

Final thesis

**Title: The terminal differentiation of
midbrain dopaminergic neurons**

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Introduction:

Parkinson's disease is a central nervous system disorder characterized by the gradual loss of motor control. This neurodegenerative disorder affects 1-2% of the people over 65 and 3-5% of the population over 85.[1] The most obvious motor symptoms include resting tremor, difficulty with initiation of movement and rigidity. This disease also has non-motor symptoms including depression, constipation, pain and sleep disorder.[2] Treatments with levodopa and dopamine agonists are effective in managing the early motor symptoms of the disease.[3] These treatment options are effective in alleviating the cardinal motor symptoms of the disease, that are caused by the degeneration of dopamine-generating neurons in the substantianigra pars compacta, a subpopulation of the mesencephalic dopaminergic neurons. Dopamine is neurotransmitter, a chemical product that is released by a nerve cell to send a signal to another nerve cell. Its name is derived from its chemical structure, it is an amine that formed by removing a carboxyl group from a molecule of L-DOPA.[4] This process initially involves conversion of the essential amino acid tyrosine to L-dihydroxy-phenylalanine (L-Dopa) by Tyrosin hydroxylase, the first and rate limited enzyme in this pathway. L-Dopa is then decarboxylated by the enzyme L-aromatic amino acid decarboxylase (Aadc/Ddc) to produce dopamine.[5, 6] In the brain dopamine plays an important role in motor control and motivation. Although the dopaminergic neurons constitute a small area of the brain but project to many other area of brain and exert powerful effect on their target tissue.[4]

Neural tissue is generated from the embryonic ectodermal layer, during gastrulation.[7] During this stage, Otx2 (the early anterior neuroectoderm marker gene) restricted to the anterior region of embryo in all three germ layers including the anterior neural plate and Gbx2 (another homeobox-containing transcription factor) is expressed through all three germ layers in the

posterior part of the embryo. Shortly after gastrulation, neural plate is subdivided into four distinct regions that include forebrain, midbrain, hindbrain and spinal cord.[8] This division occurs through the expression of special transcription factors, Otx2 and Gbx2. Otx2 expression defines forebrain and midbrain, whereas Gbx2 expression defines the hindbrain and spinal cord shortly after polarization of neural tissue. [9]The first specified region after the induction of the neuroectoderm is the midbrain region. This region is specified and developed by the expression of the transcription factor, Otx2 in the anterior neuroectoderm and also establishment of signaling center at the boundary of midbrain and hindbrain.[10] Midbrain-hindbrain organizer (MHO) or isthmus border is a region that separates mesencephalon from hindbrain and also plays a key role in controlling of the size and location of mesencephalic dopaminergic neurons (mesDA).[9, 10]MHO is established by the mutual repression of two opposing transcription factors, otx2 and Gbx2 that are required for the correct positioning of MHO genes expression.(Figure 1)[9]

The precursor cells of mesDA neurons are induced in ventral midbrain by an interaction of two diffusible factors, sonic hedgehog (shh) and the fibroblast growth factor (fgf8), that are secreted by the floor plate and by MHO, respectively. Shh function-blocking antibodies and recombinant Shh protein were used to examine the role of Shh in the induction of DA neurons in the midbrain and rostral forebrain. E9 ventral midbrain (v3) or ventro-rostral forebrain (v1) explants containing endogenous Shh were exposed to the Shh-blocking antibody from day 0 in culture and monitored for the presence of DA neurons 6 days later. DA neurons were detected in control explants v1 and v3 but were absent when these explants were cultured with Shh-blocking antibody. Shh antibody added to the explants after 24 or 48 hrs prevented the appearance of DA in the forebrain but not in the midbrain, indicating that the critical period for Shh signaling is

within the first 24 hrs of somitogenesis in the midbrain and 48 hrs after somitogenesis in the forebrain. Another experiment revealed a requirement for fgf8 expression in induction of ectopic Th-expressing neurons in ventral forebrain explants, while the appearance of mesDA neurons in ventral midbrain explants can be stopped by a soluble blocking receptor for fgf8. This result indicates that Fgf8 **protein may** provide positional information for the specification of mesDA neurons along the anteroposterior (A-P) axis of neural tube and shh is physiological inducer of DA neuron in the ventral midbrain and ventrorostral forebrain and it can control the position of DA neuron along the dorsoventral (D-V) axis. Taken together, both shh and fgf8 are required for specification of DA neuron along both axes, because DA neurons were not detected in explant cultures with shh alone or with fgf8 alone.**[11]**

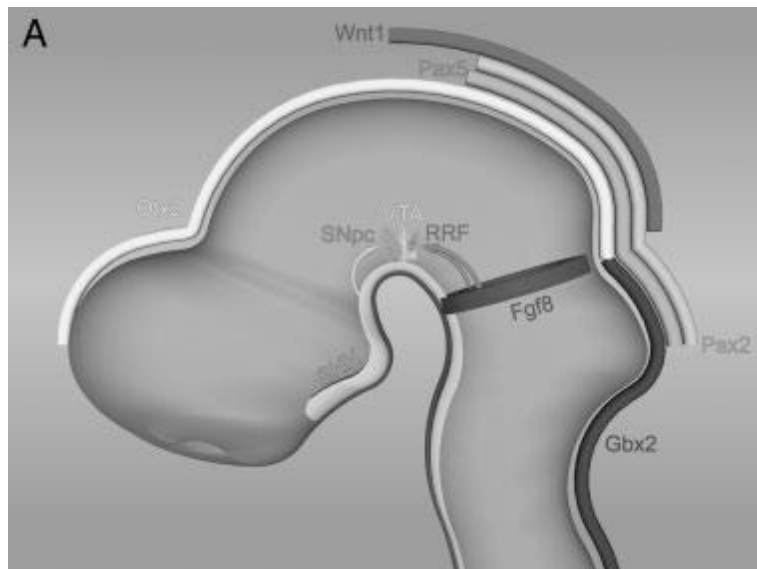


Figure 1, early development, regional identity and induction of mesDA neuron.A) Interaction of sonic hedgehog (Shh), secreted by the floor plate, and the fibroblast growth factor 8 (Fgf8), released from the mid-hindbrain organizer (MHO), induces mesDA neurons in the ventral midbrain. These neurons will later give rise to three distinct nuclei, retrorubral field (RRF), ventral tegmental area (VTA)

and substantia nigra pars compacta (SNpc). The location of mesDA neurons is dependent upon the MHO, which is defined by the expression of two opposing factors, Otx2 and Gbx2, and also by Pax2, Pax5, and Wnt1, which participate in the regional specification of midbrain tissue.**[9]**

Before induction of mesencephalic DA neurons a second group of transcription factors participate in regionalization of mid-hindbrain. These include *fgf8*, *Pax2/5*, *Lmx1b*, *Wnt1* and *En1/2.Fgf8* plays a key role in midbrain/hindbrain patterning activity. The induction of *Fgf8* is regulated by *pax2.Pax2* and *Pax5* cooperate in establishment and maintenance of MHO.(Figure 1)[12-14]*Lmx1b* is another transcription factor that is expressed for determining the dorsal and ventral patterning in the developing limb bud and is also known as a regulator of neuronal cell fate, due to its essential role in regulating the expression of *Fgf8*, in the MHB, and *Wnt1*. [15] *Wnt1*, on the other hand is required for growth of the posterior midbrain/ anterior hindbrain during development.[9]After induction of mesencephalic dopaminergic neurons, they localize in three distinct nuclei: Substantia nigra pars compacta (SNpc), ventral tegmental area (VTA), and retrorubral field (RRF). Historically these nuclei were designated as A9 (SNpc), A10 (VTA) and A8 (RRF). Neurons of SNpc innervate the dorsolateral striatum and caudate putamen and constitute the nigro-striatal pathway to control motor function. DA neurons of VTA project to the ventral striatum and cortical area, forming mesolimbocortical pathway to regulate mood and emotional behavior. It is important to know that dopaminergic neurons in VTA and RRF are less susceptible to degeneration and remain unaffected during the Parkinson's disease. [1, 16]

Although Transcription factors are essential in determining the fate of neuronal population and control many process such as localization in the ventral midbrain, they play important role in defining neurotransmitter phenotype, axon guidance and synapse formation as well as regulation of survival and cell death. (Figure 2)[1, 9]

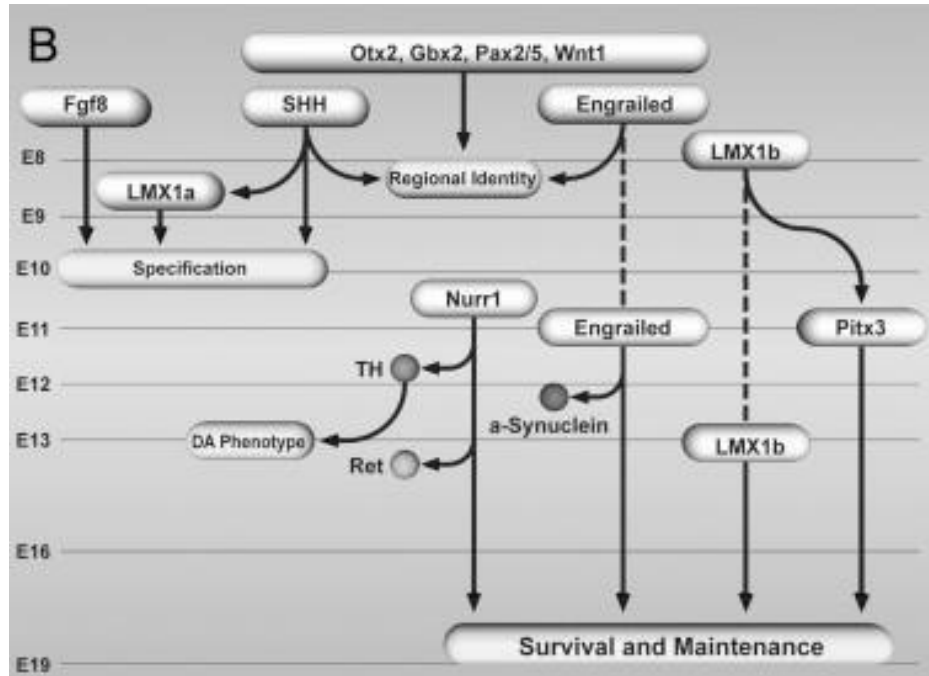


Figure 2, late development of mesDA neurons.(B)

Genes involved in the late development of mesDA neurons. After the early midbrain development and induction of mesDA neurons, the development of postmitotic neurons is controlled by at least three distinct pathways under the influence of Nurr1, the

Engrailed genes, Lmx1b, and Pitx3. These factors start to be expressed in mesDA neurons around the same time (E10.5-13) and are important for their neurotransmitter phenotype, their survival and maintenance throughout the lifetime of the mouse. Expression of several vital genes is controlled by these transcription factors. In mice lacking Engrailed genes, the expression of α -synuclein is significantly reduced. The expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and of the GDNF receptor component, Ret, which is required for long-term survival of nigral DA neurons,⁴⁷ is regulated by Nurr1. The Engrailed genes and Lmx1b are expressed in the region before induction of mesDA neurons (dotted lines); yet, their expression becomes relatively specific to postmitotic mesDA neurons during late embryogenesis.^[9]

Foxa1/2 are two transcription factors which participate in both early development and late development of mDA neurons. Foxa1 and Foxa2 initially play an important role in specification of mDA progenitors by regulating the Sonic hedgehog (Shh) expression. They also participate in mDA neurons differentiation by controlling LMX1a and LMX1b expression. Besides their activity in early development of mDA; Foxa1/2 are playing a crucial role in maturation of mDA

neurons by regulation of Nurr1 and En1 expression in immature mDA neurons. LMx1b, also participate in late development of mDA neurons by preceding the expression of Nurr1 and Pitx3.

In this work we will discuss the transcription factors, Nurr1, Pitx3 and the engrailed genes(En1/2) those are essential for the late development, and the lifelong survival and maintenance of mesencephalic dopaminergic neurons.(Figure 2) We will discuss the role of these transcription factors in regulation of differentiation, maturation and survival of mesDA neurons and also the relation of each factor to the normal physiological function of postmiotic mesDA neurons, as well as their possible connection with Parkinson's disease.

Nurr1

Development of mesDA neurons depends on expression and activity of many genes and their protein products. Orphan nuclear receptor Nurr1 or NrHa2 is one of the first nucleic receptors that was found to be involved in the specification and differentiation of mesDA neurons.[17] Nurr1 is a member of steroid/thyroid hormone nuclear receptors and is related to a super family of transcription factors that have a role in modulating gene transcription in response to small lipophilic molecules. [18] Structurally, Nurr1 has a DNA-binding domain, a ligand binding domain and two transcription activation function domains, AF1 and AF2, at N and C termini, respectively, which are responsible for binding to specific nuclear receptors.[19, 20] DNA binding domain is highly conserved and consists of two Zinc finger modules. This specific domain, functionally binds to an NGFI-B response element (NBRE) to activate transcription.[21] Two structural features for Nurr1 nuclear receptor are noteworthy. First is that, Nurr1 ligand binding domain does not have any cavity because the tight packing of the side chains from

hydrophobic residue in the ligand binding domain prevents the molecule from having a ligand-binding cavity. The second feature is that Nurr1 lacks a classical binding site for coactivators, yet it can promote transcriptional activity with the help of standard protein fold resembling the agonist-bound ligand domain. [18]Nurr1 activates transcriptional process via its ligand-binding domain and heterodimerization with the retinoid X receptor in response ligand.[22, 23]Retinoid X receptor is a type of nuclear receptor that is activated by 9-cis retinoic acid.

Nurr1 transcription factor is encoded by an immediate early gene and is predominantly expressed in the brain.[24, 25]Human Nurr1 gene, mapped on chromosome 2q22-23.[26, 27], is approximately 7 kb long and contains 8 exons and 7 introns, it also possesses an untranslated exon. This gene is expressed in embryonic ventral midbrain around the embryonic day 10.5 (E10.5) and continues to be expressed in adulthood because of its critical role in postmitotic development and the lifelong maintenance of mesencephalic dopaminergic neurons.[28-30] Nurr1 also has a crucial role during early postmitotic differentiation of DA neuron since it directly controls the expression of *TH*, *Vmat2/Slc8a2* and *Dat/Slc6a3* genes, that are required for dopamine and neurotransmitter biosynthesis. The vesicular monoamine transporter 2, is a protein that is encoded by *Slc8a2* gene in humans, that work as an integral membrane protein which is responsible for monoamines and neurotransmitter; such as dopamine; transportation from cellular cytosol into synaptic vesicle.[31, 32]Defining of neurotransmitter phenotype of mesDA neurons is one of the important roles of Nurr1 because it regulates the expression of genes involved in the synthesis, packaging, transport and reuptake of dopamine.[33, 34]Nurr1 induces the expression of tyrosine hydroxylase (TH) (the rate-limiting enzyme in dopamine synthesis) directly by transactivating the promoter activity of the TH gene because the TH promoter contains multiple sequence motifs homologous to the Nurr1 binding motif.[35] Another key role of Nurr1 is related to regulation of

dopamine transporter gene. Dopamine active transporter gene (Dat) is another membrane – spanning protein which is responsible for dopamine uptake.[36] Because of this important feature, Dat is one of the specific markers for dopaminergic neurons. Transcription of Dat is regulated by the high affinity binding of Nurr1 to an extended half-hormone response element, NGF1-B, in its 5'-untranslated region.[37, 38] Nurr1 is also responsible for transformation of L-DOPA to DA and packaging of DA to synaptic vesicles by regulating the expression of aromatic L-amino acid decarboxylase (AADC) and vesicular monoamine transporter-2 (VMAT2).[33] In later development, Nurr1 has an essential role in survival and maintenance of DA neurons by regulating the expression of survival factors such as Ret (rearranged in transfection). Ret tyrosine kinase is the high affinity ligand-binding component of the glial cell line derived neurotrophic factor (GDNF) receptor complex that is attached to the cell surface through aglycosyl phosphatidylinositol anchor.[39] Activity of this component directly related to Nurr1 expression, because this gene has a special role for activation of Ret-dependent survival pathway and also activation of pathways that are related to autophosphorylation of the tyrosine domains of Ret trigger like PI3K, MAPK which are essential for neuronal survival and neurite outgrowth.[40] GDNF and its relatives are responsible for protecting mesDA neurons against neurotoxic insult and cell death through this pathway.[41, 42] In Nurr1-deficient mice, DA precursor cells fail to migrate normally or they are unable to innervate target areas, possibly through downregulation of the receptor tyrosin kinase Ret.[40]

The function of Nurr1, in differentiation and survival of mDA neurons was mainly discovered through mouse knockout studies. These studies showed, neuroepithelial cells that give rise to the mesDA neurons, ventralize normally and differentiate into neurons without the dopaminergic phenotype. Although haploinsufficient animals do not show any special deficiency

in mesDA neurons number or motor function, they show vulnerability and cell death in response to neurotoxic insult.[43, 44] Nurr1^{+/-} mice also show decreasing in the number of nigral mesDA neurons and motor activities that all of them related to reducing in the mesolimbic and mesocortical dopamine levels. [28, 44]

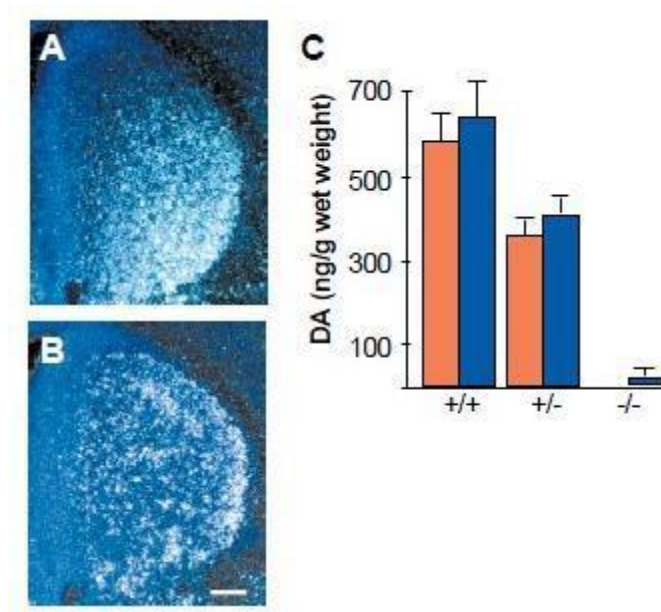


Figure 3, (A and B) In situ hybridization of coronal sections at the striatal level of newborn brains shows normal distribution of enkephalin (A) and substance P (B) mRNA in Nurr12/2 mice. Scale bar, 300 mm. (C) DA was absent in Nurr12/2 mice and reduced in Nurr11/2 mice. Shown is HPLC analysis (18) of dissected striata (orange bars) and ventral midbrains (blue bars) of wild-type (1/1), heterozygote (1/2), and Nurr1(2/2) mice. The decreases of DA are

statistically significant ($P \leq 0.01$). (DA detected in nigra areas in two of five Nurr12/2 brains in all probability corresponds to contamination from adjacent nonaffected DA neurons.)

[45]

Beside the important role of Nurr1 in indicating neurotransmitter phenotype of DA neurons and their special role in survival and maintenance of mesDA neurons during life, genetic association studies have established a connection between Nurr1 and the etiology of Parkinson's diseases. Mutations in the first and sixth exons of Nurr1 are more common among sporadic and familial cases of PD.[46-49] Mutation in the 5' first exon also has an important role in decreasing the

Nurr1 mRNA levels and further in the expression of TH.[47] Nurr1 deficiency affected Neurons of the substantianigrapars compacta more than cells in the ventral tegmentum.

According to the crucial role of Nurr1 in specification, differentiation, postmioticdevelopment and lifelong maintenance of mDA neuron; deficiency and mutation in this gene may contribute to pathogenesis of Parkinson's disease.

Pitx3

Another gene that has key role, in development of postmitotic DA neurons as well as the long-term survival and maintenance of mesDA neurons is Pitx3. Pitx3 is a unique transcription factor that has important roles in differentiation, survival and maturation of midbrain dopaminergic neurons.[50]In the brain, the expression of Pitx3 is limited to MesDA neurons, in substantianigrapars compacta and in about half of the VTA DA neurons. This expression of Pitx3 is maintained throughout adult life in both rodent and human.[51] Structurally this gene is a member of the Rieg/ pitxhomeobox family and is considered a paired – like homedomain transcription factor. [9]The expression of Pitx3 starts at E11.5 just after expression of Nurr1 (around E10.5),[52]. Both Nurr1 and Pitx3 regulate a subset of phenotypic markers and play a cooperative role in the late maturation of midbrain DA neurons. [53]

Pitx3 has many developmental functions , for example it express in the developing eye lens and it is critical for lens formation, it's been found that reduction in expression of Pitx3 leads to change in proliferation, differentiation and survival of lens cells.[54] It also has important roles in development of skeletal muscle,[55] central nervous system and mesDA neurons, during embryogenesis.

Pitx3 has a regulatory function for **MesDA** neurons by regulating **TH [56] expression**. By activation of TH promoter through direct interaction with a single high-affinity binding site.[56] It been shown that pitx3 deficiency have direct connection with loss of the density of dopamine nerve cells in adult brain by reduction of TH expression in **substantianigra** neurons, because total TH-positive cells **are reduce** by 71% in the SN and 52% in VTA of mutant mice.[51] There are **many** data that suggest that SN TH- positive neurons disappear during the fetal period **where as** VTA neuron are disappear later during postnatal **period.(Figure 4)[51]**

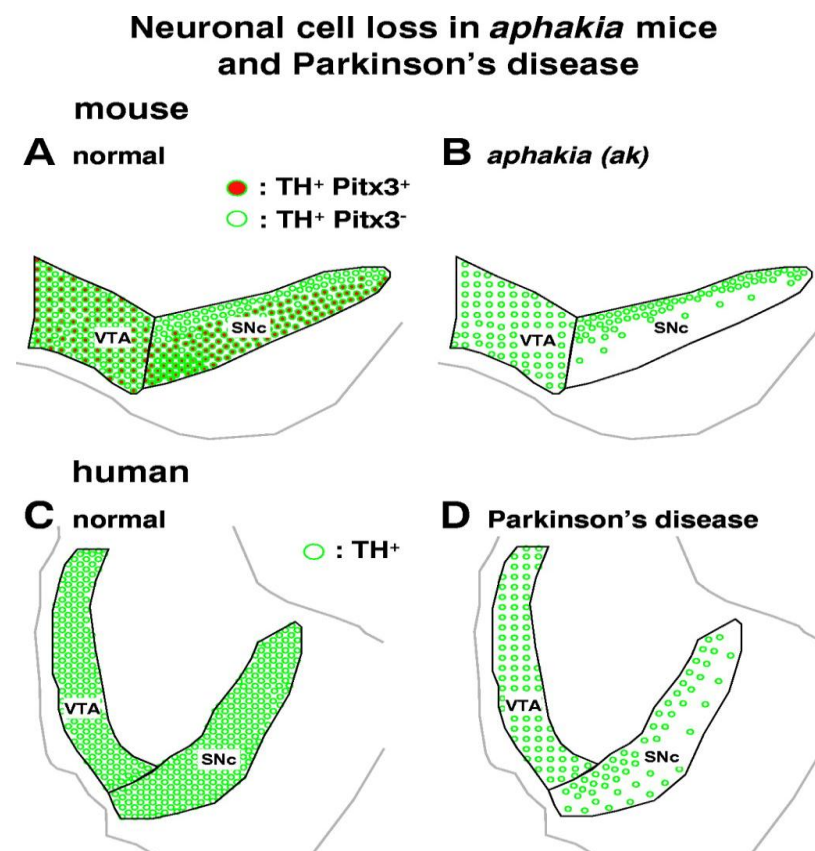


Figure 4,(A) The right midbrain of a normal mouse showing the distribution of TH-positive Pitx3-positive (green with red core) and TH-positive Pitx3-negative neurons (green) in SNpc and VTA. Most ventral SNpc TH-positive neurons are Pitx3 positive, whereas the dorsal SNpc largely contains Pitx3-negative TH-positive neurons. About half of the VTA TH-positive neurons are Pitx3 positive, and both populations are intermingled. (B) In ak mice, SNpc

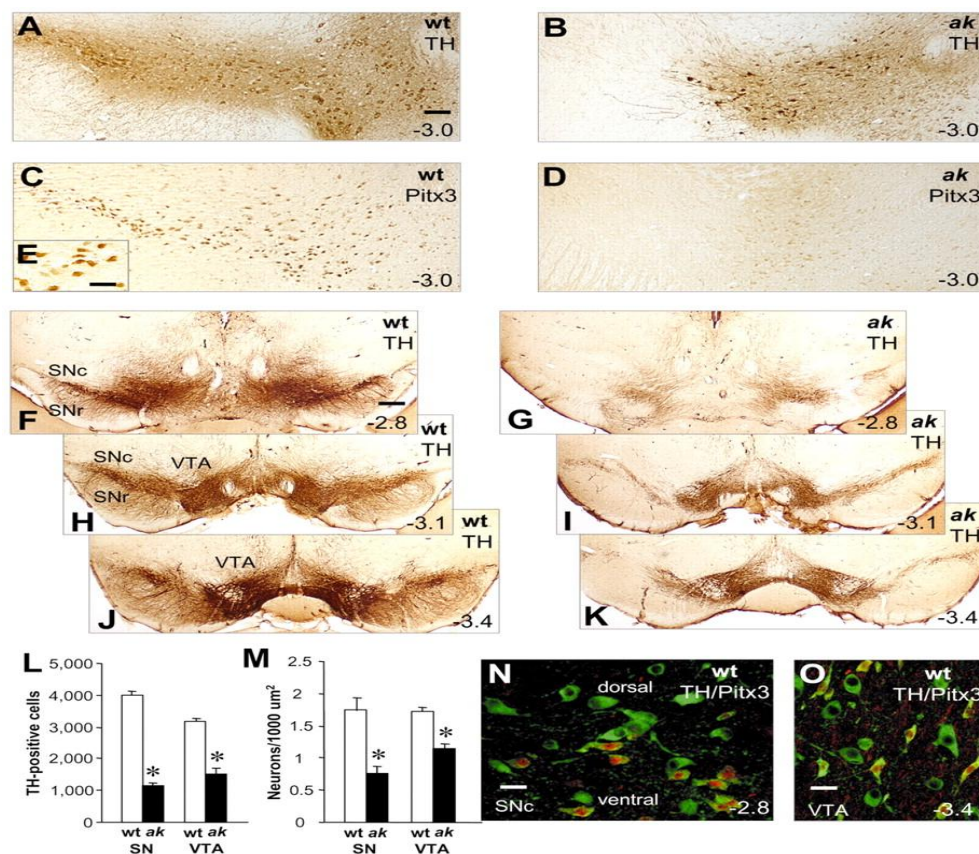
Pitx3-positive neurons are lost between E12.5 and P1, whereas VTA cells are lost postnatally. (C) Outline of the right MesDA system of a normal human showing the distribution of TH-positive neurons in SNpc and VTA. (D) Individuals with PD typically have the most severe cell depletion in ventral SNpc, followed by dorsal SNpc and VTA [modified, with permission, from. Although a decrease of PITX3-positive neurons was shown in samples from individuals with PD, the regional distribution of human PITX3-positive neurons remains to be established.[51]

Pitx3 has another regulatory task in dopaminergic neurons development. Aldehyde dehydrogenase (AHD2) is a gene that highly expresses by Pitx3 positive dopaminergic neurons in SN. The existence of AHD2 is so important for DA neurons differentiation, patterning and survival because of its regulatory function for retinoic acid.[57] retinoic acid (RA), the active derivative of vitamin A (retinol) is made by three retinaldehyde dehydrogenases RALDH1-3 after initial retinol oxidation by alcohol dehydrogenases.[58] Study on The expression of RALDH1 (also known as AHD2) in the midbrain in early developing and adult mesDA neuron, suggest that RA is locally synthesized during all steps in development, generation and differentiation of mesDA neuron.[57]

Another function of this gene is manipulation of DA neurons. with these reasons we can understand that this gene play apart in the development of dopaminergic cell type rather than in axonal path finding because there are some new working suggest that single homeodomain transcription factor like Pitx3 can determine single neuronal identity [52], so they involved in induction of neurotransmitter phenotype.[59] Axonal path finding and connection with their target are another important aspect of development of DA neurons that occur with the help of another gene like Pax6. [59]

Knockout and knock in experiments have provided more information on the role of Pitx3 in embryonic development. They demonstrated that when Pitx3 gene knocked out in aphakia mouse by deletion in upstream promoter area and parts of exon 1,[60] mice show severe disorder of motor function, including inability to walk, chorea, and diplegia with flexion contractures of the lower limbs.[61] Homozygote mice also show, reduction in DA levels in nigrostriatal pathway

and dorsal striatum. In these mice DA neurons in substantianigra compacta (SNPC) and nigrostrial pathway fail to develop and display reduced expression of dopamine transport regulators and vesicle release protein and an increase in developmental cell death.[59, 62] In knock-in experiment for Pitx3,eGFP reporter was used to make heterozygote mouse, after in vitro differentiation, they demonstrated that 95% of TH positive cells were Pitx3eGFP/+ in SNpc and VTA neurons, suggesting an essential developmental role for Pitx3 in these neuronal populations.(Figure 5)[60]



Figure

5,The aphakia (ak) mice have no detectable Pitx3 and a markedly reduced midbrain dopaminergic system. (A-D) Adjacent coronal midbrain sections containing the SN

in P50 wild-type (A,C) and ak (B,D) mice immunostained for TH (A,B) and Pitx3 (C,D). Rostrocaudal positions are indicated as millimeters relative to bregma in the lower right corner. (E) High-power view of the SN shown in C, highlighting the nuclear staining. By contrast, none of the weak background staining in D was nuclear. (F-K) Equivalent rostral-to-caudal coronal midbrain sections of P100 wild-type (F,H,J) and ak (G,I,K) mice immunostained for TH. (L) Stereological analysis of TH-positive cells of the left SN

and VTA in wild-type and ak mice. The data are represented as the means $\pm$ s.e.m. (n=4). (M) Density of Nissl-stained cell bodies in the left SN and VTA of wild-type and ak mice (n=4). A statistically significant decrease in TH-positive cell bodies and density of Nissl-stained cell bodies was detected in the SN and VTA of ak mice compared with controls ($P<0.01$, t-test). (N,O) Coronal sections through the right SN (N) and VTA (O) of a P50 wild-type mouse immunostained for TH (fluorescein-labeled, green) and Pitx3 (rhodamine-labeled, red) analyzed by confocal microscopy. Scale bars: in A, 125 μ m for A-D; in F, 250 μ m for F-K; in E, 30 μ m for E; in N and O, 30 μ m. [51]

Genetic variation or polymorphism of Pitx3 gene may influence the risk of Parkinson's disease. It was shown that polymorphism in rs3758549 in promoter of pitx3, rs2281983 in exon3 of Pitx3 and rs4919621 in intron1 of pitx3 are associated with PD. While some studies show association with early onset PD, another set of studies demonstrates lack of association. In this case they demonstrate that polymorphism in both rs2281983 allele C and rs4919621 allele A were associated with higher risk in early onset of PD.[63] Because of the crucial role of Pitx3 gene in long-term survival and maintenance of midbrain dopaminergic neuron[64] and also its direct connection with TH expression, deficiency in this gene shows severe reduction of dopamine level and loss of dopaminergic cells in SNpc and it can cause of lower motor activity, because SN dopaminergic neurons project primarily to the dorsal striatum to regulate motor control.[51] With these reason and many in situ analysis researcher can demonstrate that reduce in density of Pitx3 expression neurons can cause of Parkinson disease, since for symptoms of PD, it is assumed that 50-70% dopaminergic neurons must have degenerated.[51]

En1/2

En1 and En2 are members of homeobox transcription factors family that including a highly conserved DNA binding, helix-turn-helix and homeodomain protein fold.[65] They are express in both SN and VTA DA neurons and are required for the development, survival and maintenance of mesDA neuron.[66, 67]The engrailed genes have two special functions during early and late development of midbrain and mesDA neurons. In early development of midbrain, the expression of En1/2 can modulate comprising of the neuroepithelium of posterior midbrain and anterior hind brain, which will later give rise to the cerebellum, the colliculi and the ventral midbrain nuclei.[68] At embryonic day 8 (E8) in mice, EN1 and EN2 start their expression in midbrain/hindbrain boundary region or isthmic organizer.[69] In this time, these transcription factors are important for maintaining and regulating the isthmic expression of Fgf8 by direct interaction with DNA-binding intronic fragment on FGF8. [11, 70]The expression of both Fgf8 and sonic hedgehog in the floor plate are required for development and induction of mesDA neurons in the ventral midbrain. [11] After neuronal induction, the specific expression of engrailed genes in mesDA neurons starts between E11.5 and E14. [71]After this time, EN1 and EN2 are important for the survival and maintenance of mesDA neurons throughout life. These genes regulate expression of alpha-synuclein gene.[71]alpha-synuclein is a 14KD phosphoprotein of 140 amino acids that was first defined as a presynaptic protein involved in synaptic plasticity, maintenance of the synaptic vesicle and regulation of dopamine biosynthesis. Several mutations(including the Ala53Thr and Ala30P point mutations) in this gene cause PD. Additionally, alpha-synuclein accumulations have been observed in lewy-bodies (abnormal aggregates of protein that develop inside degenerating SNpc neurons). It has been hypothesized that alpha-synuclein is synthesized in cell bodies of mesDA neurons and is

transported along axons to synaptic terminals. It has been shown that alpha-synuclein moves in a slow component of axonal transport and binds to vesicles for moving in fast component of axonal transport. In Ala30p mutation, binding of alpha-synuclein to vesicles is decreased, resulting in less alpha-synuclein exiting from the cell body along axon, cause local over expression of alpha-synuclein and altering its normal cellular localization, leading to its accumulation in **lewybodyies**. For its axonal transportat, phosphorylation of the serine or tyrosine residues of alpha-synuclein is important. In conclusion, most mutations on alpha-synuclein **genereduce** its rate of transport in neurons and phosphorylation has an important role in the regulation of alpha-synuclein axonal transport.[72] All of these regulations are important for dopamine release in synapses of DA neuron, due to the essential role of alpha-synucleinin dopamine release.

Mouse knockout studies have revealed the critical role of the En1/2 genes in the dopaminergic pathways. Homozygote mutant null mice lacking all four alleles of the two transcription factors have a problem in induction of DA neurons in ventral midbrain and in differentiation of neurons and becoming **postmitotic .MesDA** neurons show some developmental defects including lack of axonal outgrowth, and the Th-positive cells die between E12 and E14, the exact time of engrailed expression in wild type mesDA **neurons.[71, 73]** EN1-/-; EN2+/+ mice, display abnormal distribution of DA neurons in ventral midbrain and all the offspring die at birth. This mutation shows that the postmiotic survival effect of EN1 is more dominant than EN2 because mice lacking EN2 show less severe phenotype. EN1+/+; EN2-/- , mice do not show significant deficiencies in their dopaminergic **neurons.[71, 73]** In En1+/-, En2-/- ,**mesDA** grow normally but after 2 month display a gradual loss **of 70% of their SNpc neurons**. In this situation, loss of dopaminergic neurons results in diminished dopamine content and release

within the dorsal striatum, that leads to reduction in forward locomotion, an increase in freezing episodes in the swim test and weight loss. [9, 74] Another study reveals the importance of Engrailed1/2 in regulation of dopamine storage and release in caudate putamen (Cpu). Putamen works as a regulatory part for movement and influence of learning zone by using dopamine. [74] In En1^{-/-}, En2^{+/-}, both SNpc and VTA have fewer numbers of TH-positive neurons and the expression of alpha-synuclein is reduced. (Figure 6) [71]

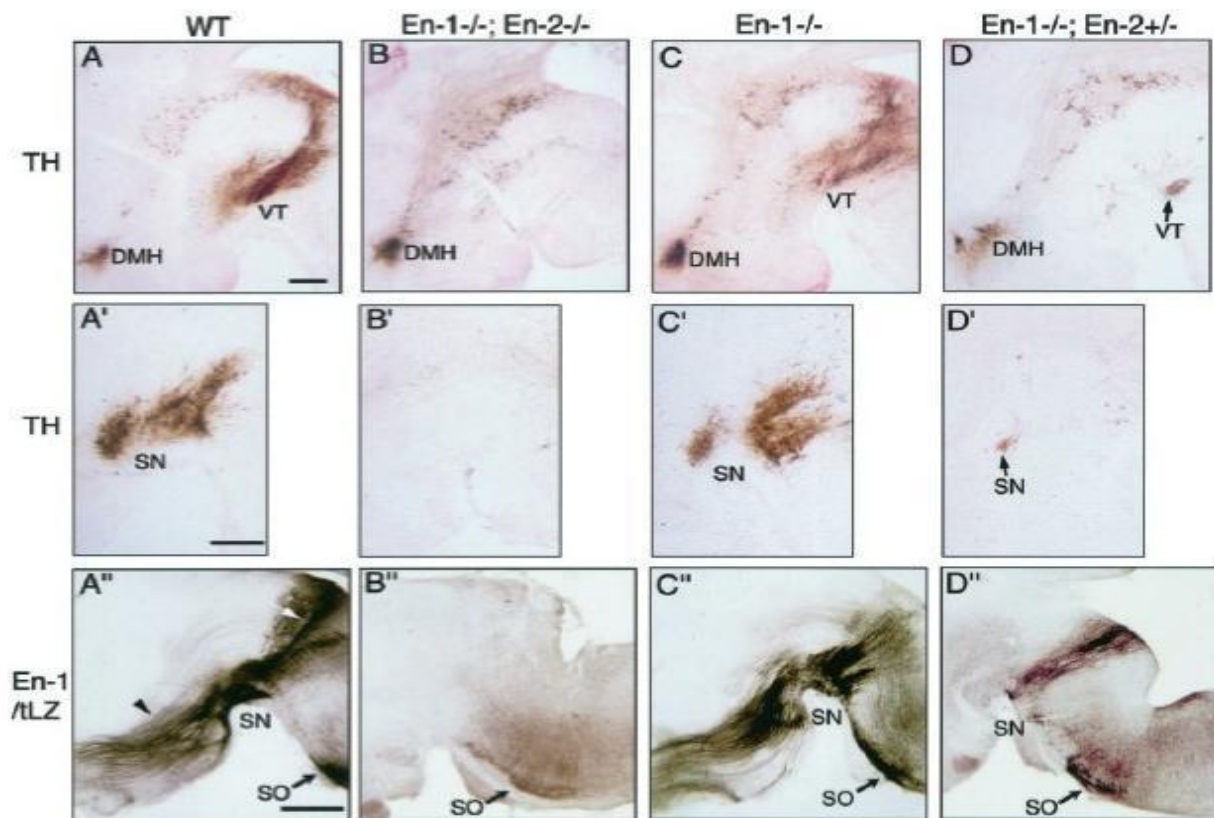


Figure 6,Midbrain dopaminergic neurons require *En-1* and *En-2* for their survival in a gene dose-dependent manner. Immunohistochemistry on sagittal sections of P0 mouse brains using antibodies against tyrosine hydroxylase (TH) to identify dopaminergic neurons (A, A', B, B', C, C', D, D'), or against *tb-gal* (A0, B0, C0, D0) to identify *En-1*/tau-LacZ (*En-1*/tLZ)-expressing neurons in the ventral midbrain of wild-type (WT) mice (A–A0),

engrailed doublemutants (En-12/2/En-22/2) (B–B0), En-12/2 mutants (C–C0), and En-12/2/En-21/2 mutants (D–D0). In A0–D0, mice with the En-1/tau-LacZ (En-1/tLZ)reporter construct were used. Therefore, in A0–D0, wild type is actually En-11/tLZ, and En-12/2 is actually En-1tLZ/tLZ. A–A0, Wild type. THimmunostaining reveals the normal distribution of the midbrain dopaminergic neurons. In the midline (A), the neurons of the ventral tegmentum(VT)are labeled, and in a more lateral section (A9), those of the substantianigra (SN) are labeled. The b-gal immunostaining of a section from an En-11/tLZreveals a similar labeling pattern as TH for both the VT and SN. In addition, SN axons projecting rostrally to the forebrain (A0, arrowhead) andEn-1-expressing radial glia (white arrow) are labeled. DMH, dorsomedial hypothalamic nucleus. B–B0, Engrailed double mutant. Dopaminergic neurons of the SN and VT are not detected by immunostaining with either TH (B, B9) or b-gal (B0). However, dopaminergic neurons of the DMH (a diencephalicnucleus) remain TH-positive (B–B0), indicating that their dopaminergic phenotype does not depend on En-1 or En-2. In addition, neurons in the superior olive (SO) that normally express En-1 are marked by the En-1/tau-LacZ reporter in engrailed double mutants (B0), as well as in the other mutantgenotypes examined (C0, D0), indicating that En-1 expression is not auto-regulated. C–C0, En-12/2 mutant. Distribution of the dopaminergic neuronsappears normal with the exception of those located in the VT that appear more loosely clustered (compare C with A). D–D0, En-12/2/En-21/2 mice. The dopaminergic neurons of the SN and VT are reduced to a small cluster of cells in the ventral midbrain, demonstrating an engrailed gene dose dependent effect on the development of these neurons. Rostral to the left, dorsal to the top. Scale bars, 200 mm.[71]

Development, survival and maintenance role of the engrailed genes in mesDA neurons is mediated by several signaling pathways. Pann-eurotroph receptor (P75NTR) and mitochondria are the two main survival/death-mediating targets of the engrailed transcription factors. In neuronal injury and degeneration, the expression of the neurotrophin receptor p75 increases.[75] P75NTR receptor and Tropomyosin receptor kinase (TrK) A/B/Care transmembrane proteins that work as a receptor for neurotrophins, consisting of nerve growth factor (NGF), brain-derived neurotrophic factor(BDNF), neurotrophin4/5 and 3. In Nervous system, P75 has a dual role in

both survival and cell death, depending on cellular context and molecular form of ligand, while Trk **A,B,C** work purely as survival receptors. The survival activities of P75 and the Trk receptors is mainly mediated by phosphatidylinositol-3 **kinase(PI3K)** and extracellular-signal-regulated kinase1/2 (Erk1/2) pathway. P75NTR works as a co-receptor of the TRK receptors and helps them to conduct the NT signal and the neurotrophin-induced neurite outgrowth for neuronal survival. P75NTR enhances the affinity and specificity of TRK receptors for neurotrophins. Experiments showed, mice which are lacking P75NTR in their hippocampal cells did not show any detectable TRKA activation **orneuro protection** against toxicity by **NGF.[76]** Death signaling via P75 is mediated by phosphorylation of c-jun N-terminal kinase. In Engrailed-deficient mesDA neurons, cell death occurs by high expression of P75NTR and loss of the Erk1/2 activity. EN1/2 in mesDA neurons has an important role as suppressor of the P75NTR death signaling. In engrailed-deficient mesDA neurons, BDNF, NT3/4 are prefer to bind P75NTR instead of TRkB or TrkC because of high amount of P75NT, due to En1/2 **deficiency.[77]**The engrailed genes also induce their survival effect by **inhibit in** neurotoxic insult and the mitochondrial pathway of apoptosis by regulating the anti-apoptotic members of **theBCL2** family. In engrailed-deficient mice, mesDA neurons are sensitized to mitochondria permeability transition and inhibition of the mitochondrial complex-I activity, resulting in their vulnerability to neurotoxic insult and cell death.**[77]**

These studies suggest an indirect link between the engrailed genes and the etiology of Parkinson's disease, since mitochondrial dysfunction is believed to be involved in pathogenesis and progression of neuronal loss in PD. Some other studies have attempted demonstrating a more direct relationship between Parkinson's disease and single nucleotide polymorphisms in an intronic region of EN1, on the 3' downstream of EN1 and the promoter region of EN2.

[78]En1/2 has a crucial role in maintenance and survival of mesDA neurons by regulation of alpha-synuclein and P75NTR. So, deficiency in these genes may enhance the specific vulnerability of the midbrain dopaminergic neurons to cell death and PD-like motor deficits.

Discussion:

Parkinson's disease is a neurodegenerative disorders, characterized by the loss of dopaminergic neurons in the substantia nigra par compacta of the midbrain, resulting in decreased dopamine levels. The main function of this neuronal population is in control of movement. The development, long-term survival and the neurotransmitter identity of the mesDA neurons of the SNpc is regulated by the expression, activation and interaction of three developmental transcription factors, Engrailed, Nurr1 and Pitx3. Among, all of these important genes, the Engrailed genes have a crucial role in regulation of alpha- synuclein expression.

Alph-synuclein is an important factor that was identified as the first PD gene and has a role in regulating dopamine release. The expression of alpha-synuclein is regulated by the engrailed genes. The engrailed genes may be indirectly involved in regulating the expression of tyrosine hydroxylase, the rate-limiting enzyme in production of dopamine, by modulating the phosphorylation of two mitogen activated protein kinase (MAPK) family, Erk1/2.[77] Erk2 and its relative Erk5 also contribute to differentiation and survival of mesDA neurons by increasing the transcriptional activity of Nurr1. They elevate Nurr1 activity by mediating its phosphorylation and nuclear localization, which in turn affects the expression level of tyrosin hydroxylase. (Figure 8)[79, 80]. Nurr1 and Pitx3 are other two transcription factors that have an important role in the development and maintenance of post-mitotic mesDA neurons by

regulating several functions, including the expression of Th⁺ [53]. The expression of both En1/2 and Nurr1 in the mesDA neurons can prevent mitochondrial impairment, a known part of the etiology of PD. Engrailed and Nurr1 prevent mesDA neurons from undergoing mitochondrial permeability transition that occurs in response to death stimuli including neurotoxic insult. The mitochondrial function of Engrailed and Nurr1 is likely to be mediated by the pro and anti-apoptotic members of the Bcl2 family. (Figure 7)[77, 81, 82]

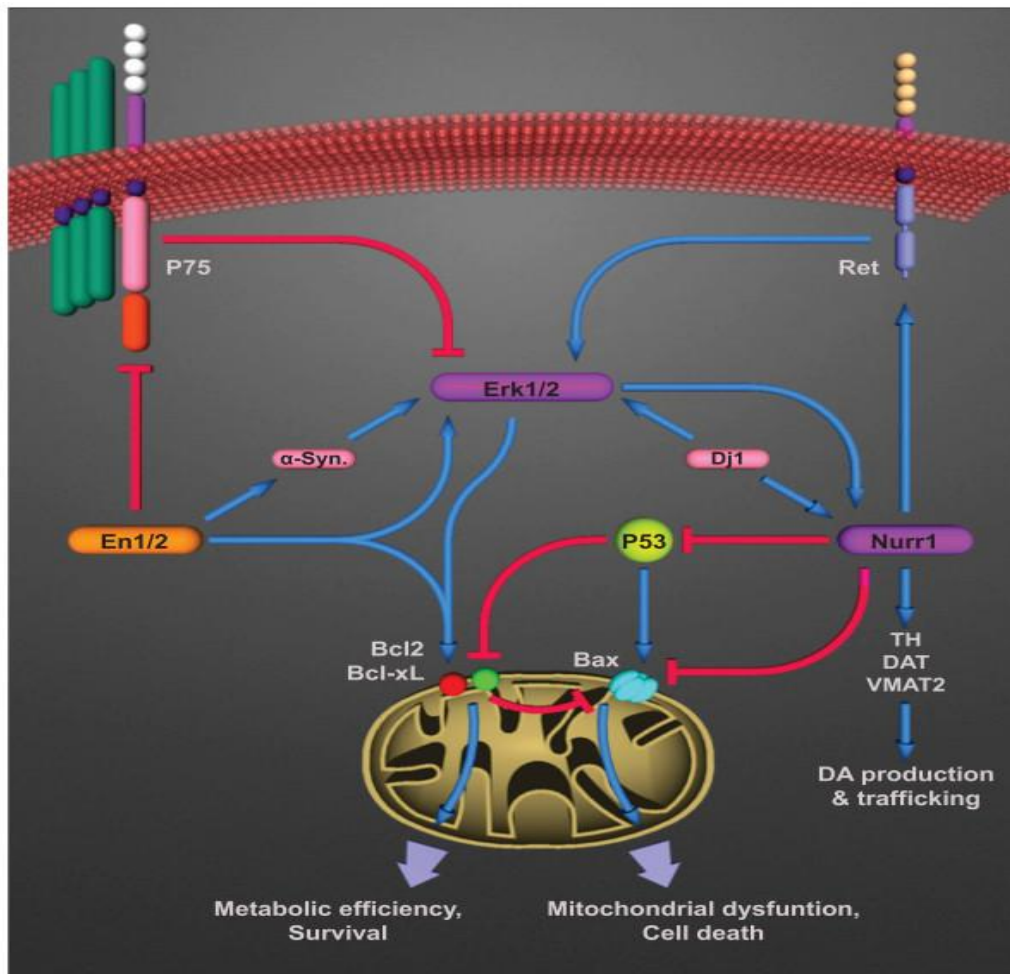


Figure 7: Signaling pathways involved in regulation of cellular survival and death by engrailed and Nurr1 in mesencephalic dopaminergic

neurons. Transcriptional regulation of several survival/death-regulating genes including the pan-neurotrophin receptor P75 and protooncogene Ret, as well as modulation of downstream survival mechanisms, such as MAPK (Erk1/2) by engrailed and Nurr1 leads to the long-term regulation of survival and death of mesDA neurons. Down-

regulation of P75 expression by the engrailed genes results in disinhibition of Erk1/2, resulting in survival of mesDA neurons. The effect is similar to the Ret-mediated activation of Erk1/2 by Nurr1. The survival and degeneration pathways regulated by engrailed and Nurr1 converge on mitochondria and are mediated by the anti- and pro-apoptotic members of the Bcl-2 family, Bcl2, Bcl-xL and Bax.[1]

While these transcription factors have a general role in development of midbrain and the dopaminergic neurons, they possess functions specific to the SNpc neurons. In the Pitx3 knockout mice the mesDA neurons are lost only in substantia nigra while the dopaminergic neurons in the VTA remain unaffected. Similarly, the Engrailed haploinsufficient mice display a 70% degeneration of mesDA neurons in SNpc while VTA is not affected. These studies suggest the involvement of the developmental transcription factors in determining the specific vulnerability of the substantia nigra neurons to environmental and genetic risk factors in comparison to the VTA neurons. This inherent vulnerability and susceptibility of the SNpc neurons, which is a key contributing element in development of the motor symptoms during the course of PD, is likely to be defined early on during the time that cells find their regional identity, express their dopaminergic phenotype and become postmitotic. Engrailed, Nurr1 and Pitx3 are essential transcription factors during this phase and are likely to be involved in defining this property of the neurons.

Understanding the molecular mechanisms essential for the generation and differentiation of mesDA neurons during development may be beneficial in developing better in vitro protocols for differentiation of stem cells to mesDA neurons for cell replacement therapeutic purposes. The ultimate goal of this process is to differentiate embryonic and induced pluripotent stem cells into the dopaminergic neurons with the right midbrain identity and to incorporate them into the brains

of the PD patients to replace the missing nigral neurons. Replicating the right developmental cues is essential to the proper and efficient in vitro differentiation of mesDA neurons. The engrailed genes, Nurr1 and Pitx3 have been utilized in this process, as they are key to the terminal differentiation of mesDA neuron.[83]

Nurr1, Pitx3 and engrailed are important survival genes for mesDA neurons because they are work as crucial regulating factors for expression of other essential genes like alph-synuclein, tyrosin hydroxylase, AHD2, DAT and Ret. These genes are responsible for promoting survival of mesDA neurons. The direct use of the developmental genes in gene therapy may, therefore, alter disease progression and be of benefit to the patients.

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