, 2006)

4.2.2 Designing and cloning the Foxa1 targeting vector

Applying the technique described in the previous section, I used restriction digest fragments of BAC clones RPCI-23-91E17, AZ244633 and AZ246635 to design a targeting construct which included the entire coding sequence of exon 2 and approximately 7.5 kb of flanking intronic sequence cloned into a modified pFLRT vector (kindly provided by Prof. Rüdiger Klein, Munich). The multiple cloning site in this pFLRT.m2 vector contain additional unique restriction sites. The positive selection marker PGKneo was flanked by two Flipase Recognition Target (FRT) recombination sites.

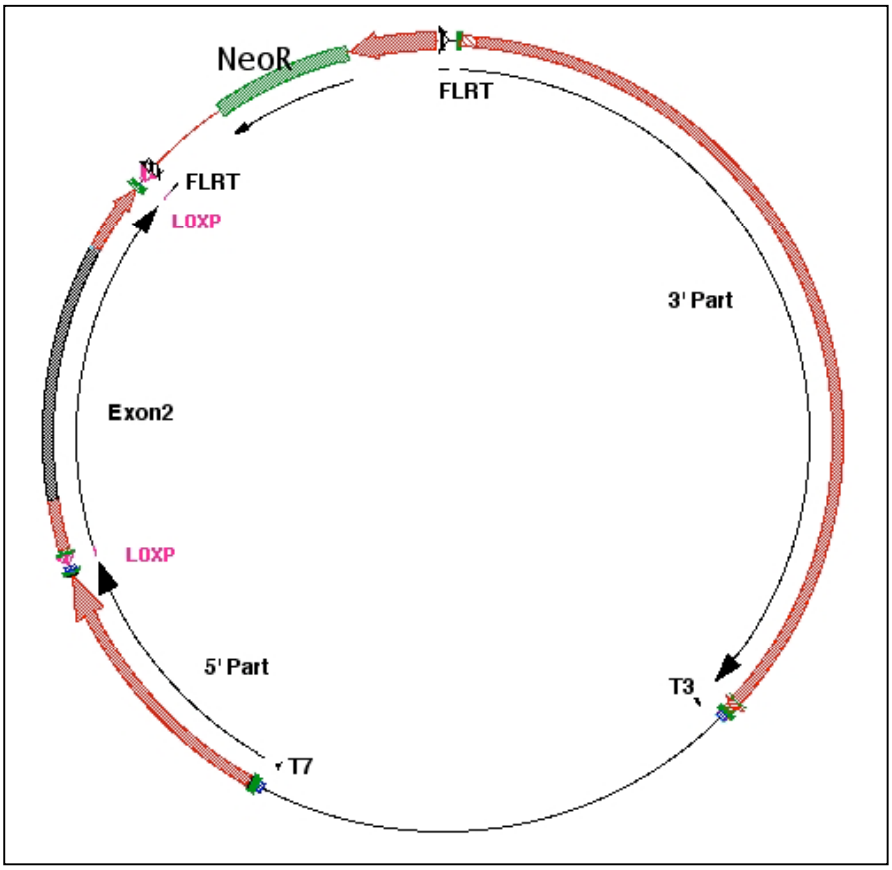


Figure 4.6: The Foxa1 loxP knock-in construct. Exon 2 of the Foxa1 gene is flanked by two loxP sites. The Neo sequence is flanked by two FLRT sites making a removal of Neo possible, if necessary. 4.2 kb of 5′ and 7 kb of 3′ flanking sequence complete the construct. The total size is 14.3 kb.

In a process analogous to the one used in the CRE/loxP system, the PGKneo sequence could have been removed by crossing the FRT carrying mice with a strain universally expressing recombination enzyme (FLP or Flp). This strategy is similar to the one described by (Long *et al.*, 2003) where a mammary gland specific deletion of both ErbB4 alleles was generated. A low-passage aliquot of TBV-2 embryonic stem cells (generously provided by Prof. Wolfgang Wurst, Munich) was then grown on MitoC

growth inhibited fibroblasts and maintained in an undifferentiated state by the addition of LIF (leukaemia inhibitory factor). The first electroporation of $22.5x10^6$ cells with a NotI linearized Foxa1 loxP construct yielded close to 100 colonies after 10 days of G418 (neomycin) selection, out of which 60 were picked based on their apparently undifferentiated state and size. They were analyzed via Southern blot to detect cells in which the construct had replaced the wild type sequence.

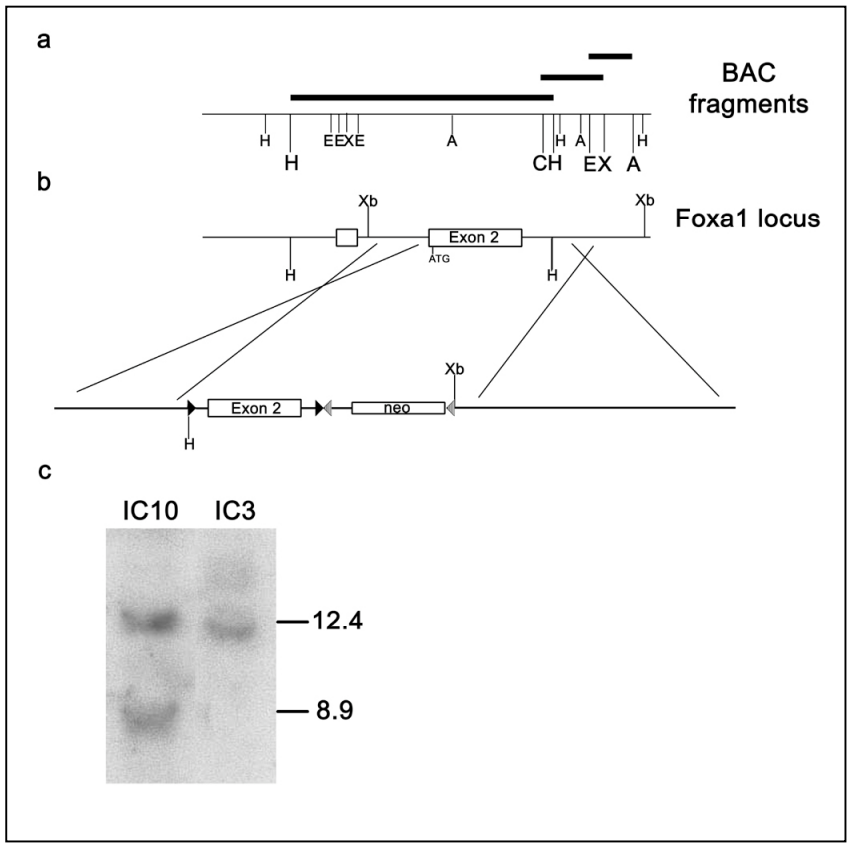


Figure 4.7: Generation of Foxa1 loxP knock-in mice. (a) BAC restriction digest fragments used for designing the targeting vector. Three BACs were digested and screened for. Together, the three genomic fragments cover exon 2 of the Foxa1 alpha gene as well as 4.2 kb of 5´ and 7 kb of 3´flanking sequence. (b) Structure of the mouse Foxa1 locus. The Foxa1 loxP knock-in targeting vector is shown below. Abbreviations: A, AscI; B, XbaI; C, ClaI; E, EagI; H, HindIII; X, XhoI; neo, PGKneo G418 resistance cassette used for positive selection flanked by FRT recombination sites (shaded triangles). Black triangles, loxP recombination sites flanking exon 2. (c) Radioactive Southern blots of XbaI digested genomic DNA from Tbv-2 ES cells that have undergone recombination. An 3´external probe was used to screen for correctly targeted integration by Southern Blotting. The wild-type allele of Foxa1 generates a band with the predicted size of 12.4 kb with this probe, whereas the mutated allele gives a band of 8.9 kb. The 12.4 kb wild-type band is present in both samples. A 8.9 kb band is present in the left sample, showing a correctly targeted Foxa1 alpha locus.

Day 0:	plating of MitoC treated feeder cells (fibroblasts)
Day 1:	plated $2.5x10^6$ TBV-2 ES cells on 6 cm MitoC feeder plate
Day 2:	grew to confluency
Day 3:	split 1:6 on 9 cm MitoC feeder plates
Day 4 and 5:	grew to confluency
Day 6:	pooled 3 plates for electroporation, pooled 3 plates for later use, $22.5x10^6$ cells were used for electroporation
Day 7:	begin of neomycin selection of ES cells
Day 10:	cells without recombinant DNA begin to die
Day 16:	picking of ES cell clones of appropiate size and shape into 96 well plates for storage and 24 well plates for growing and use for genomic Southern blots
Day 20+:	isolation of genomic DNA, begin of Southern blots

Figure 4.8: Timeline for the ES cell culture and Foxa1 loxP electroporation
ES cells were maintained in an undifferentiated state by the addition of LIF to the medium. Electroporated ES cells were given 24 hours time before begin neomycin selection. After selection, colonies were picked according to their undifferentiated state and appropriate size.

Radioactive Southern blots were performed on XbaI digested genomic DNA obtained from the colonies. A 3´ external probe was used to screen for correctly targeted integration. The wild-type allele of Foxa1 generates a band with the predicted size of 12.4 kb with this probe, whereas the mutated allele gives a band of 8.9 kb.

Out of 60 colonies picked, 5 colonies showed a correctly positioned insert on one arm of the sequence, however, blots with probes against the 5' arm were all negative. After reducing the probes size and using different parts of the probe sequence, I came to the conclusion that the construct, while correctly inserted, had an unknown sequence next to it. Although I repeated the experiment and picked another number of colonies, I could not analyze and proceed with the project, and shifted my focus on the research of the K_{ATP} channels and their function under oxidative stress.

ES colonies after electroporation	ca. 100
ES cell colonies picked	60
Positive 3' Southern blot	5
Positive 5' Southern blot	0

Figure 4.9: Summary of ES clones analyzed
Of about 100 ES cell colonies present after neomycin selection, 40 colonies contained or consisted of differentiated cell. These were not picked.

4.3 ChIP assay to verify binding of Pbx protein to AADC promoter

The TALE class transcription factor Pbx1a has long been implicated in altering engrailed protein function in the midbrain (Gemel *et al*., 1999). In an in-silico promotor study performed in our lab, a putative binding site of Pbx1a on the promotor of the l-aromatic amino acid decarboxylase (AADC) gene was found (unpublished results).

AADC is part of the biosynthesis chain of dopaminergic neurons and converts L-dihydroxyphenylalanine to dopamine. It is regulated by Nurr1 (Hermanson *et al*., 2003; Smits *et al*., 2003).

To verify in vitro binding of Pbx1a to the AADC promoter, I performed a chromatin immunoprecipitation assay (ChIP assay) on DNA/protein harvested from cells of the murine neuroblastoma cell line N2A. To assure Pbx is expressed, we used a transfected N2A clone which expresses both engrailed and Pbx1a under the control of the TetON system (Gossen and Bujard, 1992). The TetOn system comprises a plasmid which expresses a tetracycline-controlled transactivator which in turn is able to drive the expression of a target gene in the plasmid in the presence of an effector, doxycycline.

To compensate for low DNA concentrations which may arise from low cell numbers, two nested primer pairs were designed (see Materials and Methods). The specifity of the primers was tested on mouse genomic DNA. 1 µl of a 50 µg/ml sample was used in PCRs with an annealing temperature ranging from 58 to 64°C. Both primer pairs produced a product of expected length at every temperature, although at 64°C it was weaker. Therefore and to guarantee optimal annealing, the annealing temperature was set to 57°C in all following PCRs.

N2A cultures were incubated with 5 mg/ml doxycycline (D9891, Sigma-Aldrich, Germany) for 24 hours, after which +dox culture and –dox control were harvested and processed according to manufacturers instructions (Cat.No.: 17-295, Upstate, USA). In brief, cells are fixed and lysed, then the DNA (with bound proteins) is sonically shreddered and incubated with an anti-Pbx antibody (sc888, SantaCruz, USA) bound to an agarose matrix. Bound DNA-protein-antibody-matrix is collected and the DNA is purified. The histones were removed via a high-salt/high-temperature step. Before and after sonification, samples were set aside to test for presence of AADC sequence via PCR, to be able to see whether the sequence was still contained in the solution. 2

and 4 µl of each step, including the final eluation, were used in a PCR with the two outer primers. 2 µl of these first two reactions were then used in a PCR with the two inner primers, producing four product lanes per product set aside.

In both –Dox and +Dox samples, the AADC sequence could be amplified from all samples at the expected size of 209 bp, but not from the eluate, suggesting that Pbx1a does not bind to the AADC promotor under these conditions. However, lacking an appropriate positive control, a failure of the assay itself can not be excluded. The use of the Pbx-overexpressing N2A cell line might also be problematic as it could theoretically produce secondary effects of protein aggregation and steric blocking or might contain methylated and therefore unresponsive DNA sequences.

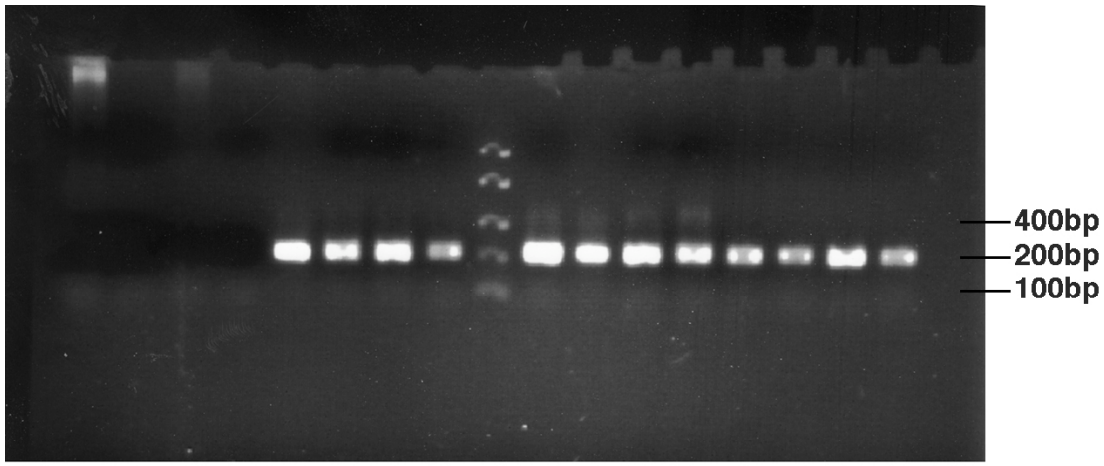


Figure 4.10: Gel of PCR after ChIP assay

Before and after sonification samples of the solution were set aside and run in a PCR with outer and inner AADC primers. DNA not bound to the column was used as well. In both –Dox and +Dox samples, the AADC sequence could be amplified from all samples at the expected size of 209 bp, but not from the eluate, suggesting that Pbx1a does not bind to the AADC promotor under these conditions.

4.4 Lmx1b heterozygous phenotype of aged animals

Ever since the LIM homeobox transcription factor Lmx1b had been implicated to be important for mesDA development (Burbach *et al.*, 2003), a role for this factor as a survial factor could not be excluded. As homozygous mutant mice die within 24 hours after birth (Chen *et al.*, 1998), I therefore investigated aged animals heterozygous for Lmx1b (-/+) for a loss of TH+ neurons in the ventral midbrain. Animals of about 22 months of age were sacrificed, the brains were isolated, cut as floating sections and stained with an anti-TH antibody (as described in the Materials and Methods section of this work). On a gross morphological level, no differences in the amount of the cells in the SNpc and VTA or the density of axons in the striatum could be seen. This suggests that unlike the engrailed haploid insufficiency, a reduced expression of Lmx1b does not lead to a loss of mesDA neurons in mice. Since I was not interested in a possible slight change in cell numbers, they overall mesDA cell number of the mice was not determined.

Figure 4.11: Lmx1b -/+ mice have a normal mesDA system
Lmx1b+/- mutant mice exhibit a normal number and distribution of DA neurons in the SNpc and VTA. Below as comparison the reduced SNpc of a EnHT mouse and normal SNpc of a wild type mouse. Scale bar is 1mm, dorsal is up

Discussion

5 Discussion

The nigral dopaminergic neurons exhibit a specific vulnerability to cellular stressors, which becomes apparent in the degeneration of these neurons in Parkinson's Disease. The Fox genes are expressed by mesDA neurons and regulate the expression of K_{ATP} channels in this neuronal population. Given their well-known role as regulatory elements in cellular stress and energy metabolism, they are likely candidates for having an influence on mesDA cell death. Study of their effect under defined conditions of mesDA challenge may provide insights into the molecular mechanism of the nigral cell death.

In this work, I present evidence for a negative effect of K_{ATP} channel activation on mesDA neurons under oxidative stress. On E12 mouse primary cultures of ventral mesencephalon, the opening of K_{ATP} channels via pinacidil increased the rate of mesDA cell death under oxidative stress, no matter if 6OHDA, MPTP or rotenone was used. Conversely, a forced blocking of the K_{ATP} channels by tolbutamide or by an unspecific potassium channel blocker, tetraethylammonium, is leading to an increased rate of cellular mesDA survival.

Furthermore, the differential effect the drugs have on the cultures treated with the three toxins provides evidence that distinct molecular mechanisms of inducing cell death are activated by each toxin, even if all induce apoptosis.

An in vivo model of gradual mesDA loss, the En^{HT} mouse, provided me with a markedly different type of environment. Here, I demonstrate that inactivation of the K_{ATP} channel by crossing En^{HT} mice with the K_{ATP} channel defective SUR1 mutant rescues the progressive mesDA cell loss occurring in these animals.

Taken together, the data provided in this work highlights the importance of K_{ATP} channel function in mesDA neuron stress and further understanding of the specific vulnerability of this cell population to stressors. This mechanism might help in the development of treatments against the neuronal degeneration occurring in Parkinson's Disease.

5.1 K_{ATP} channel function in cellular stress

5.1.1 pmK_{ATP} activation in hypoxia and ischemia

In the work I present here, blocking of K_{ATP} channels of the dopaminergic neurons of the ventral midbrain, leads to a partial rescue when these cells are under oxidative stress caused by MPTP or rotenone. This mechanism seems not to be present in other cell types or might depend on the circumstance of the challenge.

There, activation of K_{ATP} channels on the plasma membrane (pm K_{ATP}) at low ATP levels is known to lead to a hyperpolarization and consequently to a reduced frequency of action potentials, which are very energy consuming (Nichols, 2006). In short, the cell saves energy by becoming more unresponsive. Intuitively, one might think that opening of pmK_{ATP} channels has therefore a positive effect on cell survival, especially in a situation where the cellular energy supply is impaired, as is the case in ischemia or hypoxia.

Indeed, after hypoxia in cardiac myocytes has taken place, ATP levels drop and the opening of pmK_{ATP} channels on the cell shortens the its action potentials (Nichols *et al*., 1991). K_{ATP} channel opening in vascular smooth muscle cells in reaction to oxygen shortage leads to vasodilatation (Quayle *et al*., 1997). Both mechanisms show a compensation of the open K_{ATP} channel in reaction to energy demand. The cardiac cell saves energy through less work, the blood vessel dilates to enhance the rate energy and oxygen can be supplied to muscle tissue. This has been used to strengthen cells against a strong ischemic insult. A short withdrawal of oxygen followed by reperfusion leads to an improved cell survival rate after a subsequent long ischemic pulse (Gok *et al*., 2006). Pharmaceutically, this ischemic preconditioning (IPC) can be triggered by K_{ATP} channel openers (KCOs) like pinacidil and others.

5.1.2 m K_{ATP} channels in hypoxia and ischemia

Similarly, K_{ATP} channels on the inner membrane of the mitochondria are known to affect cellular survival by opening under conditions of cellular energy shortage. The mechanism of this effect is still a matter of discussion. When the mitochondrial membrane potential is collapsing due to outside influence like oxygen deprivation or toxins, the potassium diffusion into the matrix will be inhibited, causing the

mitochondrion to contract (Garlid and Paucek, 2003). There is evidence that mK_{ATP} channels may buffer changes in matrix volume and inter-membrane space, as well as modulating the accumulation of free calcium, which would help to overcome mitochondrial stress (Rousou *et al*., 2004).

To my best knowledge, there are only two publications, by Tai and colleagues (Tai and Truong, 2002) (Tai *et al*., 2003), who investigated m K_{ATP} channel function in a toxin assay similar to what is shown in this work.

This group demonstrated a positive effect of mitochondrial K_{ATP} openers on rotenone treated undifferentiated PC12 cells. In order to separate a possible mitochondrial K_{ATP} (mK_{ATP}) channel contribution and a plasma membrane one (pmK_{ATP}), Tai et al. used patch clamping and various K_{ATP} openers and blockers and found that pmK_{ATP} channel activity is not detectable under any of the conditions tested. This means that the positive effect of the K_{ATP} channel openers had to come from an activation of mK_{ATP} channels. However, Tai et al. used undifferentiated cells of the PC12 cell line for their experiments, which was isolated from rat pheochromocytoma of the adrenal medulla. Therefore, these cells are likely not similar to mesDA neurons in their molecular makeup and if their reaction K_{ATP} channel mirrors something occurring in mesDA neurons is questionable.

There is also evidence that activation of mK_{ATP} channels *alone* may lead to a) a decrease of membrane potential by increasing the permeability of the mitochondrial membrane, b) mitochondrial swelling which will hinder ATP synthesis and finally, c) to a release of cytochrome c (Scatena *et al*., 2007). Therefore, the effect of mK_{ATP} activation most likely depends on if the mitochondria are already challenged by e.g energy depletion or ROS, or not.

Research on mK_{ATP} channels is hindered by the fact that the subunits of the mK_{ATP} have yet to be identified. It is assumed but as of now unproven that the mK_{ATP} channels have the same or at least very similar subunit composition as the pmK_{ATP} channels. There is evidence for other Sur-like proteins, some of which are much smaller than the known isoforms (Szewczyk *et al*., 1997). It has been speculated that this size-reduction might reflect an adaptation to the more limited space on mitochondrial membranes. An interesting finding has the been the identification of functional mK_{ATP} channels in myocytes of Kir6.1 and 6.2 mutant mice (Miki *et al*., 2002) (Suzuki *et al*., 2002), which do not express any K_{ATP} channel on the plasma

membrane. This shows that other proteins aside from Kir6.1 and 6.2 are present and able to form a mK_{ATP} channel. Whether they are compensating for a lack of Kir6.x or representing actual wild-type mK_{ATP} channel subtypes is unknown.

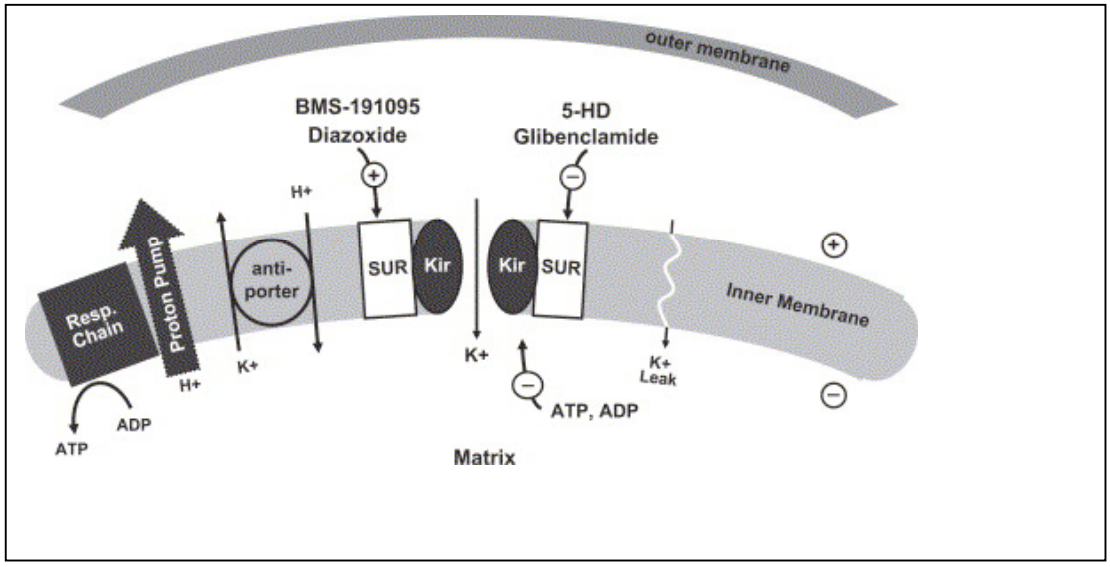


Figure 5.1: Physiological and pharmacological agents which affect mK_{ATP} channels
It is assumed but as of yet unclear if mK_{ATP} channels consist of similar subunits as pmK_{ATP} channels. Other factors, aside from the ones shown in the figure, such as reactive oxygen species or protein kinase C, can also affect function of the mK_{ATP} channel.
(Kir, inwardly rectifying potassium channel subunit; SUR, sulfonylurea receptor subunit; 5-HD, 5-hydroxydecanoate; Resp. Chain, Respiratory Chain; ATP, adenosine triphosphate; ADP, adenosine diphosphate) (Busija *et al*., 2004).

In summary, it is likely that both m and pmK_{ATP} channels are activated during oxidative stress, but the available information on mK_{ATP} structure and indeed, function, is scarce. Tolbutamide and pinacidil bind to and activate both m and pm K_{ATP} channels. Experimentally, one would need m or pm K_{ATP} specific blockers and openers to separate their function, e.g. the mK_{ATP} specific activator diazoxide and the blocker 5-hydroxydecanoate. It remains to be seen what the effect of these substances on mesDA neurons is. Moreover, taken into account the relative short time frame in which hypoxia and ischemia are experimentally studied (usually under 24 hours) and the long time period PD models need to cause neurodegeneration (24 hours to months), it is possible that an initially beneficial effect like the activation of K_{ATP} channels in hypoxia, turns into something causing additional stress and cell death after a certain time point, as can be seen in the presented results.

5.1.3 K_{ATP} blocking and neurodegeneration

There is some more evidence for K_{ATP} channel blocking as a protective measure against oxidative stress in mesDA neurons. Liss and colleagues applied MPTP and rotenone to patch-clamped mesDA neurons and discovered that the neurons are completely electrically silenced under these conditions. When tolbutamide was added to the solution, the normal firing pattern of the cell was restored (Liss *et al*., 2001) (Liss *et al*., 2005). When they injected MPTP and into Kir6.2 deficient mice, which lack a pmK_{ATP} channel, they discovered that the mesDA neurons were significantly more resistant to the effect of MPTP than wild type mice (Liss *et al*., 2005 368). Evidence for a positive effect of pmK_{ATP} blocking also comes from experiments done in the lab of Gang Hu and others. Ironically, this group investigated the effect of a novel K_{ATP} channel *opener*, iptakalim, on rat midbrain slice cultures and detected a protective effect of iptakalim in the haloperidol model of PD which also targets the respiratory chain in the mitochondria (Wang *et al*., 2005) (Yang *et al*., 2005). Since none of their experiments provided direct evidence that iptakalim actually opened K_{ATP} channels, eventually mesDA neurons were patch-clamped and an increase in spontaneous firing was detected upon addition of iptakalim, which was mirrored by the application of the blocker tolbutamide. Iptakalim turned out to be a rather specific pmK_{ATP} channel blocker, not opener (Wu *et al*., 2006).
Similarly, the data presented here shows that this positive effect is present in primary cultures treated with rotenone and either tolbutamide and TEA, and MPTP where there cell survial is mediated by TEA only. Consequently I propose the following mechanism for K_{ATP} function in oxidative stress on mesDA neurons.

5.1.4 Hypothesis of K_{ATP} function in mesDA oxidative damage

One of the tasks of K_{ATP} channels is to tie the excitability of a cell to the state of its energy supply. Dropping ATP to ADP levels lead to an activation of pmK_{ATP} and mK_{ATP} channels. Consequently, in a normal in vivo situation with sufficient glucose supply, both pmK_{ATP} and mK_{ATP} channels are closed (Garlid and Paucek, 2003; Nichols, 2006).
Insult with either 6OHDA, MPTP or rotenone will lead to the generation of large amounts of ROS via inhibition of the electron transport chain of complex I in

mitochondria, which is one predominant feature that is shared between the three toxins. MPTP and rotenone affect even the same protein, the NADH-ubiquinone reductase, while 6OHDA may generate ROS through a variety of mechanisms (Schober, 2004) (Testa *et al.*, 2005).

5.1.4.1 Effect of pmK_{ATP} channels

The inhibition of mitochondrial ATP production leads to the activation of the pmK_{ATP} channels (Liss *et al.*, 2005). The open pmK_{ATP} channel then hyperpolarizes the cell by leading to an efflux of potassium ions from the cell against the electrical gradient but following the concentration gradient. This hyper-polarization is electrophysiologically silencing the cell. Disregarding the possible parallel effect of mK_{ATP} channels, a lack of neuronal excitability alone can promote cell death in immature mesDA neurons of the rat (Salthun-Lassalle *et al.*, 2004). If this is also the case for mature neurons is, however, unknown.

5.1.4.2 Effect of mK_{ATP} channels

As both ROS and low ATP levels are known to activate mK_{ATP} channels and they in turn lead to the production of ROS, a toxin such as MPTP will lead to a massive generation of reactive oxygen species, causing damage to proteins, forming of the mitochondrial transition pore (MTP), efflux of cytochrome c into the cytosol and finally apoptosis (Kukreja, 2006). As stated earlier, the effect of the mK_{ATP} channel in regards to having a protective effect is most likely dependent on the state of the cell and the strength of the insult to the mitochondria. Given that most ischemic insults are transient and are causing primarily a depletion of ATP and only as secondary effect the production of ROS, I propose that the direct binding to and inactivation of complex I proteins such as NADH-ubiquinone reductase confers damage to the mitochondria on a scale the activation of mK_{ATP} channels is not able to buffer. Even more, the activation and following additional production of ROS might very well add to the already high level of ROS present caused by inhibiting complex I and enhance cell death as seen with the pinacidil treatment. In this mechanism, time might also be a factor, in the sense that a certain threshold of ROS has to be reached before cell death is triggered.

5.1.5 Differential effect of drugs and toxins

While the toxins used in this work are known to target complex I of the mitochondrial respiratory chain, each has additional mechanisms to cause cellular stress and apoptosis. Rotenone is known to inhibit microtubule formation and there is evidence that monomeric tubulin causes cell death (Brinkley *et al.*, 1974; Marshall and Himes, 1978). MPTP can inhibit complex III and IV of the respiratory chain as well as interacting with a number of cyotosolic proteins (Mizuno *et al.*, 1988). Finally, 6OHDA has a wide range of possible ways to cause cell death. Once in the cell, 6-OHDA can undergo spontaneous non-enzymatic auto-oxidation to give rise to secondary toxins, such as quinones (Saner and Thoenen, 1971) and superoxide radicals, in addition to hydrogen peroxide. In the presence of iron II, 6-OHDA can also produce harmful hydroxyl radicals via the Fenton reaction (Cohen and Heikkila, 1974). Taken together the various mechanisms by which each used toxin can induce cell death, it is not surprising that the three used drugs have similar different effects on cellular survival.

Tolbutamide and pinacidil both bind to and block/activate pmK_{ATP} as well as mK_{ATP} channels. In that light it is interesting that both drugs have little or no effect on MPTP treated cells, but a rather distinct effect on cells which were treated with rotenone. As said before both toxins have additional secondary mechanism of triggering cell death, furthermore there is evidence that the intra-cellular apoptosis pathway of between these two is different as well (Choi *et al.*, 1999).

5.1.6 TEA: different mode of apoptosis?

The strong effect TEA has on rotenone and MPTP treated primary cultures might be evidence for other nolecular mechanisms of apoptosis. TEA blocks almost every type of potassium channel on the plasma membrane, but does not enter the cell and therefore is unable to block mK_{ATP} channels (Garlid and Paucek, 2003). While it cannot be excluded that TEA binds more strongly to pmK_{ATP} channels or is more resistant to degradation than tolbutamide, the available literature suggests another possibility. Several ischemic insults on cortical and CA1 hippocampal neurons cause an NMDA receptor-mediated excessive K+ efflux from the cells and subsequently a depletion of intracellular potassium. This is thought to trigger apoptosis and can be

partially alleviated by the addition of TEA in vitro (Yu *et al.*, 1997) (Wei *et al.*, 2003).

Another more PD-related example of involvement of other types of potassium channels comes again from the work of Liss et al. who investigated the molecular mechanism of mesDA cell death in the *weaver* mouse. In this naturally occurring mutation of the inwardly rectifiying potassium channel Girk2, the channels lose their ion-specifity and allow a non-selective cation influx which causes cell death (Liss *et al.*, 1999b). In the same work, evidence is presented that the ion influx activates K_{ATP} channels. Interestingly, after crossing the *weaver* mouse with Kir6.2 mutant mice, which do not express a K_{ATP} channel on the plasma membrane, only a weak rescue of the *weaver* phenotype was be observed, again pointing to a different pathway of apoptosis (Liss *et al.*, 2005). Taken together, the effect of TEA on the toxin assays likely represents involvement of several potassium channel-related mechanisms triggering cell death. Whether this is representing an artefact connected to the additional effects the toxin have on cells or mirrors a PD-like in vivo situation, is unclear.

5.1.7 Towards specifity: cellular differences between VTA and SNpc

The presence of K_{ATP} channels alone does not explain why DA neurons from the SNpc subpopulation in the ventral mesencephalon are more vulnerable to insults than the neighbouring DA neurons from the VTA. As stated earlier, the molecular make up of mK_{ATP} in general is unclear, and most likely contains additional as of yet unidentified sub-units of at least the SUR family (Miki *et al.*, 2002) (Suzuki *et al.*, 2002). The expression of pmK_{ATP} channels, however, has been studied extensively. In over 95%, the mesDA neurons of both SNpc and VTA express the same type of channel, built from Kir6.2 and Sur1 (Liss *et al.*, 2005). Therefore, the presence of this channel alone is not explaining the differential vulnerability of the SNpc neurons to cellular stressors. One difference between the two populations are the different expression levels of the uncoupling protein 2 (UCP-2) (Andrews *et al.*, 2005). As mild uncoupling is known to reduce the amount of reactive oxygen species (ROS) (Krauss *et al.*, 2005), it was speculated that a higher level of uncoupling in the VTA might make these cells more resistant to oxidative stress. Lack of UCP-2 was shown to sensitize mesDA neurons against MPTP insult, whereas a two-fold over-expression

of the protein reduced the number of dying cells by 30%, using the same experimental setup (Andrews *et al.*, 2005). Subsequently, a three-fold higher expression of the neuronal uncoupling protein UCP-2 in the VTA was found (Liss *et al.*, 2005), although this was not confirmed by a genetic screen (Chung *et al.*, 2005). Additional artificial uncoupling of the mesDA neurons made the SNpc neurons more resistant to complex I inhibition. The VTA became more sensitive to inhibition of complex I, the pmK_{ATP} channels were activated and the cells ceased their spontaneous firing (Liss *et al.*, 2005). As proposed earlier, this could be interpreted in a way that mitochondrial uncoupling up to certain degree protects against oxidative stress, as can be seen in the normal higher expression of UCP-2 in the VTA and the artificially level of uncoupling in the SNpc under experimental conditions. Is the level of uncoupling too high, the cells is put under more stress and reacts by opening the pmK_{ATP} channels. To further study this mechanism, it would be interesting to compare the expression levels of other neuronal populations which are affected in PD, like e.g. the neurons in locus coeruleus, raphe nuclei or olfactory bulb.

5.1.8 Involvement of the engrailed genes in oxidative stress

The effect of the Sur1 mutation on En^{HT} mutants suggests a convergence of engrailed and K_{ATP} function. The lack of engrailed leads to an increasingly drastic loss of mesDA neurons depending on the number of engrailed 1 and engrailed 2 alleles which are missing (Sgado *et al.*, 2006). MesDA cells dying in these mutants enter apoptosis via the caspase-dependent mitochondrial pathway. Caspases 3 and 9 are activated, there is an increase of cytosolic cytochrome C, most likely because of an opening of the mitochondrial transition pore (MTP) and subsequently, cell death. Primary cultures of double mutant E12 embryos were shown to be more sensitive to rotenone treatment than littermates carrying only the En2-/- mutation. Conversely, N2A cells over-expressing engrailed were more resistant to MPTP treatment than wild type cells (Alavian, submitted). Therefore, the role of engrailed seems to converge with K_{ATP} channel function on the level of the formation of the MTP when the cell is under stress. Insofar, it is conceivable that a lack of K_{ATP} channel function has a protective effect on the progressive loss in En^{HT} mice. However, more animals carrying both Sur1 and En^{HT} mutation need to be analysed. Given that En^{HT} mice lose their SNpc neurons over a fixed period of time, it also would be interesting to

compare Sur1-/+En^{HT} animals to En^{HT}, to see whether the deletion of a single allele is able to slow the degeneration of the DA neurons.

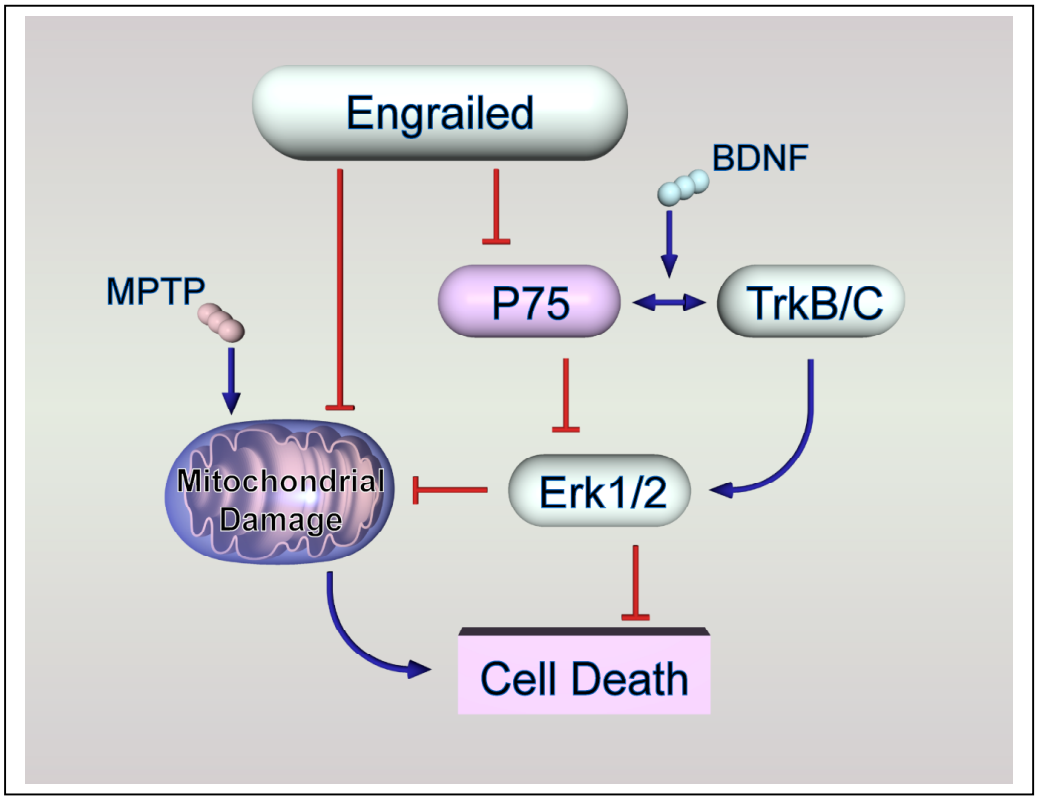


Figure 5.2: Role of engrailed in oxidative stress and apoptosis
Loss of engrailed expression leads to instability of mitochondrial membrane, and increased sensitivity to mitochondrial damage by (for example) MPTP (Alavian, submitted).

5.2 Requirement of engrailed for DRN and LC

Mutant mice deficient for both engrailed genes show a specific loss of the DRN and LC, whereas two adjacent nuclei, the MRN and NSC, which belong to the same pool of neurotransmitter-specific neurons, are not affected. Our study and the data I present here, comprises the first report that these nuclei require different positional information for their development. Other transcription factors like Otx2 and Gbx2 define the neuroepithelium rostral or caudal to the isthmus, respectively, but do not have such a distinct function for these neurons. The finding that En1 mutant mice develop almost normally in respect to DRN and LC, indicates that the En1 and En2 genes are largely functionally redundant in respect to the development of ventral mid/hindbrain.

The expression of Lmx1b and Pet1 in the 5-HT precursor cells and the post-mitotic neurons (Hendricks *et al.*, 1999) (Ding *et al.*, 2003) suggests a cell-autonomous requirement, as the neurons fail to differentiate and are absent in the respective

mutant mice. However, the loss of DRN in the engrailed mutants, the lack of engrailed expression in the post-mitotic neurons, suggests that En1 and En2 provide positional information in the neuroepithelium acting prior to Lmx1b and Pet1.

The location of NSC noradrenergic neurons just directly ventral to the LC, its scattered distribution and an afferent projection which is often indistinguishable from the LC led occasionally to the conclusion that the noradrenergic neurons of the NSC are just a low density extension of the LC and not a distinctive population of neurons. Our finding that the NSC is not affected in En1/2 double mutants suggests that LC and NSC are of different ontogenetic origin and are therefore two separate cell populations in the same way as serotonergic neurons of the MRN and DRN. The physical vicinity of LC and NSC is either a mere coincidence or reflects the fact that they both require Fgf8 for their induction (Ye *et al.*, 1998).

5.3 One step cloning – a fast way to obtain DNA fragments of defined size

Recently, the nucleotide sequences of entire genomes became available. This information combined with older sequencing data discloses the exact chromosomal location of millions of nucleotide markers stored in the databases at NCBI, EMBO or DDBJ. Despite having resolved the intron/exon structures of all described genes within these genomes, the sequencing data opens up other interesting possibilities. The isolation of smaller genomic fragments from larger genomic clones like BACs (bacterial artificial chromosomes) or others is usually a time consuming process and requires a significant amount of resources. It normally involves screening of libraries either by PCR or hybridization (Campbell and Choy, 2002) followed by a fragmentation process and re-screening until a plasmid is isolated containing the targeted DNA.

I could demonstrate that combining genome and BES (BAC end sequence) data substantially reduces the time necessary to isolate defined genomic fragments from BAC clones of species with a fully sequenced genome. The method reduces the process to a single cloning step requiring only common reagents and equipment. Using standard restriction enzymes, I cloned specific DNA fragments of 1 to 10 kb in

size from a given BAC in less than 48 hours. This method should be applicable to similar sources of subcloned genomic DNA like PACs (Sternberg, 1992), YACs (Burke *et al*., 1987) and others, if sequences of the ends as well as of the underlying genome are known as in the cases of the here described BACs. The used Bluescript vector can be substituted for any vector with a comparable number of specific restriction sites within its multiple cloning site. This method works with very high fidelity compared to another widely used alternative procedure, long-range PCR, which is notoriously unreliable, even on low-complexity templates, e.g. BAC clones. The strategy presented here may become the method of choice to isolate defined genomic DNA out of larger genomic clones.

5.4 Future perspectives

There is little data available today on K_{ATP} channel function in regard to PD. Groups working on stroke usually operate in a time-frames of a few hours for their experiments and not over the course of 24 hours or even longer as it is necessary to emulate a progressive loss like seen in Parkinson's Disease. Depending on their respective model system, the K_{ATP} channels have different sub-unit compositions, and the sub-units of the mK_{ATP} channels have yet to be identified in a definitive way. Even when focusing on a certain type of cell and condition, most groups looking to manipulate K_{ATP} channel state have used substances which exert their effect on both mK_{ATP} and pmK_{ATP} channels, if the effect on mK_{ATP} channels is considered at all.
In this work, I used the K_{ATP} channel blocker tolbutamide and the opener pinacidil on E12 primary cultures. Both drugs affect pmK_{ATP} and mK_{ATP} channels and the positive effect of tolbutamide and negative effect of pinacidil on mesDA survival needs to be studied in more detail.
Therefore, it is advised to built upon the existing data on K_{ATP} function in PD models with agents specific for m and pmK_{ATP} channels, respectively. As noted, e.g. iptakalim was identified as a specific pmK_{ATP} blocker, whereas BMS 191095 or diazoxide activate solely mK_{ATP} channels.

Furthermore, it would be interesting to compare the effect of these drugs to the ones used here and in other chemical and animal models of nigral degeneration, like the haloperidol model mentioned above and the e.g. the *aphakia* mouse/Pitx3 mutant. This especially goes for the unspecific potassium channel blocker TEA, since my work points to the possible involvement of additional non-K_{ATP} potassium channels in other molecular mechanisms of apoptosis.

The rescue of the En^{HT} phenotype by the addition of the homozygous Sur1 mutant points to a convergence of the pathway by which engrailed is influencing mesDA survival and the action of the K_{ATP} channels. More animals need to be studied to define this connection further, possibly by the additional investigation of dopamine levels in the striatum and an electrophysiological comparison of En^{HT} and $Sur1En^{HT}$ mice.

The literature data on UCP2 levels and its effect on ROS levels is one possible explanation for the differential vulnerability of the nigral neurons. However, it is inconclusive as long as it is not clear whether other neuronal populations affected by PD have similar level of UCP2 expression as the DA neurons of the SNpc.

Taken together, the data presented here further cements the role of K_{ATP} channels in oxidative stress and cell death and may provide new insight for the development of drugs for treatment of Parkinson's Disease.

6 Abbreviations

AADC	l-Aromatic amino acid decarboxylase
Aldhd2	Aldehyde dehydrogenase 2
AP	Anterior-posterior
ATP	Adenosine Triphosphate
AVE	Anterior visceral endoderm
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogen protein
bp	base pairs
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
DA	Dopamine
DAT	Dopamine transporter
DNA	Deoxyribonucleic acid
DMEM	Dulbecco modified eagle medium
dNTP	Desoxyribonucleotide
dpc	Days post coitum
DV	Dorsal-ventral
E	Embryonic day
EDTA	Ethylene diamine tetracetic acid
En	Engrailed
En^{HT}	En1+/-;En2-/-
En1	Engrailed-1
En2	Engrailed-2
Fgf	Fibroblast growth factor
FCS	Fetal calf serum
Foxa1	Forkhead containing transcription factor I (formerly HNF3α)
FP	Floor plate
Gbx	Gastrulation brain homeobox gene
GDNF	Glial cell line derived factor
Kir6.2	Rectifying potassium channel element
LB (medium)	lysogeny broth

L-DOPA	L-3,4-dihydroxyphenylalanine (also called Levodopa)
Lmx	LIM homeodomain-containing transcription factor
MAO	Monoamine oxidase
MHB	Mid-hindbrain boundary
mK_{ATP}	Mitochondrial K_{ATP} channel
MPT	Mitochondrial permeability transition
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
6-OHDA	6-hydroxydopamine
Otx	Orthodenticle homologue
Park-1	Parkin-1
Park-2	Parkin-2
Pax	Paired box genes
PC12	Rat pheochromocytoma cell line
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
PD	Parkinson's disease
PFA	Paraformaldehyde
Pitx3	Paired-like homeodomain transcription factor
pmK_{ATP}	Plasma membrane K_{ATP} channel
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRF	Retrorubral field
RT	Reverse transcriptase or room temperature
Shh	Sonic Hedgehog
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
Sur1	Sulfonyl receptor I
Tgfβ	Transforming growth factor beta
TH	Tyrosine hydroxylase
TGF	Transforming growth factor
TLZ	tauLacZ
μCi	micro Curie

μF	micro Farad
V	volt
VE water	de-mineralized (“vollentsalztes”) water
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area
Wnt	Wingless homologue transcription facto

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Abeliovich, A. and Flint Beal, M. (2006). Parkinsonism genes: culprits and clues. J Neurochem 99, 1062-72.

Abeliovich, A., Schmitz, Y., Farinas, I., Choi-Lundberg, D., Ho, W. H., Castillo, P. E., Shinsky, N., Verdugo, J. M., Armanini, M., Ryan, A. *et al.* (2000). Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron 25, 239-52.

Acampora, D., Gulisano, M., Broccoli, V. and Simeone, A. (2001). Otx genes in brain morphogenesis. Prog Neurobiol 64, 69-95.

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A. and Brulet, P. (1995). Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation. Development 121, 3279-90.

Adams, K. A., Maida, J. M., Golden, J. A. and Riddle, R. D. (2000). The transcription factor Lmx1b maintains Wnt1 expression within the isthmic organizer. Development 127, 1857-67.

Alavian, K. N., Scholz, C. and Simon, H. H. (2007). Transcriptional regulation of mesencephalic dopaminergic neurons: The full circle of life and death. Mov Disord.

Alberi, L., Sgado, P. and Simon, H. H. (2004). Engrailed genes are cell-autonomously required to prevent apoptosis in mesencephalic dopaminergic neurons. Development 131, 3229-36.

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

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. J Mol Biol 215, 403-10.

Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., Perlmann, T. and Ericson, J. (2006). Identification of intrinsic determinants of midbrain dopamine neurons. Cell 124, 393-405.

Andrews, Z. B., Horvath, B., Barnstable, C. J., Elsworth, J., Yang, L., Beal, M. F., Roth, R. H., Matthews, R. T. and Horvath, T. L. (2005). Uncoupling protein-2 is critical for nigral dopamine cell survival in a mouse model of Parkinson's disease. J Neurosci 25, 184-91.

Araki, I. and Nakamura, H. (1999). Engrailed defines the position of dorsal di-mesencephalic boundary by repressing diencephalic fate. Development 126, 5127-35.

Aranda, A. and Pascual, A. (2001). Nuclear hormone receptors and gene expression. Physiol Rev 81, 1269-304.

Asano, M. and Gruss, P. (1992). Pax-5 is expressed at the midbrain-hindbrain boundary during mouse development. Mech Dev 39, 29-39.

Ashcroft, F. M. (1988). Adenosine 5'-triphosphate-sensitive potassium channels. Annu Rev Neurosci 11, 97-118.

Bally-Cuif, L., Cholley, B. and Wassef, M. (1995). Involvement of Wnt-1 in the formation of the mes/metencephalic boundary. Mech Dev 53, 23-34.

Baloh, R. H., Tansey, M. G., Golden, J. P., Creedon, D. J., Heuckeroth, R. O., Keck, C. L., Zimonjic, D. B., Popescu, N. C., Johnson, E. M., Jr. and Milbrandt, J. (1997). TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. Neuron 18, 793-802.

Bayer, S. A., Wills, K. V., Triarhou, L. C. and Ghetti, B. (1995). Time of neuron origin and gradients of neurogenesis in midbrain dopaminergic neurons in the mouse. Exp Brain Res 105, 191-9.

Beal, M. F. (2001). Experimental models of Parkinson's disease. Nat Rev Neurosci 2, 325-34.

Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K. and Seitelberger, F. (1973). Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. J Neurol Sci 20, 415-55.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat Rev Neurosci 3, 517-30.

Besnard, V., Wert, S. E., Hull, W. M. and Whitsett, J. A. (2004). Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. Gene Expr Patterns 5, 193-208.

Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V. and Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3, 1301-6.

Bjorklund, L. M., Sanchez-Pernaute, R., Chung, S., Andersson, T., Chen, I. Y., McNaught, K. S., Brownell, A. L., Jenkins, B. G., Wahlestedt, C., Kim, K. S. ***et al.*** (2002). Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc Natl Acad Sci U S A 99, 2344-9.

Blaess, S., Corrales, J. D. and Joyner, A. L. (2006). Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. Development 133, 1799-809.

Bouchard, M., Pfeffer, P. and Busslinger, M. (2000). Functional equivalence of the transcription factors Pax2 and Pax5 in mouse development. Development 127, 3703-13.

Braak, H. and Braak, E. (2000). Pathoanatomy of Parkinson's disease. J Neurol 247 Suppl 2, II3-10.

Brennan, J., Lu, C. C., Norris, D. P., Rodriguez, T. A., Beddington, R. S. and Robertson, E. J. (2001). Nodal signalling in the epiblast patterns the early mouse embryo. Nature 411, 965-9.

Brinkley, B. R., Barham, S. S., Barranco, S. C. and Fuller, G. M. (1974). Rotenone inhibition of spindle microtubule assembly in mammalian cells. Exp Cell Res 85, 41-6.

Britto, J., Tannahill, D. and Keynes, R. (2002). A critical role for sonic hedgehog signaling in the early expansion of the developing brain. Nat Neurosci 5, 103-10.

Broccoli, V., Boncinelli, E. and Wurst, W. (1999). The caudal limit of Otx2 expression positions the isthmic organizer. Nature 401, 164-8.

Brown, T. P., Rumsby, P. C., Capleton, A. C., Rushton, L. and Levy, L. S. (2006). Pesticides and Parkinson's disease--is there a link? Environ Health Perspect 114, 156-64.

Burbach, J. P., Smits, S. and Smidt, M. P. (2003). Transcription factors in the development of midbrain dopamine neurons. Ann N Y Acad Sci 991, 61-8.

Burke, D. T., Carle, G. F. and Olson, M. V. (1987). Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236, 806-12.

Burke, R. E., Antonelli, M. and Sulzer, D. (1998). Glial cell line-derived neurotrophic growth factor inhibits apoptotic death of postnatal substantia nigra dopamine neurons in primary culture. J Neurochem 71, 517-25.

Busija, D. W., Lacza, Z., Rajapakse, N., Shimizu, K., Kis, B., Bari, F., Domoki, F. and Horiguchi, T. (2004). Targeting mitochondrial ATP-sensitive potassium channels--a novel approach to neuroprotection. Brain Res Brain Res Rev 46, 282-94.

Cabin, D. E., Shimazu, K., Murphy, D., Cole, N. B., Gottschalk, W., McIlwain, K. L., Orrison, B., Chen, A., Ellis, C. E., Paylor, R. ***et al.*** (2002). Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. J Neurosci 22, 8797-807.

Callahan, C. A. and Thomas, J. B. (1994). Tau-beta-galactosidase, an axon-targeted fusion protein. Proc Natl Acad Sci U S A 91, 5972-6.

Campbell, T. N. and Choy, F. Y. (2002). Approaches to library screening. J Mol Microbiol Biotechnol 4, 551-4.

Canet-Aviles, R. M., Wilson, M. A., Miller, D. W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M. J., Ringe, D., Petsko, G. A. and Cookson, M. R. (2004). The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proc Natl Acad Sci U S A 101, 9103-8.

Castelo-Branco, G., Sousa, K. M., Bryja, V., Pinto, L., Wagner, J. and Arenas, E. (2006). Ventral midbrain glia express region-specific transcription factors and regulate dopaminergic neurogenesis through Wnt-5a secretion. Mol Cell Neurosci 31, 251-62.

Castelo-Branco, G., Wagner, J., Rodriguez, F. J., Kele, J., Sousa, K., Rawal, N., Pasolli, H. A., Fuchs, E., Kitajewski, J. and Arenas, E. (2003). Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. Proc Natl Acad Sci U S A 100, 12747-52.

Castillo, S. O., Baffi, J. D., Palkovits, M., Goldstein, D. S., Kopin, I. J., Witta, J., Magnuson, M. A. and Nikodem, V. M. (1998). Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene. Mol Cell Neurosci 11, 36-46.

Chan, P., DeLanney, L. E., Irwin, I., Langston, J. W. and Di Monte, D. (1991). Rapid ATP loss caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mouse brain. J Neurochem 57, 348-51.

Chen, H., Lun, Y., Ovchinnikov, D., Kokubo, H., Oberg, K. C., Pepicelli, C. V., Gan, L., Lee, B. and Johnson, R. L. (1998). Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat Genet 19, 51-5.

Chi, C. L., Martinez, S., Wurst, W. and Martin, G. R. (2003). The isthmic organizer signal FGF8 is required for cell survival in the prospective midbrain and cerebellum. Development 130, 2633-44.

Choi, W. S., Yoon, S. Y., Oh, T. H., Choi, E. J., O'Malley, K. L. and Oh, Y. J. (1999). Two distinct mechanisms are involved in 6-hydroxydopamine- and MPP+-induced dopaminergic neuronal cell death: role of caspases, ROS, and JNK. J Neurosci Res 57, 86-94.

Chung, C. Y., Seo, H., Sonntag, K. C., Brooks, A., Lin, L. and Isacson, O. (2005). Cell type-specific gene expression of midbrain dopaminergic neurons reveals molecules involved in their vulnerability and protection. Hum Mol Genet 14, 1709-25.

Clark, L. N., Wang, Y., Karlins, E., Saito, L., Mejia-Santana, H., Harris, J., Louis, E. D., Cote, L. J., Andrews, H., Fahn, S. *et al.* (2006). Frequency of LRRK2 mutations in early- and late-onset Parkinson disease. Neurology 67, 1786-91.

Clarkson, E. D., Zawada, W. M. and Freed, C. R. (1995). GDNF reduces apoptosis in dopaminergic neurons in vitro. Neuroreport 7, 145-9.

Cohen, G. and Heikkila, R. E. (1974). The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. J Biol Chem 249, 2447-52.

Costa, R. H., Grayson, D. R. and Darnell, J. E., Jr. (1989). Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and alpha 1-antitrypsin genes. Mol Cell Biol 9, 1415-25.

Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. Nature 380, 66-8.

Cuervo, A. M., Stefanis, L., Fredenburg, R., Lansbury, P. T. and Sulzer, D. (2004). Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science 305, 1292-5.

Danielian, P. S. and McMahon, A. P. (1996). Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. Nature 383, 332-4.

Dauer, W. and Przedborski, S. (2003). Parkinson's disease: mechanisms and models. Neuron 39, 889-909.

Davis, C. A. and Joyner, A. L. (1988). Expression patterns of the homeo box-containing genes En-1 and En-2 and the proto-oncogene int-1 diverge during mouse development. Genes Dev 2, 1736-44.

De Keyser, J., Ebinger, G. and Vauquelin, G. (1990). Age-related changes in the human nigrostriatal dopaminergic system. Ann Neurol 27, 157-61.

de Lau, L. M. and Breteler, M. M. (2006). Epidemiology of Parkinson's disease. Lancet Neurol 5, 525-35.

de Rijk, M. C., Launer, L. J., Berger, K., Breteler, M. M., Dartigues, J. F., Baldereschi, M., Fratiglioni, L., Lobo, A., Martinez-Lage, J., Trenkwalder, C. *et al.* (2000). Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. Neurology 54, S21-3.

De Robertis, E. M. and Kuroda, H. (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. Annu Rev Cell Dev Biol 20, 285-308.

Ding, Y. Q., Marklund, U., Yuan, W., Yin, J., Wegman, L., Ericson, J., Deneris, E., Johnson, R. L. and Chen, Z. F. (2003). Lmx1b is essential for the development of serotonergic neurons. Nat Neurosci 6, 933-8.

Duvoisin, R. C. (1992). Overview of Parkinson's disease. Ann N Y Acad Sci 648, 187-93.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell 75, 1417-30.

Episkopou, V., Arkell, R., Timmons, P. M., Walsh, J. J., Andrew, R. L. and Swan, D. (2001). Induction of the mammalian node requires Arkadia function in the extraembryonic lineages. Nature 410, 825-30.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell 90, 169-80.

Fabre, E., Monserrat, J., Herrero, A., Barja, G. and Leret, M. L. (1999). Effect of MPTP on brain mitochondrial H2O2 and ATP production and on dopamine and DOPAC in the striatum. J Physiol Biochem 55, 325-31.

Fahn, S. (2003). Description of Parkinson's disease as a clinical syndrome. Ann N Y Acad Sci 991, 1-14.

Farkas, L. M., Dunker, N., Roussa, E., Unsicker, K. and Krieglstein, K. (2003). Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo. J Neurosci 23, 5178-86.

Fasano, S. and Brambilla, R. (2002). Cellular mechanisms of striatum-dependent behavioral plasticity and drug addiction. Curr Mol Med 2, 649-65.

Ferri, A. L., Lin, W., Mavromatakis, Y. E., Wang, J. C., Sasaki, H., Whitsett, J. A. and Ang, S. L. (2007). Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner. Development 134, 2761-9.

Forno, L. S., DeLanney, L. E., Irwin, I. and Langston, J. W. (1993). Similarities and differences between MPTP-induced parkinsonsim and Parkinson's disease. Neuropathologic considerations. Adv Neurol 60, 600-8.

Fortin, D. L., Nemani, V. M., Voglmaier, S. M., Anthony, M. D., Ryan, T. A. and Edwards, R. H. (2005). Neural activity controls the synaptic accumulation of alpha-synuclein. J Neurosci 25, 10913-21.

Fuccillo, M., Joyner, A. L. and Fishell, G. (2006). Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development. Nat Rev Neurosci 7, 772-83.

Funahashi, J., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H. and Nakamura, H. (1999). Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity. Dev Growth Differ 41, 59-72.

Funayama, M., Hasegawa, K., Kowa, H., Saito, M., Tsuji, S. and Obata, F. (2002). A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. Ann Neurol 51, 296-301.

Galter, D., Westerlund, M., Carmine, A., Lindqvist, E., Sydow, O. and Olson, L. (2006). LRRK2 expression linked to dopamine-innervated areas. Ann Neurol 59, 714-9.

Garlid, K. D. and Paucek, P. (2003). Mitochondrial potassium transport: the K(+) cycle. Biochim Biophys Acta 1606, 23-41.

Gemel, J., Jacobsen, C. and MacArthur, C. A. (1999). Fibroblast growth factor-8 expression is regulated by intronic engrailed and Pbx1-binding sites. J Biol Chem 274, 6020-6.

German, D. C., Manaye, K., Smith, W. K., Woodward, D. J. and Saper, C. B. (1989). Midbrain dopaminergic cell loss in Parkinson's disease: computer visualization. Ann Neurol 26, 507-14.

Gibb, W. R. and Lees, A. J. (1988). The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. J Neurol Neurosurg Psychiatry 51, 745-52.

Giguere, V. (1999). Orphan nuclear receptors: from gene to function. Endocr Rev 20, 689-725.

Gilks, W. P., Abou-Sleiman, P. M., Gandhi, S., Jain, S., Singleton, A., Lees, A. J., Shaw, K., Bhatia, K. P., Bonifati, V., Quinn, N. P. ***et al.*** (2005). A common LRRK2 mutation in idiopathic Parkinson's disease. Lancet 365, 415-6.

Gok, S., Vatansever, S., Vural, K., Sekuri, C., Izanli, A., Tezcan, A. and Cilaker, S. (2006). The role of ATP sensitive K+ channels and of nitric oxide synthase on myocardial ischemia/reperfusion-induced apoptosis. Acta Histochem 108, 95-104.

Goldberg, M. S., Fleming, S. M., Palacino, J. J., Cepeda, C., Lam, H. A., Bhatnagar, A., Meloni, E. G., Wu, N., Ackerson, L. C., Klapstein, G. J. ***et al.*** (2003). Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. J Biol Chem 278, 43628-35.

Gossen, M. and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A 89, 5547-51.

Greggio, E., Jain, S., Kingsbury, A., Bandopadhyay, R., Lewis, P., Kaganovich, A., van der Brug, M. P., Beilina, A., Blackinton, J., Thomas, K. J. ***et al.*** (2006). Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. Neurobiol Dis 23, 329-41.

Gregory, S. G., Sekhon, M., Schein, J., Zhao, S., Osoegawa, K., Scott, C. E., Evans, R. S., Burridge, P. W., Cox, T. V., Fox, C. A. *et al.* (2002). A physical map of the mouse genome. Nature 418, 743-50.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B. and Joyner, A. L. (1995). Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. Science 269, 679-82.

Hasegawa, E., Takeshige, K., Oishi, T., Murai, Y. and Minakami, S. (1990). 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. Biochem Biophys Res Commun 170, 1049-55.

Hendricks, T., Francis, N., Fyodorov, D. and Deneris, E. S. (1999). The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. J Neurosci 19, 10348-56.

Hendricks, T. J., Fyodorov, D. V., Wegman, L. J., Lelutiu, N. B., Pehek, E. A., Yamamoto, B., Silver, J., Weeber, E. J., Sweatt, J. D. and Deneris, E. S. (2003). Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. Neuron 37, 233-47.

Hermanson, E., Joseph, B., Castro, D., Lindqvist, E., Aarnisalo, P., Wallen, A., Benoit, G., Hengerer, B., Olson, L. and Perlmann, T. (2003). Nurr1 regulates dopamine synthesis and storage in MN9D dopamine cells. Exp Cell Res 288, 324-34.

Hughes, A. J., Daniel, S. E., Kilford, L. and Lees, A. J. (1992). Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 55, 181-4.

Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P. A. and Rosenthal, A. (1995). Induction of midbrain dopaminergic neurons by Sonic hedgehog. Neuron 15, 35-44.

Inagaki, N., Gonoi, T., Clement, J. P., Wang, C. Z., Aguilar-Bryan, L., Bryan, J. and Seino, S. (1996). A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K+ channels. Neuron 16, 1011-7.

Ishibashi, M. and McMahon, A. P. (2002). A sonic hedgehog-dependent signaling relay regulates growth of diencephalic and mesencephalic primordia in the early mouse embryo. Development 129, 4807-19.

Itier, J. M., Ibanez, P., Mena, M. A., Abbas, N., Cohen-Salmon, C., Bohme, G. A., Laville, M., Pratt, J., Corti, O., Pradier, L. *et al.* (2003). Parkin gene inactivation alters behaviour and dopamine neurotransmission in the mouse. Hum Mol Genet 12, 2277-91.

Javitch, J. A., D'Amato, R. J., Strittmatter, S. M. and Snyder, S. H. (1985). Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6 -tetrahydropyridine:

uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proc Natl Acad Sci U S A 82, 2173-7.

Joyner, A. L. (1996). Engrailed, Wnt and Pax genes regulate midbrain--hindbrain development. Trends Genet 12, 15-20.

Joyner, A. L., Herrup, K., Auerbach, B. A., Davis, C. A. and Rossant, J. (1991). Subtle cerebellar phenotype in mice homozygous for a targeted deletion of the En-2 homeobox. Science 251, 1239-43.

Joyner, A. L., Kornberg, T., Coleman, K. G., Cox, D. R. and Martin, G. R. (1985). Expression during embryogenesis of a mouse gene with sequence homology to the Drosophila engrailed gene. Cell 43, 29-37.

Joyner, A. L. and Martin, G. R. (1987). En-1 and En-2, two mouse genes with sequence homology to the Drosophila engrailed gene: expression during embryogenesis. Genes Dev 1, 29-38.

Kaestner, K. H., Katz, J., Liu, Y., Drucker, D. J. and Schutz, G. (1999). Inactivation of the winged helix transcription factor HNF3alpha affects glucose homeostasis and islet glucagon gene expression in vivo. Genes Dev 13, 495-504.

Kawagoe, R., Takikawa, Y. and Hikosaka, O. (2004). Reward-predicting activity of dopamine and caudate neurons--a possible mechanism of motivational control of saccadic eye movement. J Neurophysiol 91, 1013-24.

Kim, K. S., Kim, C. H., Hwang, D. Y., Seo, H., Chung, S., Hong, S. J., Lim, J. K., Anderson, T. and Isacson, O. (2003). Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner. J Neurochem 85, 622-34.

Kish, S. J., Shannak, K. and Hornykiewicz, O. (1988). Uneven pattern of dopamine loss in the striatum of patients with idiopathic Parkinson's disease. Pathophysiologic and clinical implications. N Engl J Med 318, 876-80.

Kitai, S. T., Shepard, P. D., Callaway, J. C. and Scroggs, R. (1999). Afferent modulation of dopamine neuron firing patterns. Curr Opin Neurobiol 9, 690-7.

Krauss, S., Zhang, C. Y. and Lowell, B. B. (2005). The mitochondrial uncoupling-protein homologues. Nat Rev Mol Cell Biol 6, 248-61.

Kubis, N., Faucheux, B. A., Ransmayr, G., Damier, P., Duyckaerts, C., Henin, D., Forette, B., Le Charpentier, Y., Hauw, J. J., Agid, Y. *et al.* (2000). Preservation of midbrain catecholaminergic neurons in very old human subjects. Brain 123 (Pt 2), 366-73.

Kukreja, R. C. (2006). Mechanism of reactive oxygen species generation after opening of mitochondrial KATP channels. Am J Physiol Heart Circ Physiol 291, H2041-3.

Lai, E., Prezioso, V. R., Smith, E., Litvin, O., Costa, R. H. and Darnell, J. E., Jr. (1990). HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev 4, 1427-36.

Lai, E., Prezioso, V. R., Tao, W. F., Chen, W. S. and Darnell, J. E., Jr. (1991). Hepatocyte nuclear factor 3 alpha belongs to a gene family in mammals that is homologous to the Drosophila homeotic gene fork head. Genes Dev 5, 416-27.

Lang, A. E. and Lozano, A. M. (1998). Parkinson's disease. First of two parts. N Engl J Med 339, 1044-53.

Langston, J. W., Ballard, P., Tetrud, J. W. and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219, 979-80.

Langston, J. W., Forno, L. S., Tetrud, J., Reeves, A. G., Kaplan, J. A. and Karluk, D. (1999). Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann Neurol 46, 598-605.

Larsen, C. N., Krantz, B. A. and Wilkinson, K. D. (1998). Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. Biochemistry 37, 3358-68.

Lebel, M., Gauthier, Y., Moreau, A. and Drouin, J. (2001). Pitx3 activates mouse tyrosine hydroxylase promoter via a high-affinity binding site. J Neurochem 77, 558-67.

Lee, C. S., Friedman, J. R., Fulmer, J. T. and Kaestner, K. H. (2005). The initiation of liver development is dependent on Foxa transcription factors. Nature 435, 944-7.

Lesage, S., Durr, A., Tazir, M., Lohmann, E., Leutenegger, A. L., Janin, S., Pollak, P. and Brice, A. (2006). LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. N Engl J Med 354, 422-3.

Li, J. Y. and Joyner, A. L. (2001). Otx2 and Gbx2 are required for refinement and not induction of mid-hindbrain gene expression. Development 128, 4979-91.

Liss, B., Bruns, R. and Roeper, J. (1999a). Alternative sulfonylurea receptor expression defines metabolic sensitivity of K-ATP channels in dopaminergic midbrain neurons. Embo J 18, 833-46.

Liss, B., Franz, O., Sewing, S., Bruns, R., Neuhoff, H. and Roeper, J. (2001). Tuning pacemaker frequency of individual dopaminergic neurons by Kv4.3L and KChip3.1 transcription. Embo J 20, 5715-24.

Liss, B., Haeckel, O., Wildmann, J., Miki, T., Seino, S. and Roeper, J. (2005). K-ATP channels promote the differential degeneration of dopaminergic midbrain neurons. Nat Neurosci 8, 1742-51.

Liss, B., Neu, A. and Roeper, J. (1999b). The weaver mouse gain-of-function phenotype of dopaminergic midbrain neurons is determined by coactivation of wvGirk2 and K-ATP channels. J Neurosci 19, 8839-48.

Liu, A. and Joyner, A. L. (2001a). Early anterior/posterior patterning of the midbrain and cerebellum. Annu Rev Neurosci 24, 869-96.

Liu, A. and Joyner, A. L. (2001b). EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region. Development 128, 181-91.

Liu, A., Li, J. Y., Bromleigh, C., Lao, Z., Niswander, L. A. and Joyner, A. L. (2003). FGF17b and FGF18 have different midbrain regulatory properties from FGF8b or activated FGF receptors. Development 130, 6175-85.

Liu, A., Losos, K. and Joyner, A. L. (1999). FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. Development 126, 4827-38.

Liu, Y., Fallon, L., Lashuel, H. A., Liu, Z. and Lansbury, P. T., Jr. (2002). The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. Cell 111, 209-18.

Long, W., Wagner, K. U., Lloyd, K. C., Binart, N., Shillingford, J. M., Hennighausen, L. and Jones, F. E. (2003). Impaired differentiation and lactational failure of Erbb4-deficient mammary glands identify ERBB4 as an obligate mediator of STAT5. Development 130, 5257-68.

Lundell, M. J., Chu-LaGraff, Q., Doe, C. Q. and Hirsh, J. (1996). The engrailed and huckebein genes are essential for development of serotonin neurons in the Drosophila CNS. Mol Cell Neurosci 7, 46-61.

Mandir, A. S., Przedborski, S., Jackson-Lewis, V., Wang, Z. Q., Simbulan-Rosenthal, C. M., Smulson, M. E., Hoffman, B. E., Guastella, D. B., Dawson, V. L. and Dawson, T. M. (1999). Poly(ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism. Proc Natl Acad Sci U S A 96, 5774-9.

Marshall, L. E. and Himes, R. H. (1978). Rotenone inhibition of tubulin self-assembly. Biochim Biophys Acta 543, 590-4.

Martinat, C., Bacci, J. J., Leete, T., Kim, J., Vanti, W. B., Newman, A. H., Cha, J. H., Gether, U., Wang, H. and Abeliovich, A. (2006). Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. Proc Natl Acad Sci U S A 103, 2874.

Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. and Martin, G. R. (1999). FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. Development 126, 1189-200.

Maruoka, Y., Ohbayashi, N., Hoshikawa, M., Itoh, N., Hogan, B. L. and Furuta, Y. (1998). Comparison of the expression of three highly related genes, Fgf8, Fgf17 and Fgf18, in the mouse embryo. Mech Dev 74, 175-7.

Mastick, G. S., Davis, N. M., Andrew, G. L. and Easter, S. S., Jr. (1997). Pax-6 functions in boundary formation and axon guidance in the embryonic mouse forebrain. Development 124, 1985-97.

Mata, I. F., Taylor, J. P., Kachergus, J., Hulihan, M., Huerta, C., Lahoz, C., Blazquez, M., Guisasola, L. M., Salvador, C., Ribacoba, R. *et al.* (2005). LRRK2 R1441G in Spanish patients with Parkinson's disease. Neurosci Lett 382, 309-11.

Matsuo, I., Kuratani, S., Kimura, C., Takeda, N. and Aizawa, S. (1995). Mouse Otx2 functions in the formation and patterning of rostral head. Genes Dev 9, 2646-58.

Maxwell, S. L. and Li, M. (2005). Midbrain dopaminergic development in vivo and in vitro from embryonic stem cells. J Anat 207, 209-18.

Mayer, R. A., Kindt, M. V. and Heikkila, R. E. (1986). Prevention of the nigrostriatal toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by inhibitors of 3,4-dihydroxyphenylethylamine transport. J Neurochem 47, 1073-9.

McCormack, A. L., Di Monte, D. A., Delfani, K., Irwin, I., DeLanney, L. E., Langston, W. J. and Janson, A. M. (2004). Aging of the nigrostriatal system in the squirrel monkey. J Comp Neurol 471, 387-95.

McGeer, P. L., Schwab, C., Parent, A. and Doudet, D. (2003). Presence of reactive microglia in monkey substantia nigra years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration. Ann Neurol 54, 599-604.

McMahon, A. P. and Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 62, 1073-85.

McMahon, A. P., Joyner, A. L., Bradley, A. and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of Wnt-1-/Wnt-1- mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. Cell 69, 581-95.

Menzies, F. M., Yenisetti, S. C. and Min, K. T. (2005). Roles of Drosophila DJ-1 in survival of dopaminergic neurons and oxidative stress. Curr Biol 15, 1578-82.

Meulener, M., Whitworth, A. J., Armstrong-Gold, C. E., Rizzu, P., Heutink, P., Wes, P. D., Pallanck, L. J. and Bonini, N. M. (2005). Drosophila DJ-1 mutants are selectively sensitive to environmental toxins associated with Parkinson's disease. Curr Biol 15, 1572-7.

Miki, T., Minami, K., Zhang, L., Morita, M., Gonoi, T., Shiuchi, T., Minokoshi, Y., Renaud, J. M. and Seino, S. (2002). ATP-sensitive potassium channels participate in glucose uptake in skeletal muscle and adipose tissue. Am J Physiol Endocrinol Metab 283, E1178-84.

Miki, T. and Seino, S. (2005). Roles of KATP channels as metabolic sensors in acute metabolic changes. J Mol Cell Cardiol 38, 917-25.

Millen, K. J., Wurst, W., Herrup, K. and Joyner, A. L. (1994). Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse Engrailed-2 mutants. Development 120, 695-706.

Miller, D. W., Hague, S. M., Clarimon, J., Baptista, M., Gwinn-Hardy, K., Cookson, M. R. and Singleton, A. B. (2004). Alpha-synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication. Neurology 62, 1835-8.

Millet, S., Campbell, K., Epstein, D. J., Losos, K., Harris, E. and Joyner, A. L. (1999). A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. Nature 401, 161-4.

Mizuno, Y., Sone, N., Suzuki, K. and Saitoh, T. (1988). Studies on the toxicity of 1-methyl-4-phenylpyridinium ion (MPP+) against mitochondria of mouse brain. J Neurol Sci 86, 97-110.

Muthane, U., Ramsay, K. A., Jiang, H., Jackson-Lewis, V., Donaldson, D., Fernando, S., Ferreira, M. and Przedborski, S. (1994). Differences in nigral neuron number and sensitivity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in C57/bl and CD-1 mice. Exp Neurol 126, 195-204.

Nichols, C. G. (2006). KATP channels as molecular sensors of cellular metabolism. Nature 440, 470-6.

Nichols, C. G., Ripoll, C. and Lederer, W. J. (1991). ATP-sensitive potassium channel modulation of the guinea pig ventricular action potential and contraction. Circ Res 68, 280-7.

Nichols, W. C., Pankratz, N., Hernandez, D., Paisan-Ruiz, C., Jain, S., Halter, C. A., Michaels, V. E., Reed, T., Rudolph, A., Shults, C. W. ***et al.*** (2005). Genetic screening for a single common LRRK2 mutation in familial Parkinson's disease. Lancet 365, 410-2.

Nicklas, W. J., Vyas, I. and Heikkila, R. E. (1985). Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci 36, 2503-8.

Nunes, I., Tovmasian, L. T., Silva, R. M., Burke, R. E. and Goff, S. P. (2003). Pitx3 is required for development of substantia nigra dopaminergic neurons. Proc Natl Acad Sci U S A.

Ohama, E. and Ikuta, F. (1976). Parkinson's disease: distribution of Lewy bodies and monoamine neuron system. Acta Neuropathol (Berl) 34, 311-9.

Okafuji, T., Funahashi, J. and Nakamura, H. (1999). Roles of Pax-2 in initiation of the chick tectal development. Brain Res Dev Brain Res 116, 41-9.

Orban, P. C., Chui, D. and Marth, J. D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. Proc Natl Acad Sci U S A 89, 6861-5.

Osoegawa, K., Tateno, M., Woon, P. Y., Frengen, E., Mammoser, A. G., Catanese, J. J., Hayashizaki, Y. and de Jong, P. J. (2000). Bacterial artificial chromosome libraries for mouse sequencing and functional analysis. Genome Res 10, 116-28.

Ozelius, L. J., Senthil, G., Saunders-Pullman, R., Ohmann, E., Deligtisch, A., Tagliati, M., Hunt, A. L., Klein, C., Henick, B., Hailpern, S. M. *et al.* (2006). LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews. N Engl J Med 354, 424-5.

Palacino, J. J., Sagi, D., Goldberg, M. S., Krauss, S., Motz, C., Wacker, M., Klose, J. and Shen, J. (2004). Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. J Biol Chem 279, 18614-22.

Park, J., Lee, S. B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., Kim, J., Shong, M., Kim, J. M. *et al.* (2006). Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature 441, 1157-61.

Parkinson, J. (2002). An essay on the shaking palsy. 1817. J Neuropsychiatry Clin Neurosci 14, 223-36; discussion 222.

Peirano, R. I. and Wegner, M. (2000). The glial transcription factor Sox10 binds to DNA both as monomer and dimer with different functional consequences. Nucleic Acids Res 28, 3047-55.

Perez, F. A. and Palmiter, R. D. (2005). Parkin-deficient mice are not a robust model of parkinsonism. Proc Natl Acad Sci U S A 102, 2174-9.

Periquet, M., Corti, O., Jacquier, S. and Brice, A. (2005). Proteomic analysis of parkin knockout mice: alterations in energy metabolism, protein handling and synaptic function. J Neurochem 95, 1259-76.

Perlmann, T. and Wallen-Mackenzie, A. (2004). Nurr1, an orphan nuclear receptor with essential functions in developing dopamine cells. Cell Tissue Res 318, 45-52.

Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R. *et al.* (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276, 2045-7.

Prakash, N. and Wurst, W. (2006). Genetic networks controlling the development of midbrain dopaminergic neurons. J Physiol 575, 403-10.

Przedborski, S., Jackson-Lewis, V., Kostic, V., Carlson, E., Epstein, C. J. and Cadet, J. L. (1992). Superoxide dismutase, catalase, and glutathione peroxidase activities in copper/zinc-superoxide dismutase transgenic mice. J Neurochem 58, 1760-7.

Przedborski, S., Jackson-Lewis, V., Naini, A. B., Jakowec, M., Petzinger, G., Miller, R. and Akram, M. (2001). The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. J Neurochem 76, 1265-74.

Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S. L., Wurst, W. and Simeone, A. (2004). Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. Development 131, 2037-48.

Quayle, J. M., Nelson, M. T. and Standen, N. B. (1997). ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. Physiol Rev 77, 1165-232.

Rhinn, M., Picker, A. and Brand, M. (2006). Global and local mechanisms of forebrain and midbrain patterning. Curr Opin Neurobiol 16, 5-12.

Riddle, R. and Pollock, J. D. (2003). Making connections: the development of mesencephalic dopaminergic neurons. Brain Res Dev Brain Res 147, 3-21.

Rinne, J. O., Lonnberg, P. and Marjamaki, P. (1990). Age-dependent decline in human brain dopamine D1 and D2 receptors. Brain Res 508, 349-52.

Ristoratore, F., Carl, M., Deschet, K., Richard-Parpaillon, L., Boujard, D., Wittbrodt, J., Chourrout, D., Bourrat, F. and Joly, J. S. (1999). The midbrain-hindbrain boundary genetic cascade is activated ectopically in the diencephalon in response to the widespread expression of one of its components, the medaka gene Ol-eng2. Development 126, 3769-79.

Robertson, K. and Mason, I. (1997). The GDNF-RET signalling partnership. Trends Genet 13, 1-3.

Ross, O. A., Toft, M., Whittle, A. J., Johnson, J. L., Papapetropoulos, S., Mash, D. C., Litvan, I., Gordon, M. F., Wszolek, Z. K., Farrer, M. J. *et al.* (2006). Lrrk2 and Lewy body disease. Ann Neurol 59, 388-93.

Rousou, A. J., Ericsson, M., Federman, M., Levitsky, S. and McCully, J. D. (2004). Opening of mitochondrial KATP channels enhances cardioprotection through the modulation of mitochondrial matrix volume, calcium accumulation, and respiration. Am J Physiol Heart Circ Physiol 287, H1967-76.

Roussa, E. and Krieglstein, K. (2004). Induction and specification of midbrain dopaminergic cells: focus on SHH, FGF8, and TGF-beta. Cell Tissue Res 318, 23-33.

Rowitch, D. H. and McMahon, A. P. (1995). Pax-2 expression in the murine neural plate precedes and encompasses the expression domains of Wnt-1 and En-1. Mech Dev 52, 3-8.

Sacchetti, P., Mitchell, T. R., Granneman, J. G. and Bannon, M. J. (2001). Nurr1 enhances transcription of the human dopamine transporter gene through a novel mechanism. J Neurochem 76, 1565-72.

Sakurada, K., Ohshima-Sakurada, M., Palmer, T. D. and Gage, F. H. (1999). Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. Development 126, 4017-26.

Salthun-Lassalle, B., Hirsch, E. C., Wolfart, J., Ruberg, M. and Michel, P. P. (2004). Rescue of mesencephalic dopaminergic neurons in culture by low-level stimulation of voltage-gated sodium channels. J Neurosci 24, 5922-30.

Saner, A. and Thoenen, H. (1971). Model experiments on the molecular mechanism of action of 6-hydroxydopamine. Mol Pharmacol 7, 147-54.

Sasai, Y. (2002). Generation of dopaminergic neurons from embryonic stem cells. J Neurol 249 Suppl 2, II41-4.

Saueressig, H., Burrill, J. and Goulding, M. (1999). Engrailed-1 and netrin-1 regulate axon pathfinding by association interneurons that project to motor neurons. Development 126, 4201-12.

Scatena, R., Bottoni, P., Botta, G., Martorana, G. E. and Giardina, B. (2007). The role of mitochondria in pharmacotoxicology: a reevaluation of an old, newly emerging topic. Am J Physiol Cell Physiol 293, C12-21.

Schober, A. (2004). Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. Cell Tissue Res 318, 215-24.

Schoenberg, B. S., Osuntokun, B. O., Adeuja, A. O., Bademosi, O., Nottidge, V., Anderson, D. W. and Haerer, A. F. (1988). Comparison of the prevalence of Parkinson's disease in black populations in the rural United States and in rural Nigeria: door-to-door community studies. Neurology 38, 645-6.

Scholz, C., Doderlein, G. and Simon, H. H. (2006). One step cloning of defined DNA fragments from large genomic clones. J Biochem Mol Biol 39, 464-7.

Schulte, G., Bryja, V., Rawal, N., Castelo-Branco, G., Sousa, K. M. and Arenas, E. (2005). Purified Wnt-5a increases differentiation of midbrain dopaminergic cells and dishevelled phosphorylation. J Neurochem 92, 1550-3.

Schulz, J. B. and Falkenburger, B. H. (2004). Neuronal pathology in Parkinson's disease. Cell Tissue Res 318, 135-47.

Schwarz, M., Alvarez-Bolado, G., Urbanek, P., Busslinger, M. and Gruss, P. (1997). Conserved biological function between Pax-2 and Pax-5 in midbrain and cerebellum development: evidence from targeted mutations. Proc Natl Acad Sci U S A 94, 14518-23.

Seghers, V., Nakazaki, M., DeMayo, F., Aguilar-Bryan, L. and Bryan, J. (2000). Sur1 knockout mice. A model for K(ATP) channel-independent regulation of insulin secretion. J Biol Chem 275, 9270-7.

Seidler, A., Hellenbrand, W., Robra, B. P., Vieregge, P., Nischan, P., Joerg, J., Oertel, W. H., Ulm, G. and Schneider, E. (1996). Possible environmental, occupational, and other etiologic factors for Parkinson's disease: a case-control study in Germany. Neurology 46, 1275-84.

Seniuk, N. A., Tatton, W. G. and Greenwood, C. E. (1990). Dose-dependent destruction of the coeruleus-cortical and nigral-striatal projections by MPTP. Brain Res 527, 7-20.

Sgado, P., Alberi, L., Gherbassi, D., Galasso, S. L., Ramakers, G. M., Alavian, K. N., Smidt, M. P., Dyck, R. H. and Simon, H. H. (2006). Slow progressive degeneration of nigral dopaminergic neurons in postnatal Engrailed mutant mice. Proc Natl Acad Sci U S A 103, 15242-7.

Shamim, H., Mahmood, R., Logan, C., Doherty, P., Lumsden, A. and Mason, I. (1999). Sequential roles for Fgf4, En1 and Fgf8 in specification and regionalisation of the midbrain. Development 126, 945-59.

Shendelman, S., Jonason, A., Martinat, C., Leete, T. and Abeliovich, A. (2004). DJ-1 is a redox-dependent molecular chaperone that inhibits alpha-synuclein aggregate formation. PLoS Biol 2, e362.

Simeone, A., Puelles, E. and Acampora, D. (2002). The Otx family. Curr Opin Genet Dev 12, 409-15.

Simon, H. H., Saueressig, H., Wurst, W., Goulding, M. D. and O'Leary, D. D. (2001). Fate of Midbrain Dopaminergic Neurons Controlled by the Engrailed Genes. J Neurosci 21, 3126-3134.

Simon, H. H., Scholz, C. and O'Leary, D. D. (2005). Engrailed genes control developmental fate of serotonergic and noradrenergic neurons in mid- and hindbrain in a gene dose-dependent manner. Mol Cell Neurosci 28, 96-105.

Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R. *et al.* (2003). alpha-Synuclein locus triplication causes Parkinson's disease. Science 302, 841.

Sirinathsinghji, D. J., Kupsch, A., Mayer, E., Zivin, M., Pufal, D. and Oertel, W. H. (1992). Cellular localization of tyrosine hydroxylase mRNA and cholecystokinin mRNA-containing cells in the ventral mesencephalon of the common marmoset: effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Brain Res Mol Brain Res 12, 267-74.

Smidt, M. P., Asbreuk, C. H., Cox, J. J., Chen, H., Johnson, R. L. and Burbach, J. P. (2000). A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. Nat Neurosci 3, 337-41.

Smidt, M. P. and Burbach, J. P. (2007). How to make a mesodiencephalic dopaminergic neuron. Nat Rev Neurosci 8, 21-32.

Smidt, M. P., Smits, S. M., Bouwmeester, H., Hamers, F. P., Van Der Linden, A. J., Hellemons, A. J., Graw, J. and Burbach, J. P. (2004). Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. Development 131, 1145-55.

Smidt, M. P., van Schaick, H. S., Lanctot, C., Tremblay, J. J., Cox, J. J., van der Kleij, A. A., Wolterink, G., Drouin, J. and Burbach, J. P. (1997). A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. Proc Natl Acad Sci U S A 94, 13305-10.

Smits, S. M., Ponnio, T., Conneely, O. M., Burbach, J. P. and Smidt, M. P. (2003). Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons. Eur J Neurosci 18, 1731-8.

Solano, S. M., Miller, D. W., Augood, S. J., Young, A. B. and Penney, J. B., Jr. (2000). Expression of alpha-synuclein, parkin, and ubiquitin carboxy-terminal hydrolase L1 mRNA in human brain/ genes associated with familial Parkinson's disease. Ann Neurol 47, 201-10.

Spanagel, R. and Weiss, F. (1999). The dopamine hypothesis of reward: past and current status. Trends Neurosci 22, 521-7.

Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R. and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. Nature 388, 839-40.

Spraul, M., Streeck, A., Nieradzik, M. and Berger, M. (1989). Uniform elimination pattern for glibenclamide in healthy Caucasian males. Arzneimittelforschung 39, 1449-50.

Staropoli, J. F., McDermott, C., Martinat, C., Schulman, B., Demireva, E. and Abeliovich, A. (2003). Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. Neuron 37, 735-49.

Stefanis, L., Larsen, K. E., Rideout, H. J., Sulzer, D. and Greene, L. A. (2001). Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. J Neurosci 21, 9549-60.

Sternberg, N. L. (1992). Cloning high molecular weight DNA fragments by the bacteriophage P1 system. Trends Genet 8, 11-6.

Sund, N. J., Ang, S. L., Sackett, S. D., Shen, W., Daigle, N., Magnuson, M. A. and Kaestner, K. H. (2000). Hepatocyte nuclear factor 3beta (Foxa2) is dispensable for maintaining the differentiated state of the adult hepatocyte. Mol Cell Biol 20, 5175-83.

Sund, N. J., Vatamaniuk, M. Z., Casey, M., Ang, S. L., Magnuson, M. A., Stoffers, D. A., Matschinsky, F. M. and Kaestner, K. H. (2001). Tissue-specific

deletion of Foxa2 in pancreatic beta cells results in hyperinsulinemic hypoglycemia. Genes Dev 15, 1706-15.

Suzuki, A., Aizawa, K., Gassmayr, S., Bosnjak, Z. J. and Kwok, W. M. (2002). Biphasic effects of isoflurane on the cardiac action potential: an ionic basis for anesthetic-induced changes in cardiac electrophysiology. Anesthesiology 97, 1209-17.

Szewczyk, A., Wojcik, G., Lobanov, N. A. and Nalecz, M. J. (1997). The mitochondrial sulfonylurea receptor: identification and characterization. Biochem Biophys Res Commun 230, 611-5.

Tai, K. K., McCrossan, Z. A. and Abbott, G. W. (2003). Activation of mitochondrial ATP-sensitive potassium channels increases cell viability against rotenone-induced cell death. J Neurochem 84, 1193-200.

Tai, K. K. and Truong, D. D. (2002). Activation of adenosine triphosphate-sensitive potassium channels confers protection against rotenone-induced cell death: therapeutic implications for Parkinson's disease. J Neurosci Res 69, 559-66.

Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S. M., Takahashi, K. and Ariga, H. (2004). DJ-1 has a role in antioxidative stress to prevent cell death. EMBO Rep 5, 213-8.

Talpade, D. J., Greene, J. G., Higgins, D. S., Jr. and Greenamyre, J. T. (2000). In vivo labeling of mitochondrial complex I (NADH:ubiquinone oxidoreductase) in rat brain using [(3)H]dihydrorotenone. J Neurochem 75, 2611-21.

Tanaka, Y., Engelender, S., Igarashi, S., Rao, R. K., Wanner, T., Tanzi, R. E., Sawa, A., V, L. D., Dawson, T. M. and Ross, C. A. (2001). Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. Hum Mol Genet 10, 919-26.

Taylor, J. P., Mata, I. F. and Farrer, M. J. (2006). LRRK2: a common pathway for parkinsonism, pathogenesis and prevention? Trends Mol Med 12, 76-82.

Testa, C. M., Sherer, T. B. and Greenamyre, J. T. (2005). Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures. Brain Res Mol Brain Res 134, 109-18.

Thomas, K. R. and Capecchi, M. R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature 346, 847-50.

Thuret, S., Bhatt, L., O'Leary, D. D. and Simon, H. H. (2004). Identification and developmental analysis of genes expressed by dopaminergic neurons of the substantia nigra pars compacta. Mol Cell Neurosci 25, 394-405.

Tomac, A., Lindqvist, E., Lin, L. F., Ogren, S. O., Young, D., Hoffer, B. J. and Olson, L. (1995). Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. Nature 373, 335-9.

Tretiakoff, C. (1919). Contribution a l'etude de l'anatomie pathologique du locus niger
de Soemmering avec quelques dedutions relatives a la pathogenie des troubles du tonus musculaire et de la maladie de Parkinson. . These de Paris.

Tzschentke, T. M. and Schmidt, W. J. (2000). Functional relationship among medial prefrontal cortex, nucleus accumbens, and ventral tegmental area in locomotion and reward. Crit Rev Neurobiol 14, 131-42.

Unsicker, K., Meier, C., Krieglstein, K., Sartor, B. M. and Flanders, K. C. (1996). Expression, localization, and function of transforming growth factor-beta s in embryonic chick spinal cord, hindbrain, and dorsal root ganglia. J Neurobiol 29, 262-76.

Urbanek, P., Fetka, I., Meisler, M. H. and Busslinger, M. (1997). Cooperation of Pax2 and Pax5 in midbrain and cerebellum development. Proc Natl Acad Sci U S A 94, 5703-8.

Valente, E. M., Salvi, S., Ialongo, T., Marongiu, R., Elia, A. E., Caputo, V., Romito, L., Albanese, A., Dallapiccola, B. and Bentivoglio, A. R. (2004). PINK1 mutations are associated with sporadic early-onset parkinsonism. Ann Neurol 56, 336-41.

Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M. and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. Neuron 31, 743-55.

Varastet, M., Riche, D., Maziere, M. and Hantraye, P. (1994). Chronic MPTP treatment reproduces in baboons the differential vulnerability of mesencephalic dopaminergic neurons observed in Parkinson's disease. Neuroscience 63, 47-56.

Veronese, F. M., Sartore, L., Schiavon, O. and Caliceti, P. (1990). A comparative study of enzymatic, structural, and pharmacokinetic properties of superoxide dismutase isolated from two sources and modified by monomethoxypolyethylene glycol using different methods of coupling. Ann N Y Acad Sci 613, 468-74.

Volakakis, N., Malewicz, M., Kadkhodai, B., Perlmann, T. and Benoit, G. (2006). Characterization of the Nurr1 ligand-binding domain co-activator interaction surface. J Mol Endocrinol 37, 317-26.

Wallen, A., Zetterstrom, R. H., Solomin, L., Arvidsson, M., Olson, L. and Perlmann, T. (1999). Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice. Exp Cell Res 253, 737-46.

Wallen, A. A., Castro, D. S., Zetterstrom, R. H., Karlen, M., Olson, L., Ericson, J. and Perlmann, T. (2001). Orphan nuclear receptor Nurr1 is essential for Ret expression in midbrain dopamine neurons and in the brain stem. Mol Cell Neurosci 18, 649-63.

Walther, C. and Gruss, P. (1991). Pax-6, a murine paired box gene, is expressed in the developing CNS. Development 113, 1435-49.

Wan, H., Dingle, S., Xu, Y., Besnard, V., Kaestner, K. H., Ang, S. L., Wert, S., Stahlman, M. T. and Whitsett, J. A. (2005). Compensatory roles of Foxa1 and Foxa2 during lung morphogenesis. J Biol Chem 280, 13809-16.

Wang, S., Hu, L. F., Yang, Y., Ding, J. H. and Hu, G. (2005). Studies of ATP-sensitive potassium channels on 6-hydroxydopamine and haloperidol rat models of Parkinson's disease: implications for treating Parkinson's disease? Neuropharmacology 48, 984-92.

Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N. P. and Perlmann, T. (2003). Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature 423, 555-60.

Wassarman, K. M., Lewandoski, M., Campbell, K., Joyner, A. L., Rubenstein, J. L., Martinez, S. and Martin, G. R. (1997). Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. Development 124, 2923-34.

Wei, L., Yu, S. P., Gottron, F., Snider, B. J., Zipfel, G. J. and Choi, D. W. (2003). Potassium channel blockers attenuate hypoxia- and ischemia-induced neuronal death in vitro and in vivo. Stroke 34, 1281-6.

Werner, P., Mytilineou, C., Cohen, G. and Yahr, M. D. (1994). Impaired oxidation of pyruvate in human embryonic fibroblasts after exposure to L-dopa. Eur J Pharmacol 263, 157-62.

West, A. B., Moore, D. J., Choi, C., Andrabi, S. A., Li, X., Dikeman, D., Biskup, S., Zhang, Z., Lim, K. L., Dawson, V. L. *et al.* (2007). Parkinson's disease-associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity. Hum Mol Genet.

Witta, J., Baffi, J. S., Palkovits, M., Mezey, E., Castillo, S. O. and Nikodem, V. M. (2000). Nigrostriatal innervation is preserved in Nurr1-null mice, although dopaminergic neuron precursors are arrested from terminal differentiation. Brain Res Mol Brain Res 84, 67-78.

Wu, J., Hu, J., Chen, Y. P., Takeo, T., Suga, S., Dechon, J., Liu, Q., Yang, K. C., St John, P. A., Hu, G. *et al.* (2006). Iptakalim modulates ATP-sensitive K(+) channels in dopamine neurons from rat substantia nigra pars compacta. J Pharmacol Exp Ther 319, 155-64.

Wurst, W., Auerbach, A. B. and Joyner, A. L. (1994). Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. Development 120, 2065-75.

Xu, J., Lawshe, A., MacArthur, C. A. and Ornitz, D. M. (1999). Genomic structure, mapping, activity and expression of fibroblast growth factor 17. Mech Dev 83, 165-78.

Yang, Y., Liu, X., Long, Y., Wang, F., Ding, J. H., Liu, S. Y., Sun, Y. H., Yao, H. H., Wang, H., Wu, J. *et al.* (2005). Systematic administration of iptakalim, an ATP-sensitive potassium channel opener, prevents rotenone-induced motor and neurochemical alterations in rats. J Neurosci Res 80, 442-9.

Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. Cell 93, 755-66.

Yu, S. P., Yeh, C. H., Sensi, S. L., Gwag, B. J., Canzoniero, L. M., Farhangrazi, Z. S., Ying, H. S., Tian, M., Dugan, L. L. and Choi, D. W. (1997). Mediation of neuronal apoptosis by enhancement of outward potassium current. Science 278, 114-7.

Zetterström, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L. and Perlmann, T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. Science 276, 248-250.

Zetterstrom, R. H., Williams, R., Perlmann, T. and Olson, L. (1996). Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. Brain Res Mol Brain Res 41, 111-20.

Zhao, S. (2001). A comprehensive BAC resource. Nucleic Acids Res 29, 141-3.

Zhao, S., Shatsman, S., Ayodeji, B., Geer, K., Tsegaye, G., Krol, M., Gebregeorgis, E., Shvartsbeyn, A., Russell, D., Overton, L. *et al.* (2001). Mouse BAC ends quality assessment and sequence analyses. Genome Res 11, 1736-45.

Zhou, W., Zhu, M., Wilson, M. A., Petsko, G. A. and Fink, A. L. (2006). The oxidation state of DJ-1 regulates its chaperone activity toward alpha-synuclein. J Mol Biol 356, 1036-48.

Zigova, T., Barroso, L. F., Willing, A. E., Saporta, S., McGrogan, M. P., Freeman, T. B. and Sanberg, P. R. (2000). Dopaminergic phenotype of hNT cells in vitro. Brain Res Dev Brain Res 122, 87-90.

Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., Hulihan, M., Uitti, R. J., Calne, D. B. *et al.* (2004). Mutations in LRRK2 Cause Autosomal-Dominant Parkinsonism with Pleomorphic Pathology. Neuron 44, 601-7.