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Kambiz Nassirpour Alavian
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Mitochondrial Instability, Lack of Erk1/2 Activation and High Expression of P75NTR in Engrailed-Deficient Mesencephalic Dopaminergic Neurons

Referees: Professor Dr. Konrad Beyreuter

Professor Dr. Hilmar Bading

To My Parents

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Summary

Progressive loss of mesencephalic dopaminergic (mesDA) neurons is the main pathological feature of Prakinson's Disease. The homeobox transcription factors, Engrailed-1 and Engrailed-2, are essential for survival and long-term maintenance of this neuronal population. In mutant embryos, null for both genes, the mesDA neurons are generated and start to express their neurotransmitter phenotype, but fail to differentiate and die during midgestation. The mice, heterozygous for Engrailed-1 and homozygous null for Engrailed-2, suffer a Parkinson's Disease-like postnatal degeneration of their nigral DA neurons. Prior studies had shown that the Engrailed-deficient mesDA neurons die by apoptosis, evident from activation of caspase-3 and presence of pyknotic nuclei. The subject of this study was to elaborate more on the molecular pathway of death and to find out the mechanisms of survival by interfering with this process.

In this work, I demonstrate that reduction in expression of Engrailed in mesDA neurons results in a higher sensitivity to mitochondrial apoptotic insult. The increased vulnerability to treatment with MPTP or specific inhibitors of Bcl-2 and Bcl-X_L causes release of cytochrome C from the mitochondrial inter-membrane space into the cytosol and subsequent activation of caspases. I also show that the Engrailed deficiency results in increased expression of P75 neurotrophin receptor, which is causal to death of the neurons. The death of Engrailed double mutant mesDA neurons, as a direct result of high expression of P75, can be inhibited by application of TrkB/C-specific neurotrophins, BDNF, NT4 or NT3, by siRNA knockdown (of P75) or by addition of an inhibiting antibody for P75. The rescue effect of these survival factors is diminished by concurrent treatment of the primary cell cultures with an inhibitor of the extracellular signal regulated kinase1/2 (Erk1/2) pathway. Additionally, there is an inverse correlation between expression of P75 and phosphorylation of Erk1/2, suggesting that Erk1/2 deactivation is critical to the death signaling of P75.

This study shows a connection between the etiology of PD, exemplified by mitochondrial instability, and the dose dependent survival effect of the Engrailed genes. In addition to causing mitochondrial vulnerability, the absence of engrailed genes activates a death pathway, instigated by the high expression of P75 and inhibition of activation of Erk1/2 pathway.

Zusammenfassung

Progressiver Verlust der mesencephalen dopaminergen (mesDA) Neurone ist das bedeutenste pathologische Kennzeichen von Morbus Parkinson. Die Homedomänen-Transkriptionsfaktoren Engrailed-1 und Engrailed-2 sind notwendig für das Überleben dieser neuronalen Population. In Mausembryonen die eine Nullmutation beider Gene tragen, entstehen diese Neurone und beginnen ihren Neurotransmittertyp zu exprimieren, differenzieren jedoch nicht und sterben während der Schwangerschaft ab. Mäuse, die heterozygot für Engrailed-1 und homozygot für Engrailed-2 sind, erleiden eine Parkinson-ähnliche postnatale Degeneration ihrer nigralen DA Neurone. Vorhergehende Studien haben gezeigt, dass die mesDA Neurone Apoptose durchlaufen, deutlich sichtbar an der Aktivierung von Caspase-3 und des Vorkommens von pyknotischen Nuklei. Das Ziel dieser Arbeit war die weitere Erforschung dieses molekularen Wegs zum Zelltod, der Mechanismus und Möglichkeiten, diesen zu stören oder zu unterbrechen.

In dieser Arbeit zeige ich, dass die Reduktion der Expression von Engrailed in mesDA Neuronen zu einer höheren Empfindlichkeit gegnüber mitochondrialer apoptotischer Störung führt. Die höhere Sensivität gegenüber einer Gabe von MPTP oder spezifischen Inhibitoren für Bcl-2 und Bcl-X_L verursacht die Freisetzung von Cytochrom C aus dem mitochondrialen inter-Membranraum in das Zytosol und eine darauffolgende Aktivierung von Caspasen. Ich zeige ausserdem, dass der Verlust von Engrailed zu einer erhöhten Expression von P75 Neurotrophin Rezeptor führt, der den Zelltod dieser Neurone verursacht. Der Tod der mesDA Neurone in Engrailed Doppelmutanten als eine Ursache von hoher P75 Expression kann durch TrkB/C spezifische Neurotrophine wie BDNF, NT4 oder NT3, durch siRNA knockdown von P75 oder durch Antikörper gegen P75 verhindert werden.Der positive Effekt dieser Überlebensfaktoren wird durch eine Behandlung der primären Zellkulturen mit einem Inhibitor des Signalweges der extrazellulären Signalkinase 1/2 (Erk1/2) verringert.Zusätzlich besteht eine inverse Korrelation zwischen der Expression von P75 und der Phosphorylierung von Erk1/2, die es wahrscheinlich erscheinen lässt, dass die Erk1/2 Deaktivierung notwendig für den Signalweg von P75 ist.

Diese Studie zeigt eine Korrelation zwischen der Ätiologie von Morbus Parkinson, dargestellt durch eine mitochondriale Instabilität, und des dosisabhängigen Überlebenseffekts der Engrailed Gene. Ausser eine mitochondriale Verwundbarkeit hervorzurufen, führt die Abwesenheit der Engrailed Gene zur Aktivierung eines apoptotischen Signalwegs, der durch die hohe Expression von P75 und der Inhibition der Aktivierung des Erk1/2/ Signalwegs ausgelöst wird.

Introduction

1. General Introduction

Mesencephalic dopaminergic (mesDA) neurons are the main source of dopamine in the central nervous system. The specific loss of mesDA neurons of the substantia nigra pars compacta (SNpc) is the main characteristic of one of the most prominent neurodegenerative disorders, Parkinson's disease (PD). The homeodomain transcription factors Engrailed-1 (En1) and Engrailed-2 (En2) play pivotal roles in differentiation survival and maintenance of the mesDA neurons during the embryonic development and throughout adulthood. In mice lacking both Engrailed genes, mesDA neurons are generated and start expressing their neurotransmitter phenotype, but they die by apoptosis between E12 and E14, the stages when the expression of Engrailed in wild type embryos sets in (Alberi et al., 2004; Simon et al., 2001). The intermediate genotypes between complete lack of the Engrailed genes and wild type show various degrees of cell loss in the mesDA system. Most interestingly, mice heterozygous null for Engrailed-1 and homozygous null for Engrailed-2 (En1+/-;En2-/-), which are viable and fertile, exhibit a progressive degeneration, specific to the nigral DA neurons within the first three months after birth, resulting in a phenotype reminiscent of PD.

Although the causes of vulnerability and degeneration of mesDA neurons are still unknown, the evidence like involvement of the death receptors and their ligands, mitochondrial dysfunction and an increase in activation of caspases suggest the extrinsic and intrinsic pathways of apoptosis as the executioners of neuronal cell death. Mitochondria is a point of convergence for several deleterious processes, involved in pathogenesis of PD. Reduced activity of mitochondrial complex-I and production of reactive oxygen species (ROS) has been observed in postmortem brain tissue of PD patients and can cause specific loss of nigral mesDA neurons in three major neurotoxin-based models of the disease, MPTP, Rotenone and 6-OHDA (Schober, 2004). This inhibition results in permeability of the mitochondrial membrane and release of pro-apoptotic molecules, like cytochrome C, into the cytoplasm and activation of caspases, leading to apoptotic cell death. In animal models of PD, This effect could be ablated by high expression of Bcl-2 and Bcl-X_L the anti-apoptotic members of the Bcl-2 family (Vila et al., 2001; Yang et al., 1998).

Neuronal cell death can be a result of neurotrophin deficiency. Action of the neurotrophins, consisting of NGF, BDNF, NT4/5 and NT3, is mediated via a set of specific tyrosine kinase receptors (TrkA, B, C) and a common receptor, P75 (P75NTR/NGFR). While the Trk receptors signal survival(Lu et al., 2005), P75 can have a survival or apoptotic function, dependent on the cellular context and the molecular form of the ligand(Lu et al., 2005). The pro-survival role of neurotrophins and their receptors has been mainly attributed to the downstream effect of phosphotydil inositol-3 kinase (PI3K) and the extracellular receptor kinase 1/2 (Erk1/2) pathway(Krieglstein et al., 2002), whereas apoptosis by P75 alone is triggered by phosphorylation of JNK and of the BH3-only members of the Bcl-2 family(Bhakar et al., 2003).

In this work, I have studied the mechanism of loss of the Engrailed-deficient mesDA neurons, in correlation with the death machinery of P75, one of its transcriptional regulatory targets, and in the context of etiology of PD, mitochondrial dysfunction and apoptosis.

2. Development of mesDA Neurons and Transcription Factors

2.1. Early Development

The central nervous system develops from the neural ectoderm. After gastrulation, thickening of the neural ectoderm results in formation of neural plate. This process takes place under the inductive control of signaling molecules released from the notochord and the dorsal mesoderm. The ectoderm's default cell fate is neural but the neural differentiation is mainly inhibited by activity of the bone morphogenic protein (BMP) pathway and to some degree, members of the FGF and Wnt families. The neural fate is a result of blocking of these inhibitory forces or, in other words, de-repression. Blocking of the BMP pathway by factors like follistatin, noggin and chordin or inhibition of the Wnt pathway by the activity of FrzB, dickkopf, and Cerberus can induce neural fate. During neurulation, the rostrocaudal neural plate is formed and bent to shape the neural groove; then, it folds up on its anterioposterior axis and eventually closes to form the neural tube (Smith and Schoenwolf, 1997). Then, by the rostrocaudal patterning, the neural tube is subdivided into four major

parts of forebrain, midbrain, hindbrain, and the spinal cord (Lumsden and Krumlauf, 1996; Rubenstein and Beachy, 1998; Rubenstein et al., 1998). This subdivision relies on the pattern of expression of transcription factors within the neural ectoderm (Prakash and Wurst, 2004). After rostrocaudal patterning, under the ventralizing induction of the sonic hedgehog (SHH) and the dorsalizing induction of BMPs, the neural tube becomes dorsoventrally polarized (Simon et al., 2003).

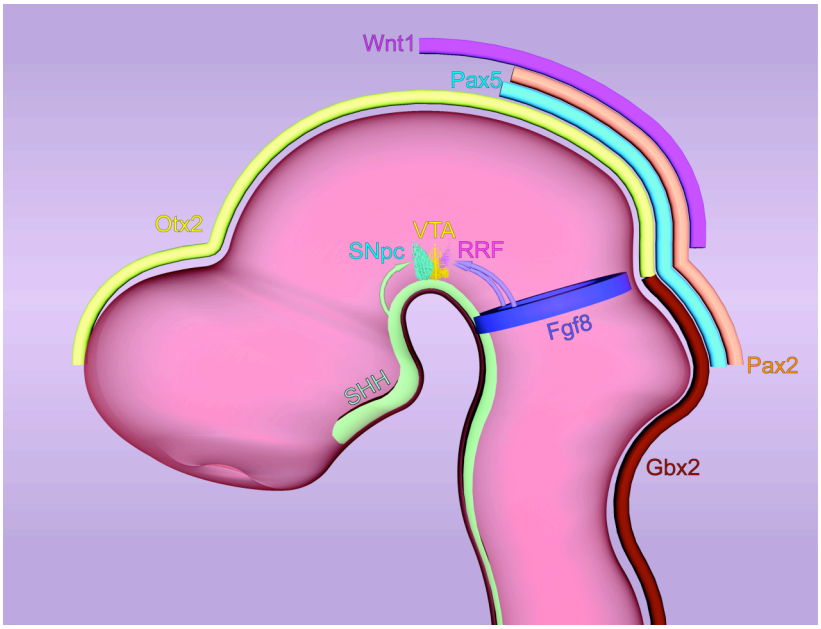


Figure-1: Schematic view of an E12 embryo; Induction of mesDA neurons by Fgf8 and SHH and approximate domains of expression of the factors, involved in regional identity of the midbrain and specification of mesDA neurons.

At this stage, the expression of genes in the boundary between midbrain and hindbrain forms the midbrain-hindbrain organizing structure (MHO), which is essential to the fate of mesDA neurons by regulation of further development of this region and controlling generation of specific cell populations in the ventral midbrain and rostral hindbrain (Liu and Joyner, 2001). One of the first positional signals to appear is the drosophila orthodenticle protein, Otx2, which is expressed in the epiblast and in the anterior visceral endoderm before gastrulation (Simeone et al., 2002). The expression of Otx2 becomes progressively limited to the anterior ectoderm and from E7 onward, sharply defines the region of forebrain and midbrain (Broccoli et al., 1999). In the Otx2 mutant mice, the forebrain and midbrain regions are deleted (Acampora et al., 1995). At the same time, similar to Otx2, the expression of Gbx2 becomes limited from all three germ layers of the posterior embryo to the anterior hindbrain by E8.5. Since in Gbx2-/- mutants, domain of Otx2 expands posteriorly during early somite stages, it is likely that Gbx2 defines the caudal border of Otx2 domain (Millet et al., 1999). In mice embryos, deficient for both Otx2 and Gbx-2 genes, the temporal expression of Fgf8 and other genes in the midbrain-hindbrain region is unaltered but

the position of expression of this set of genes is shifted to the anterior region of the neuroectoderm, suggesting that the expression of Otx2 and Gbx2 is required only for the proper positioning, and not the induction, of the midbrain/hindbrain organizer MHO (Li and Joyner, 2001).

Between E8 and E9, functionally equivalent transcription factors, Pax2 and Pax5, are expressed in a domain around the Otx2/Gbx2 boundary (Bouchard et al., 2000) and are required in a gene-dose dependent manner for proper development of the midbrain and the cerebellum (Urbanek et al., 1997). Then Wnt1, a homolog of the drosophila segmentation gene, wingless, is expressed within the Pax-2 domain and is restricted to the Otx2 positive midbrain cells. The main function of Wnt1 is development of the midbrain and in Wnt1 mutant mice, the entire midbrain and cerebellum is lost (McMahon et al., 1992). Wnt1 regulates the expression of the Engrailed and their expression domains overlap (Bally-Cuif et al., 1992).

The next factors, important in early development of mesDA neurons, are the Engrailed genes with Engrailed-1, being expressed in the entire mesencephalon-met encephalon and the Engrailed-2 expression domain being around the Otx2/Gbx2 boundary (Davis and Joyner, 1988). In early stages, the En1 mutant mice, show cerebellar and patterning defects. These effects are minor in the En2-deficient mice (Millen et al., 1994) (Wurst et al., 1994). Later on, expression of the Engrailed genes in the mesDA neurons imposes a gene dose-dependent survival effect on these neurons (Simon et al., 2001). Two other structurally related proteins, Lmx1a and Lmx1b, encoding a LIM homeodomain-containing transcription factor, are expressed after E9.5. Lmx1b is expressed in the caudal forebrain, midbrain, and hindbrain but its expression domain eventually becomes restricted to the Wnt1 and Fgf8 expression domain at the isthmus (Adams et al., 2000). On the other hand, the expression of Lmx1a is specific to dopaminergic progenitor cells and essential for induction of mesDA neurons (Andersson et al., 2006).

The mesDA neurons are induced by a combinatorial effect of the fibroblast growth factor 8 (Fgf8), which is locally produced at the mid/hindbrain boundary and in the rostral forebrain, and the sonic hedgehog (SHH), which is expressed along the ventral neural tube (Ye et al., 1998)(Fig. 1). Mice, lacking Fgf8, have gastrulation defects and do not have DA neurons (Ye et al., 1998). Also, the Fgf8 soaked beads can induce ectopic generation of midbrain and cerebellar structures along with expression of

marker genes in the diencephalon of the chick brain (Crossley et al., 1996; Liu and Joyner, 2001). Studies in zebra fish confirm the pivotal role of Fgf8 in formation of isthmic organizer and development of the region around it(Guo et al., 1999; Reifers et al., 1998).

SHH is considered a general ventralizing signal in the CNS. The amino terminal product of SHH autoproteolysis (SHH-N), produced by the floor plate, can induce different cell types within the neural tube in a dose dependent manner and is capable of inducing dopaminergic neurons in vitro (Hynes et al., 1995; Wang et al., 1995). SHH can also coordinate tissue size and shape in addition to cell type identity (Agarwala et al., 2001).

The factors involved during the transition period, between the time of induction of dopaminergic progenitor dells and when mesDA neurons become post mitotic, are mainly unknown. Two homeodomain proteins, important during this period, are Lmx1a and Msx1, which are induced by SHH. The role of Lmx1a has been extensively studied and shown to be important in formation of roof plate and dorsal-ventral patterning of the neural tube (Kruger et al., 2002; Millonig et al., 2000). While Lmx1a and Msx1, both, are selectively expressed in the DA progenitor cells, only the expression of Lmx1a can induce ectopic dopaminergic cells in chick embryos and can cause expression of Msx1(Andersson et al., 2006). The essential function of Lmx1a, as an intrinsic determinant of the mesDA neurons has been confirmed by its ability to differentiate embryonic stem cells into mesDA neurons. The evidence, including ability of Lmx1a to induce dopaminergic neurons of correct identity, expressing Nurr1, En1, Pitx3, Lmx1b and DAT, and also reduction of mesDA neurons, when Lmx1a is knocked down by RNA interference, show that Lmx1a is required and sufficient for differentiation of mesDA neurons from DA progenitor cells (Andersson et al., 2006).

After induction by Fgf8 and SHH, the mesDA neurons are generated and detectable by the rate-limiting enzyme of the dopamine production, tyrosine hydroxylase (TH). There are time differences between generation of the subpopulations of mesDA neurons, with higher proportions of the substantia nigra pars compacta (SNpc) and retrorubral field (RRF) being generated earlier (E11) than ventral tegmentum (VT). Around the same time, the neurons start innervating their respective targets in the basal ganglia and forming two of the most important DA pathways of the brain. Innervation of axons from VT to nucleus accumbens and the olfactory tubercle forms the

mesolimbic pathway while neurons of SNpc project their axons to the dorsal striatum, forming the nigrostriatal pathway (Nelson et al., 1996).

At this stage, the expression of Lmx1b, Nurr1, Pitx3, and the Engrailed genes (En1 and En2), all crucial survival factors (Simon et al., 2003; Wallen and Perlmann, 2003), in is detectable in mesDA neurons (Fig. 3). These factors represent at least three molecular cascades, essential for survival of the postmitotic mesDA neurons throughout the life of a species (Fig. 2)(Smidt et al., 2000).

2.2. Post-mitotic Transcription Factors

2.2.1. Nurr-1

Nurr-1 is a member of the orphan nuclear receptors. The structural analysis of the ligand-binding domain shows that there is neither a cavity within the structure nor a binding site for co-activators, meaning that Nurr1 is ligand independent (Wang et al., 2003). The expression of Nurr1 starts in the ventral midbrain at E10.5, around the time

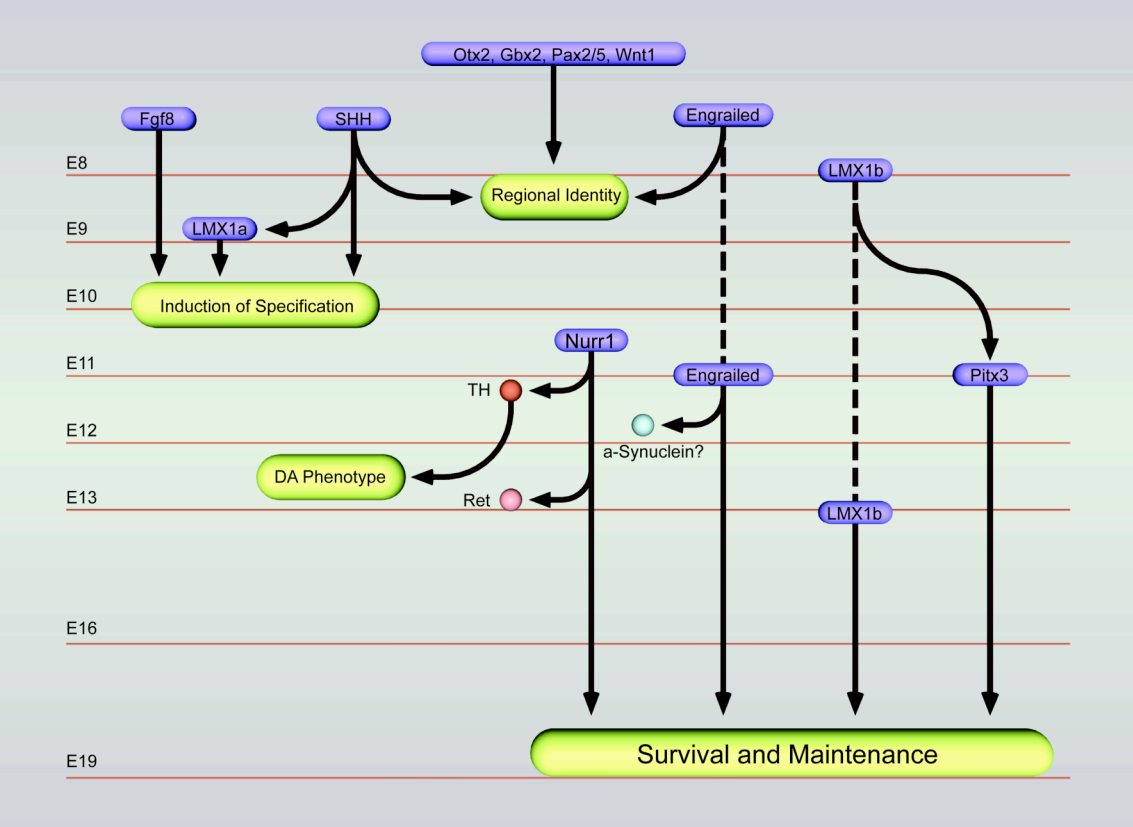


Figure-2: Time of expression and main function of transcription factors in development of the midbrain and mesDA neurons. Dashed lines represent expression in the region and not necessarily in the mesDA neurons.

the mesDA neurons express TH and become post mitotic (Zetterstrom et al., 1996). One of the main functions of Nurr1 is the control over synthesis and storage of dopamine in the midbrain. Mice, lacking Nurr1, do not express TH (Castillo et al., 1998) due to a direct trans-activation of the promoter of TH (Kim et al., 2003). Furthermore, expression of Nurr1 in dopaminergic MN9D cells increases DA content and the expression of aromatic L-amino acid decarboxylase (AADC) and vesicular monoamine transporter-2 (VMAT2). In mesDA cells of Nurr1 knockout embryos AADC and VMAT2 are deregulated (Hermanson et al., 2003; Smits et al., 2003). Study of the Nurr1 knockout mice also show that at P0, the dopaminergic phenotypic markers are absent from the mesDA neurons, while mesDA specific transcription factors like Pitx3, Engrailed1/2 and Lmx1b are present (Perlmann and Wallen-Mackenzie, 2004).

Nurr1 is important for migration of the mesDA neurons and their innervation to their respective forebrain targets (Perlmann and Wallen-Mackenzie, 2004; Wallen et al., 1999; Witta et al., 2000). Additionally, the Nurr1 heterozygous null mice show a postnatal, age dependent loss of nigral neurons resulting in reduced dopamine levels in the striatum and motor dysfunction (Jiang et al., 2005). An important factor, deregulated in mesDA neurons of Nurr1 knock-out mice is Ret, a subunit of receptors for glial derive neurotrophin factor (GDNF) (Baloh et al., 1997; Robertson and Mason, 1997; Wallen et al., 2001). GDNF promotes survival of mesDA neurons in various in vivo and in vitro conditions, including against neurotoxin treatment or the naturally occurring postnatal cell death (Burke et al., 1998; Clarkson et al., 1995; Tomac et al., 1995). Although Nurr1 is considered a postmitotic survival factor for the mesDA neurons, its expression in neuronal stem cells, can induce a general dopaminergic phenotype and in combination with expression of Pitx3 can result in terminal maturation of ES cell cultures into midbrain dopaminergic phenotype (Martinat et al., 2006).

Nurr1 represents a strong link between developmental paradigms and pathogenesis of PD. Several screenings of DNA from PD patients has provided a direct link of Nurr1 to PD. In such a study, about 10% of the familial PD cases had a hetero-duplex variation in the exon-1 of the Nurr1 gene while the patients with sporadic PD were unaffected (Le et al., 2003). In another study, a homozygous polymorphism of intron-6 was found in 9.5% of the familial cases and 4.2% of the sporadic patients (in

comparison to 0.9% for control individuals) (Xu et al., 2002). The heterozygote polymorphism of the same site has been associated with increased risk of PD (Zheng et al., 2003). In addition to the genetic studies, Nurr1 has been linked to the animal models of PD by studies showing a direct correlation between the level of expression of Nurr1 and resistance to MPTP induced injury (Le et al., 1999; Lee et al., 2002). Taken together, these studies show that Nurr1 is crucial for long term survival of mesDA neurons, in addition to being important in the terminal differentiation and neurotransmitter identity of these neurons.

2.2.2. Pitx3

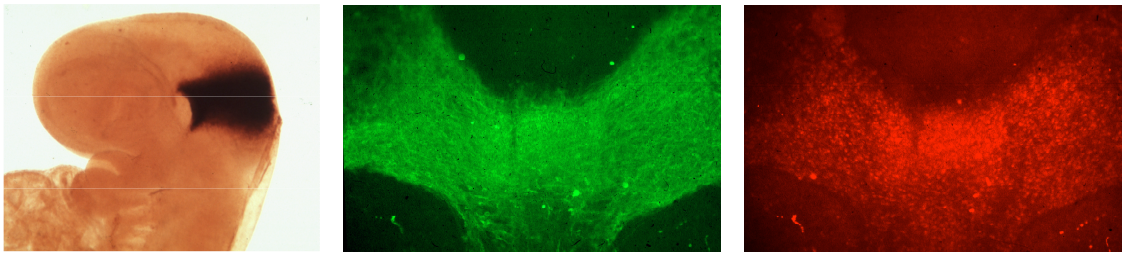
The expression of the paired-like homeodomain transcription factor 3 (Pitx3) in the CNS is limited to the ventral midbrain and specifically to the mesDA neurons, starting between E11.5 and E12.5 (Smidt et al., 1997). Since Pitx3 is also expressed in the developing lens, deletion of a region upstream of the 5' UTR of the Pitx3 gene results in Aphakia, characterized by small eyes and lack of a lens and anterior segment structures, caused by an arrest of lens development around E10-E11 (Semina et al., 2000). In the Pitx3 knockout (Aphakia) mice, loss of mesDA neurons is mostly in the rostral and lateral regions and at P0, the loss is almost exclusive to the SNpc and, with the exception of the cells bordering with SNpc, the VTA neurons remain unaltered. The loss of nigral neurons does not cause significant motor deficits, despite the considerable reduction in the dopamine level of the striatum (Nunes et al., 2003; Smidt et al., 2004; Smits et al., 2005).

Since the entire population of mesDA neurons is present in Aphakia mouse at E11.5 (the onset of expression of Pitx3), it is unlikely that Pitx3 is involved in proliferation or migration of these neurons and the specific nigral loss in these mice is due to a defect in terminal differentiation (Smidt et al., 2004). The specificity of the function of Pitx3 to the DA neurons of SNpc is more evident from the ES cells, expressing Pitx3, which show an up-regulation of the genes more specific to this region and not for the entire population of mesDA neurons. An example of such genes is aldehyde dehydrogenase 2 (AHD2), which has its highest expression level in the nigral DA neurons. This is in contrast to the expression of Nurr1 in ES cells, which lacks this

specificity and is relevant to all midbrain DA markers (Chung et al., 2005). In addition to its role in differentiation, Pitx3 controls the expression of TH in the nigral DA neurons, by activation of the TH promoter through direct interaction with a single high-affinity binding site within the promoter (Maxwell et al., 2005) (Lebel et al., 2001).

2.2.3. Lmx1b

Lmx1b and Pitx3 are part of the same molecular pathway, since in the mice, homozygous null for Lmx1b, Pitx3 expression is lost. The TH expression in the mesDA neurons of these mice is unaltered (Burbach et al., 2003). In addition to its importance in limb and kidney development (Chen et al., 1998; Kania et al., 2000), the member of the LIM homeodomain family, Lmx1b, is also critical in development and survival of the mesDA neurons. The expression of Lmx1b in mesDA neurons starts around E7.5 and continues throughout the embryonic development into adulthood (Burbach et al., 2003). The expression of Lmx1b is maintained by Fgf8 and defines the localization of Wnt1 expression (Adams et al., 2000). The mesDA neurons of the mice homozygous null for Lmx1b have normal expression of their phenotypic and transcriptional markers and TH positive neurons are generated but they are lost during embryonic maturation (Smidt et al., 2000). Lmx1b is also required for the induction of Wnt1 expression in the chicken mid/hindbrain boundary region (Adams et al., 2000).



Engrailed (E9) **TH (E14)** **En1 (E14)**

Figure-3: Pre-specification (E9) domain of expression of Engrailed and colocalization of Engrailed with TH in postmitotic (E14) neurons (Simon et al., 2004).

2.2.4. Engrailed1/2

The homeobox transcription factor, Engrailed, was first discovered in Drosophila melanogaster and has been extensively studied in this species (Eker, 1929). In drosophila, Engrailed has many major functions including development of a second sex comb, compartment and segment formation and wing development (Garcia-Bellido and Santamaria, 1972; Kornberg, 1981; Tokunaga, 1961). The cloning and sequencing of the mammalian (mouse) Engrailed has shown that it is highly conserved throughout evolution, with 75% homology to the drosophila Engrailed (Holland and Williams, 1990; Joyner et al., 1985). In fact, the Drosophila Engrailed can substitute for mouse Engrailed-1 function in the mid-hindbrain development (Hanks et al., 1998). The two mammalian homologues, Engrailed-1 (En1) and Engrailed-2 (En2) both belong to the family of homeobox transcription factors and have about 95% sequence similarity in the, DNA binding, homeodomain (Davidson et al., 1988). Genetically, En1 is located in the central portion of the mouse chromosome-1 and En2 in the proximal portion of

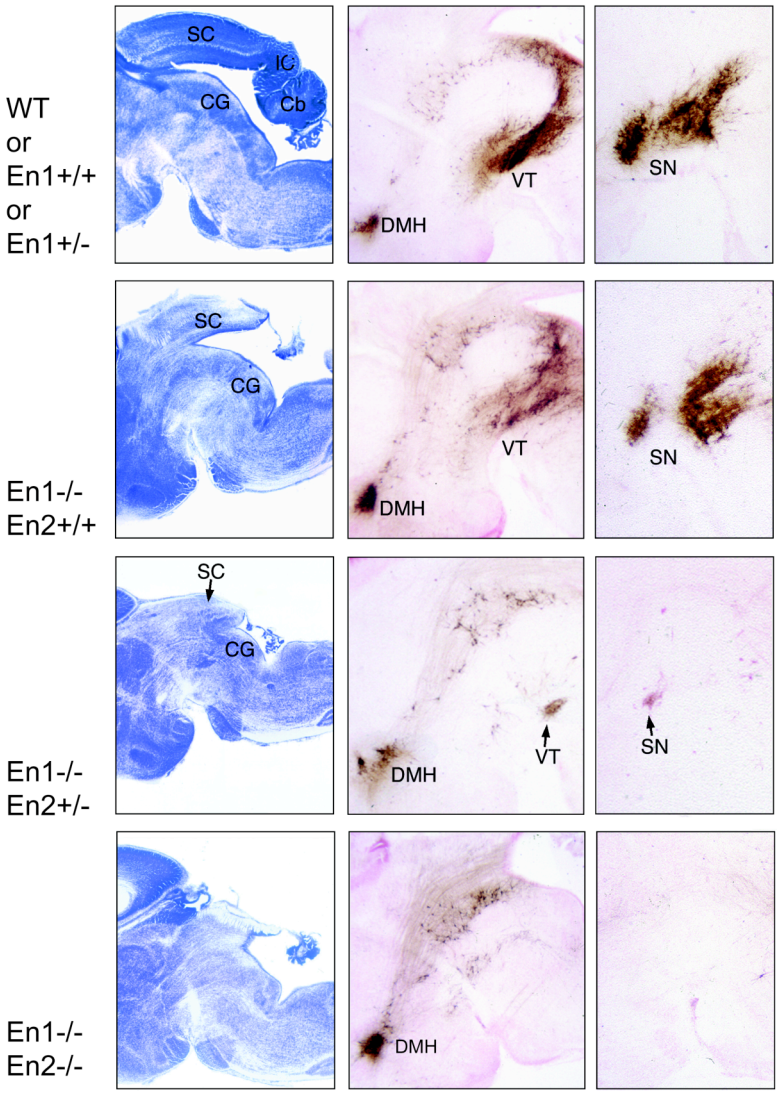


Figure-4: Gene dose dependency of mesDA neurons on the Engrailed genes and the functional redundancy of En1 and En2 (Simon et al., 2001)

mouse chromosome-5 (Joyner and Martin, 1987). The replacement of En1 coding sequence with En2 sequence, by gene targeting, shows that the two genes are functionally redundant (Hanks et al., 1995). The two genes are expressed around E8 in the anterior neuroectoderm in two distinct nuclei but eventually the expression domain of both merge into the border region between midbrain and hindbrain (Simon et al., 2004). During early embryogenesis, the Engrailed genes are involved in regionalization and also in specification of certain neuronal populations (Hidalgo, 1998; Wurst and Bally-Cuif, 2001). The expression of both genes in the mesDA neurons starts between E11 and E12 and continues throughout the life of the organism (Simon et al., 2001). The post mitotic mesDA neurons require the Engrailed genes for their survival and maintenance in a gene-dose dependent manner. In the mice lacking both genes, the mesDA neurons differentiate, become post mitotic and start expressing TH but at the time of expression of the Engrailed genes in their wild type counterparts, around E12, they start to die and by E14 the entire population of mesDA neurons is lost (Simon et al., 2001). Mice homozygous null for En1, die at birth, while En2 mutant mice only have a minor defect in cerebellar foliation (Millen et al., 1994; Wurst et al., 1994). With regard to the mesDA neurons, neither of the mutant mice, alone, shows major defects and both appear to be normal during embryonic development.

Studies on mice, homozygous null for one gene and heterozygous for the other, show that the survival of mesDA neurons is dependent on the dose of Engrailed (Fig. 4). In the En1-/-En2+/- mice, the SNpc and VT are substantially diminished and the entire

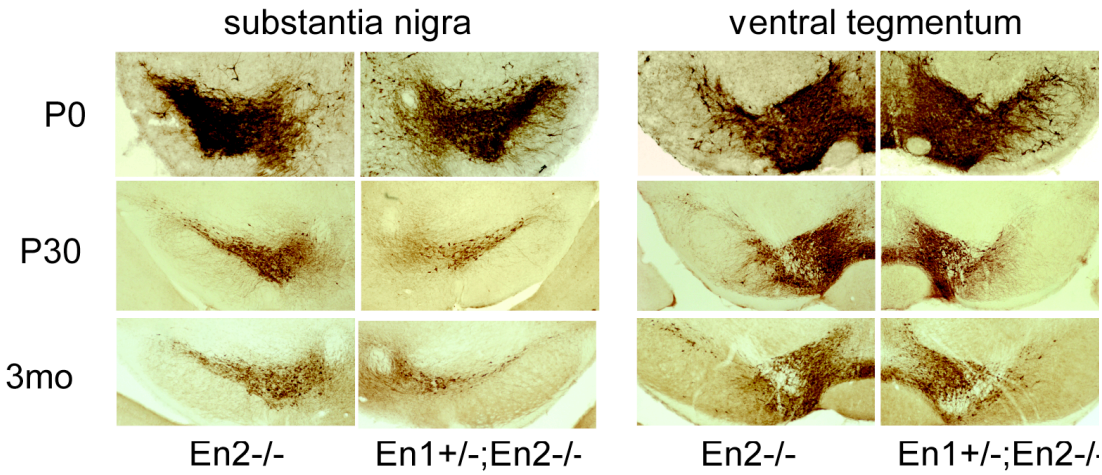


Figure-5: Postnatal, loss of DA neurons of En1+/-;En2-/- Mice. The loss happens after P15 and is limited to SN neurons and the number of mesDA neurons in VTA is unaltered (Sgadó et al., 2006).

DA population is reduced to a small cluster close to the ventral midbrain. The En1+/-En2-/- mice appear to be normal during embryonic development but show a postnatal gradual loss of 70%, specific to the SNpc during the first three months of their lives (Fig. 5). The PD-like gradual loss of the nigral DA neurons is accompanied by diminished storage and release of dopamine in the caudate putamen, motor deficits similar to akinesia and bradykinesia, and a reduction in weight, all closely resembling the symptoms of PD.

The initial studies on the mechanism of cell loss in the double mutant mice showed that the requirement for Engrailed is cell autonomous shown by cell mixing and RNAi experiments. The cultured mesDA neurons from the double mutant animals die in 72 hours even when mixed with the dissociated ventral midbrain tissue from wild type animals, indicating that the pathway of death is triggered intrinsically and the survival requirement for Engrailed is independent of the surrounding tissue. Additionally, activation of caspase-3 and appearance of pyknotic nuclei, both markers of apoptotic cell death, have been seen in mesDA neurons in vitro and in vivo, suggesting apoptosis as the mechanism of cell death (Alberi et al., 2004).

3. Parkinson's Disease, Pathology, Anatomy and Physiology of Dopamine System

3.1. Clinical features of Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, affecting approximately 1.5 to 2% of the population over the age of 55. The first set of clinical features include unilateral onset of resting tremor, muscular rigidity, and akinesia or bradykinesia which are the main clinical symptoms of PD, originally described by James Parkinson (Parkinson, 2002). In advanced stages, another set of problems appears, including dementia, depression, sleep disorders, sensory complaints, dysarthria, dysphagia, constipation and urinary dysfunction, masked facies, olfactory dysfunction, sialorrhea, seborrhea and orthostatic hypotension (Guze and Barrio, 1991; Hamani and Lozano, 2003). The first three of the symptoms are usually required and sufficient for diagnosis of PD and they become detectable when more than 50% of the mesDA neurons of the substantia nigra pars compacta (SNpc) are lost (Fearnley and Lees, 1991; Steece-Collier et al., 2002). The cell loss

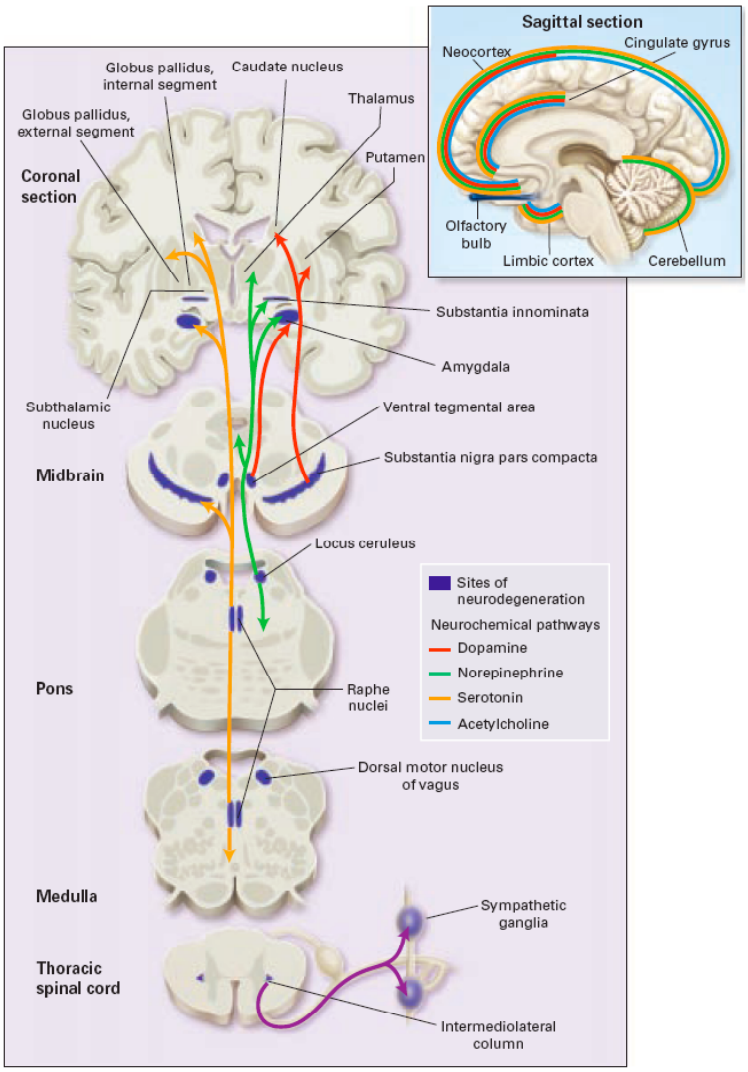


Figure-6: The sites of neurodegeneration; Dopaminergic as well as other pathways of the brain, affected by PD (Lang et al., 1998).

during the early stages is specific to the SNpc and it is only in very late stages when, dopaminergic neurons of ventral tegmentum, the neighboring cell population, are affected (Damier et al., 1999). The second set of dysfunctions could be due to loss of other neuronal populations of the brain, including locus ceruleus, raphe nuclei, and nucleus basalis (Fig. 6) (Halliday et al., 1990a; Halliday et al., 1990b; Lang and Lozano, 1998a). While eventually the noradrenergic, serotonergic and cholinergic pathways are affected, cause of initiation and progression of PD is attributed to loss of the mesDA neurons and deficiency in dopamine level of the basal ganglia, the target tissue of nigral dopaminergic neurons.

3.2. Dopamine Pathway, Pathophysiology of Parkinson's Disease

The mesDA neurons consist of the neurons located at three distinct nuclei, retrorubral field (RRF), substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA). DA neurons of VTA innervate into the ventromedial striatum, nucleus acumbens and the olfactory tubercle, forming the mesolimbic pathway, which is associated to pleasure, reward and desire (Oades and Halliday, 1987). Dysfunction in this pathway has been related to behavioral disorders, such as addiction and schizophrenia. The mesDA neurons of SNpc, on the other hand, exclusively innervate into the dorsal striatum, forming the nigrostriatal pathway, involved in motor control and implicated in Parkinson's disease (Fig. 7).

The prevailing model of pathophysiology of PD involves the structures in the basal ganglia and ventral midbrain, including regions of striatum (divided into three nuclei of putamen, caudate and nucleus

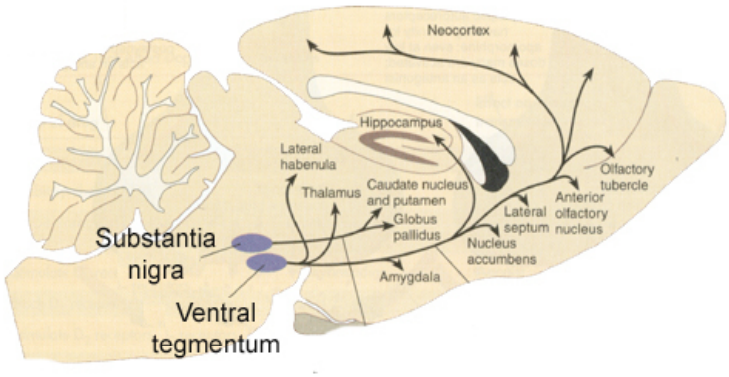


Figure-7: Dopaminergic pathways arising from midbrain; the nigrostriatal and mesolimbic pathways in rat (adapted from http://www.chemistry.emory.edu/justice/).

accumbens), the external and internal segments of the globus pallidus, the subthalamic nucleus, and substantia nigra comprised of three nuclei of pars compacta, pars reticulata and pars lateralis. In this model, loss of SNpc neurons results in diminished release of DA to two striatal nuclei, caudate and putamen. Eventually, this results in increased inhibitory γ-aminobutyric acid (GABA)-ergic output from the basal ganglia via two pathways. The neurons, expressing dopamine receptor D1, directly project to the internal segment of the globus pallidus (iGP) and substantia nigra pars reticulata (SNpr), while the neurons expressing D2 receptor project to the external segment of the globus pallidus (eGP). The diminished release of dopamine to caudate and putamen is excitatory on the D1-expressing cells and it is inhibitory on the D2 expressing cells. Therefore, the decrease in the DA level simultaneously causes a decrease in the GABAergic output to iGP and increased GABAergic output to eGP. The increased glutamatergic or decreased GABAergic input from the indirect or direct pathways, respectively, to the iGP and the SNpr results in increased thalamic inhibition, which leads to disruption of he activity of the cortical motor system, resulting in akinesia, rigidity and tremor (Fig. 8) (Albin et al., 1989; Lang and Lozano, 1998b; Lindvall and Bjorklund, 1979; Olanow and Tatton, 1999). There has been no effective treatment against loss of nigral neurons and current PD drugs relieve the

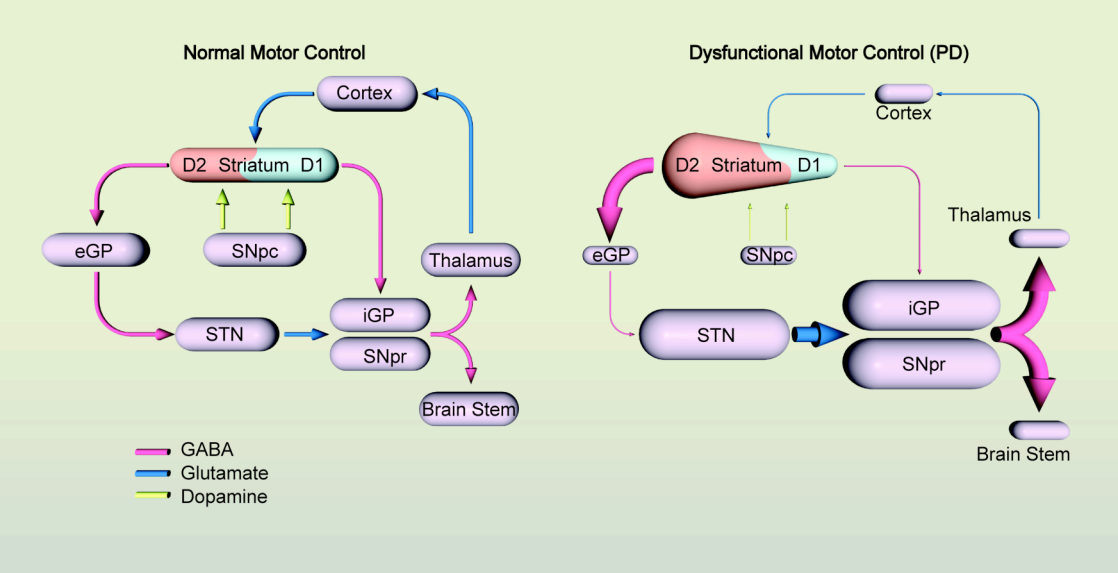


Figure-8: The basal ganglia model of pathophysiology of Parkinson's disease. Loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) causes decreased level of DA in the striatum which, depending on the receptor, causes decrease or increase of the GABAergic output and via direct or indirect pathway, causes disruption of the cortical motor system.

symptoms by overcoming the effect of dopamine deficiency in the striatum instead of stopping the cell loss in SNpc. The dopamine agonists, like apomorphine, and the dopamine precursor, L-DOPA serve such a purpose. The monoamine oxidase-B (MAO-B) inhibitors like Selegiline and Rasagiline also compensate for dopamine deficiency by inhibiting breakdown of secreted dopamine (Youdim et al., 2005).

3.3. Biochemistry and Molecular Biology of Dopamine Pathway

A neurotransmitter for slow synaptic transmission, dopamine (4-(2-aminoethyl)benzene-1,2-diol) is a member of the catecholamine family of neurotransmitters, which are synthesized from tyrosine. The steps in dopamine synthesis include hydration of tyrosine to L-DOPA (3,4-dihydroxy-L-phenylalanine) by tyrosine hydroxylase (TH) and decarboxylation of L-DOPA to dopamine by aromatic-L-amino acid decarboxylase (AADC). The rate-limiting enzyme of this process, TH, is often used to identify dopaminergic neurons. After synthesis, DA is packed into vesicles and released into the synaptic cleft, which binds to DA receptors in postsynaptic terminal. The unbound DA is taken up by DA transporter (DAT) and after reuptake, the DA inside the cell is either degraded to DOPAC by monoamine amine oxidase type B (MAO-B) or recycled into

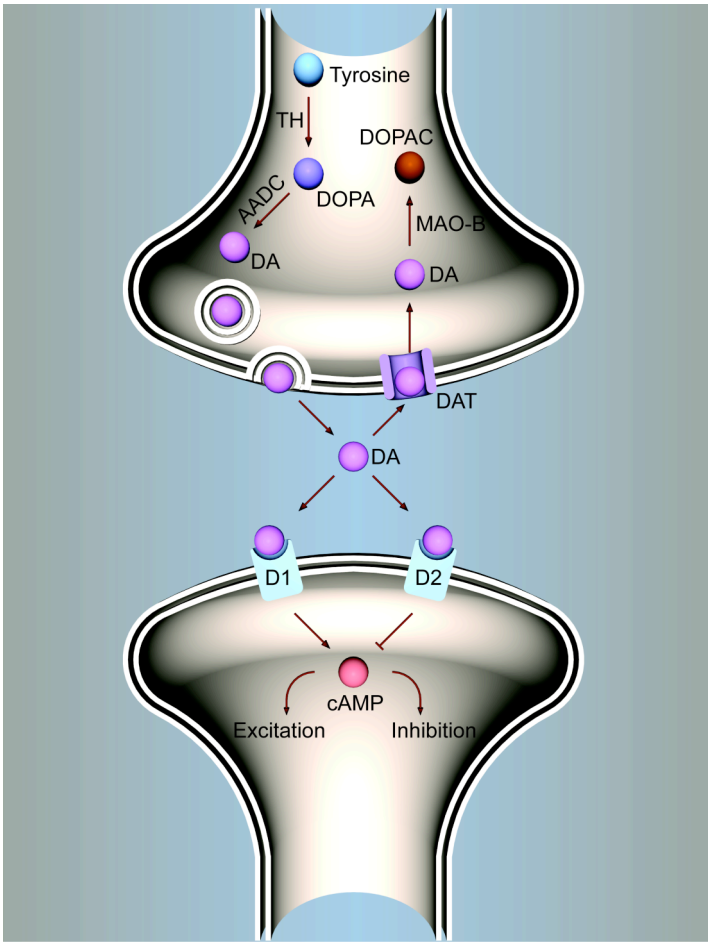


Figure-9: Synthesis, release, uptake, degradation and receptor binding of dopamine .

vesicles (Fig. 9).

The binding of DA to its receptors can cause inhibition or excitation, depending on type of the receptor. The dopamine receptors comprise 7-transmembrane domain and are metabotropic G protein coupled receptors, consisting of five members, D1-D5. The D1-like receptors, D1 and D5, are excitatory and induce their function by increasing the intracellular level of cyclic-AMP (cAMP) via adenylyl cylcase and subsequently, the level of cAMP-dependent protein kinase-A (PKA) The D2 like receptors, D2, D3 and D4, have inhibitory function with their activation resulting in reduction of cAMP levels. One important pathway of D1 signaling includes phosphorylation of DARPP32 by PKA which causes enhanced NMDA response (Girault and Greengard, 2004; Greengard, 2001a; Greengard, 2001b).

3.4. Experimental Models of Parkinson's Disease

The most common method to simulate the events of basal ganglia is to induce death of nigral DA neurons by specific neurotoxins. The widely used substances for this purpose are 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which induce specific loss of nigral neurons or agricultural toxins like rotenone and maneb. 6-OHDA is a hydroxylated analogue of DA which can cause catecholamine depletion in the brain after application into the basal ganglia or lateral ventricles (Ungerstedt, 1968). The mode of action of 6-OHDA is via production of reactive oxygen species (ROS). Generation of hydrogen peroxide and hydroxyl radicals and auto-oxidation by involvement of monoamine oxidase (MAO), iron, and Fenton reaction are some of the chemical events, causing oxidative stress (Blum et al., 2001). 6-OHDA also induces mitochondrial dysfunction by direct inhibition of complex-I of the mitochondrial respiratory chain in cell-free environments (Glinka and Youdim, 1995). This effect is reversible and independent of ROS and could not be replicated in the whole cell experiments, suggesting that inhibition of complex-I is not causal to production of ROS (Blum et al., 2001). However, the mitochondrial membrane damage occurs upon application of 6-OHDA, where ROS is likely to be responsible for the impairment of the mitochondrial membrane (Lotharius et al., 1999).

An analogue of the narcotic meperieine, MPTP, was first discovered in heroin addicts who displayed clinical characteristics of Parkinson's disease (Langston et al., 1983) and has been widely used, ever since, on different species to model nigral cell loss and PD symptoms. MPTP passes the blood brain barrier and is transformed (by MAO) to its active form, MPP^+, which can be taken up by DAT and accumulated inside dopaminergic cells. The cytosolic MPP^+ can enter mitochondria and inhibit complex-I, which in turn leads to inhibition of oxidation of NAD+ linked substrates (Mizuno et al., 1989). The mitochondrial events lead to decreased ATP levels inside the cell, loss of mitochondrial membrane potential, alteration of calcium homeostasis, and free radical formation. The inhibition of complex-I is only partially responsible for production of ROS, since mitochondria deprived Rho0 cells also respond to MPP^+ (Przedborski and Jackson-Lewis, 1998). Similar to 6-OHDA, MPTP also causes an increase in iron levels in nigral neurons, resulting in added oxidative stress (Blum et al., 2001; Przedborski and Jackson-Lewis, 1998). In both, 6-OHDA and MPTP models, the mitochondrial impairment has been linked to the classical pathways of apoptosis, especially the mitochondrial pathway (Vila and Przedborski, 2003b).

4. Apoptosis and Parkinson's Disease

4.1. Extrinsic and Intrinsic Pathways of Apoptosis

The term apoptosis, as it is known today, was first used in 1972 by Kerr et al. (Kerr et al., 1972) to describe a novel form of dying which differed in many ways from other modes of programmed cell death. Since then, it has become the main focus of research concerning normal developmental cell death and also too little or too much unwanted cell loss in disorders ranging from cancer to neurodegenerative diseases. The morphological characteristics of apoptosis include cell shrinkage, chromatin degradation and condensation, membrane blebbing and nuclear fragmentation (Kerr et al., 1972). Traditionally, apoptosis has been classified into caspase-dependent or independent mechanisms and, based on the mode of initiation, arbitrated via two major interconnected pathways, the death receptor-mediated, or extrinsic, and the mitochondrial or intrinsic cascades.

4.1.1. Receptor Mediated/ Extrinsic Pathway

The extrinsic pathway of apoptosis is activated upon interaction of the tumor necrosis family (TNF) members with the cysteine-rich, extracellular sub-domain of their respective death receptors, which belong to the TNF receptor (TNFR) gene family. The eight-member family of receptors includes CD95 (Fas or APO-1) and TNFR1 (p55 or CD12a). The ligand-receptor interaction leads to recruitment of the adaptor molecules and formation of signaling complexes and activation of caspase-8 and caspase-3. For example, in the case of CD95, the interaction of the Fas ligand with its receptor leads to recruiting of the adapter molecule, Fas associated death domain (FADD), and procaspase-8. Association of the death domains of the Fas receptor and FADD plus interaction of the death effector domains (DED) of FADD and procaspase-8 from one side and FADD with the Flice inhibitory protein (FLIP) from the other side (CD95L&R, FADD, procaspase-8 and FLICE make up DISC), lead to cleavage of caspase-8 and subsequent activation of an apoptotic cascade, plus instigation of an inherent inhibitory mechanism opposing the initiation of this deleterious process (Smith et al., 1994) (Ashkenazi and Dixit, 1998) (Peter and Krammer, 2003) (Lavrik et al., 2005). Activation of caspase-8 leads to activation of the effector caspases like caspase-3, 6 or 7 (Fig. 10). The TNFR1 apoptotic signaling pathway employs a similar mechanism of receptor-ligand interaction, formation of death signaling complex and activation of caspase-8 and subsequently, activation of the executioner caspases. This mechanism could directly lead to destruction of the cell or to activation of the, otherwise independent, machinery of mitochondrial pathway.

4.1.2. Mitochondrial/ Intrinsic Pathway

Although mitochondrial apoptosis could be considered a magnifying step for caspase activation and other pathways of apoptosis, intrinsic stimuli such as reactive oxygen species cause mitochondrial dysfunction and, eventually, lead to apoptosis. A crucial event in this process is the release of cytochrome C and other pro-apoptotic molecules (like AIF and SMAC/Diablo) from the mitochondrial outer membrane. About 90% of cytochrome C is stored in vesicles formed from mitochondrial inner membrane and

10% is located at the intermembrane space (Mayer and Oberbauer, 2003). Cytochrome C has a defined role as being an electron transfer protein in mitochondrial oxidative phosphorylation. The apoptotic role of cytochrome-C involves its release into the cytosol and being an essential component in formation of apoptosome, the structure required for activation of caspase-9. Although the exact mechanism of the cytochrome C release into the cytosol is not known, there are several hypotheses about the structural changes in mitochondrial membrane permeability, due to interplay of several molecules and mechanisms, with the members of the Bcl-2 family acting as the main regulators of the overall process. The changes in the membrane permeability are associated with formation of the mitochondrial permeability transition pore and other membrane channels, involving the members of the Bcl-2 family and the upstream regulatory proteins, such as p53 (Polster and Fiskum, 2004).

The multipeptide channel, mitochondrial permeability transition (MPT) pore forms at the contact site of the two membranes, consisting of peptides like voltage dependent anion channel (VADC) on the outer membrane and adenine nucleotide translocator (ANT) on the inner membrane plus other molecules from the matrix and intermembrane space (Zoratti and Szabo, 1995). Bax, a pro-apoptotic member of the Bcl-2 family, interacts with individual components of the mitochondrial permeability transition pore (Adachi et al., 2004; Shimizu et al., 1999). Binding of the anti-apoptotic members of this family can close the VDAC and inhibit apoptosis (Shimizu et al., 2000). Despite these interactions, the permeability transition pores can cause apoptosis on their own and the Bcl-2 family members can regulate release of cytochrome C independent of these pores. Therefore, the interaction of members of the Bcl-2 family with VDAC, ANT and other components of the MPT pore is only one of the ways these proteins induce their pro and anti apoptotic functions.

4.1.3. Bcl-2 Family Members

Members of the Bcl-2 family are classified into three groups of pro-apoptotic, anti-apoptotic and BH3-only, sharing at least one conserved Bcl-2 homology (BH) domains (Borner et al., 1994). Each of the BH domains is involved in a specific function. The BH3 domain is responsible for progression of apoptosis while BH4 domain induces

anti-apoptotic activity. BH1 and BH2 are used in formation of the structures necessary for interaction and oligomerizatin of the members of this family. Therefore, the anti-apoptotic members, including Bcl-2, Bcl-X_L, Bcl-w and Mcl-1 have all four domains where BH4 domain is missing from the pro-apoptotic members like Bax and Bak. Other members like Bid, Bok, Bim and Bad only have the BH3 domain (Huang and Strasser, 2000).

Beside the interactions with mitochondrial transition permeability pore, the pro-apoptotic members of the Bcl-2 family, can also form new channels and induce membrane lipid alterations (Sharpe et al., 2004). In non-apoptotic cells, Bax exists as an inactive monomer either in the cytosol or loosely bound to the mitochondrial outer membrane. Upon activation by apoptotic stimuli, including interaction with truncated Bid (t-Bid), Bax undergoes a conformational change, which allows the disengagement of its C-terminal anchor domain from the hydrophobic groove and insertion into the mitochondrial outer membrane (Nechushtan et al., 2001). Insertion into the membrane is followed by Bax oligomerization, which requires further conformational change and release of the BH3 domain, required for Bax-Bax homodimerization or heterodimerization of Bax with pro-survival members of the family (Chan and Yu, 2004).

Studies on the structure of the Bcl-X_L show that the BH-1 and BH-2 domains, along with the BH3 domain, form a hydrophobic groove on the surface of the anti-apoptotic members of the Bcl-2 family to which the alpha helical BH3 domain binds (Muchmore et al., 1996) (Sattler et al., 1997). The exact molecular mechanism of protection by the pro-survival members of the Bcl-2 family is not known but dimerization with their pro-apoptotic relatives (by interaction of the amphipathic alpha-helical BH3 domain of one member with the hydrophobic surface groove of another) is essential to their function. Therefore, the ratio of pro apoptotic to the anti apoptotic members is essential in regulation of mitochondrial sensitization and the rate of death via the intrinsic pathway. Likewise, the BH3-only proteins can induce apoptosis by direct interaction with the pro-apoptotic members (synergistic and activating effects) or neutralizing the effect of anti-apoptotic members (inhibition of heterodimerization with pro-apoptotic members).

4.1.4. Downstream of the Mitochondria

After the release of cytochrome C from the mitochondrial intermembrane space into the cytoplasm, it binds to and oligomerizes the apoptotic protease activator factor 1 (Apaf-1) and procaspase-9 to form the apoptosome (Li et al., 1997). The main mode of activation of caspase-9 is through conformational changes of procaspase-9 via association with the other two molecules (Rodriguez and Lazebnik, 1999). Formation of the activating protein complexes (apoptosome for caspase-9 and DISC for caspase-8) is a common molecular event in activation of initiator caspases, while the effector caspases are activated through proteolytic cleavage by their respective initiator caspases (Shi, 2004). In the same way, activation of caspase-9 mainly leads to activation of caspase-3, which is also the effector caspase of the death receptor mediated apoptosis.

Beside the caspase-dependent pathways, mitochondria could be involved in several other apoptotic processes including release of the apoptosis inducing factor (AIF) and activation of the caspase-independent pathway (Joza et al., 2001). The mitochondrial pathway is considered as a magnifying step for the extrinsic apoptosis in certain cell types with low levels of activation of caspase-8 (Scaffidi et al., 1998). In such cells, the Bcl-2 family member, Bid, is truncated by caspase-8 and the truncated Bid (tBid) will mediate release of cytochrome C and activation of caspases 9 and 3 (Li et al., 1998; Luo et al., 1998). The activation of caspase-3, downstream of caspase-8 and/or caspase-9 (Stennicke et al., 1998), leads to cell death due to its cleavage and, therefore, degradation and inactivation of proteins (like the caspase activated DNAse, CAD, or Bcl-2 proteins) and destruction of cellular structures (like lamin) (Thornberry and Lazebnik, 1998).

4.2. PD and Apoptosis

Although the causes of vulnerability and degeneration of the mesDA neurons are still unknown, the evidence like involvement of the death receptors and their ligands, mitochondrial dysfunction and increase in activation of caspases suggest the extrinsic and intrinsic pathways of apoptosis as the executioners of neuronal cell death

(Hartmann and Hirsch, 2001; Hartmann et al., 2000; Tatton et al., 2003; Vila and Przedborski, 2003b). Mitochondria is the site of action for several deleterious processes involved in pathogenesis of PD. Reduced activity of mitochondrial complex-I and production of reactive oxygen species are found in postmortem brains of PD patients and can cause specific loss of mesDA neurons of SNpc in the three major neurotoxin-based models of the disease, MPTP, Rotenone and 6-OHDA (Schapira et al., 1990; Schober, 2004). Additionally, the inhibition of complex-I results in permeability of the mitochondrial membrane and release of pro-apoptotic molecules, including cytochrome C, into the cytoplasm and subsequent activation of caspases, leading to apoptotic cell death (Kroemer et al., 1998; Perier et al., 2005). In animal models of PD, this effect could be ablated by high expression of Bcl-2 and Bcl-X_L the anti-apoptotic members, or genetic ablation of Bax, the pro-apoptotic member of the Bcl-2 family (Vila et al., 2001; Yang et al., 1998).

4.2.1. The Death Receptor Mediated Pathway and PD

Human postmortem studies implicate the involvement of death receptor induced cell death in PD. The levels of pro-inflammatory cytokines like TNF-α are elevated in the brain and cerebrospinal fluid of PD patients (Mogi et al., 1994) and the density of TNF positive glial cells in SNpc of PD patients is higher than the healthy individuals (Boka et al., 1994). The human studies also suggest an increase in the soluble form of Fas/CD95 in PD patients (Mogi et al., 1996). Furthermore, the expression of Fas associated death domain (FADD), in dopaminergic neurons is limited to mesDA neurons and especially to the SNpc (Hartmann et al., 2002a). Despite these observations, the data from the neurotoxin based animal models is controversial. For instance, the studies from CD95 knockout mice assume both, neuroprotective and apoptotic roles for this ligand-receptor pathway (Hayley et al., 2004; Landau et al., 2005). Similarly, results of the studies about the role of TNF receptors have been are contradicting and both in favor or against their involvement in loss of mesDA after neurotoxin treatment (Rousselet et al., 2002; Sriram et al., 2002).

4.2.2. The Mitochondrial Pathway and PD

Mitochondria are the site of convergence of many deleterious processes. Some of the mitochondrial events, supporting the notion of involvement of the intrinsic pathway of apoptosis in PD, observed in postmortem brains of PD patients and most (if not all) of the animal and cellular models of this disease, are activation of the effector and initiator caspases of the mitochondrial apoptosis, caspase-3 and caspase-9, release of

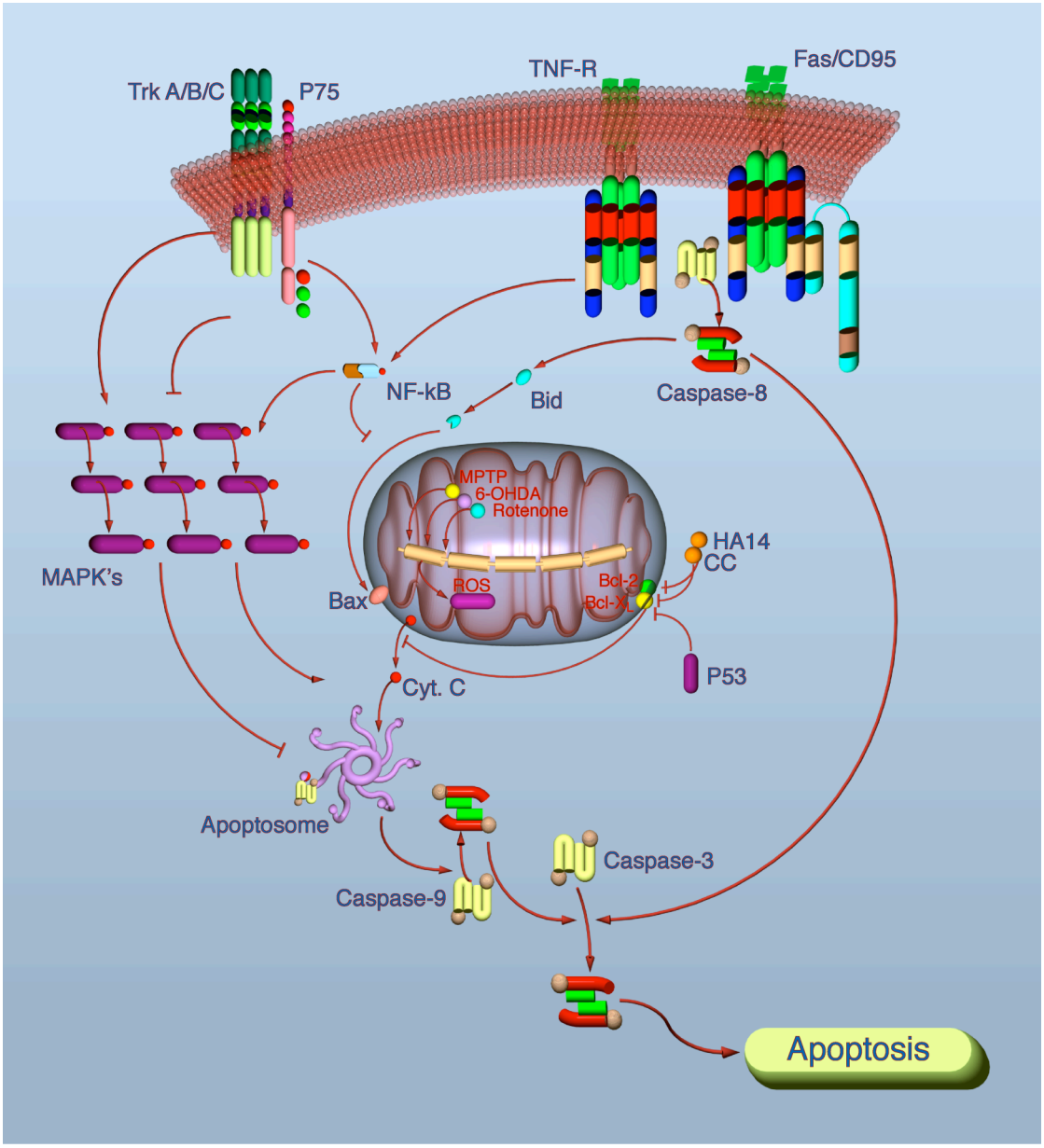


Figure-10: Extrinsic and Mitochondrial pathways of apoptosis, neurotoxin induced vulnerability of mitochondria and the anti-apoptotic role of neurotrophins.

cytochrome C and AIF from mitochondria to the cytosol, differential expression and regulation of members of the Bcl-2 family, alterations in the activity of complex-I of the electron transport chain and excessive production of the reactive oxygen species (ROS). Therefore, mitochondrial dysfunction is considered a decisive point in etiology of PD.

The activity of the mitochondrial complex-I (NADH/ubiquinone oxidoreductase) is reduced specifically in the substantia nigra of the PD patients (Schapira et al., 1990). This biochemical defect is also present in three major neurotoxin induced models of PD, MPTP, 6-OHDA and Rotenone (Schober, 2004). In addition to causing oxidative damage to DNA, proteins and membrane lipids, (similar to the effect of excessive $Ca2^{+}$) excessive production of ROS by inhibition of complex-I, can trigger release of the pro-apoptotic proteins from mitochondria by interacting with classical mitochondrial apoptosis (Fiskum et al., 2003). This interaction has been demonstrated in the mouse MPTP model where complex I deficiency, caused by chronic application of MPTP, increases the level of cytochrome C in the mitochondrial intermembrane space and triggers mitochondrial localization of Bax (Perier et al., 2005).

In postmortem brains of PD patients, expression of the BCl-2 family members is affected by the disease. Bax is expressed and present in higher percentages in the Lewy body containing (PD suffering) neurons than other mesDA neurons of the same subjects (Hartmann et al., 2001). Also, the expression of Bcl-X_L is doubled in the mesDA neurons of PD patients (Hartmann et al., 2002b). The role of the members of this family, also, has been demonstrated in the animal models. The neurons from transgenic mice, over-expressing human Bcl-2, are resistant to neurotoxicity, induced by 6-OHDA and MPTP (Offen et al., 1998; Yang et al., 1998) while Bax ablation can have similar results in MPTP treated animals (Vila et al., 2001). In a genetic model of PD, where alpha-synuclein has a dual function, Bcl-2, Bax and Bcl-X_L contribute to survival as well as death (Seo et al., 2002). A comprehensive picture about different functions of the Bcl-2 family members is yet to emerge, since in addition to regulation of the oxidative stress (Hochman et al., 1998), members of the Bcl-2 family are considered to be involved in the general survival and maintenance of the mesDA neurons (Savitt et al., 2005), further associating the pivotal role of mitochondrial apoptosis to the vulnerability of this neuronal population.

The events downstream of the mitochondria, like release of cytochrome C, Smac/DIABLO or AIF, have not been studied in human tissue and the activation and expression of caspase-3 is the only event that happens in the SNpc of PD patients (Hartmann et al., 2000). On the other hand, in the animal models of PD, there is growing evidence suggesting the activation of caspase-dependent and independent pathways, downstream of the mitochondria. In these models, activation of caspases arbitrates the death signal from the mitochondria or other upstream pathways, like P53 or the death-mediating MAPK pathways, P38 and c-Jun N-terminal kinase (JNK) (Blum et al., 1997; Choi et al., 2004; Lotharius et al., 2005; Wang et al., 2004).

5. Trophic Factors in Survival of MesDA Neurons

Neurotrophic factors regulate many essential processes including neuronal survival, death and plasticity in the CNS. Three major families of neurotrophic factors are the neurokines, including the cilliary neurotrophic factor (CNTF), glial derived neurotrophic factor (GDNF) family and the nerve growth factor (NGF) related neurotrophins. GDNF and the brain derived neurotrophic factor (BDNF), belonging to the two latter super-families, are the most effective survival factors for mesDA neurons during the course of development or in preventing neurotoxin-induced cell loss in animal models of PD (Henderson et al., 1994; Hyman et al., 1991; Spina et al., 1992; Tomac et al., 1995).

5.1. Glial Derived Neurotrophic Factor

A member of the transforming growth factor-ß (TGF- ß) super family, GDNF is a general anti-apoptotic and survival factor for mesDA neurons in vivo and in vitro (Clarkson et al., 1997; Tomac et al., 1995). Unlike other TGF-ß family members where the signaling happens through the receptor serine-tyrosine kinases, the neurotrophic function of GDNF family ligands (GFL's) is activated when these ligands bind to their cognate GDNF-family receptor-α (GFRα), resulting in activation of the Ret (rearranged during transformation) tyrosine kinase (Airaksinen and Saarma, 2002). The high affinity binding of GFLs to one of the GFRα receptors dimerizes Ret,

resulting in phosphorylation of tyrosine domains on the Ret molecules which become the binding sites for Src homology-2 SH2 domain of the interacting intracellular signaling molecules(Cacalano et al., 1998). The intracellular pathways, activated through this mechanism include phosphatidylinositol 3 kinase (PI3K), the mitogen activated protein kinase (MAPK) pathway inducing processes like proliferation, differentiation, migration, neurite outgrowth and survival (Airaksinen and Saarma, 2002; Koeberle and Ball, 2002; Neff et al., 2002; Saarma and Sariola, 1999). In mesDA neurons, GDNF induces its anti-apoptotic function through mechanisms such as, over-expression of Bcl-2 and Bcl-X_L(Sawada et al., 2000), enhancement of TH phosphorylation(Kobori et al., 2004) and activation of Erk1/2 and PI3K pathways (Ugarte et al., 2003).

5.2. Neurotrophins and Their Receptors

5.2.1. Neurotrophin Receptors

Neurotrophins, consisting of BDNF, NGF, NT3 and NT4/5, induces their action via a set of specific tyrosine kinase receptors (Trk) and a common receptor (P75). The induction of the kinase activity of the (Trk) tyrosine receptor kinase family members, TrkA, TrkB and TrkC, by their respective neurotrophins, NGF, BDNF or NT4/5 and NT3 is mainly pro-survival. This survival function is through homodimerization of the Trk receptors upon binding of neurotrophins and trnasphosphorylation of tyrosine residues in their cytoplasmic domains which (similar to Ret) become the site of interaction with the Src homology domain-containing adaptor protein (SHC) which, via recruitment of other specific adaptor proteins, causes involvement of the Ras pathway, resulting in activation of PI3K and MAPK/Erk1/2 (Atwal et al., 2000; Liu and Meakin, 2002; Obermeier et al., 1993)(Fig. 11). A similar mechanism has been envisioned for P75-Trk regulated survival while the exact underlying mechanism is not known yet.

The dual (pro-survival or pro-apoptotic) function of P75 neurotrophin receptor depends on the neurotrophins, its dimerizing partner and the factors binding to its intracellular death domain (Fig. 11). While the type of neurotrophins could be a factor in deciding the function of P75, it is form of the ligand, i.e. the pro or mature form,

that is the key to determining the apoptotic or survival mediating function of p75 (Lee et al., 2001). Neurotrophins in their pro form have a high affinity for p75 than Trk receptors and upon binding, force p75 to dimerize with Sortilin while the mature neurotrophins have a high affinity for Trk receptors and cause homodimerization of them or their heterodimerization with P75 (Nykjaer et al., 2004). On the other hand, the mere over-expression of P75 can result in apoptosis, mediated by phosphorylation of JNK and BH3-only members of the Bcl-2 family (Fig. 11) (Bhakar et al., 2003). The binding of intracellular proteins, like NRAGE to the death domain of P75 can also induce cell death via activation of MAPK's and mitochondrial dysfunction(Salehi et al., 2002). In this paradigm the survival mediating role of Trk's can be viewed as inhibition of the death machinery of P75 with the decision of life and death being made by activation of the signaling pathway of MAPK's, Erk1/2 and JNK, respectively.

5.2.2. Neurotrophins

While there has been some evidence for NGF protective role against MPP^+ treatment in PC12 cells, (Shimoke and Chiba, 2001) there is not much evidence for NGF as a survival factor for mesDA neurons, probably due to the lack of expression of its cognate receptor, TrkA in these neurons (Melchior et al., 2003). Among neurotrophins, the survival effect of BDNF in mesDA neurons and its protective role against neurotoxin-induced cell death is the most pronounced (Hyman et al., 1991). Reduced level of BDNF in nigral mesDA neurons and their surrounding tissue in PD patients (Howells et al., 2000) may provide a link between lack of BDNF and the human condition. Additionally, observations from gain and loss of function experiments in the animal models support the notion of BDNF as a potent survival factor for mesDA neurons. While the mRNA level of BDNF decreases upon application of neurotoxins, (Venero et al., 1994), application of BDNF protects cells from toxicity induced by 6-OHDA and MPTP (Frim et al., 1994; Shults et al., 1995). On the other hand, BDNF knockdown by antisense oligonucleotides or conditional knock out causes loss of mesDA neurons (Baquet et al., 2005; Porritt et al., 2005).

Other members of the neurotrophin family have a lesser, yet significant, effect on mesDA neurons. Because of binding to the same Trk receptor (TrkB), the function of NT4 is very similar to that of BDNF to the degree that it can rescue the BDNF deficient mice when knocked into the BDNF locus (Fan et al., 2000). In mesDA neurons, NT4, is protective against toxic effects of iron and nitric oxide(Lingor et al., 2000). It is also beneficial to nigral explant cultures, when combined with GDNF (Altar et al., 1994). Furthermore, NT4 could be involved in locomotor control since in the D2 receptor-deficient mice, which display motor dysfunction, its mRNA level is lower (Bozzi and Borrelli, 1999). NT3 has a lesser, yet significant effect (in comparison to BDNF and NT4) as a survival factor for mesDA neurons and in eliminating the behavioral deficits caused by partial 6-OHDA lesions (Altar et al., 1994).

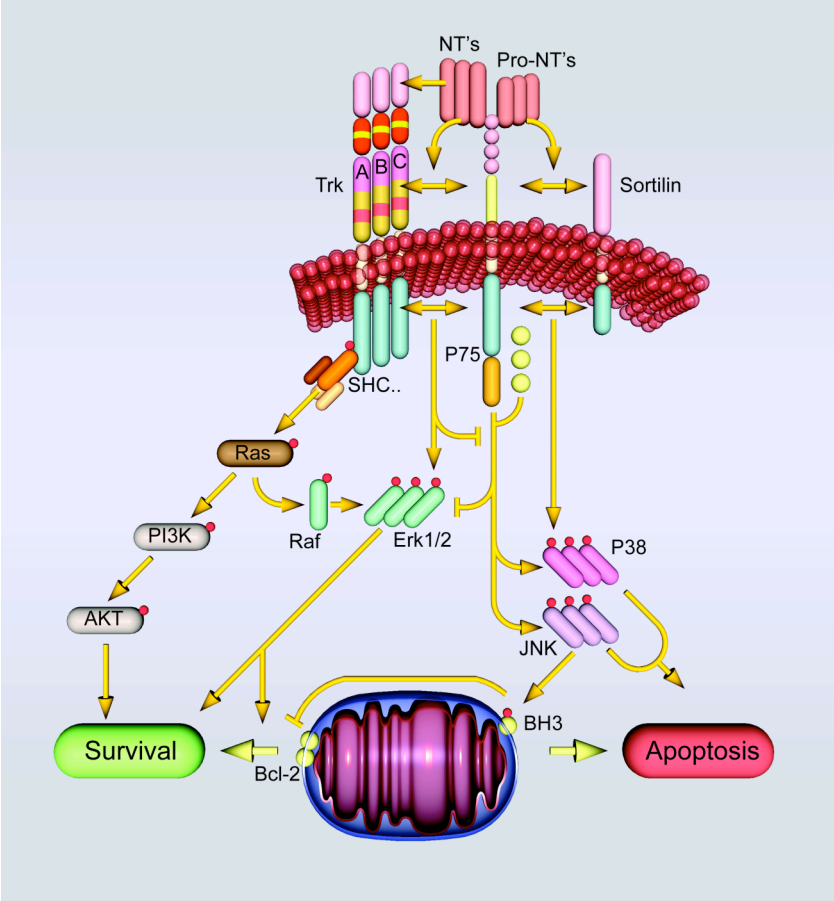


Figure-11: The molecular pathway of neurotrophins, their receptors and downstream pathways. Mere overexpression of P75 or its binding to sortilin induces apoptosis via JNK or P38, as opposed to the pro-survival role of the Trk receptors which are pro-survival and activate the Etrk1/2 pathway.

5.3. Downstream Effectors of Neurotrophins

The pro-survival role of GDNF, BDNF and other neurotrophins has been attributed to activation of two downstream pathways, the phosphotydil inositol-3 kinase (PI3K) and the extracellular receptor kinase 1/2(Erk1/2) (Krieglstein et al., 2002). PI3K is mainly considered a survival pathway, being activated by many factors including expression of α-synuclein, presence of GDNF, BDNF, and other neurotrophins (Salinas et al., 2003; Seo et al., 2002; Ugarte et al., 2003).

Mitogen activated protein kinases (MAPKs) consist of three distinct molecular pathways with opposing functions. While JNK and P38 are classically known as stress induced pathways, triggering apoptosis, Erk1/2 pathway is generally considered pro-survival and a mediator of action of neurotrophins (Xia et al., 1995). Mechanism of activation of Erk, like the other two MAPK, is dependent upon its upstream G-protein, MAPKKK, and MAPKK activities. Therefore, a mechanism consisting of Ras, Raf and MEK is responsible for activation of Erk. Activation of the proto-oncogene, Ras, is dependent upon GDP-GTP exchange (McCormick, 1993). The activation of Ras can result in activation of all three types of mammalian Raf, Raf-1, A-Raf and B-Raf (Kolch, 2000). Mek1/2 is an activation target for all three homologues of Raf, which, in turn, phosphorylates a motif of Thr-Glu-Tyr in the activation loop of Erk1/2 (Kolch, 2000). Erk1/2 has also been considered independent of its upstream MAPKK molecule (MEK) and, for example, P75 can directly interact with Erk1/2, upon addition of NGF (Volente et al., 1993). While the kinase activity of the Trk receptors, via its intracellular domain, clearly signals survival, the exact molecular mechanism and outcome of P75-Trk interaction with regard to activation of the downstream pro-survival or pro-apoptosis MAPKs is not well understood.

Contrary to the traditional view about the role of Erk1/2, there is also evidence, suggesting a pro-apoptotic function for Erk1/2. This function depends on the site and duration of activation and has been observed only in vitro. While sustained, cytoplasmic activation of Erk1/2 is consistent with its pro-survival role, its short term, nuclear activation seems to cause apoptosis via a caspase-independent mechanism (Oh-hashi et al., 1999; Subramaniam et al., 2004; Torii et al., 2004; Troadec et al., 2002). In mesDA neurons, Erk1/2 is involved in many processes including regulation

of trophic factor survival and neurotransmitter synthesis. With regard to neurotrophic factors and mesDA neurons, Erk1/2 is known as one of the downstream effector of GDNF signaling (Nicole et al., 2001; Ugarte et al., 2003). Erk1/2 is also important to the neurotransmitter synthesis in mesDA neurons. The activation of tyrosine hydroxylase is a result of its phosphorylation at three distinct sites (ser-19, 31, 40) of which, two, ser-31 and ser-40, are responsible for synthesis of dopamine and regulation of these two sites has been linked to activation of Erk1/2 and protein kinase A (Haycock et al., 1992; Kobori et al., 2004).

Methods and Material

1. Generation of the Mice and Genotyping

1.1. Generation and Maintenance of the Mice

The En1/tau-lacZ mice were generated by a knock in strategy, where the first 71 codons of the En1 gene, including the first codon, including the start codon, were replaced by the Tau-lacZ (Callahan and Thomas, 1994) sequence, as described in (Saueressig et al., 1999). Generation of the En2-deficient mice, done by targeted gene deletion, has been described in (Joyner et al., 1991). The two lines were crossed for obtaining En^{DM}, En^{HT} and En2-/- animals.

1.2. Extraction of Genomic DNA

About 1cm tail biopsy from 4-6 weeks old mice were collected and stored at -20°C. For digestion of the tails, each tail was incubated overnight at 55°C in 550ul TNES buffer (50 mM Tris, 0.4 M NaCl, 100 mM EDTA, and 0.5 % SDS) with 350ug of proteinase-K (Applichem, Germany). After digestion, 180ul 5M NaCl was added to the samples following by a 15min. centrifugation at 14000rpm. The supernatant was transferred to a new tube. An equal volume of 96% ethanol (750ul) was added to the supernatant and centrifuged at 14000rpm for 30min. The supernatant was drained and the pellet was air-dried. The pellet was dissolved in 300ul H_2O or TE (10mM Tris pH=8, 1mM EDTA) and stored at 4°C for PCR.

1.3. Genotyping

For producing three genotypes of En1 i.e. wild type, heterozygous and mutant, all in En2-/- background, crossing was made using parents with En1+/- En-/- genotype. The genotyping for En2 wild type and mutant alleles was performed by PCR using the following three primers in the same mix, the common primer, TTGAGAAGAGAGGCCCTGTA, the wild type primer, specific for En2 homeobox, CTGGAACAAAAGGCCAGTGT, and the, TCTCATGCTGGAGTTCTTCG in the neomycin gene, to detect the En2 mutated allele/s. The primers for detecting the knocked in Tau-LacZ allele were GTGTCCGGCAGCTTGGTCTT on the Tau-LacZ

gene, and TTCGCTGAGGCTTCGCTTTG, starting at position 224 of the exon-1 of En1. The PCR settings for En were 5 min at 94°C, 36 cycles (45 sec at 94°C, 1min at 54°C, 1 min at 72°C), 5 min at 72°C and for En1 they were 4 min at 94°C, 30 cycles (45 sec at 94°C, 1min at 54°C, 1 min at 72°C), 4 min at 72°C. The PCR mix consisted of 2.5 mM $MgCl_2$, 10x buffer, 1mM dNTP and 2% primer mix and 1% Taq polymerase (all from Fermentas, Germany) plus 1µg/µl DNA (2% of the total volume). Alternatively, for detection of the tau-lacZ, X-gal staining was used by incubating the toes of embryos/animals in the solution, containing 5 mM $K_3Fe(CN)_6$ (Sigma, Germany), 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$ (Sigma, Germany), 2 mM $MgCl_2$ and with 1mg/ml X-Gal (AppliChemGmbH, Germany) for 30min at 37ºC.

2. Primary Culture of Ventral Midbrain

2.1. Preparation of Laminin-Coated Cover slips

12mm glass cover slips (NeoLab, Germany) were boiled in 80% ethanol and sterilized by rinsing in 100% ethanol and flaming. Alternatively, cover slips were placed in front of the ultraviolet lamp overnight. Then, they were placed into the 24 well culture dishes and covered with 500µl polyornithin solution (polyornithin 0.1 mg/ml (Sigma) in 15mM borate buffer (Sigma, Germany) pH 8,4.) for (at least) one hour at room temperature. The cover slips were washed for three times successively with sterile water. 1mg/ml aliquots of laminin (Sigma, Germany) were thawed slowly on ice and dissolved (1:1000) in DMEM-F12 (Invitrogen, Germany). The cover slips were covered by 500µl Laminin dissolved in DMEM (final concentration: 1µg/ml) and incubated at 37° from 2 hours to overnight. Right before culturing, the cover slips were washed (once) and covered by DMEM-F12 to avoid drying.

2.2. Dissection, Dissociation and Culturing of Ventral Midbrain

The embryos were collected in the L15 (Leibovitz) medium (Cambrex, Belgium). Ventral mesencephalon from E12 WT, HZ, En2-/- and MT embryos were dissected using scissors and forceps. The meningis were removed and the extra pieces were cut using a sterilized blade. The dissected ventral midbrain pieces were put into the L15

medium on ice for up to 45min. The L15 medium was aspirated and 0.5ml 0.25% trypsine-EDTA (Invitrogen/Gibco, Germany) was added following by 15min. incubation at 37°C. The 0.25% trypsine-EDTA was removed and 0.5ml L15 plus 0.5ml fetal calf serum (FCS) (Invitrogen, Germany) was added. The combination of L15 and FCS was removed and the pieces of tissue were washed twice with 1 ml complete medium containing DMEM-F12 with 1% glutamine (Invitrogen, Germany), 1% Penicillin/Streptomycin (final concentration: 50U/mlPen. -50µg/ml Strep.) (Invitrogen, Germany), 0.36% (or 33mM) glucose (Sigma, Germany), 0.25% BSA (Sigma, Germany), 1% N2 medium (Invitrogen, Germany) and 5% FCS. After the washes, the cells were dissociated using gentle triturating with a flamed Pasteur pipette (with restricted tip). The tubes were centrifuged at 1000g for 10min. in an ultracentrifuge (Beckmann TL, rotor TLS 55). The medium was removed and replaced with 500µl fresh complete medium. The number of cells was adjusted to 150000 per cover slip and the cells were plated on polyornithin/laminin pre-coated cover slips. After one hour at 37°C, when the cells were attached, the cover slips were placed into 24 well culture dishes with 500µl complete medium. 500µl medium was added after 24 hours and the cells would survive for up to 30 days. For treatment with neurotoxins, transfection with siRNA or with the inhibitory substances, serum free medium was used, containing the complete medium without the FCS.

3. Cell lines

Cells were maintained at 37°C and 5% CO2 in Dulbecco's modified Eagle's medium/F-12 (Invitrogen, Germany) 100units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Germany) and 10% Tet System Approved Fetal Bovine Serum (Clontech, Germany) and grown on poly-D-lysine-coated plates. The growth conditions for N2A cells were similar to that of MN9D cells, except that they did not need coating. The growth conditions of the SH-SY-5Y cell lines were also the same except for the concentration of FBS, which was 20% instead of 10%. The PC12 cells were grown in RPMI medium supplemented with 10% horse serum, 5% FBS and 1% Penicillin/ Streptomycin (final concentration: 50U/mlPen. -50µg/ml Strep.) (Invitrogen, Germany). The freezing of the cells was done after detachment and centrifugation, followed by addition of freezing solution containing 10% DMSO

(Sigma, Germany) in FBS. Initially, cells were incubated on ice for 30min., frozen at -20˚C for two hours in a paper towel, kept at -80˚C overnight to one week and then, they were transferred into liquid nitrogen at -180˚C for long-term storage. Thawing of the cells was done by immediate shaking in a 37˚ water bath and the cells were always centrifuged at 1000rpm for 5min. before re-growing.

4. Expression of Engrailed in MN9D Cells, Tet-ON System

For expression of En1, the TetON expression system was used (Gossen and Bujard, 1992; Hermanson et al., 2003). Cells were transfected with the pTet-On vector using Lipofectamine PLUS (Invitrogen, Germany). An individual G418-resistant colony was isolated, expanded, and screened for expression of the Tet-inducible transcription factor. The En1 cDNA was cloned into the pRevTRE vector downstream of the Tet-regulated promoter elements. MN9D-Tet-On cells were co-transfected with pRevTRE-En1 and pTK-Hyg vectors using Lipofectamine2000 (Invitrogen, Germany). An individual hygromycin-resistant colony was isolated, expanded, and screened for the induction of En1 by doxycycline. En1 expression and GFP expression were checked by western blot or by immunohistochemisty. En1 expression was induced by addition of 5µg/ml doxycycline

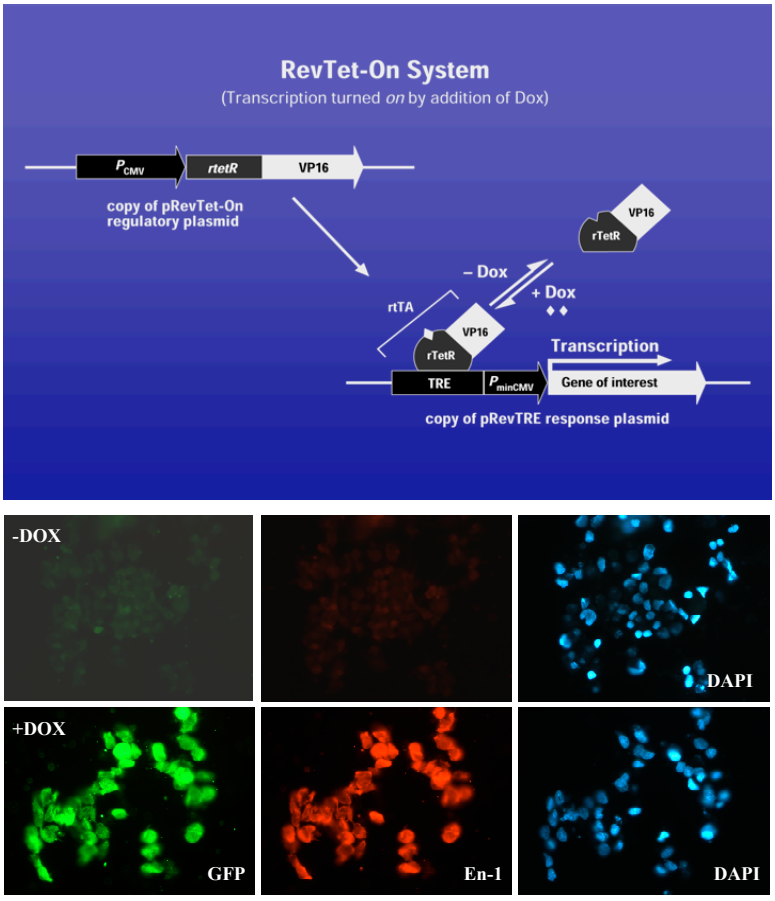


Figure-1: Outline of the Tet-ON system (top)(adapted from www.clontech.com/clontech/tet/index.shtml) and expression of Engrailed and GFP in MN9D cells by Tet-ON (bottom).

(Clontech) to the culture medium. Neurotoxin treatment was done on ±Dox MN9D cells. The expression of En1 and GFP was checked, by immunohistochemistry (Fig. 1).

5. Chemical Treatment

List of the compounds and the toxins used in the pathway study, stock concentrations, using concentrations, storage conditions and treatment times are listed in Table-1.

	Compound	Solvent	Stock Conc.	Store at	Final Conc	Time	Mechanism of Interest
1	Apoptosis Activator 2	DMSO	10mM	-80°	200nM	24h	Apaf-1 oligomerization
2	Apigenin	DMSO	27mM	-20°	270µM	30min+	MAPK inhibitor/P53 Inducer
3	BHA	EtOH	100mM	-20°	10µM	>30h	Antioxidant/Autophagy
4	Caffeine	H2O	83mM	4°	1mM	72h	E2F1 Inhibitor
5	Chelarythrine Chloride	DMSO	1mM	-80°	4µM	18h	Bcl-Xl Inhibiting factor
6	Curcumin	DMSO	13.5mM	-20°	7µM	24h	p53 Inhibitor
7	Cyclophosphamide monohydr.	DMSO	3.5mM	Fresh	3.58µM	30min	FAS Apoptosis Inducer
8	HA14	DMSO, PBS	13mM	-80°	30µM	12-18h	Bcl2 Inhibiting peptide
9	Parthenolide	DMSO	402.7mM	-20°	20µM	18h	NF-κB/MAPK Inhibitor
10	PDTC	H2O	101.4mM	-80°	5µM	2h	NF-κB Inhibitor
11	Prima-1	H2O	10.8mM	-20°	70µM	18h	p53 Activator
12	Rapamycin	DMSO	100µM	-80°	20nM	24h	mTOR Inhibitor
13	Staurosporine	DMSO	1mM	4°	10µM	1-6h	Protein Kinase Inhibitor
14	SU9516	DMSO	4.14mM	-80°	5µM	24h	pRb-E2F Dissociator
15	EGTA	H2O	5M	-20°	5mM	72h	Ca2+ Chelator
16	MPP+	H2O	10mM	Fresh	10µM	24h	mDA specific neurotoxin
17	6-OHDA	Medium	200µM	Fresh	200µM	24h	mDA specific neurotoxin
18	Bax-Inhibiting Peptide, V5	H2O	3.4mM	-20°	100µM	72h	Ku70 Bax inhibiting
19	pifithrin-α	DMSO	9.7mM	-20°	10µM	72h	p53 inhibitor
20	JNK inhibitor	DMSO	18.18	-20°	7µM	72h	JNK inhibitor
21	Rotenone	DMSO	253.5µM	-20°	40nM	24h	Complex-1 inhibitor
22	Tolbutamide	DMSO	185 mM	-20°	1mM	24h	Potassium Channel blocker
23	K252A	DMSO	200µM	-20°	200nM	72h	Trk Inhibitor
24	TEA	H2O	1M	-20°	1mM	24	Potassium Channel blocker
25	PD9	DMSO	20mM	-20°	50µM	24	Erk inhibitor
26	Bcl-XL BH4-TAT	DMSO	261.5µM	-20°	200nM	24	Anti apoptotic domain of Bcl-XL

Table-1: Specification of the chemicals, used in study of the pathway of cell death. The serum was withdrawn 24-2hrs., before application of the chemicals. The chemicals remained in the medium for entire duration of the treatment except for 6-OHDA where the treatment time was less than 15 minutes but the cells were analyzed after 24h.

6. Cell Death/Proliferation Assay

The cell death was quantified using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Germany). 40µl of the 96 AQueous One Solution was directly added to cells with 1ml medium and after incubation at 37° for one to four

hours, 100µl of the medium was transferred to 96 well plates and the absorbance was measured at 490nm using the Bio-Rad-550 Microplate reader.

7. Small Interfering RNA

7.1. Design and Preparation of siRNA Oligos

For RNA interference experiments, either ready to use dsRNA oligos were used or they were produced by the Dicer method. For this method, after designing and cloning a 450-500bp fragment from the coding region of the desired gene into the pBluescript II SK(-) vector, which was digested from both sides (in separate tubes) and two sets of transcriptions from the two sides of the fragment were carried out for two hours in a total mix of 20µl, containing 1µg digested DNA (adjusted to 7µl), 4µl Trans 5X transcription buffer (Promega, Germany), 2 µl dNTP mix (New England Biolabs, Germany), 2µl 0.1M DTT (Promega, Germany), 1 µl T7 (or T3 or SP6) RNA Polymerase 2500 U 50 U/µl (GIBCO BRL) and 3.5 µl DEPC treated H2O. After transcription, the RNAse-free DNAse was added and the tubes were kept at 37°C for 15min. The ssRNA were cleaned, using Qiagen RNeasy kit and the amount of ssRNA was measured. Equal amounts of the ssRNA were added to the annealing buffer (100 mM NaCl, 20 mM Tris at pH 8.0, 1mM EDTA) and the mixture was heated up to 95°C for 3min. and then at 68° for 30min. followed by overnight cooling down to room temperature. The dicer digestion reaction to produce 21mers was carried out at 37°C by addition of the dicer enzyme and 10x reaction buffer (both from Stratagene, Germany). The cut dsRNA were purified, using a purification column specific for fragments smaller than 50bps (Millipore, Germany). The RNAi oligos were stored at -20°C until transfection.

The design of the 21mer ready to use RNAi oligos was carried out by using the online software at biomers.net in accordance with the protocol of Elbashir et al. (Elbashir et al., 2001). The sequence lied within the coding sequence of pbx-1, p75NTR, and NRAGE (Mage-D1). For p75, the sense sequence being 5'-ACAGAACACAGUGUGUGAA(dTdT) and the anti-sense sequence, 5'-UUCACACACUGUGUUCUGU(dTdT). The sense sequence for NRAGE oligonucleotide was 5'-UAACUUGAAUGUGGAAGAG(dTdT) and the anti-sense was 5'-CUCUUCCACAUUCAAGUUA(dTdT). For coupling with Penetratin-One

(QBiogene, France), the sense strand was modified with a thiol group on the 5' end of the sense strand and for the purpose of detection of transfection, the anti-sense strand was labeled with FITC.

7.2. siRNA Transfection

The transfection reagents, HiPerfect (Qiagen, Germany), Ribojuice (Calbiochem, Germany), and Lipofectamine (Invitrogen, Germany) were used according to the manufacturers' protocols and except for HiPerfect with a transfection efficiency of 50% for primary neuronal cultures, these reagents could not achieve optimum transfection and therefore, Penetratin-1 was used.

Penetratin-1 solution was reconstituted to 2mg/ml (=0.8mM) in sterile water. Stock solution of 20mM TCEP (tris-2-carboxyethyl phosphine from Pierce, Germany) in sterile RNAse/DNAse free water was made. SiRNA oligos were dissolved in RNAse/DNAse free sterile annealing buffer to a concentration of 900µM. 1µl TCEP was added to 224µl siRNA and incubated for 15min at room temperature. Then, 25µl Penetratin-1 was added, mixed and incubated for 5 min. at 65°C followed by 1 hour at 37 C. The aliquots were frozen at -80°C. The Penetratin coupled siRNA oligos were heated to 65°C for 15min. and dissolved in complete growth medium. Then, it was added to cultures in different concentrations to minimize the toxicity with maximized transfection efficiency. For the siRNA of p75, 20µl of the Penetratin-siRNA mix was dissolved in 500µl medium.

8. Immunocytochemistry and Immunohistochemistry

The cover slips were fixed with 4% PFA and washed thoroughly with PBS. Fixed Cells were blocked in the blocking solution (10% FCS, 0.5% Triton, in 0.1M PB.) for one hour at room temperature. For DAB staining, 0.006% H_2O_2 was added to the blocking solution. After blocking, the cells were washed with PBS and put into the primary antibody/s, diluted into the blocking solution, overnight at 4°. After washing the cells with PBS, the cells were placed into the secondary antibody/s (also diluted in the blocking solution) for 2 hrs. at room temperature. After another wash with PBS, in some cases, to enhance the signal, the biotin-streptavidin system was used and

therefore the cells were covered with the tertiary, streptavidin conjugated, antibody diluted in PBS for one hour at room temperature. After washing the cells with PBS, they were treated for 5 min. with 4'-6-Diamidino-2-phenylindole (DAPI), diluted (1:50,000 in 0.1M PB). For the fluorescent staining, after a final wash with PBS, the cover slips were mounted using the Aqua Poly/Mount solution (Polysciences, Germany). In case of sheep anti-TH primary antibody, For the DAB color reaction, cells were treated with 0.1% DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma-Germany) added to 0.1M PB solution in presence of 0.006% H_2O_2.

For immunohistochemistry on the PFA fixed sections, the same procedure was used except for the number and duration of the washes, which were three times five minutes after each step. The antibodies, used regularly, were rabbit anti TH, at a dilution between 1:500 and 1:1000 (Chemicon, Germany), sheep anti TH at a dilution of 1:200 (Chemicon, Germany), purified mouse anti Engrailed-4G11 at a dilution of 1:100 (Developmental studies, Hybridomas Bank). The using concentration of polyclonal rabbit antibodies for most of the antigens was 1:500.

9. Protein Lysis and Preparation

Cells from cell lines were lysed and stored in 2x Laemmli buffer (2.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 710 mM beta-mercaptoethanol). The midbrain total protein from was extracted from dissociated tissue using 2x Ripa buffer (50mM Tris-HCl, pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150mM sodium chloride, 1mM EDTA, 1mM PMSF, 1ug/ml aprotinin, 1ug/ml leupeptin, 1ug/ml pepstatin, 1mM sodium orthovanadate, 1mM NaF) and diluted into Laemmli sample buffer. The subcellular protein fractions (from cytosol, membranes, nucleus, or cytoskeleton) were extracted using Qiagen's Qproteome Cell Compartment kit in presence of protease inhibitor. 5 x 106 cells were centrifuged at 500xg for 10min at 4°C. The cell pellet was re-suspended in 2ml ice cold PBS and centrifuged again (twice). The pellet was re-suspended in 1ml Extraction buffer CE1, incubated at 4°C for 10 min, and centrifuged at 1000xg for 10 min at 4°C. The supernatant was transferred and marked cytosolic protein and the pellet was re-suspended in 1ml ice-cold extraction buffer CE2 and Incubated for 30 min at 4°C on an end-over-end shaker. The suspension was centrifuged 6000xg for 10 min at 4°C.

The supernatant was transferred and marked membrane/mitochondrial protein. 7µl Benzonase and 13µl distilled water were added to the pellet and incubated for 15 min at room temperature. 500µl ice-cold extraction buffer CE3 was added into the tube and Incubated for 10 min at 4°C on end-over-end shaker. The insoluble was centrifuged at 6800xg for 10 min at 4°C. The supernatant was marked nuclear protein and the pellet was re-suspended in 500µl extraction buffer CE4. This suspension contained cytoskeletal fraction. For the first three portions, the extraction buffers CE1-3 contained protease inhibitor. For precipitation of the protein, four volumes of ice cold acetone was added to protein samples and incubated for at least 15 min. at -20°C. The samples were centrifuged for 10 min at 12,000 x g at 4°C. The supernatant was discarded and the pellet was air-dried. The pellet was re-suspended in 2X Laemmeli buffer. All protein samples were stored at -80°C.

10. Western Blot Analysis

Stacking and running SDS polyacrylamide gels were prepared and placed in the Laemmeli running buffer (0.192M Glycine, 0.25M Tris base, 0.1% SDS). The samples were ran at 80v and then transferred to membrane at 4°C, using the semidry or wet transfer methods. The blot was blocked in (5% milk added to PBSt) for one hour to overnight at room temperature or 4°C, respectively. After three 5min. washes, primary antibodies, dissolved in 5% albumin fraction V solution (in PBSt) were added and left shaking from two hours to overnight at 4°C. The blots were washed with PBSt for three times for 5 to 10 minutes each and then they were placed into the secondary, peroxidase conjugated antibody solution for one hour at room temperature. After three times washing with PBSt, the blots were covered by ECL solution (GE Biomedicals, USA) consisting of 1x solution A and 40x solution B. After more than one-minute exposure to ECL solution, the blots were placed on the Heperfilm (GE Biomedicals, USA) and developed and fixed using respective solutions (Kodak, Germany). The noncommercial ECL was carried by using 10x the solution of 0.25mM luminal in 0.1M Tris-HCl (PH 8.6) and 1x of 0.1mM Coumaric acid solution in DMSO mixed with 1.5ul per 5ml, 30% H_2O_2 solution.

11. Immunoprecipitation

The basic protocol of the Protein-G Immunoprecipitation Kit (Sigma, Germany) was used. About 150000 cells were lysed, using 300µl IP buffer. The primary antibody was added to the lysis, the volume was adjusted to 600µl by addition of IP buffer, and transferred to the spin column. The column was placed in a end-over-end shaker overnight at 4°C. 30µl of the protein-G agarose beads were washed in cold 1X IP buffer by centrifugation at 12000 x g for 30 seconds. The supernatant was removed and the beads were re-suspended in 50µl IP buffer and added to the cell lysate. After 2hrs of incubation at 4°C in the end-over-end shaker, the column was broken and centrifuged at 12000 x g for 30 seconds. The beads inside the column were washed nine times, using 700µl 1X IP buffer and centrifugation at 12000x g. The last wash was done using 0.1X IP buffer. 100µl 2x Laemmeli buffer was added to the beads and the beads were boiled (95°C) inside the closed column for 5min. The sample was spinned down at 12000 x g and stored at -80°C.

12. In Situ Hybridization

12.1. Digoxygenin Labeled In Situ

12.1.1. Preparation of Digoxigenin labeled RNA Probes

Sense and anti-sense RNA probes were generated by PCR amplification of 400 to 1000bp fragments. After cloning of the desired fragment into the pBSK vector, it was linearized and incubated for 2hrs. at 37°C in 20μl transcription mix consisting of T7 polymerase (Ferentas, Germany), Trans 5x buffer (Fermentas, Germany), 10x DIG labeling mix (Roche, Germany), 0.01 M DTT (Promega, Germany), and 1U/μl RNAse inhibitor (Promega, Germany). After transcription, 10U/μl RNAse free DNAse was added to the mix for 15min at 37°C. The DIG-probes were purified using the RNAeasy purification columns (Qiagen, Germany) or they were precipitated in 100μl TE, 1M LiCl, and 300μl 100% ethanol, for 30 min at -70˚C and then centrifuged at 14000rpm for 10 min. The pellet was washed with 80% ethanol, and re-suspended in TE/formamide (50μl: 50μl). After 10min. at 60˚C, the amount of RNA was measured by spectrophotometer (RNA 230 and 260 nm) and stored at –20°C.

12.1.2. Digoxigenin (DIG) Labeled In Situ

The embryonic tissue were fixed overnight at 4°C in 4%PFA following by incubation in 30% sucrose in 0.1M PB solution at 4°C. The tissue was placed in OCT Tissue Tek, frozen, and cryostat sectioned. The adult tissue were fresh frozen in isopentane, pre-cooled at -70°C and then, sectioned. After air-drying for at least 20min. (up to 3hrs), the sections were put into 4% PFA for 10min. then washed three time with PBS for 3min. each, following by a 5 min. treatment with 1ug/ml proteinase-K in 50mM Tris and 5mM EDTA. The time of proteinase-K treatment for fresh frozen sections was not more than 1min.; The fixation and washing steps were repeated and the sections were treated for 10min. with acetylation mix, consisting of 3.25ml triethanolamine, 0.7ml acetic anhydride, in 250ml H_2O. Then, the sections were washed three times for 5min., each, with PBS.

The 400ng/ml digoxygenin labeled RNA probes were dissolved in hybridization buffer, consisting of 50%formamide, 5x SSC, 5x Denhardt's, 250ug/ml baker yeast RNA, and 500ug/ml herring sperm DNA in H_2O. Then, the probes were heated at 80°C for 5min. and were put on ice. 100ul probe was used for incubation with each slide at 72°C overnight in a 5x SSC and 50% formamide humidified chamber. After overnight incubation, slides were submerged into 5x SSC to remove the cover slips and were placed into 0.2x SSC for 2 hours at 72°C followed by a room temperature wash with 0.2x SSC for 5min. The sections were treated with B1 buffer (100mM Tris PH=7.5, 150mM NaCl, in H_2O) for 5min and then blocked for 1 hour with 10% goat serum in B1 Buffer. They were incubated overnight at 4°C with 1:5000 dilution in B1 buffer plus 1% goat serum. The overnight anti-Digoxygenin, antibody treatment, was followed by three 5 min. washes with B1 buffer and one 5 wash with B2 buffer (100mM Tris PH=9.5, 100mM NaCl, 50mM $MgCl_2$ in H_2O). Then, the sections were left in NBT/BCIP solution (0.338ug/ml NBT and 0.35ug/ml BCIP in B2 buffer) for 3h to several days for color reaction. The DPX mounting preceded by air-drying the sections, and 3min. dehydration steps with 30%, 50%, 70%, 95% and 100% ethanol.

12.2. Radioactive Labeled In Situ

12.2.1. Preparation of the Radioactive RNA Probes

The transcription mix for radioactive in situ consisted of 4µl 5X transcription factor (Fermentas, Germany), 1µl 0.1M DTT, 1.5µl nucleotide mix (without UTP), 0.5µl RNAse inhibitor, 0.5µl digested DNA, 0.5µl T7 or T3 RNA polymerase, and S35 labeled UTP (50UCi) and added H_2O for a total volume of 20µl. After incubation at 37˚C for 2hrs., 47µl H_2O, 20µl yeast tRNA (10mg/ml), 1µl 1M DTT, and 1µl RNAse free DNAse were added to the mix and incubated for 15min at 37˚C. The RNA probes were then precipitated by addition of 100µl H_2O, 70µl 7.5M NH_4OAc, 700µl 100% ethanol, and incubation at -70˚C for 10 min. After centrifugation at 14000rpm for 10min, the supernatant was removed and the pellet was dried. The pellet was dissolved in a mix of 70µl formamide, 70µl H_2O and 10µl 1M DTT. 1µl of the probe was used for measurement of the radioactivity, which had to be at least 400,000cpm/µl.

12.2.2. Radioactive In Situ

The mounted sections were pretreated with 4%PFA in PBS, and then they were washed with PBS for 3min twice. The slides were incubated at 2XSSC at 65˚C for 30min. followed by a 1min. H_2O wash. The sections were treated for 10min. with 20µg/ml Proteinase-K, dissolved in TE (10mM Tris-PH=8 and 1mM EDTA, 200µl proteinase-K-20mg/ml, 400µl EDTA 0.5M and 2ml Tris 1M with added H_2O up to 200ml). The slides were put into a solution of 0.2% glycine in PBS for 30s. and washed for one min. twice in PBS. Then, they were fixed with 4% PFA for 20min. following by a wash of PBS for 3min. They were incubated in the acetylation mix (3.25ml triethanolamine and 0.7ml acetic anhydride in 250ml H_2O) for 10min. After a three-minute wash with PBS, the sections were dehydrated through consecutive, 2min. treatments with 30%, 60%, 80%, 95% and 100% ethanol. After drying for 1hr, the slides were treated with 50,000,000cpm/ml RNA probe, dissolved in the hybridization mix containing 50% formamide, 10% Dextran, 1X Denharts, 0.3M NaCl, 10mM Tris PH=7.5, 10mM sodium phosphate PH=6.8, 5mM EDTA, 25mM DTT, and 50mM ß-mercaptoethanol. The mixture was heated to 80˚C for 5min. and centrifuged for 1min

and then added on top of the sections. The slides were incubated at 55˚C overnight in a humidified chamber. After hybridization, the slides were washed with 5X SSC and 20mM ß-mercaptoethanol, pre-heated at 55˚C for 30min. The cover slips were removed and the slides were washed twice with 2X SSC, 50% formamide, and 20mM ß-mercaptoethanol for 45min. at 65˚C. The slides were placed at 0.5M NaCl/TE solution at 37˚C for 15min., followed by addition of 20µg/ml RNAse-A and treatment for 15 min. The slides were placed in a solution of 50% formamide, 1mM EDTA and 20mM ß-mercaptoethanol at 55˚C for two hours, following by washes of 0.2X and 0.2X SSC at 55˚C for 15 min. each. The pre-hybridization dehydration steps were repeated and the slides were dried for one hour. Hyperfilm (Kodak, Germany) exposure time was between one day and up to four weeks.

13. Identification and Analysis of Transcription Factor Binding Sites

The transcription factor binding site analysis and identification of the interspecies conserved sequences of mouse and human P75 (up to -10kb) was carried out, using the online computational tools at http://pga.lbl.gov/rvista.html. The global alignment file was generated by the AVID program. The TRANSFAC matrix library and the MATCH program were used to identify all transcription factor binding site (TFBS) matches in each sequence. Then, the global alignment and the sequence annotations were used to identify all aligned TFBSs present in the non-coding DNA. A second file was generated, containing aligned non-coding TFBS's. DNA sequence conservation was determined by the hula-hoop module, identifying the TFBS's, surrounded by conserved sequences and generated a data table. Finally, the data was customized by choosing for En1 as the transcription site to be visualized the TRANSFAC parameters were set to rVISTA's default, 0.75/0.8.

14. Luciferase Assay

The luciferase reporter vector was constructed, using the suspected P75 promoter region. An 8kb piece, containing the first axon and 5000bp upstream, was cut, using

XhoI/BglII from the RP24-347P1 BAC clone. This piece was placed into the MCS of the pBluescript II (KS)+ vector. 6.4kb, between XhoI and XbaI restriction sites was cut and placed into pGL3 vectors with and without the SV40 enhancer element. En1 containing N2A Tet-ON cells were grown in 24 well culture dishes, 48h after addition of Dox, the cells were transfected, using Lipofectamine2000, with the 6.4kb piece, cloned into PGL3 basic vector or into the vector containing SV40 enhancer and simultaneously with the Renilla control vectors. 24h after transfection, cells were washed with PBS and lysed by addition of 250µl passive lysis buffer to each well (PLB; Promega, Mannheim). After shaking the cells horizontally for 15 min at room temperature, an equal volume (250µl) of the Dual-Glo Luciferase reagent was added to the medium. After 10min. incubation at room temperature, the firefly luminescence was measured with a luminometer. For measuring the Renilla luciferase activity, Dual-Glo, Stop and Glo Luciferase reagent was added within 4hrs after addition of Dual-Glo and the luminescence was measured within 2hrs. The activity of firefly (*Photinud pyralis*) was normalized against the activity from *Renilla (Renilla reniformis*) luciferase and then normalized against the reading from the empty vector.

Results

1. Involvement of the Mitochondrial Pathway of Apoptosis

We had previously reported that loss of Engrailed expression causes activation of caspase-3 in mesDA neurons(Alberi et al., 2004; Sgado et al., 2006). Caspase-3 is the effector caspase of the extrinsic and intrinsic (mitochondrial) pathways of apoptosis(Li et al., 1997; Stennicke et al., 1998). Since multiple in vitro and in vivo experiments have shown that mesDA neurons, and in particular, the subgroup in the substantia nigra, are sensitive to apoptosis induced by mitochondrial dysfunction(Li et al., 1997), I examined the contribution of this pathway to the loss of mesDA neurons in En^{DM} embryos and nigral neurons of postnatal En^{HT} mice. I detected small, round TH-positive cells with active caspase-9 and pyknotic, fragmented nuclei in the ventral midbrain of dying Engrailed-deficient E13 embryos. Similar to the null mutant embryos, mesDA neurons of En^{HT} mice also showed condense, fragmented nuclei along with activated caspase-9 during the postnatal stages of nigral cell loss (P21). The P21 En^{HT} nigral neurons also showed activation of caspase-3, similar to the En^{DM} mesDA neurons (Fig. 1)(Alberi et al., 2004; Sgado et al., 2006).

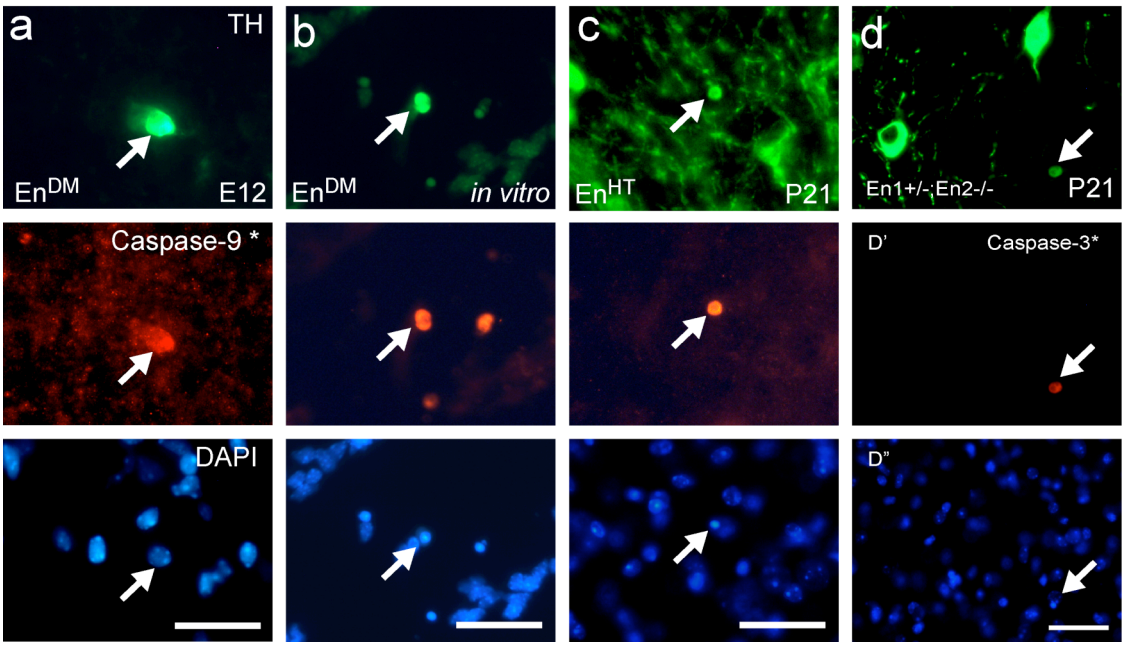


Figure-1: Activation of caspases in TH positive mesDA neurons of Engrailed-deficient mice. Caspase-9 is activated in mesDA neurons of En^{DM} E13 embryos (a), E12 primary cultures of ventral midbrain (b) and P21 En^{HT} SN neurons (c). Caspase-3 is also activated in En^{HT} P21 nigral neurons. All TH positive cells with pyknotic nuclei showed caspase 3 and 9 activation. Scalebar=25µm.

To further investigate whether cell death in Engrailed-deficient mesDA neurons involves the mitochondrial pathway, I applied apoptosis-inhibiting peptides to E12 primary cultures derived from ventral midbrain. In culture, untreated En^{DM} mesDA neurons die between 24h and 72h after dissociation. The survival rate of En^{DM} mesDA neurons did not differ significantly from the untreated controls whether specific inhibitors for caspases-3, -9, or -8, or a general caspase inhibitor (Fig. 2) were applied. Additionally, the inhibition of Bax, a pro-apoptotic member of the Bcl-2 family (Sawada et al., 2003) also failed to rescue the Engrailed-deficient mesDA neurons (Fig. 2). To examine other mechanisms related to mitochondrial apoptosis in mesDA neurons, I also applied inhibitors to JNK and P53 (Anderson and Tolkovsky, 1999). Both did not change the survival rate significantly.

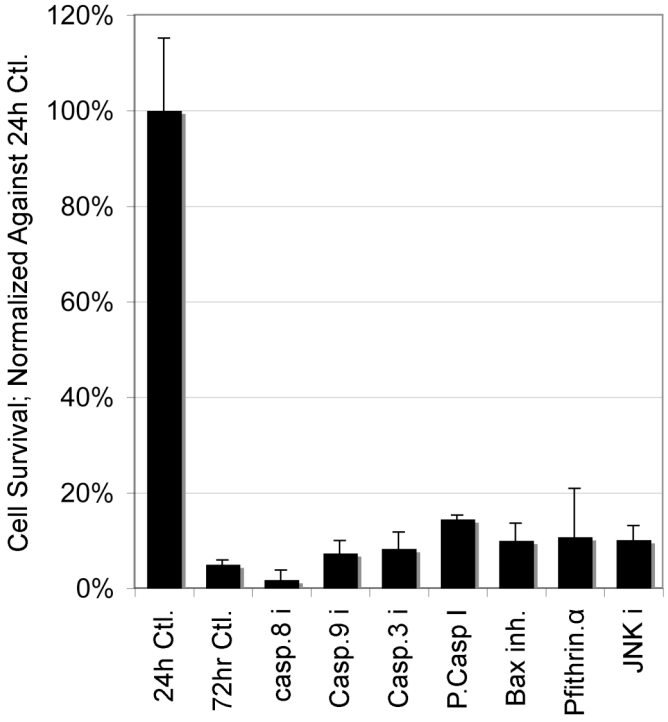


Figure-2: Effect of anti apoptotic reagents on E12 En^{DM} mesDA neurons. Treatment of En^{DM} cultures with inhibitors for caspases 3,8 and 9 as well as a pan-caspase inhibitor, the Bax mitochondrial translocation inhibitor, Ku70, a specific inhibitor of P53, Pfithrin-α, and JNK inhibitor, SP600125, induce no significant improvement on the survival rate of TH positive cells. The period of DA cell loss for untreated mutant cultures is between 24h and 72h (Alberi et al., 2004). The number of surviving cells in each case was normalized against the number of untreated En^{DM} cells at 24h.

2. Mitochondrial Dysfunction is Contributing but not the Sole Mechanism of Cell Loss

Failure of the specific inhibitors of the intrinsic pathway to rescue Engrailed-deficient mesDA neurons from cell death suggested either lack of involvement of apoptosis or participation of one or more parallel independent molecular pathways. To elaborate on

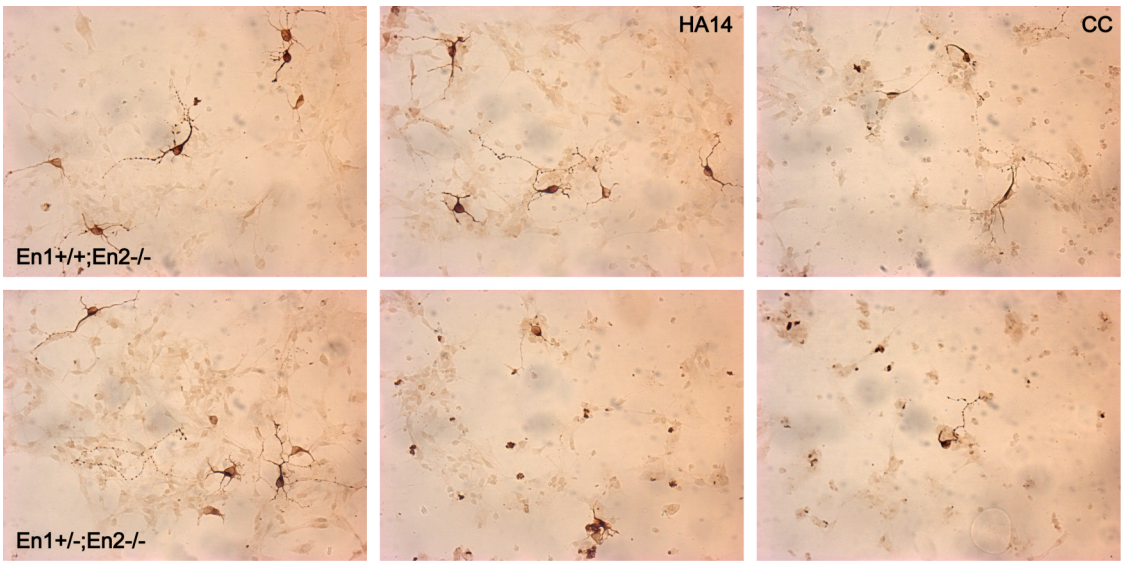


Figure-3: Sensitivity of mesDA neurons to inhibitors of Bcl-2 and Bcl-X_L. The rate of survival of the En2-/- mesDA neurons is more than their En1+/-;En2-/- (En^{HT}) littermate counterparts, in presence of HA14 and Chelerythrine chloride, the inducers of mitochondrial apoptosis via inhibition of Bcl-2 and Bcl-X_L, respectively. For the exact numbers see Fig. 5.

these possibilities, I correlated the sensitivity of mesDA neurons to the induction of different pathways of programmed cell death to the level of Engrailed expression by using mesDA neurons, derived from embryos of two Engrailed genotypes, with wild-type-like survival rate in vitro, i.e. En2-/- and En^{HT} (En1+/-;En2-/-). The ratio of pro- to anti-apoptotic members of the Bcl-2 family of proteins is believed to play a critical role in the intrinsic pathway by determining stability of the mitochondrial outer membrane (Hengartner, 2000). I, therefore, induced mitochondrial apoptosis via application of HA14-1 and chelerythrine chloride, specific inhibitors of the anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-X_L, respectively(Tanaka et al., 2004; Wang et al., 2000). 24 hours after treatment with HA14-1 and chelerythrine chloride, the rate of survival of mesDA neurons derived from E12 En2-/- embryos was on average 75.3 ± 6.8% and 92.7 ± 2.7% ($p<0.001$, $n>15$ for both), respectively, higher than their counterparts derived from En^{HT} littermates (Fig. 3, 5).

The three most common reagents to induce cell death in mesDA neurons, MPP^+, 6-OHDA and rotenone(Dauer and Przedborski, 2003), cause cell death by induction of the mitochondrial pathway of apoptosis. They cause activation of caspase-9 and caspase-3(Kaul et al., 2003; Liang et al., 2004; Watabe and Nakaki, 2004). Having confirmed these observations in our culture system (Fig. 4), I explored the possibility that the sensitivity to these toxins may be linked to the level of Engrailed expression,

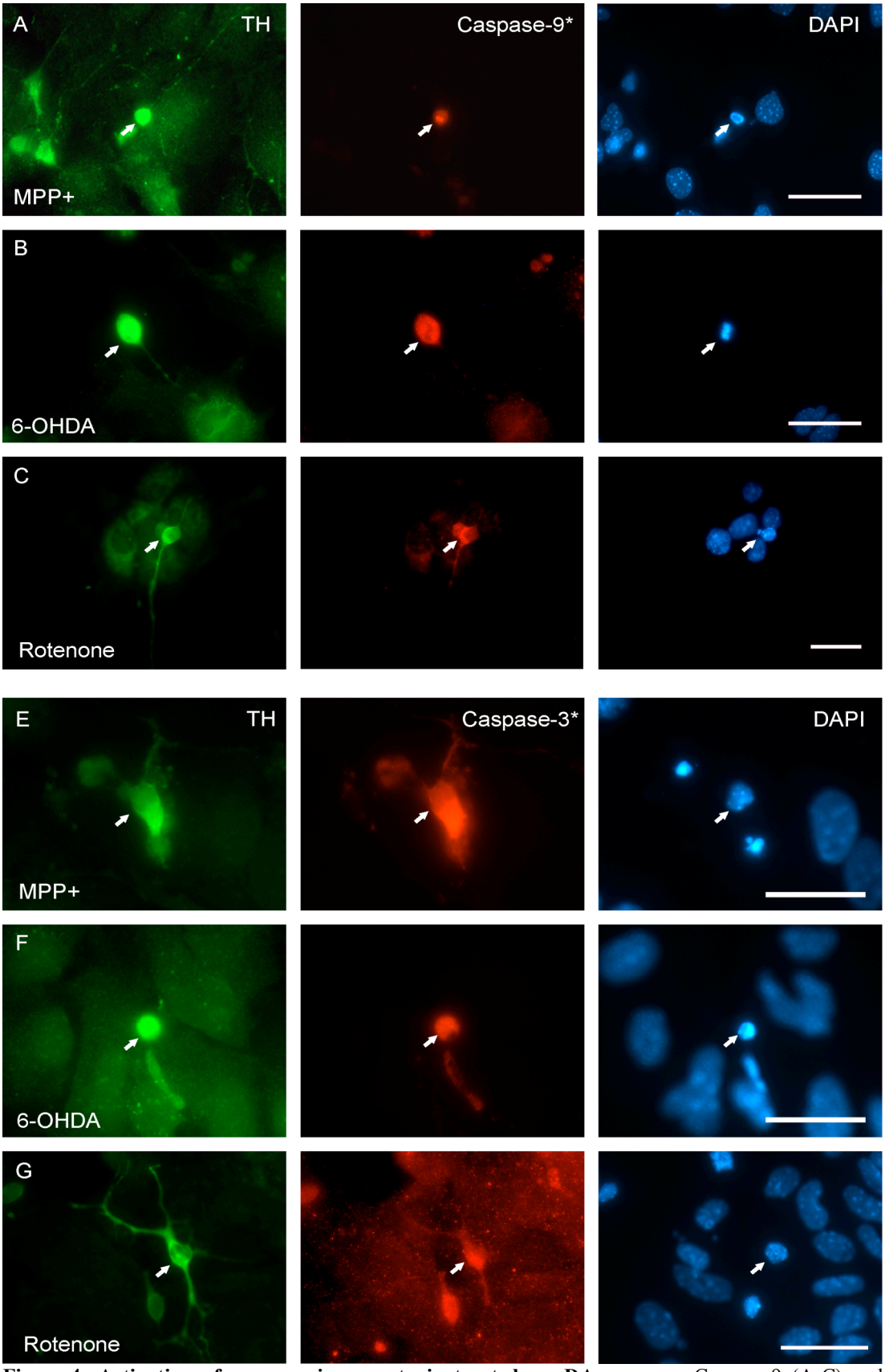


Figure-4: Activation of caspases in neurotoxin treated mesDA neurons. Caspase-9 (A-C) and caspase-3 (E-G) are activated in wild type mesDA neurons, treated with MPP^{+}, 6-OHDA or rotenone. Scalebar = 25µm.

comparable to what I observed for HA14-1 and chelerythrine chloride. After 48 hours in culture, the rate of survival of mesDA neurons derived from En2-/- embryos was on average 124.5 ± 5.0% ($p<0.001$, n=12) higher than for their En^{HT} littermates after treatment with MPP^+, while the higher dose of En1 had no or only a, comparatively, little effect after treatment with 6-OHDA or rotenone (3,4 ± 8,9% p=0.91, n=8 and 20,8 ± 3,6% p=0.03 n=8, respectively) (Fig. 5). These data suggest that MPTP and Engrailed may act upon the same molecular pathway upstream of caspase-9, which differs from that of 6-OHDA or rotenone. To determine whether the protection exerted by the Engrailed proteins is specific to the mitochondrial pathway of apoptosis, I also employed the Apoptosis Activator-2(Nguyen and Wells, 2003), Prima-1(Bykov et al., 2002), and the tumour necrosis factor (TNFα)(Adams, 2003). The first induces cell death by triggering apoptosome formation, the second by activation of p53 and the third, as a ligand of the TNF receptors, induces the extrinsic, receptor-mediated, pathway of apoptosis. In all three cases, the level of En1 expression did not have a significant influence on the survival rate of the neurons (-8.1 ± 6.0% (p=0.67) for AA2, 4.6 ± 6,2% (p=0.82) for Prima-1 and 5.1 ± 3.1% (p=0.48) for TNFα (n≥7 for all) (Fig. 5).

3. Protective Effect of Engrailed Against Mitochondrial Insult and MPP^+ Induced Cell Death in MN9D Cells

To confirm the Engrailed dose dependent sensitivity of mesDA neurons to mitochondrial insult and to death, induced by MPP^+, I carried out gain of function experiments, using the inducible Tet-On system to express En1 in the dopaminergic cell line MN9D(Choi et al., 1992). As in the primary cultures, En1 was protective against cell death, caused by application of MPP^+ (42.4 ± 0.7% $p<0.0001$, n=23), chelerythrine chloride (46.7 ±1.3%, $p<0.0001$, n=10) and HA14-1 (32.4 ± 1.3%, $P<0.0001$, n=10). Contrary to the results for the dissociated primary neurons, En1 improved the survival rate of 6OHDA and rotenone treated MN9D cells (36.9 ± 2.1%; $p<0.0001$, n=24 and 39.0 ± 2.0%; $p<0.0001$, n=32, respectively). The difference to the primary culture may be attributable to induction of high, non-physiological expression of En1 in the cell lines. For the other three reagents, the effect was, as in the primary

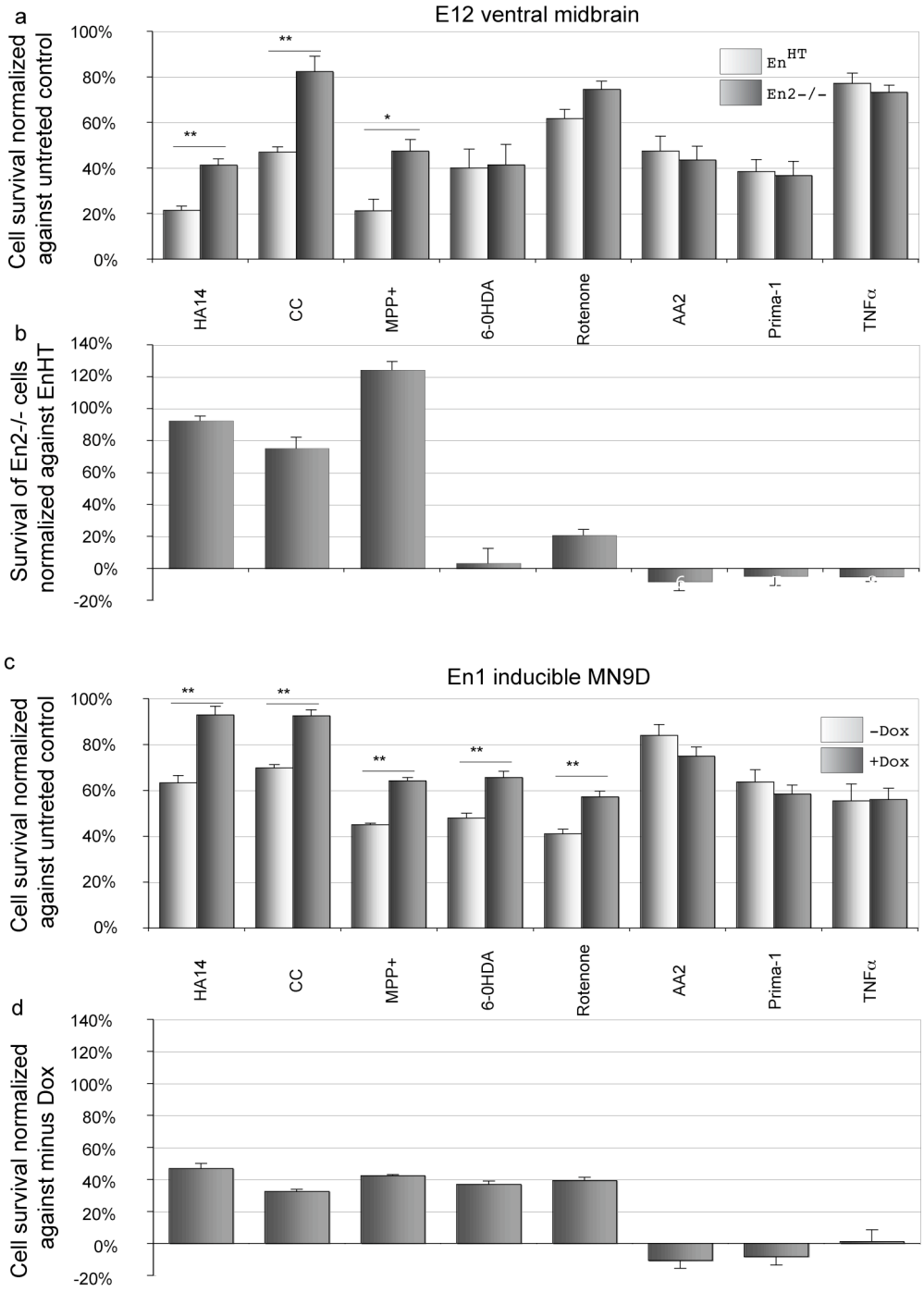


Figure-5: Sensitivity of mesDA neurons to inhibitors of Bcl-2 and Bcl-X_L. Charts of primary cell cultures of En1+/+;En2-/- and En1+/-;En2-/- E12 ventral midbrain (a,b) and En1 inducible MN9D cells (c,d) after application of apoptosis inducing compounds are shown. Number of cells are normalized against untreated controls (a,c) or normalized against treated En1+/-;En2-/- cells (b) or treated non-induced MN9D cells (d) to show relative rescue effect in correlation with the level of En1 expression. Level of Engrailed expression inversely determines the sensitivity to MPP^+ and to inhibition of Bcl-2 (by HA14) and Bcl-X_L (by CC = chelerythrine chloride) in primary mesDA neurons and MN9D cells, whereas the outcome after application of the tumor necrosis factor alpha (TNFα), prima-1 and the apoptosis activator 2 (AA2) is independent of the level of En1 expression. 6-OHDA and rotenone behave differently in primary mesDA neurons (a,b) and MN9D cells (c,d). Error bars standard error. $6 \leq n \leq 27$, $*p \leq 0.001$, $**p \leq 0.0001$.

cultures, negligible (-10.7 ± 4.7%, p=0.26 for AA2, -8.3 ± 5.2%, p=0.50 for Prima-1 and 1.0 ± 7.4%, p= 0.95 for TNFα; n≥12 for all) (Fig. 5).

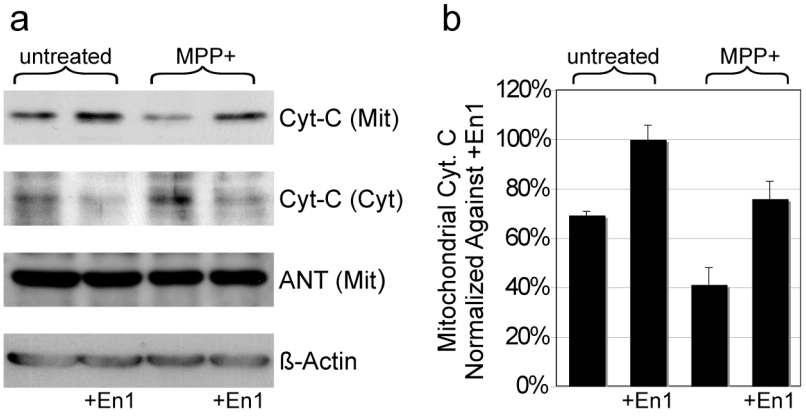


Figure-6: Inhibition of the release of cytochrome-C by Engrailed. Western blot analysis (a) of protein derived from mitochondrial vs. cytoplasmic compartments of MN9D cells, 72h after dox. induction of En1 expression and MPP^+ treatment for 24h. (starting at 48h) show that in MN9D cells, the release of cytochrome-C from the mitochondria is increased upon application of MPP^+ and decreased by expression of Engrailed. The quantification of the bands from 3 individual experiments are shown in (b).

Functional inhibition of Bcl-2 and Bcl-X_L, and application of MPP^+ can induce apoptosis by release of cytochrome C from the mitochondrial inter-membrane space into the cytosol(Cai et al., 1998; Deshmukh and Johnson, 1998). The protective effect of En1 against HA14-1, chelerythine chloride and MPP^+ may be attributable to higher mitochondrial stability. To test this hypothesis, I compared the cytosolic and mitochondrial protein fractions of En1 expressing MN9D cells to non-expressing cells. The proportion of cytochrome C in the mitochondria was always significantly higher after induction of En1 in MPP^+ and untreated cells (76.1 ± 7.3%, p=0.03, n=3 and 41.6 ± 7.0%, p=0.005, n=3 respectively), whereas the total amount of cellular cytochrome C was unaltered (Fig. 6). In contrast, the proportional levels, in mitochondria and cytosol, of the apoptosis inducing factor (AIF), which causes caspase-independent mitochondrial apoptosis(Lorenzo et al., 1999), were independent of the level of En1 expression (data not shown), suggesting that Engrailed participates in regulation of mitochondrial stability via the cytochrome-C/caspase-dependent pathway of apoptosis rather than the caspase-independent pathway, represented by AIF.

4. Elevated P75 Expression in Absence of Engrailed Causes Cell Death

To identify the genes downstream of the Engrailed genes and to further elaborate on the death pathway in mesDA neurons, a genome-wide expression analysis was performed, using microarrays on En1 inducible N2A cell line (data not shown). The data suggested that the nerve growth factor receptor (Ngfr or P75) was a regulatory target of the Engrailed transcription factors. To confirm these preliminary results, I measured the amount of P75 in the cell line as well as in ventral midbrain tissue by quantitative RT-PCR. As a result of En1 induction in N2A cells, the endogenous expression of P75 decreased by 10 fold (9.9 ±1.9% $p<0.001$ n=8). Likewise, in the embryo, ventral midbrain tissue derived from control littermates (En2-/-) expressed almost 2.5 fold less P75 (42% ± 14.0% $p<0.001$ n=6) than Engrailed double mutants (Fig. 7). The Western blot analysis confirmed these results; En2-/- control littermates expressed -74,4 ± 4.4% ($p<0.001$, n=3) and En^{HT} expressed -60,2 ± 8.8% (p=0.002, n=3) less P75 than the tissue from En^{DM} embryos (Fig. 7).

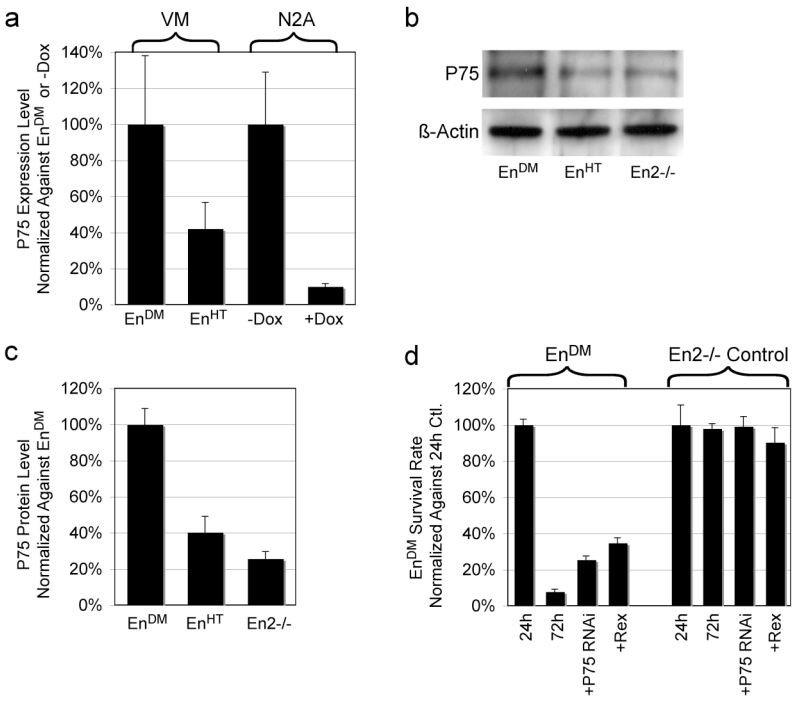


Figure-7: Differential expression of P75 and expression of trk Receptors. Quantitative RT-PCR on the En1 expressing N2A cells vs. control, as well as ventral midbrain tissue of En^{DM} vs. En2-/- E12 embryos, shows down-regulation of P75 expression in presence of En1 (a). Western blot analysis of the ventral midbrain tissue shows a similar effect, where the amount of P75 protein in the En^{DM} ventral midbrain is significantly more than the En^{HT} or En2-/- (b,c). Addition of an inhibiting antibody of P75 (Rex) or siRNA knockdown of P75 rescued the En^{DM} mesDA neurons (d). The wild type-like neurons were unaffected (d). Numbers for En^{DM} or En2-/- are normalized against 24h controls.

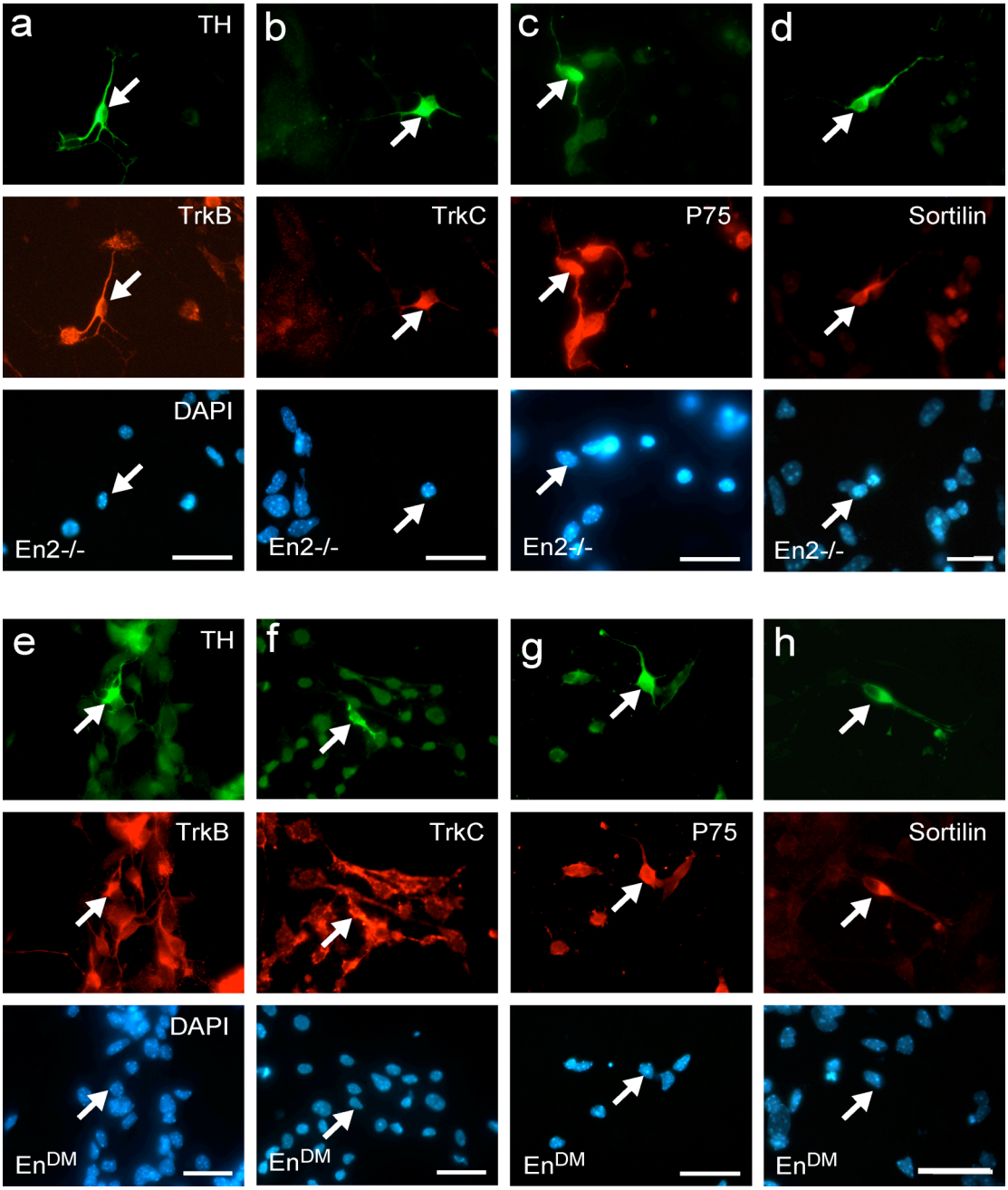


Figure-8: Expression of neurotrophin receptors in mesDA neurons. P75, TrkB, TrkC and sortilin are expressed in the primary mesDA neurons derived from En^{DM}, or En2-/- embryos. The differential expression, detectable by Western blot, can not be detected by immunohistochemistry. Scalebars = 25µm.

To test whether P75 is directly regulated by Engrailed, the genomic sequence of mouse and human P75 gene was checked, using the rVISTA and MatInspector computational tools (Cartharius et al., 2005; Loots et al., 2002; Quandt et al., 1995) for possible evolutionarily conserved binding sites of Engrailed. Two binding sites were found, between -140-180 and -250-340bp, within the human and mouse consensus promoter sequence (95% homology). Then, I performed a luciferase assay after cloning this

sequence into the reporter vector and transfecting the vector into Engrailed expressing MN9D cells. The comparison between the luciferase signals, normalized against the internal Renila controls, showed a very small and insignificant difference in presence or absence of En1 (Fig. 9), suggesting a secondary, indirect, mechanism for regulation of expression of P75.

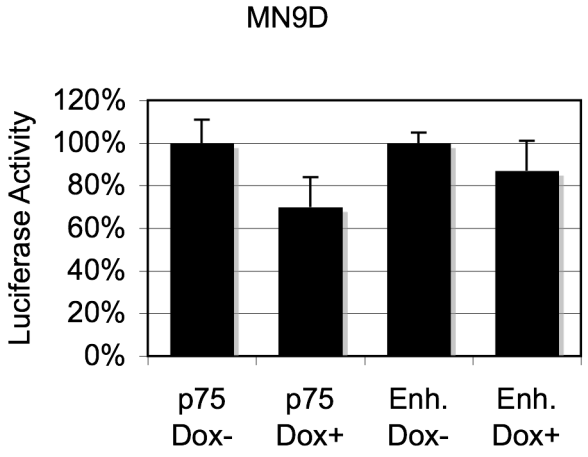


Figure-9: Luciferase activity of P75 consensus sequence in presence or absence of the En1. The renila vector was co-cotransfected and the value of luciferase activity was normalized against renila value. In addition to the renila, the values from a basic vector without the enhancer or P75 consensus sequence was used as second control which were subtracted from the values of luciferase activity. The final values from the enhancer and normal vectors were normalized against the –Dox samples.

Since P75 can mediate cell death in neurons(Lu et al., 2005), I began to investigate whether its up-regulation in

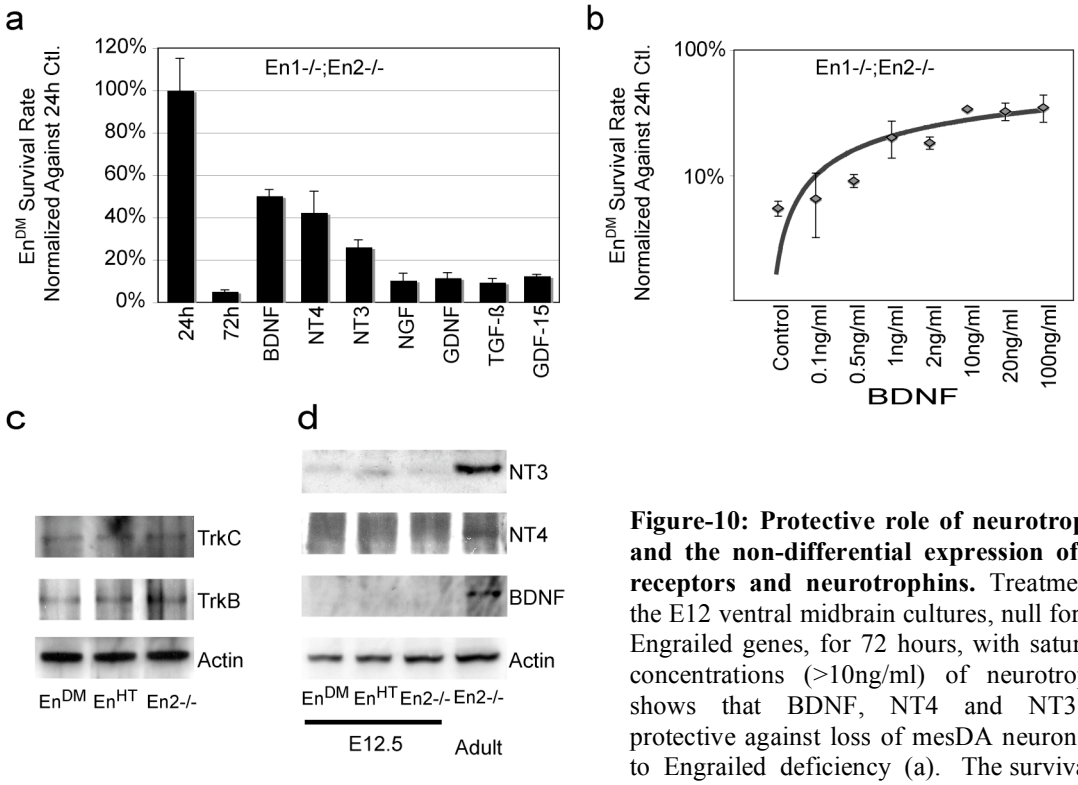


Figure-10: Protective role of neurotrophins and the non-differential expression of Trk receptors and neurotrophins. Treatment of the E12 ventral midbrain cultures, null for both Engrailed genes, for 72 hours, with saturating concentrations (>10ng/ml) of neurotrophins shows that BDNF, NT4 and NT3 are protective against loss of mesDA neurons due to Engrailed deficiency (a). The survival of Engrailed double mutant mesDA neurons under BDNF is dose dependent, with a kD of 2.5 (b). Western blot of ventral midbrain, derived from En^{DM} En^{HT} or en2-/- E12 embryos shows that the expression of Trk receptors is not differential and the neurotrophins are not expressed at this age.

Engrailed mutants is causal to the cell death. To lower P75 expression in Engrailed double mutant mesDA neurons, I applied P75 specific Penetratin-coupled siRNA duplexes(Davidson et al., 2004) to the ventral midbrain cultures. 72h after the transfection, the total P75 protein was reduced by 83.2 ± 6.3% (p=0.05, n=3) and the survival rate increased from 7.5 ± 1.24% to 25.1 ± 2.1% (p<0.001 n=16) (Fig. 3d). To complement this experiment, I interfered directly with the function of P75 by applying an inhibiting antibody (Rex)(Kruttgen et al., 1998) to the medium. This antibody also increased the survival rate to 34.8 ± 4.6% (p<0.001, n=6). These data suggest that up-regulation of P75 is a direct cause for the apoptotic cell death of Engrailed-deficient mesDA neurons.

P75 mediates dual, opposing, functions of survival and death, controlled by presence or absence of neurotrophins. For anti-apoptotic function, neurotrophins require their cognate Trk receptors as heterodimerization partners for P75(Lu et al., 2005). In order to assess a potential role of the Trk/P75 system during the course of cell loss, I determined the expression of Trk-receptors in E12 mesDA neurons. TrkC and TrkB and , but not TrkA, were detectable by immunohistochemistry and Western blot at equal levels in wildtype and En^{DM} mutants (Fig. 8).

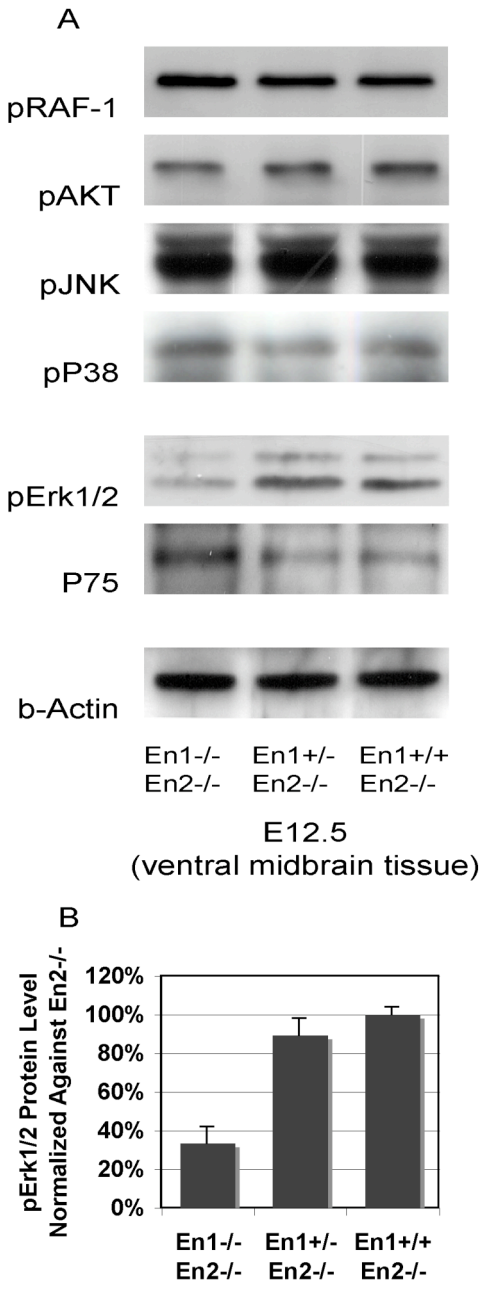


Figure-11: Differential activation of Erk1/2. Western blot on the protein, derived from ventral midbrain of mutant E12.5 embryos shows a significant reduction in phosphorylation of Erk1/2 in absence of the Engrailed genes (A,B), while the level of expression of Erk1/2 is unaltered in different genotypes (I). The antibodies against phosphorylated form of other MAPK's, JNK and P38 or the upstream RAF-1, also did not detect any significant difference amongst different genotypes of Engrailed (I).

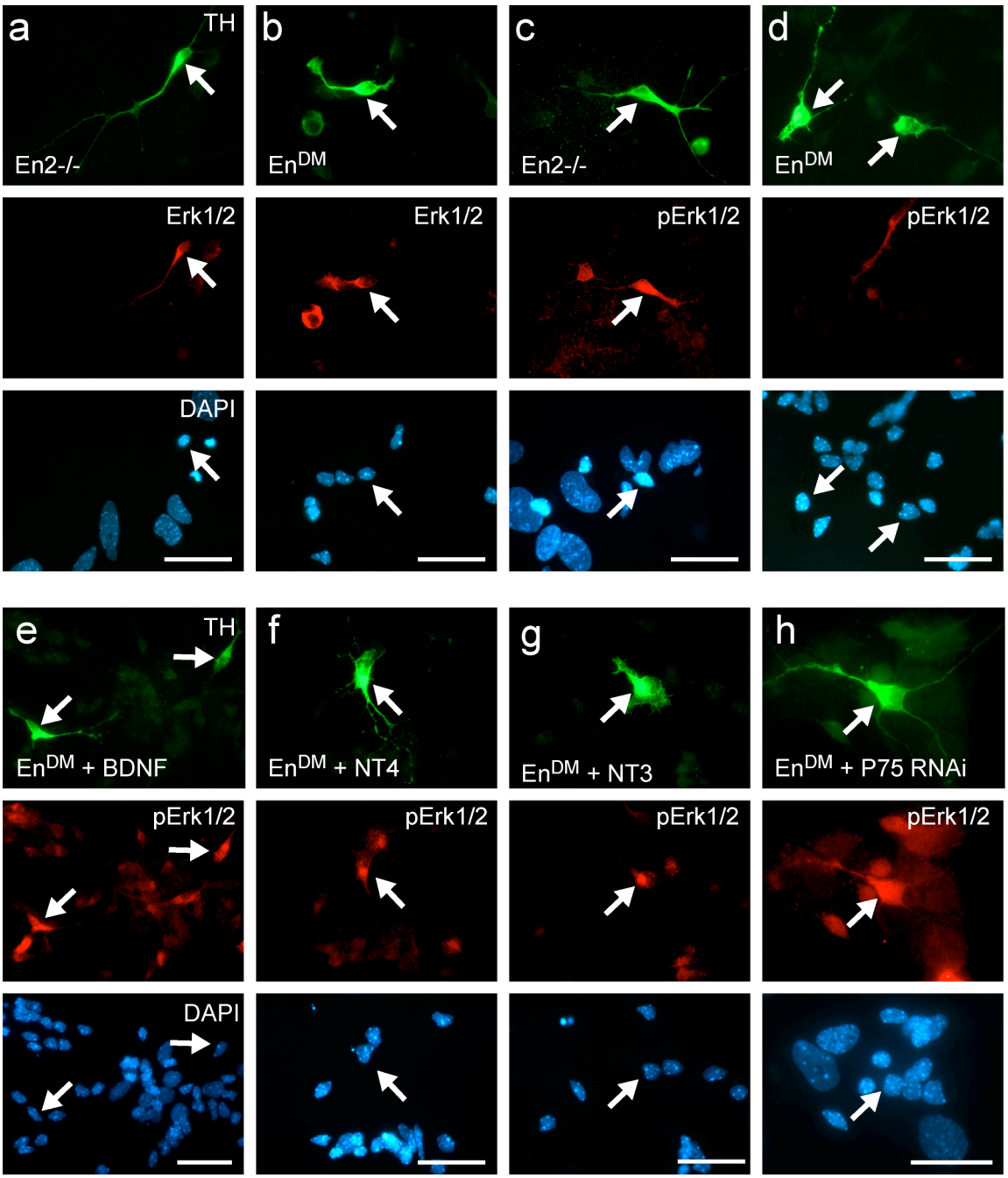


Figure-12: Differential phosphorylation of Erk1/2 in surviving vs. dying mesDA neurons and the survival mediating role of Erk1/2. While total Erk1/2 protein is present in mesDA neurons of both genotypes (a,b), P44/42 (Erk1/2) is phosphorylated in mesDA neurons, derived from En2-/- E12 ventral midbrain (c) but the activation of Erk1/2 is absent in mesDA neurons of their Engrailed double mutant (En^{DM}) littermates (d),. Erk1/2 becomes active in En^{DM} mesDA neurons, after treatment with the survival inducing neurotrophins, BDNF, NT4 and NT3 (e-g) or in mesDA neurons, where P75 is knocked down by Penetratin-coupled oligonucleotides (h). Presence of GDNF, TGF-ß or GDF15 did not induce activation of Erk1/2 in En^{DM} mesDA neurons (data not shown). Scale bars = 25µm.

The up-regulation of P75 and presence of the Trk-receptors suggested that the Engrailed deficiency introduces a neurotrophin requirement to the E12 mesDA neurons, which cannot be satisfied at this age, since the neurotrophins, specific to TrkB and TrkC, i.e. BDNF, NT4 and NT3, are not expressed in the E12 ventral midbrain as opposed to the adult (Fig. 10). To test this hypothesis, I applied saturating concentrations of BDNF, NT4 and NT3 to ventral midbrain cultures. After 72 hours, 50.2 ±2.9% ($p<0.0001$, n=27), 42.3 ±10.1% ($p<0.001$, n=9) and 26.0 ±3.5% ($p<0.001$, n=9), respectively, of the otherwise dying En^{DM} mesDA neurons survived (Fig. 10). In contrast, application of the TrkA specific ligand, NGF, did not change the survival rate significantly. To validate the specificity of BNDF, NT3 and NT4, I applied to the mutant cultures GDNF, GDF-15 and TGF-ß, all of which are demonstrated survival factors for mesDA neurons(Blum, 1998; Strelau et al., 2000; Tomac et al., 1995). Similar to NGF, none of them could prevent death of the Engrailed-deficient mesDA neurons (Fig. 10). Furthermore, the linear dose-response trend-line of the survival effect of BDNF (Fig. 10) indicated a Kd value of approximately 2.5nM which corresponds with the reported affinity of BDNF to TrkB and P75(Barker, 2004).

5. The Survival-Mediating Role of Extracellular Signal Regulated Kinase1/2 (Erk1/2)

The survival function of neurotrophins has been mainly attributed to two downstream pathways, the extracellular-signal-regulated kinase-1/2 (Erk1/2) and phosphatidylinositol-3 kinase (PI-3K) pathways(Hetman and Xia, 2000). As the next step, I examined whether these pathways play a role in arbitrating the effect of neurotrophins in Engrailed-deficient mesDA neurons and whether they intersect with the molecular events regulated by Engrailed expression. Therefore, I studied activation of the two pathways, using antibodies against the phosphorylated forms of Erk1/2 and Akt(Hetman and Xia, 2000). Immunohistochemistry showed that while total Erk1/2 protein was present in, both, En2-/- and En^{DM} mesDA neurons, it was activated only in the wild type-like cells and not in En^{DM} mesDA neurons (Fig12). Western blot analysis of ventral midbrain tissue showed a similar effect; Erk1/2 activity in E12 ventral midbrain tissue of En^{DM} embryos was reduced by 66.8 ± 18.2% ($p=0.02$, n=3) in comparison to their En2-/- littermates. In contrast to Erk1/2, I did not detect

differential activation of AKT in the Western blot (Fig. 11). Additionally, signs of differential activation of the other components of the MAPK pathways, i.e. JNK or P38 could not be detected (Fig. 11).

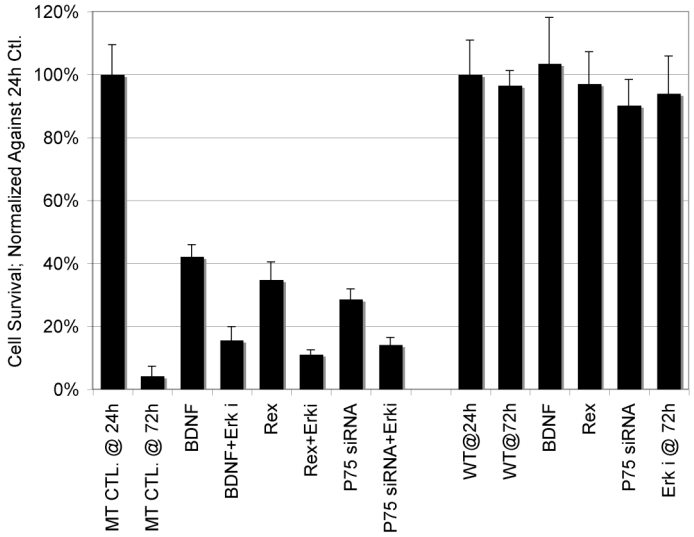


Figure-13: Erk1/2 inhibition and survival. The survival effect of neurotrophins, Rex or siRNA of P75 is abolished when the double mutant cultures are concurrently treated with 400nM of a specific inhibitor of MEK1/2, U0126 (K). The inhibitor of MEK did not have any effect on En2-/- mesDA neurons at this concentration. The increase in the amount of inhibitor to 1µM also killed the wild type-like neurons.

The loss of Erk1/2 phosphorylation in the Engrailed double mutant mesDA neurons suggested that the neurotrophin survival- and p75 death-signals lead to differential activation of this pathway. To investigate this, I examined the state of activation of Erk1/2 in En^{DM} mesDA neurons after application of the survival inducing neurotrophins. The addition of BDNF, NT4 and NT3, and the knock-down of p75 by siRNA oligonucleotides all caused phosphorylation of Erk1/2 in En^{DM} mesDA neurons (Fig. 12), however, Erk1/2 was not activated after application of NGF, GDNF, TGF-ß or GDF-15 (data not shown). If the activation of Erk1/2 is part of the molecular cascade, essential for

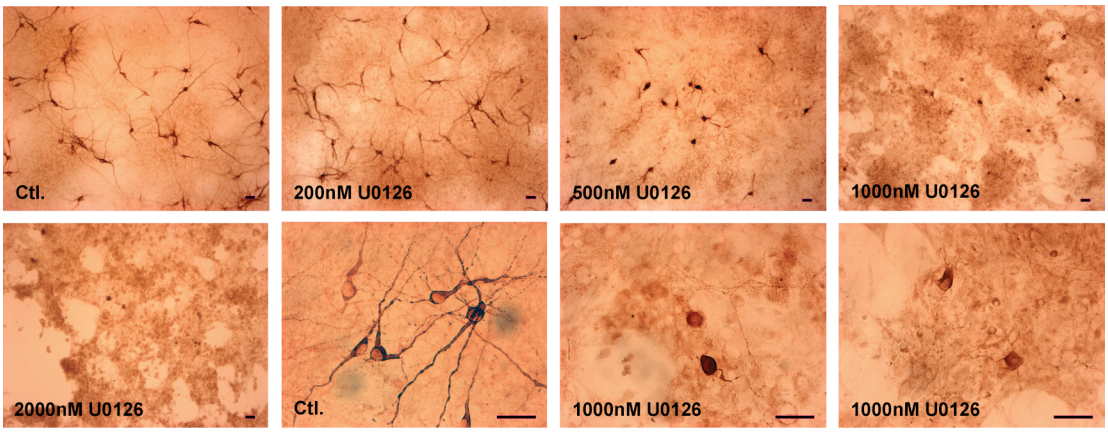


Figure-14: Inhibition of Erk1/2 by U0216. The inhibition of Erk1/2 by U0126 for three days, at concentrations as low as 500nM is detrimental to mesDA neurons. A marginal dose of U0126 was chosen for inhibition of Erk1/2 in En^{DM} mesDA neurons.

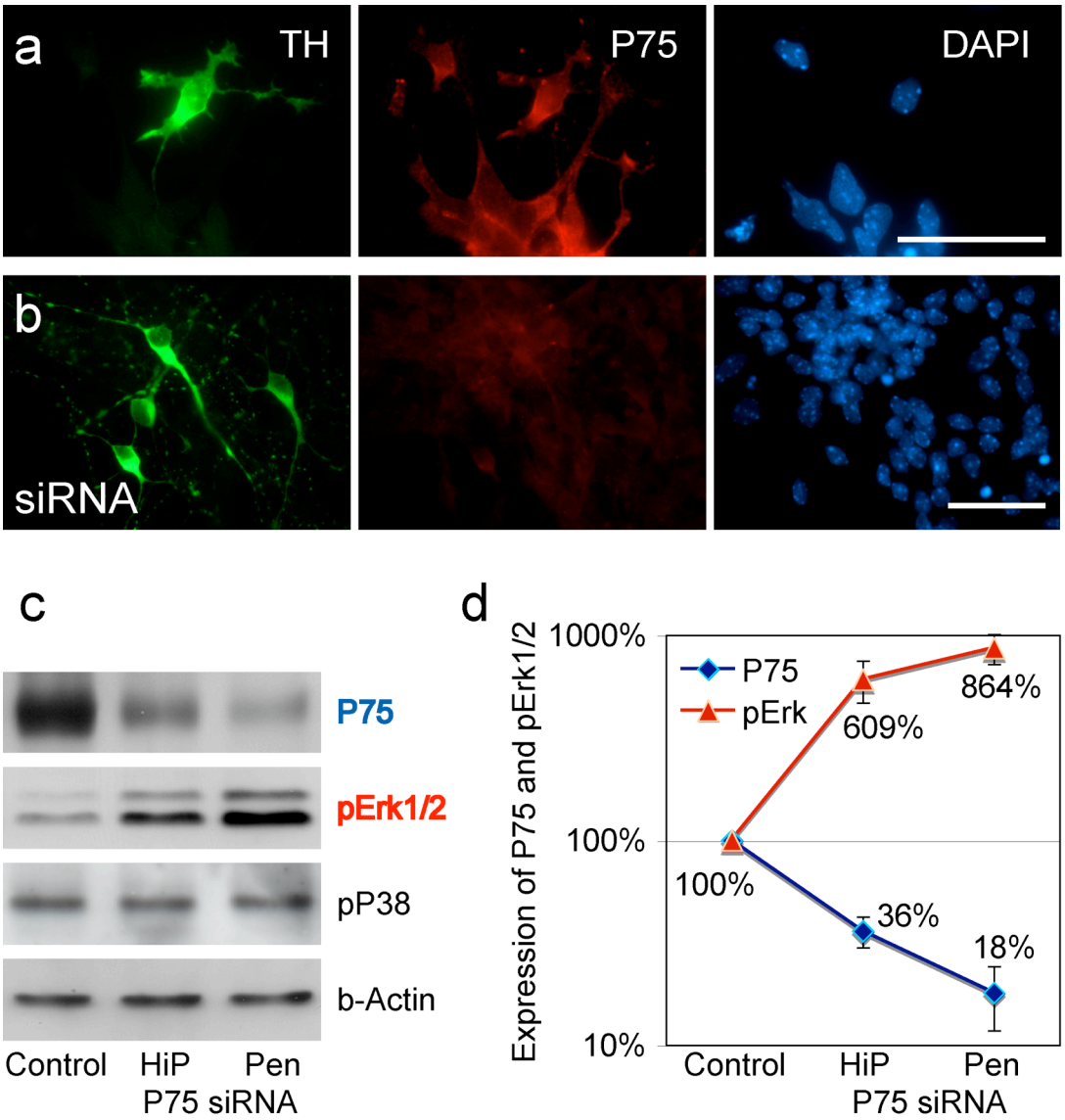


Figure-15: P75 knockdown by siRNA causes Erk1/2 phosphorylation. Immunohistochemistry on E12 primary cultures, shows expression of P75 in TH positive and other cell populations of En1+/-;En2-/- embryos (A). The expression is significantly reduced in siRNA in cultures where P75 is knocked down by Penetratin-coupled RNA oligonucleotides, 48h after transfection (B). Western blot analysis of P75 RNAi, using Penetratin-coupled oligonucleotides shows an 85% reduction in P75 protein level (C, right lane) or 65% reduction in uncoupled oligos, transfected by Hiperfect (C, middle lane). The decrease in the level of p75 in primary midbrain cultures causes an increase in phosphorylation of Erk1/2 (C). Measuring the bands from western blot shows the inverse correlation between expression of p75 phosphorylation of Erk1/2 (D).

survival of mesDA neurons and is inversely correlated to the cell death signaling, caused by the up-regulation of P75, then inhibition of the Erk1/2 pathway should interfere with the rescue effect of the neurotrophins and of P75 inhibition or silencing. To test this, I concurrently treated the cultures with U0126, an inhibitor of, the MAPKK upstream of Erk1/2, MEK1/2 (Duncia et al., 1998) at a concentration (400nM) sub-toxic to wild type-like mesDA neurons. Increase of the concentration to

1µM, was lethal to mesDA neurons after treatment for 72hrs (Fig. 13). The inhibition of Erk1/2 by U0126 significantly reduced the rescue effect for all three survival factors; from 42.2 ± 3.1% to 11.7 ± 3.5% (p=0.002) for BDNF, from 34.7 ± 4 .7% to 8.3 ± 2.7% (p<0.0001) for Rex and from 25.4 ± 2 .7% to 10.8 ± 1.9% (p=0.009) after P75 silencing (control mutant 4.2 ± 2.2% p=0.01 n ≥ 3 for all experiments) (Fig. 14).
To elaborate further on the correlation between the expression of P75 and the state of phosphorylation of Erk1/2, I silenced the P75 expression in En^{HT} ventral midbrain culture by RNAi, using two methods with different transfection efficiencies (Penetratin-coupled oligos and the lipophilic transfection reagent, HiPerfect). The former reduced the P75 expression levels on average by 82.2 ± 6.3% (p=0.05, n=3) and the latter by 63.3 ± 2.0% (p=0.011, n=3). This, in turn, caused 8.64 ± 1.45 (p=0.008, n=3) fold and 6.09 ± 1.40 (p=0.01, n=3) fold increase, respectively, in the phosphorylation of Erk1/2 (Fig. 15) demonstrating that P75 expression and activation of Erk/2 are inversely correlated to each other.

6. Reduction in Activation of Erk1/2 by Neurotoxins

Lack of activation of Erk1/2 in the En^{DM} mesDA neurons, correlated with high expression of P75 shows that Erk1/2 is a survival-inducing pathway for mesDA neurons. I also demonstrated that the specific inhibition of this pathway causes death of the neurons. To test whether deactivation of the pathway is a general phenomenon in the dying neurons, I checked the activation of Erk1/2 in cells, treated with mesDA-specific neurotoxins. Immunohistochemistry on wild type primary neurons derived from of E12 embryos showed that after 24hour neurotoxin treatment, phosphorylation of Erk1/2 was significantly reduced in mesDA neurons (Fig. 16). In MN9D cells the

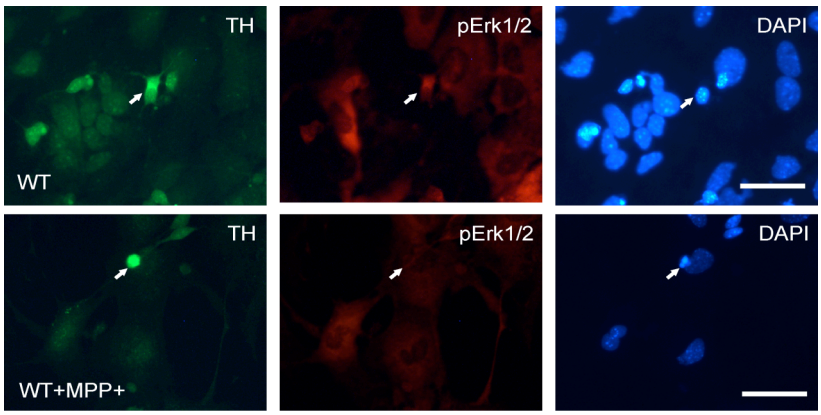


Figure-16: Deactivation of Erk1/2 in presence of neurotoxins. Treatment of primary mesDA neurons with MPP^+ decreases the Erk1/2 activation (A).

level of phosphorylated Erk1/2, comparing to that of untreated, En1 expressing cells was 47%, 46% and 36% in case of treatment by MPP^+, 6-OHDA or rotenone, respectively. The expression of Engrailed for 48hrs., prior to and during the application of neurotoxins preserved the level of activation of Erk1/2 to 71%, 62% and 54% respectively (Fig. 17). These data confirm the direct correlation between the expression of Engrailed and activation of Erk1/2 in primary mesDA neurons and suggest reduced activation of Erk1/2 in the cytoplasm, as a general vulnerability factor, common to the mesDA specific models of cell death.

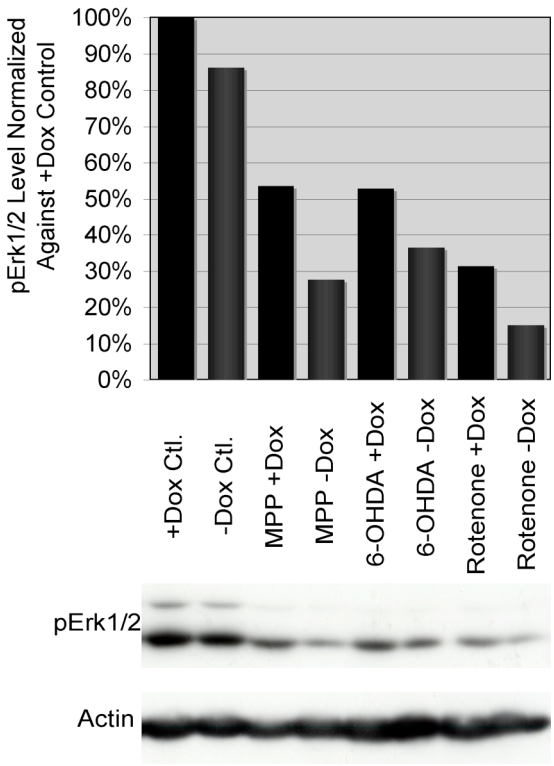


Figure-17: Deactivation of Erk1/2 in presence of neurotoxins. Treatment of primary mesDA neurons with MPP^+, 6-OHDA, and rotenone in MN9D cells decreased the activation of Erk1/2 while expression of En1 inhibited this effect (B).

7. Activation of c-Jun in Axonal Beadings

Western blot analysis showed that the death-mediating MAPK pathways were not differentially affected by the expression of Engrailed. Immunohistochemistry to detect phosphorylation of P38 and JNK also did not show differential activation, and similar

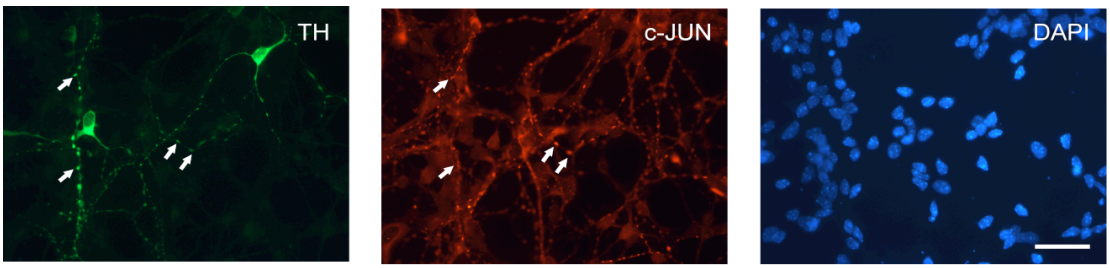


Figure-18: Activation of c-JUN in axonal beadings of mesDA neurons. While the activation of c-JUN is seen in the cell bodies of some neighboring cells, its phosphorylation in mesDA neurons happens, exclusively, in the axonal beadings. Scale bar=25µm

to phosphorylated Erk1/2, they were activated in the cell bodies and axons. However, the activation of c-Jun, downstream MAPK of JNK, in the primary cultures of mesDA neurons was exclusive to axons and to the axonal beadings of mesDA neurons, while present in the cell bodies of some neighboring cells (Fig. 18). The beadings in nerve fibers are dynamic structures, considered as signs of axonal degeneration or culture artifacts (Ochs et al., 1997). The phosphorylation of c-JUN, as a pro-apoptosis event, in axonal beadings of mesDA neurons may suggest a mechanism for axonal degeneration in mesDA neurons.

8. Miscellaneous Results

8.1. Differential Expression of NRAGE by mesDA Neurons

The interaction of intracellular factors with P75 is a determinant of its role, especially in neuronal apoptosis. Several molecules have been identified as intracellular binding partners of P75. Among the factors, interacting with the death domain of P75 and facilitating apoptosis, is a member of the melanoma associated antigen gene (MAGE)

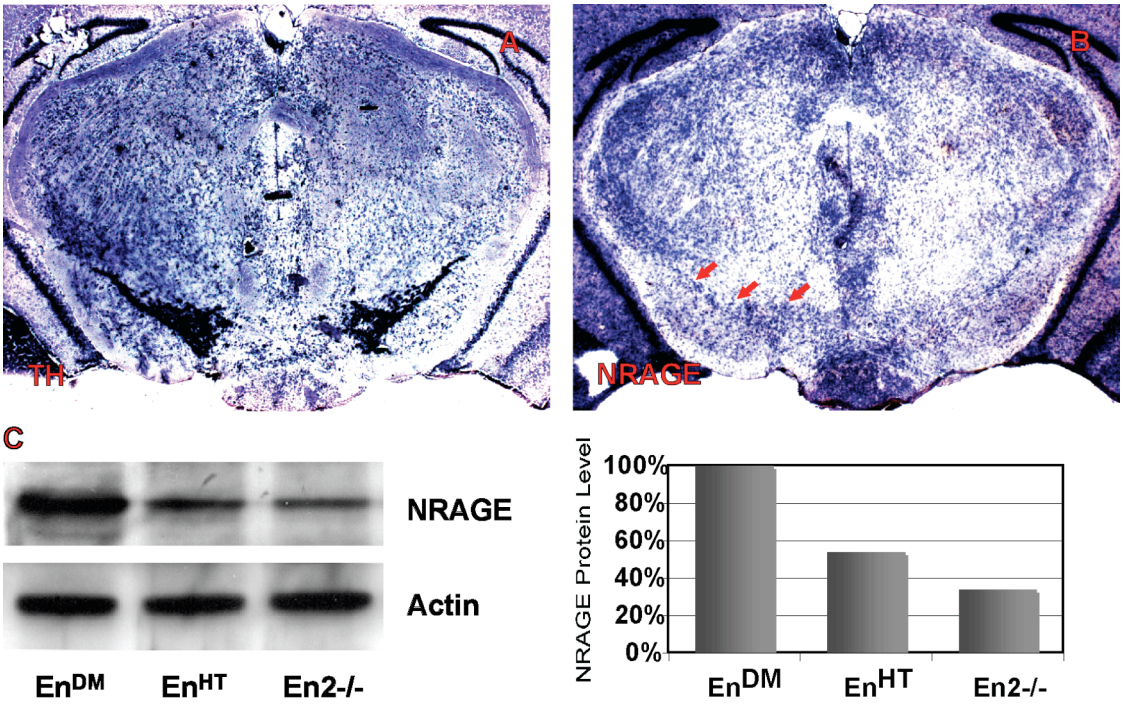


Figure-19: NRAGE expression and regulation by Engrailed. Dig In situ hybridization for TH (A)and NRAGE (B) on parallel sections of wild type adult mesDA neurons and Western blot analysis plus quantification of E12.5 ventral midbrain tissue derived from embryos with different genotypes of Engrailed (C).

family, MAGE-D1/ NRAGE (Salehi et al., 2000). The apoptotic activity of NRAGE is mediated via activation of JNK, cytosolic accumulation of cytochrome C, activation of Caspases-3, -9 and -7, and caspase-dependent cell death (Salehi et al., 2002). Although the expression, and therefore function, of NRAGE is not limited to P75 expressing neurons, all cells that express p75 also express NRAGE (Barrett et al., 2005). I could detect expression of NRAGE in a subpopulation of wild type adult mesDA neurons by in situ hybridization (Fig. 19). Western blot analysis on E12 ventral midbrain tissue showed that the expression of NRAGE in En1+/-;En2-/- and En1+/+;En2-/- was 54% and 34% of the En^{DM}, respectively. A comprehensive study of the function of NRAGE in mesDA neurons is still missing but these findings suggest an apoptotic role for NRAGE in mesDA neurons, with a possible connection to the Engrailed genes.

8.2. Expression of Faim2/Lfg in the Midbrain

The microarray study of Engrailed-expressing N2A cells showed that the expression of Fas apoptotic inhibitory moledule-2 (Faim2/LFG/NMP35) was increased in presence of En1. Localized at postsynaptic sites and in dendrites, Faim2 inhibits Fas-mediated, but not the TNF induced, extrinsic apoptosis (Schweitzer et al., 2002; Somia et al., 1999). Furthermore, Faim2 overexpression in cerebellar granule neurons makes them resistant to death by Fas ligand via activation of PI3K (Beier et al., 2005). Here I show that Faim2 is widely expressed in the midbrain and in mesDA neurons (Fig. 20). A study about the possibility of involvement of Fas mediated apoptosis and Faim2 in the

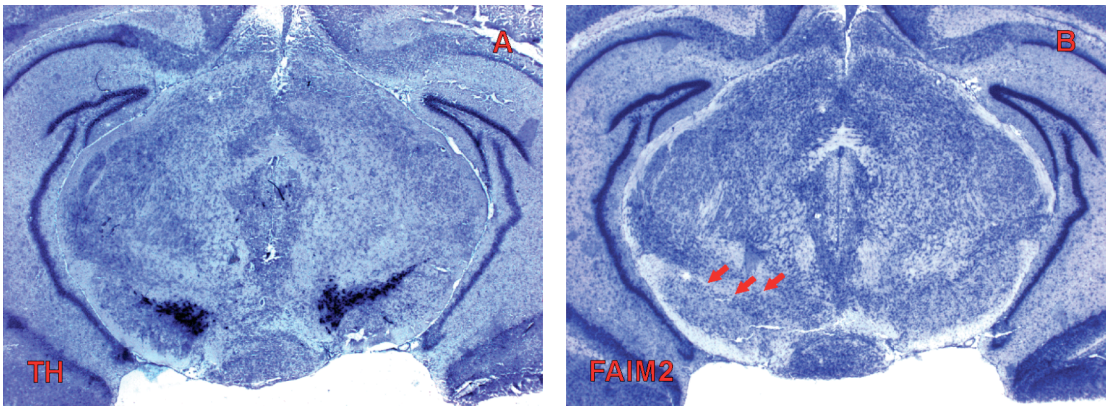


Figure-20: Expression of Faim2 in mesDA neurons and in Midbrain. Dig In situ hybridization for TH (A)and Faim2 (B) on parallel sections of wild type adult midbrain shows that Faim2 is expressed in the nigral neurons as well as the neighboring tissue.

death of Engrailed-deficient mesDA neurons is still missing.

8.3. Expression of ErbB4 in Nigral mesDA neurons

A transmembrane receptor tyrosine kinase (RTK), erythroblastic leukemia viral oncogene homolog 4 (ErbB4), is expressed in the mesDA neurons of rats. Its expression is diminished upon application of 6-OHDA (Steiner et al., 1999). The ligand, which preferentially binds to ErbB4, Neuregulin1-beta, evokes overflow of striatal dopamine, upon injection supranigrally (Yurek et al., 2004). To study the role of ErbB4 in the midbrain, in addition to development of a brain specific conditional mouse, we studied the expression of erbB4 in the mouse ventral midbrain. I used DIG and radioactive in-situ hybridization to study the expression of ErbB4 in 6 month-old mice ventral midbrain. ErbB4 was present in the SN and a subpopulation of mesDA neurons in the VTA. The expression was missing from RRF (Fig. 21). Despite the lack of deficits in the cellular, physiological or biochemical composition of nigro-striatal system of conditional mice for ErbB4 (Thuret et al., 2004),

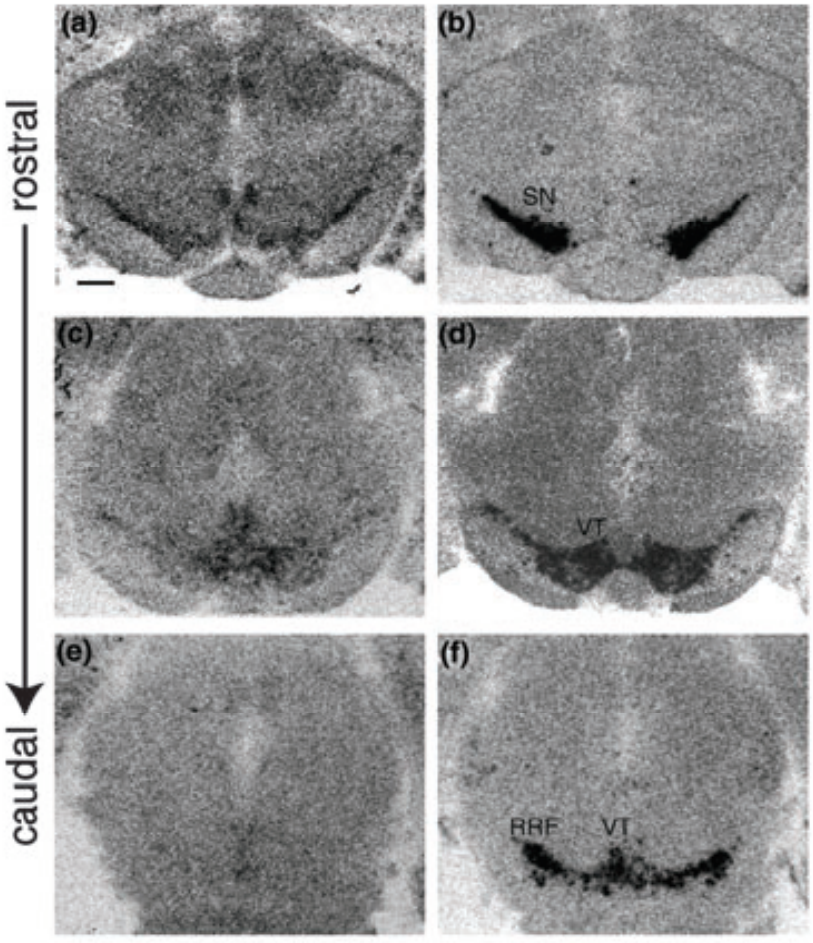


Figure-21: Expression of ErbB4 in mouse mesDA neurons. The radioactive in situ hybridization shows that the expression of ErbB4 is limited to SN and a subpopulation of VTA neurons(Thuret et al., 2004).

further study of ErbB4's function could be of value due to the specificity of its expression to SN.

8.4. Inhibition of K-ATP Channels and the Survival of mesDA Neurons

ATP sensitive potassium (K-ATP) channels are octarmeric proteins consisting of two types of subunits, being members of Kir6 and sulphonylurea receptor (SUR) family. They activity of these channels depends on the intracellular level of ATP/ADP and, therefore, dependent upon the metabolic state of the cell (Liss and Roeper, 2001). The

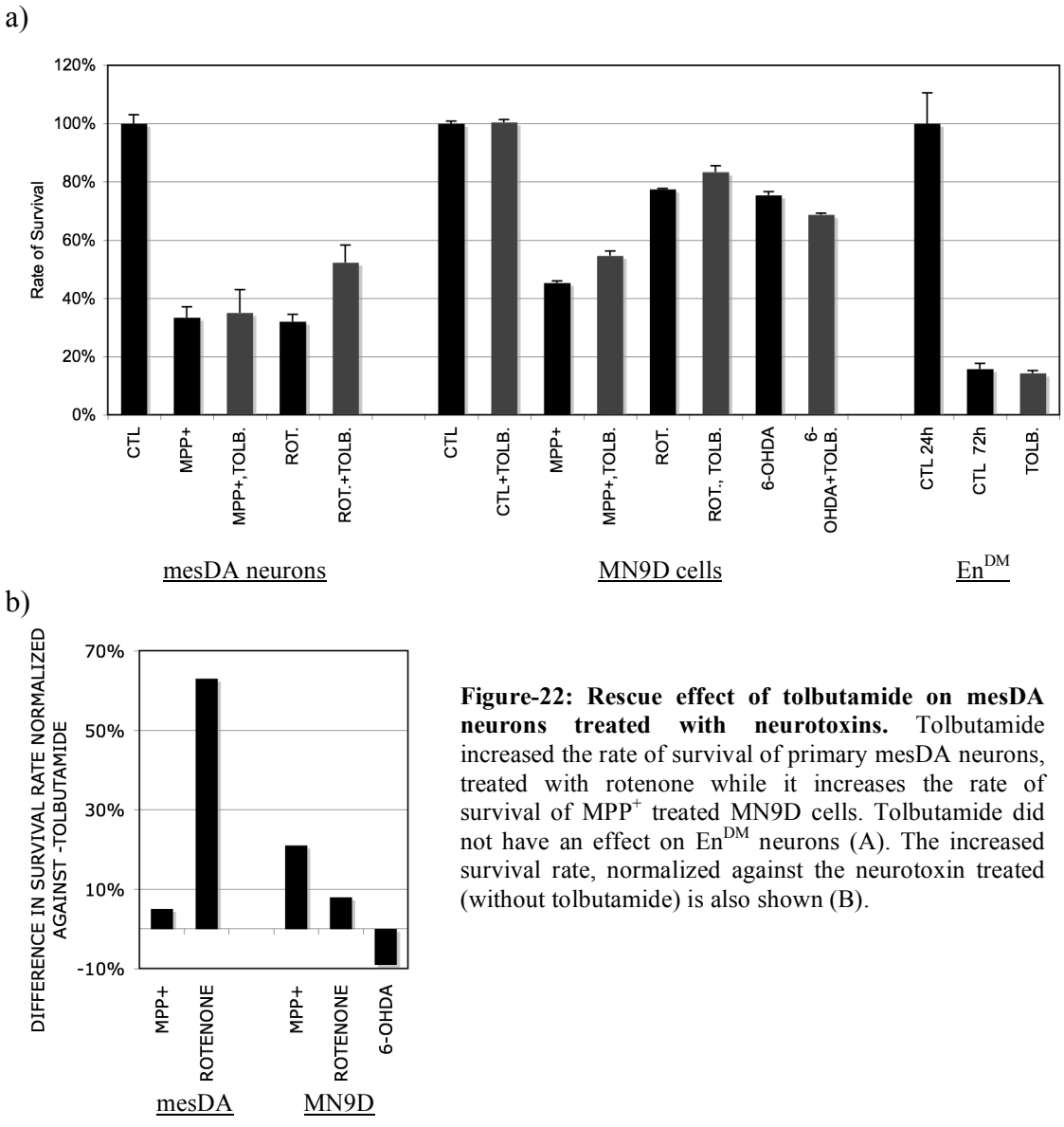


Figure-22: Rescue effect of tolbutamide on mesDA neurons treated with neurotoxins. Tolbutamide increased the rate of survival of primary mesDA neurons, treated with rotenone while it increases the rate of survival of MPP^+ treated MN9D cells. Tolbutamide did not have an effect on En^{DM} neurons (A). The increased survival rate, normalized against the neurotoxin treated (without tolbutamide) is also shown (B).

expression profile of the subunits of these channels also defines their function in different neuronal populations. For instance the DA neurons in the SN show alternative co-expression of SUR isoforms with Kir6.2 and this alternative expression defines the sensitivity of the K-ATP channels to metabolic inhibition (Liss et al., 1999). Additionally, the inactivation of Kir6.2 has been shown to selectively affect the electrophysiological activity of SN neurons and rescue of these neurons from MPTP intoxication or weaver mutation (Liss et al., 2005). I examined the effect of inhibition of K-ATP channels on the death, induced by MPP^+, 6-OHDA or rotenone in primary mesDA neurons and MN9D cells by addition of tolbutamide (Stanford and Lacey, 1995). Addition of tolbutamide to primary cultures and MN9D cells produced mixed results. In primary neurons, the significant difference was in rotenone-treated cells where in presence of tolbutamide, the rate of survival increased from 32±2% to 52±6%(P=0.05). On the other side, in MN9D cells, by presence of tolbutamide, the rate of survival of MPP^+ treated cells increased from 45±1% to 55±2% ($P<0.001$) comparing to untreated controls. The rate of survival of En^{DM} neurons did not change upon application of tolbutamide (Fig. 22). These results suggest that while inhibition of the hypo-activity of mesDA neurons via depolarization of the cells by blocking of the K-ATP channels can delay or inhibit the neurotoxin-induced cell death, it is not sufficient to rescue the mesDA neurons from dying because of Engrailed deficiency in the En^{DM} embryos. Since the cell death in En^{DM} is more acute and pronounced than other genotypes, the blocking of these channels might be beneficial to the En1+/-;En2-/- adult mice where the phenotype is restricted to the SN neurons and the cells loss is slow and progressive.

Discussion

The specific loss of mesDA neurons in En^{DM} embryos and the gradual, postnatal loss of nigral DA neurons in En^{HT} mice, is reminiscent of PD. Therefore, study of the mechanisms underlying cell death, attributable to the lack of Engrailed genes, may provide insight into the etiology of the disease. In this study, I provide evidence that the dose of Engrailed expression in mesDA neurons determines their sensitivity to mitochondrial insult, likely due to changes in the stability of the mitochondrial outer membrane and resulting in an increase in cytosolic cytochrome C and activation of caspase-9. Furthermore, I show that cell death, triggered by the loss of Engrailed expression in this neuronal population, is a result of an increase in P75 expression and, consequently, the absence of Erk1/2 activation. The apoptotic outcome of P75 up-regulation can be counteracted by addition of neurotrophins, specific to TrkB/C or siRNA knock down of p75, suggesting that the elevated P75 expression in the E12 En^{DM} embryos introduces a neurotrophin dependency, lacking from the wild type mice at this age. According to these results, Engrailed-deficiency causes cell death via two mechanisms of P75-mediated apoptosis and mitochondrial dysfunction. Taken together, in presence of the Engrailed genes, suppression of P75 and activation of Erk1/2 are parts of the same survival pathway that may, ultimately, protect cells from mitochondrial dysfunction and the intrinsic pathway of apoptosis.

1. Involvement of the Mitochondrial Pathway of Apoptosis

A strong body of evidence suggests reduced activity of the mitochondrial complex-I (Dauer and Przedborski, 2003), production of ROS and impairment of integrity of the mitochondrial membrane as parts of the process, leading to demise of mesDA neurons in PD (Fiskum et al., 2003). Apoptosis, in these neurons, is a consequence of interplay of these events from one side, and the classical mitochondrial pathway of apoptosis, involving the Bcl-2 family members, pro apoptotic molecules such as cytochrome C, AIF and the caspases, from the other side (Nicotra and Parvez, 2002). The connectivity of these two mitochondrial mechanisms is supported by the evidence that the chronic application of MPTP, as an inhibitor of complex-I which causes production of ROS, induces release of cytochrome-C from the mitochondrial intermembrane space by involvement of the Bcl-2 family members (Perier et al., 2005). The protective effect of Engrailed expression against MPP^+ as well as against inhibitors of Bcl-2 and Bcl-X_L,

shows a connection between Engrailed-deficiency and both of these mitochondrial processes and therefore, a possible relevance to etiology of PD. The activation of caspases 3 and 9 in E12 En^{DM} and P20 En^{HT} mesDA neurons and the inhibition of release of cytochrome C into the cytosol, by the Engrailed genes, further support the involvement of the mitochondrial pathway of apoptosis in death of En^{DM} mesDA neurons.

Mitochondria can give rise to both, Caspase-dependent and –independent, pathways of apoptosis, depending on the release of cytochrome C or AIF, respectively (Cai et al., 1998; Li et al., 1997). Our data are in support of the contribution of caspase-dependent, rather than caspase-independent pathway to death of the Engrailed-deficient mesDA neurons, especially since, contrary to cytochrome C, the release of AIF from the mitochondria to cytosol is not affected by the level of expression of Engrailed. If the cell death in En^{DM} neurons is caspase-dependent, failure of caspase inhibitors to rescue these neurons, suggests two possibilities that either the mitochondrial apoptosis is not essential to death of the neurons, or multiple pathways of apoptosis/cell death are involved.

Our experiments did not indicate involvement of necrosis and plasma membrane damage, detected by propidium iodide (data not shown), and the only criterion to detect cell death was the presence of pyknotic nuclei. In PD patients and in animal models of the disease, also the data strongly support apoptosis rather than other forms of cell death. If the apoptotic criterion for detection of cell death to be accepted, since all of the E13 En^{DM} and P20 En^{HT} mesDA neurons with pyknotic nucleus show activation of both caspase-3 and -9, and since the Engrailed expression clearly affects the mitochondrial events such as release of cytochrome C, caspases are part of the signaling cascade and mitochondrial apoptosis has a role in the death of mesDA neurons but, it is downstream of the survival/death decision making point and once passed that point, cells employ multiple means, and often shift to different modes or pathways of cell death, to die. This is also the prevailing view about involvement of apoptosis in PD and its animal models (Tatton et al., 2003).

2- Mechanistic Difference Between MPP^+ and 6-OHDA or Rotenone

While inhibition of complex-I of the mitochondrial respiratory chain is a key function, shared by MPTP, 6-OHDA and rotenone (Schober, 2004), and caspases-3 and -9 are activated in mesDA neurons treated by all three toxic models, there is evidence suggesting that the molecular mechanisms of cell death are different (Choi et al., 1999) and the differential effect of expression of Engrailed on death, induced by each of the toxins, suggests different mechanisms. Exclusivity of the protective effect of Engrailed against treatment with MPP^+ and not 6-OHDA or rotenone in primary mesDA neurons indicates that MPP^+ and Engrailed converge on the same molecular mechanism which is not affected or shared by the other neurotoxins. Taking the protective effect of Engrailed against Bcl-2 and Bcl-X_L inhibitors, HA14 and chelerythrine chloride, into account, also considering the opposing functions of Engrailed and MPP^+ in inhibiting and inducing the release of cytochrome C from the mitochondria, it is highly possible that the intracellular site of action of MPP^+ and Engrailed is the mitochondria and their functional point of convergence is permeability of the outer membrane.

These results were in disagreement with the outcome of the gain of function experiments in MN9D cells, where induction of Engrailed expression caused protection against treatment with all three neurotoxins. The disparity between the two sets of data can be either due to induction of abnormally high, non-physiological expression of En1 in MN9D cells, or because of the small difference in expression of En1 between En^{HT} and En2-/- (in primary cultures). The second assumption can be only tested by comparison of the rate of survival of En2-/- mesDA neurons with that of En^{DM}, but since the survival period of the En^{DM} mesDA neurons is only 72 hours, during which, the cells are undergoing cell death, this comparison is not possible. It can be concluded that in primary neurons, doubling the En1 expression level affects only the death, induced by MPP^+ and therefore, it is likely that the mechanism of cell loss, due to Engrailed deficiency, is similar to that of MPP^+ and not to the other neurotoxins, while the possibility of effectiveness of Engrailed (in high doses) as a rescue factor against all three neurotoxins is still there.

3- Lack of Involvement of TNF and P53-Mediated Apoptosis

Despite the evidence supporting involvement of the extrinsic pathway, the mitochondrial pathway of apoptosis is the prominent mode of cell death of mesDA neurons (Vila and Przedborski, 2003a). In agreement with this notion, I found that the level of expression of Engrailed in primary neurons and MN9D cells does not affect the survival rate in presence of TNF-α, representing the extrinsic pathway. The rate of survival of cells, dying by P53 activation, also, was not affected by the dose of Engrailed. Furthermore, pifithrin-α, an inhibitor of P53, a potent survival factor against MPTP treatment (Duan et al., 2002), could not rescue the En^{DM} neurons, suggesting a P53-independent role for the Engrailed genes.

P53 is a transcriptional and posttranscriptional regulator of the Bcl-2 family members and P53-induced apoptosis is mediated via regulation of the pro- and anti-apoptotic members of this family, including Bax (Tobiume, 2005; Wu and Deng, 2002). The protection of mesDA neurons and improvement of behavioral outcome in MPTP-treated mice via inhibition of P53 (by pifithrin-α) is attributed to suppression of increased expression of Bax (by MPTP) (Duan et al., 2002). Taken together with the fact that addition of KU70, an inhibitor of the mitochondrial translocation of Bax (Sawada et al., 2003), could not rescue the dying En^{DM} neurons, the expression and mitochondrial translocation of Bax, which are required for its apoptotic function (Nechushtan et al., 2001), do not contribute to the higher sensitivity of the mitochondria in Engrailed-deficient mesDA neurons.

Several studies have shown that expression of P53 and its activity is affected by 6-OHDA-treatment in dopaminergic neurons. In PC12 cells and in the SNpc of rats, the P53 cellular content increases by application of 6-OHDA (Blum et al., 2001; Blum et al., 1997). These data show that 6-OHDA-induced cell death is P53 dependent and the lack of response of cell death, induced by either of them, to the level of Engrailed expression confirms that Engrailed and 6-OHDA may act upon different molecular pathways.

4- High Expression of P75 is Causal to Loss of Engrailed-Deficient mesDA Neurons

High expression of P75 causes apoptosis in various neuronal populations via activation of the MAPK's and the mitochondrial pathway (Bhakar et al., 2003; Bunone et al., 1997; Rabizadeh et al., 1993). P75-mediated apoptosis can also be triggered extracellularly by addition of pro-neurotrophins (Lee et al., 2001; Lu et al., 2005; Teng et al., 2005). In this work, I show that the increase in the protein level of P75 is directly correlated with the decrease in expression level of Engrailed. This increase is responsible for the death of En^{DM} mesDA neurons, since the functional inhibition of P75 by Rex, or its knockdown by siRNA or addition of neurotrophins, BDNF, NT4 and NT3, significantly increased the rate of survival. The death signaling of P75 can be activated, extracellularly, by addition of pro-neurotrophins or as a result of interaction of death inducing molecules with the intracellular death domain of P75 (Barrett, 2000; Lee et al., 2001; Nichols et al., 1998). Although the cause of P75 mediated apoptosis in mesDA neurons remains unknown, one possibility is the changes in expression of intracellular molecules, such as NRAGE (Salehi et al., 2000), and their interaction with P75. In this work, I demonstrate that the expression of NRAGE is affected by expression of Engrailed, comparable to that of P75. The increased expression of NRAGE in the En^{DM} ventral midbrain may be a contributing factor to the death of these neurons, especially since the high expression of NRAGE causes apoptosis via a JNK-mediated pathway, and a similar mechanism to cell death by high expression of P75 (Kendall et al., 2002). I also show that the expression of NRAGE in the wild type adult is limited to a subpopulation of mesDA neurons in the substantia nigra. The expression of NRAGE in the En^{DM} E12 mesDA neurons and in En^{HT} P20 mice remains to be examined.

5- The Survival Effect of BDNF, NT4 and NT3 in En^{DM} mesDA Neurons, by Inhibition of P75

To induce their most prominent function, survival, neurotrophins bind to their cognate Trk receptors (Lu et al., 2005). Our results show that the survival of Engrailed-

deficient mesDA neurons by neurotrophins is dependent upon presence of Trk receptors and although a P75 ligand, in absence of TrkA, NGF does not change the rate of survival. Contrary to the primary mesDA neurons, in experiments with PC12 cell lines, which do express TrkA, application of NGF can protect the cells against MPP^+ treatment (Shimoke and Chiba, 2001). P75 and Trk receptors have been viewed as antagonizing partners and the rescue effect of BDNF, NT4 and NT3, in presence of TrkB and TrkC, is in line with previous findings showing that the P75 mediated cytotoxicity can be counteracted by Trk signaling(Davey and Davies, 1998). Our data also show that while the difference between the survival effect of NT4 and BDNF is minimal, NT3 is less effective in preventing the En^{DM} mesDA neurons. This is in agreement with previous findings, showing that NT4 and BDNF are functionally redundant and knocking in NT4 into the BDNF locus can reverse many of the phenotypes due to BDNF deficiency (Fan et al., 2000). It has been also implicated before that the NT3 (in comparison to BDNF or NT4) is less potent, as a survival factor for mesDA neurons (Altar et al., 1994; Haque et al., 1996).

The survival effect of neurotrophins can be due to activation of an intrinsic mechanism within the mesDA neurons or due to an indirect effect due to increased survival of the surrounding cells. The Kd value for binding affinity of neurotrophins to P75 or Trk is between 1 and 10nM (Barker, 2004). Since the Kd of 2.5nM, calculated from the linear dose-response trend-line of the survival effect of BDNF, corresponds to the known affinity of neurotrophins to P75 and Trk receptors, I conclude that the survival effect of neurotrophins is attributable to direct binding to the receptors on the surface of mesDA neurons as opposed to an indirect interaction with the neighboring cells, which would be reflected by a different Kd value. This is consistent with the concept that the Engrailed genes are cell-autonomously required for the survival of these neurons(Alberi et al., 2004).

6- Activity of Erk1/2 is required for Survival of mesDA Neurons

It is likely that absence of the Engrailed genes in E12 primary neurons, in absence of expression of neurotrophins, introduces a neurotrophin dependency by increasing the expression of P75. Since death of the neurons is a result of this unsatisfied requirement for neurotrophins, and addition of BDNF, NT3 and NT4 inhibits cell death, it is

possible that the downstream effectors of neurotrophins are differentially affected in En^{DM} versus En2-/- mesDA neurons. The activation of Erk1/2 in the wild type-like neurons as opposed to En^{DM} mesDA neurons, confirms this hypothesis. Addition of BDNF, NT4, NT3 and siRNA knockdown of P75 induced the activity of Erk1/2 in En^{DM} mesDA neurons, while GDNF and other neutral (to En^{DM} neurons) neurotrophic factors had no effect. On the other side, addition of 1μM U0126, an inhibitor of the upstream MAPKK of Erk1/2 pathway, Mek1/2 (Duncia et al., 1998), to the wild type-like cultures for 72 hours, is lethal to mesDA neurons, suggesting that the sustained activation of Erk1/2 is required for their survival. While addition of a lower concentration, 400nM, of this inhibitor was not toxic to the En2-/- mesDA neurons, it significantly reduced the rescue effect of all survival factors, BDNF, NT4, NT3, Rex and siRNA knockdown of P75. This shows that Erk1/2 pathway is part of the natural survival machinery of mesDA neurons, which is disrupted by ablation of the Engrailed genes and addition of neurotrophins and functional inhibition of P75, puts in place a compensatory mechanism, which up to some degree makes up for this deficiency. Since the inhibition of Erk1/2, at this dose, did not affect the survival of wild type cultures, it is possible that deactivation of Erk1/2, although an important component of the death pathway, is not the sole arbitrator of death in absence of the Engrailed genes and high level of expression of P75.

Erk1/2 and the two other MAPK pathways, JNK and P38, have opposing functions in vivo. While JNK and p38 participate in stress responses and, often, trigger apoptosis, Erk1/2 signaling regulates cell proliferation, differentiation and survival (Xia et al., 1995). With regard to neurotrophins, P75 and Trk receptors, phosphorylation of Erk1/2, has been attributed to the kinase activity of Trk receptors at two phosphorylation sites, Shc and PLC gamma, caused by addition of neurotrophins (Atwal et al., 2000; Hu and Koo, 1998; Obermeier et al., 1994), whereas the high expression of P75 causes cell death by activation of JNK pathway (Bhakar et al., 2003). In this study, I show that the activation of Erk1/2 is also controlled by expression of P75 and its activity increases when P75 is knocked down. The inhibition of activation of Erk1/2 in presence of P75 may be due to the opposing functions of P75 and the Trk receptors or direct association of P75 to Erk or the upstream molecules of the MAPK pathway (Blochl et al., 2004; Volente et al., 1993). The direct binding to P75 can be excluded as the cause of inhibition of Erk1/2, since the MEK inhibition

diminished the rescue effect of siRNA knockdown of P75, its inhibition by Rex or addition of BDNF.

Since the activation of Erk1/2 can also cause cell death (Subramaniam et al., 2004), the general role of Erk1/2 is still controversial and in neurotoxin-treated mesDA neurons, both pro-apoptotic and pro-survival roles have been implicated (Gomez-Santos et al., 2002; Jiang and Yu, 2005). In mesDA neurons, I detected long term (more than 72 hours), cytoplasmic activation of Erk1/2, only in the surviving, wild type-like neurons. Furthermore, the reduced activity of Erk1/2 by application of neurotoxins in MN9D cells and in primary mesDA neurons, death of the neurons by specific inhibition of Erk1/2, and mediating role of Erk1/2 with regard to survival inducing factors in En^{DM} neurons, i.e. neurotrophins and inhibition of P75, all support a pro-survival role for the Erk1/2 in mesDA neurons.

7- Conclusions

The ultimate question of PD is the issue of specific vulnerability of nigral DA neurons.

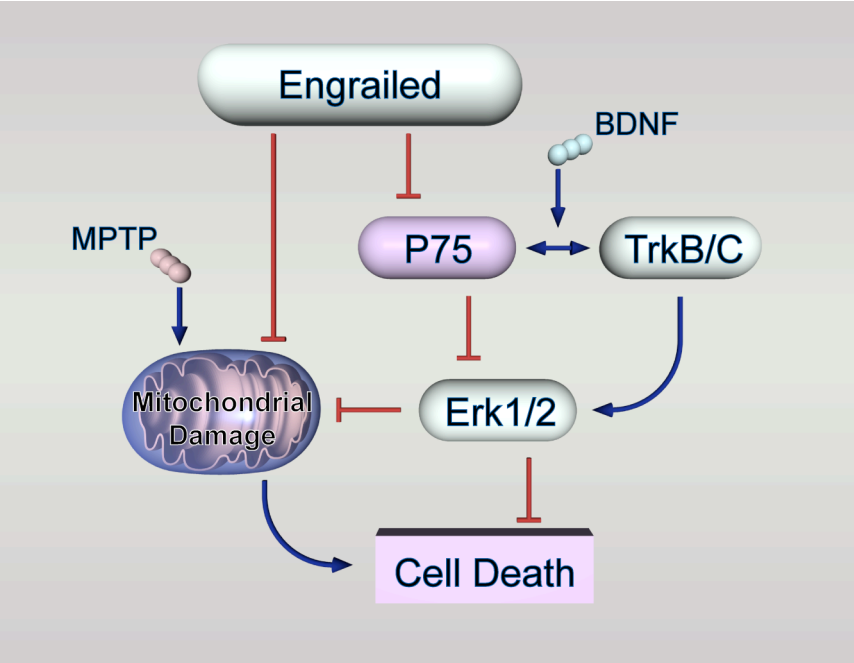


Figure-1: Engrailed genes regulate P75 expression and determine stability of mitochondria. P75 cell death signal in Engrailed deficient mesDA neurons is mediated by the suppression of the pro-survival Erk1/2 activity. The cell death signal can be counteracted by TrkB/C-specific neurotrophins. Loss of Engrailed expression also leads to instability of mitochondrial membrane, and increased sensitivity to mitochondrial damage by (for example) MPTP.

The added sensitivity of nigral neurons to cell death is manifest in knockout studies of transcription factors, such as Pitx3, Nurr1, and the Engrailed genes, showing that the neurons in substantia nigra are especially vulnerable and the most affected by lack of these genes. The specificity of the effect of absence of these transcription factors to nigral neurons suggests that the causes for vulnerability lies within the expression profile of these neurons. The prime example of differential loss of nigral neurons in absence of transcription factors is the En^{HT} mice, where the PD-like phenotypes are resembled the most. Therefore, study of the mechanisms underlying survival and death, in presence or absence of the Engrailed genes, may provide direct hints in understanding the root cause of PD. In this study, I show that mitochondrial vulnerability and regulation of expression of P75 contribute to death of the Engrailed-deficient mesDA neurons. The former is related to oxidative stress and to production of ROS, evident from the differential sensitivity of En^{HT} versus En2-/- mesDA neurons to MPTP treatment. The latter determines the activity of Erk1/2. The two mechanisms may be connected, since pharmacological inhibition of Erk1/2 increases the sensitivity to ROS(Yoon et al., 2002). The evidence, such as higher vulnerability of En^{HT} mesDA neurons to MPTP treatment, activation of caspase-9 in postnatal, dying nigral En^{HT} neurons, and the elevated expression of P75 in the ventral midbrain of En^{HT} embryos, indicate that the same mechanism may be the cause of slow, progressive PD-like degeneration of nigral DA neurons in the En^{HT} mice and therefore, abnormally low Engrailed or high P75 expression may be risk factors for PD.

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Glossary

AADC	l-Aromatic amino acid decarboxylase
AIF	Apoptosis inducing factor
Akt	Serine-threonine protein kinase
AMPA	α-amino-3-hydroxy-5-methyl4-isoxazole-propionic acid
AP	Anterior-posterior
Apaf-1	Apoptosis protease activator factor-1
ATP	Adenosine Triphosphate
BDNF	Brain-derived neurotrophic factor
BH	Bcl-2 homology domain
BME	Basal medium eagle
BMP	Bone morphogen protein
BrdU	5-Bromo-2´-deoxyuridine
cAMP	Cyclic adenosine monophosphate
CC	Chelerythrine Chloride
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanine monophosphate
CNS	Central nervous system
CREB	cAMP response element binding protein
DA	Dopamine
DAPI	4´,6-Diamidino-2-phenydole dilactate
DAT	Dopamine transporter
DNA	Deoxyribonucleic acid
DMEM	Dulbecco modified eagle medium
dNTP	Desoxyribonucleotide
DV	Dorsal-ventral
E	Embryonic day
EDTA	Ethylene diamine tetracetic acid
En	Engrailed
En^{DM}	En1-/-;En2-/-
En^{HT}	En1+/-;En2-/-
En1	Engrailed-1
En2	Engrailed-2
Erk	Extracellular signal regulated kinase
FGF	Fibroblast growth factor
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FP	Floor plate
GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
GP	Globus pallidus
GDNF	Glial cell line derived factor
HB	Homogenization buffer
HH	Hambuurger Hamilton stage
IgG	Immunoglobulin G
IL-1	Interleukin-1
L-15	Leibowitz medium-15

MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MEK	MAPKK/ERK kinase
MHB	Mid-hindbrain boundary
MPT	Mitochondrial permeability transition
MPDP+	1-methyl-4-phenyl-2,3-dihydropyridinium
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NCS	Newborn calf serum
NGF	Nerve growth factor
NT3	Neurotrophin-3
NT4	Neurotrophin-4
6-OHDA	6-hydroxydopamine
P75	Neurotrophin receptor p75/Ngfr
Park-1	Parkin-1
Park-2	Parkin-2
PC12	Rat pheochromocytoma cell line
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-3-kinase
Pitx3	Paired-like homeodomain transcription factor
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PNS	Peripheral nervous system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRF	Retrorubral field
RT	Reverse transcriptase
Shh	Sonic Hedgehog
si	Small interfering
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpl	Substantia nigra pars lateralis
SNpr	Substantia nigra pars reticulata
SOD	Superoxide dismutase
TH	Tyrosine hydroxilase
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Trk	Tyrosine kinase receptor
TUNEL	Tdt-mediated dUTP nick end labeling
UTP	Uracyl triphosphate
VMAT	Vescucular monoamine transporter
VTA	Ventral tegmental area
zVAD-fmk	Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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