Neurobiology of Parkinson’s Disease Gene Pink1 in *Danio rerio* (Zebrafish)

Madhusmita Priyadarshini

Helsinki 2013
Neurobiology of Parkinson’s Disease Gene Pink1 in *Danio rerio* (Zebrafish)

Madhusmita Priyadarshini

Neuroscience Center
and
Institute of Biomedicine, Anatomy
Faculty of Medicine
and
Finnish Graduate School of Neuroscience
University of Helsinki

ACADEMIC DISSERTATION

*To be publicly discussed with the permission of the Faculty of Medicine, University of Helsinki, in Lecture Hall 3, Biomedicum Helsinki 1, Haartmaninkatu 8, Helsinki on 3rd of December 2013 at noon*

Helsinki 2013
To My Dearest Grandma (Aei)
6. PINK1 FUNCTION ANALYSIS AND NOVEL PATHWAYS (III) 48
7. PINK1 AND NEUROPROTECTION (IV) 49

DISCUSSION 50

1. ZEBRAFISH GENOME AND GENE STRUCTURE 50
2. MOLECULAR AND GENETIC CHARACTERIZATION OF ZEBRAFISH PINK1 51
2.1. DISTRIBUTION OF PINK1 IN THE LARVAL AND ADULT BRAIN OF ZEBRAFISH 51
2.2. PINK1 KNOCKDOWN AND KNOCKOUT MODELS 51
2.3. PINK1 KNOCKDOWN AND MPTP TOXICITY 53
3. PINK1, PD, AND HIF-1 SIGNALING 53
4. THE SIGNIFICANCE OF THE PINK1 TRANSGENIC MODEL 55

CONCLUSIONS 57

ACKNOWLEDGEMENTS 59

REFERENCES 61
ABSTRACT

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease (AD). The quest for better therapies to modify the progression of PD is still ongoing. During the last two decades, the concept of the etiological basis of PD has changed, which has been driven by genetics, the recognition of familial forms, knowledge of the effects of the environment and toxins, and genome-wide association studies. Although most cases are sporadic, approximately 5–10% of PD cases are due to genetic mutations that give rise to the familial forms. Studies using neurotoxins and also genetic mutations that underlie familial PD have implicated mitochondrial dysfunction in the pathogenesis of PD.

Among the different genes associated with familial PD, PTEN-induced putative kinase1 (Pink1), responsible for the autosomal recessive type, is strongly linked to the mitochondria. To investigate in depth the underlying mechanisms of Pink1, we inhibited the function of pink1 in zebrafish using morpholino oligonucleotides (MOs). The MO was first thoroughly characterized with all necessary control experiments to avoid unspecific effects. Since the dopaminergic system is affected in PD, a marker for dopamine, tyrosine hydroxylase (TH), was used to assess damage to the system. Due to a genome duplication event that occurred early in the evolution of teleosts after the divergence from the mammals, two TH non-allelic isoforms were identified in zebrafish: th1 and th2. In the pink1 morphants, both the TH gene isoforms were altered. With in situ hybridization, the loss of th1 was found in the ventral diencephalon (dopaminergic cell groups 5, 6, 11) and th2 was reduced in the caudal hypothalamus (cell group 10b). Similar results were obtained with the cell counting method for TH1 immunoreactive cells. TH-ir indicated the loss of cells in the pretectum (group 7) and the ventral diencephalic cluster represented by cell groups 5,6,11. These pink1 morphants were exposed to subeffective doses of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This amalgamation of the toxin and genetic manipulation caused a locomotor deficit and also facilitated the loss of TH-ir in the same cell populations in the larval brains as was instigated by pink1 knockdown alone.

To investigate the involvement of pink1 in cell damage, we used a two-color gene expression-based microarray and identified a number of genes that were potentially involved in the pathogenic mechanism of the disease. After successful data analysis, the changes in critical genes were successfully validated by quantitative real-time PCR. The gene expression changes in the morphants, identified by the microarray, were rescued by pink1 mRNA
injections, suggesting the specific involvement of pink1 in the differentially expressed gene regulation. One of the significant findings was HIF signaling, an important pathway affected by pink1 knockdown. Individual factors and genes in the same pathway were validated by independent methods in the pink1 morphants to reveal whether pink1 affected hif1α or the cascade of events in the signaling pathway. Changes in the VEGF transcripts, erythropoiesis, and reactive oxygen species were observed, as were other antioxidant system genes, including cat and sod2. These pathways may provide new targets for drug development in PD.

To study the mechanisms underlying the involvement of pink1 in oxidative stress-mediated PD pathology using zebrafish as a tool, we generated a transgenic line, Tg(pink1:EGFP). The Tol2 transgenic approach was used to generate Tg(pink1:EGFP) by using the zebrafish pink1 promoter. Expression of the pink1 transgene was detected in the telencephalon, midbrain, and rhombencephalon in the CNS, and in the muscle, heart, and liver among the peripheral organs. The transgenic fish line was used to study the effect of oxidative stress. When subjected to a low concentration of hydrogen peroxide (H₂O₂), which had no effect on the mortality or phenotype of the fish, the transgenic fish showed an increase in pink1 transgene activity in the brain of the larval zebrafish. Oxidative stress-mediated changes in TH expression are valuable for PD study. H₂O₂ administration did not affect the th1 transcript levels, but it significantly increased pink1 expression and reduced the th2 transcript levels. This transgenic model will be highly useful for drug development and the screening of new potential therapeutic approaches as an in vivo model.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I–IV).

I. Chen YC, Priyadarshini M, Panula P.


III. Priyadarshini M, Tuimala J, Chen YC, Panula P.

IV. Priyadarshini M, Orosco L, Panula P
    Oxidative Stress and Regulation of PINK1 Using Zebrafish (Danio rerio). PLOS ONE (In Press)

* Authors contributed equally

Original publication II was also included in the thesis “Zebrafish as a model of Parkinson's disease ” by Dr Ville Sallinen, University of Helsinki.

The original publications are reproduced with the permission of the copyright holders.
ABBREVIATIONS

5-HT  Serotonin
6-OHDA  6-hydroxydopamine
AADC/DDC  Aromatic L-amino acid decarboxylase (EC 4.1.1.28)
AD  Alzheimer’s disease
ALS  Amyotrophic lateral sclerosis
ANOVA  Analysis of variance
Cat  Catalase
COMT  Catechol O-methyltransferase (EC 2.1.1.6)
DA  Dopamine
DAT/dat  Dopamine transporter
DBH  Dopamine beta hydroxylase (EC 1.14.17.1)
dpf  Day(s) post-fertilization
EGFP  Enhanced green fluorescent protein
EPO  Erythropoietin
H2O2  Hydrogen Peroxide
HA  Histamine
HD  Huntington’s disease
HDC/hdc  Histidine decarboxylase (EC 4.1.1.22)
HIF-1/hif-1(a)  Hypoxia inducible factor-1(a)
hpf  Hour(s) post-fertilization
ir  Immunoreactivity
LC  locus coeruleus
L-DOPA  3,4-dihydroxyphenylalanine
LGR  L-Glutathione reduced
LRRK2  Leucine rich repeat kinase 2
MAO  Monoamine oxidase A, B (EC 1.4.3.4)
MHB  Mid-hindbrain boundary
MO, mo  Morpholino oligonucleotides
MPTP  1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA  Messenger RNA
NA  Noradrenaline
NAC  N-Acetyl Cysteine
NAT  Noradrenaline transporter
PD  Parkinson’s disease
PFA  Paraformaldehyde
PINK1/ pink1  PTEN induced putative kinase 1
PTEN  Phosphatase and tensin homolog
q-RT-PCR  Quantitative real-time polymerase chain reaction
ROS  Reactive oxygen species
RT-PCR  Reverse transcriptase PCR
SNpc  Substantia nigra pars compacta
SNCA  α-synuclein
sod2  Superoxide dismutase 2
TALEN  Transcription activator-like effector nuclease
TH  Tyrosine hydroxylase (EC 1.14.16.2)
TRAP  Tremor, rigidity, akinesia and postural instability
vDC  Ventral diencephalon
VEGF  Vascular endothelial growth factor
VMAT 2  Vesicular monoamine transporter 2
WT  Wild-type
ZFN  Zinc finger nuclease
INTRODUCTION

Parkinson’s disease (PD) is the second most prevalent age-related neurodegenerative disease after Alzheimer’s disease (AD), affecting 1–2% of the world’s population over 60 years of age. The discovery of dopamine deterioration in PD by Oleh Hornykiewicz paved the way to understanding the neuropathology of the disease progression and finding alternatives for prevention and cure [1]. Many studies have suggested that central dopaminergic degeneration plays a moderate role in cognitive changes in PD patients, and other neurotransmitters and biochemical changes are also affected during disease development [2-4].

A major breakthrough in PD research was the identification of monogenic familial forms of the disease. Presently, they account for 5–10% of all PD cases [5]. The five most common PD genes that account for the majority of cases are alpha-synuclein (SNCA) [6], leucine-rich repeat kinase 2 (LRRK2) [7], PTEN-induced putative kinase 1 (PINK1) [8], dj-1 [9], and parkin [10]. Mutations in PINK1 (PARK6) are the second most common cause of autosomal-recessive PD after Parkin. An impressive body of literature and experimental data in different model systems strongly suggests that mitochondrial dysfunction plays a central role in clinically similar, early-onset autosomal recessive PD forms caused by parkin, pink1 and dj-1 gene mutations. PINK1 is a mitochondrially targeted serine/threonine kinase, which has been shown to protect cells against oxidative stress-induced apoptosis. The lack of a working mammalian model for PINK1-associated Parkinsonism has limited the scope of translating laboratory research to understanding of the mechanisms of human PD.

Catecholaminergic modulatory neurotransmitters play an important role in the pathology of PD. Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, serves as a consistent marker for the detection of all catecholaminergic neurons in the brain. Using antibodies against TH in many species, the catecholaminergic groups have been characterized [11-13]. Animal models have been fruitful in addressing fundamental questions and providing new insights into the pathogenesis of the disease. The zebrafish provides a rapid and effective means for assessing gene function in the vertebrate nervous system. The conversion of genetic and biological knowledge derived from fish systems and associated with humans has been faster than for other vertebrate models. Due to genome duplication, zebrafish researchers have faced a limitation in detecting all catecholaminergic populations in the larval and adult zebrafish. The two separate non-allelic isoforms of tyrosine hydroxylase
that exist in other species of fish should be characterized in zebrafish for a comprehensive explanation of dopaminergic dysfunction [14]. However, no previous studies have identified the second TH gene in zebrafish prior to this investigation [15-17].

Studies using the neurotoxin MPTP have implicated mitochondrial dysfunction, and PINK1 is a mitochondrial kinase. Using toxin models based on zebrafish and combining them with genetic approaches, we have worked towards developing a progressive disease model. The goal is to develop an approach in which both contributing components are present. Through the combination of both models, we aim to recapitulate essential aspects of the complexity of PD, which could contribute towards understanding the etiology of the disease.
REVIEW OF THE LITERATURE

1. Parkinson’s disease

The World Health Organization has estimated that neurological disorders hamper one in seven people worldwide, an impact that cannot be ignored. The second most prevalent neurodegenerative disease after Alzheimer’s disease (AD), affecting nearly 1% of world’s population, is Parkinson’s disease (PD). It is an age-related movement disorder, and while 5–10% of the cases are considered as early onset (occurring earlier than 50 years of age), the average age of onset of PD is around 60 years globally [18]. The prevalence of the disease increases as a function of age, reaching 4% of the world’s population in the highest age group. The nature, symptoms and treatment of PD are of ancient origin, but the true picture of the disease only came to light after the symptoms were documented in a monograph entitled An essay on the shaking palsy by Sir James Parkinson in 1817 [19, 20]. Earlier, it was known as paralysis agitans, and the name “Parkinson’s disease” was coined by Jean-Martin Charcot. The underlying biochemical changes in the brain were identified in the 1950s, largely in the work of Arvid Carlsson, who discovered that dopamine acts as a neurochemical transmitter in the brain [1,21]. The steady and progressive loss of dopamine neurons with age in the substantia nigra pars compacta (SNpc) is the main pathological characteristic of PD, along with the presence of insoluble protein inclusions termed Lewy bodies [22]. Besides these, the characteristic motor symptoms include tremor, rigidity, slowness of movement (akinesia), and postural instability, coined as the fundamental signs in the abbreviated form of TRAP [23].

The non-motor symptoms include autonomic dysfunction, cognitive and neurobehavioral problems, and sensory and sleep disturbances, which precede the motor dysfunction [23,24]. Both the motor and non-motor symptoms cause functional disability of varying degrees in PD patients that creates a complex and heterogeneous clinical picture. The non-motor symptoms in PD have also transformed the concept of the disease. The etiology of the disease still remains a mystery, but it is hypothesized that it may result from a complex interaction between environmental factors, genetic susceptibility, and ageing [24].
1.1. Genetics of PD

Parkinson's disease (PD) is classified into ‘familial’ or early-onset PD (<10% of all patients) and idiopathic or late-onset PD (>85% of all cases), which does not appear to exhibit heritability [25]. In 1996, the first genetic locus was identified in an Italian descendent with pathologically confirmed PD [26]. This study paved way for the modern genetic understanding that PD may be hereditary. Nearly 15 years later, a major breakthrough in PD research was achieved by the identification of monogenetic variants, which account for up to <10% of all PD cases [24]. Many mutations that are responsible for and cause the disease in humans have been identified, and many genetic loci have been mapped by genome-wide studies that alter the risk for PD, as listed in Table 1 [28-30]. Most PD cases are sporadic, but 10% of the cases report a family history. The genes show both dominant and recessive modes of inheritance. In the last few years, around 18 susceptibility gene loci have been identified by genome-wide studies and linkage analysis [27, 29]. So far, five validated PARK genes have conclusively been linked to PD [5, 30]. The autosomal dominant PD (ADPD) genes include SNCA (PARK1 and 4) and LRRK2 (PARK8). Autosomal recessive PD (ARPD) consists of genes such as PINK1 (PARK6), DJ-1 (PARK7), and parkin (PARK2). These genetic findings have helped immensely in understanding PD mechanisms at molecular and cellular levels using many different cell-based and animal models.

Table 1. Summary of all PARK-designated loci underlying monogenic PD [28-30].

<table>
<thead>
<tr>
<th>PARK loci/Genes</th>
<th>Position</th>
<th>Inheritance type of Parkinsonism</th>
<th>Validated loci/genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PD associated loci with conclusive evidence</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARK1/PARK4/SNCA</td>
<td>4q21</td>
<td>Dominant, rarely sporadic</td>
<td>EOPD</td>
</tr>
<tr>
<td>PARK2/parkin</td>
<td>6q25-q27</td>
<td>Recessive; sporadic</td>
<td>Juvenile and EOPD</td>
</tr>
<tr>
<td>PARK6/PINK1</td>
<td>1p35-p36</td>
<td>Recessive</td>
<td>EOPD</td>
</tr>
<tr>
<td>PARK7/DJ-1</td>
<td>1p36</td>
<td>Recessive</td>
<td>EOPD</td>
</tr>
<tr>
<td>PARK8/LRRK2</td>
<td>12q12</td>
<td>Dominant; sporadic</td>
<td>LOPD</td>
</tr>
<tr>
<td><strong>Putative loci/genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARK3/Unknown</td>
<td>2p13</td>
<td>Dominant</td>
<td>LOPD</td>
</tr>
<tr>
<td>PARK5/UCHL1</td>
<td>4p14</td>
<td>Dominant</td>
<td>LOPD</td>
</tr>
<tr>
<td>PARK9/ATP13A2</td>
<td>1p36</td>
<td>Recessive</td>
<td>Kuffor-Rakeb syndrome</td>
</tr>
<tr>
<td>PARK17/VPS35</td>
<td>16q11.2</td>
<td>Dominant; sporadic</td>
<td>LOPD</td>
</tr>
<tr>
<td>PARK10/Unknown</td>
<td>1p32</td>
<td>Unclear</td>
<td>LOPD</td>
</tr>
<tr>
<td>PARK11/ GIGYF2</td>
<td>2q36-q37</td>
<td>Dominant</td>
<td>LOPD</td>
</tr>
<tr>
<td>PARK12/Unknown</td>
<td>Xq21-q25</td>
<td>Unclear</td>
<td>Not clear</td>
</tr>
<tr>
<td>PARK13/Omi/HTRA2</td>
<td>2p12</td>
<td>Unclear</td>
<td>Not clear</td>
</tr>
<tr>
<td>PARK14/PLA2G6</td>
<td>2q21-q13</td>
<td>Recessive</td>
<td>EO dystonia-parkinsonism</td>
</tr>
<tr>
<td>PARK15/FBXO7</td>
<td>2q12-q13</td>
<td>Recessive</td>
<td>EO parkinsonian pyramidal syndrome</td>
</tr>
<tr>
<td>PARK16</td>
<td>1q32</td>
<td>Unclear</td>
<td>LOPD</td>
</tr>
<tr>
<td>PARK18/EIF4G1</td>
<td>3q27.1</td>
<td>Unclear</td>
<td>LOPD</td>
</tr>
</tbody>
</table>
1.1.1 Autosomal dominant PD genes

A. SNCA (α-synuclein, PARK1-4)

The gene α-synuclein was identified in 1994 in the human brain, and the 140 kD amino-acid protein was found to be identical to the precursor of the non-Aβ component of Alzheimer's disease amyloid [31]. It was the first causative gene to be linked with PD and was found from a study on an Italian related family and three non-related Greek descendants [6]. A study soon after this finding confirmed that α-synuclein is a major component of Lewy bodies, and a pathological hallmark of the disease [32]. Patients with point mutations in this gene had dementia and an earlier onset than sporadic cases of PD [32].

Duplications and triplications of the locus containing this gene suggest that overexpression of α-synuclein is toxic and is a major cause of the disease [33]. The intrinsic instability in the α-synuclein gene locus suggests that the genomic multiplications are de novo, since the duplication and triplication events in different PD families have different allele sizes in the 4q21-23 region [34]. The age of onset, severity of dementia and psychiatric problems appear to be associated with the number of copies of the SNCA gene. These findings have led to a simplified hypothesis of a linear dose relationship between α-synuclein levels and the severity of the disease [5]. Knowledge of most toxic levels of α-synuclein and the structural basis of aggregation are crucial for a better diagnosis and prognosis, and for developing effective therapies in PD.

B. Leucine-rich repeat kinase 2 (LRRK2, dardarin, PARK8)

In 2002, linkage analysis studies in a Japanese family with ADPD led to the discovery of a new locus in PARK8 [35]. Mutations in LRRK2 are known to be the most common cause of ADPD [5, 7]. The gene has 51 exons encoding a very large 285 kDa protein, which contains a central catalytic tridomain with GTPase, a kinase enzymatic domain and multiple protein–protein interaction domains [5]. Three other domains that have also been identified are a leucine-rich repeat (LRR), a tyrosine kinase catalytic domain (ankyrin), and a WD40 domain [36]. It has been found that mutations in different domains result in variable pathological changes. The common feature amongst all the mutations is neuronal loss and gliosis in the substantia nigra, along with Lewy bodies in most cases [36]. The LRRK2 mutations constitute around 10% of the familial PD cases, with a clear autosomal-dominant inheritance pattern [5]. LRRK2 is expressed in the brain, specifically in the cerebral cortex and putamen, and in extra-cerebral tissues such as the lungs or heart [7, 36].
1.1.2. Autosomal recessive PD genes

A. Parkin (PARK2)

The second gene to be identified as a PD gene and the first to be found responsible for ARPD was parkin. The parkin gene was first identified in a Japanese kindred with juvenile onset PD [10]. A number of small and large structural changes have subsequently been identified in the PARK2 locus, which accounts for the majority of early onset cases, depending on the population analyzed [5, 29]. Parkin mutations account for 70% of juvenile cases with an age of onset <20 years, comprising ~50% of early-onset (≤40 years) familial cases and ~20% of early-onset sporadic PD [28, 37]. The typical age of onset is before 40 years of age, and the cardinal symptoms of PD TRAP are mild. The patients usually show foot dystonia, hyperreflexia, diurnal fluctuations, and early susceptibility to levodopa-induced dyskinesias [38]. Pathological characteristics include neuronal loss and gliosis limited to the substantia nigra and locus coeruleus, but characteristic Lewy bodies are absent [10]. Recently, studies have reported neurofibrillary tangles and Lewy body pathology in some cases [39].

The parkin gene comprises 12 exons spanning over 1.4 megabases. It encodes a protein of 465 amino acids possessing a ubiquitin-like domain and a RING-finger motif, and is the second largest gene in the human genome [29]. The parkin gene is structurally divided into three parts: the amino-terminal Ubl domain, the carboxy-terminal RING box and the linker region, which connects the former two segments. The C-terminal RING box region consists of three domains termed RING0, RING1, RING2, and an IBR domain for interaction with the ubiquitination machinery [40, 41]. The differences in the pathological condition in Parkin mutants account for the variability of mutations in different regions of the gene and gene function [5].

B. PINK1 (Phosphatase and tensin homolog (PTEN)-induced putative kinase 1, PARK6)

The first direct genetic link between PD and mitochondrial dysfunction was recognized after identifying mutations in the genes encoding PINK1 that led to ARPD. PINK1 was first identified in expression profiles of cancer cells [42]. In 2004, two homozygous mutations were found in the PINK1 gene as a cause of early onset PD [8]. The PINK1 gene encodes a 581-amino-acid ubiquitous protein, consisting of an N-terminal 34-amino-acid mitochondrial targeting motif, a conserved serine–threonine kinase domain (156–509) and a C-terminal autoregulatory domain. It exhibits a mitochondrial topology where the N-terminal domain is
located inside the mitochondria and the C-terminal domain faces the cytoplasm[43]. The majority of the identified mutations are in the kinase domain, indicating the importance of PINK1 enzymatic activity in PD pathogenesis [8, 29].

PINK1 has been found to be the second most frequent causative gene after parkin. The frequency of mutations is in the range of 1–9%, with variations across different ethnicities [29]. Despite autosomal recessive transmission, 5% of the sporadic early-onset PD cases have single heterozygous pink1 mutations [28, 44]. Most of the disease characteristics are similar to those in parkin mutants, jointly with the typical TRAP cardinal signs of PD [5, 38, 44]. PINK1-linked PD resembles idiopathic PD, as it shows a good response to levodopa, frequently levodopa-induced dyskinesias, and rarely dystonia [28].

PINK1 is expressed ubiquitously, but most abundantly in the brain as well as in peripheral tissues such as the heart (especially in the myocardium), skeletal muscle, or testes in humans, mice, and rats [8, 42]. Homozygous or compound heterozygous mutations unequivocally cause ARPD in this gene. Single heterozygous mutations have been found to be a risk factor for developing PD [45]. Current findings have provided interesting evidence that pink1 and parkin function in a common pathway in mitochondrial biogenesis [46]. PINK1 binds to and phosphorylates parkin, but how it recruits parkin to mitochondria for mitophagy is unclear. This has led to some conclusions that mitochondrial quality control is necessary for the etiology of the disease [46].

C. DJ-1 (PARK7)

DJ-1 was first identified as an oncogene, but later this gene was linked by homozygosity mapping to ARPD [9]. The DJ-1 gene contains 8 exons distributed over 24 kb and encodes a 189-amino-acid protein with a still uncertain function [5, 9]. The PARK7 mutations are quite rare, with <1% early onset cases having a typical age of onset in the 20s and 30s [5].

Research into the putative function is still ongoing. DJ-1 is a redox-sensitive molecular chaperone. It regulates redox-dependent kinase signaling pathways and antioxidant gene expression by protecting mitochondria against oxidative stress [41]. It seems that DJ-1 is also involved in the pink1-parkin pathway in protecting mitochondria from oxidative stress [24].
DJ-1 is rare, even in early-onset PD. The DJ-1 protein has been found to be significantly increased and oxidatively damaged in many sporadic cases of PD [24].

1.3. Mitochondrial dynamics in PD

Changes in the number, size, and morphology of brain cells occurring during development are also accompanied by structural changes within the mitochondria, the powerhouses of the cells. Ever since the finding that exposure to the drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) leads to PD, mitochondrial dysfunction has been linked to PD pathogenesis. Mitochondrial abnormalities have been identified in the SNc of patients with PD. Studies within a decade of the discovery of the monogenic forms of PD have further strengthened the link between mitochondria and PD [38, 47, 48].

Knockdown studies on PD genes using different models point to mitochondrial dysfunction. α-Synuclein mutant mice exhibit mitochondrial degeneration associated with increased mtDNA damage and impaired activity of the electron transport chain complex IV cytochrome oxidase [49]. DJ-1 knockdown in cultures, Drosophila, zebrafish and mice sensitizes them to oxidative stress and MPTP toxicity [50, 51]. The parkin gene is involved in the autophagic clearance of mitochondria in the cell in the diseased state by a process called mitophagy [46]. PINK1 resides in the inner mitochondrial membrane space and plays a major role in mitochondrial maintenance through the phosphorylation of molecules involved in mitochondrial dynamics that project to or are located in the cytoplasm [43, 52]. Recent studies on the pink1/parkin pathway have demonstrated direct modulation of the mitochondrial fusion-fission machinery, highlighting the importance of these proteins in mitochondrial quality control in PD [53].

Biochemical findings from post-mortem brains and studies on toxin-induced animal models have revealed altered mitochondrial function in PD, particularly in complex-I of the electron transport chain [54-56]. The toxic products of oxidative damage through the disruption of complex-I are free radicals, which damage other key cellular components [57]. Mitochondria are the main source of endogenous ROS (reactive oxygen species) [58]. Overwhelming production of ROS is evident during oxidative stress, and is also a prominent cause of neurodegeneration in PD [49, 59]. To test the hypothesis that mitochondrial dysfunction in dopamine neurons can cause a progressive PD phenotype, a MitoPark mouse model was
designed [60]. In these mice, the mitochondrial transcription factor Tfam is selectively removed in midbrain dopamine (DA) neurons. The mice have reduced mtDNA expression and a respiratory chain deficiency in midbrain DA neurons. This knockout mice model exhibits a Parkinsonian phenotype with adult onset of a slowly progressive impairment of motor function accompanied by the formation of intraneuronal inclusions and dopamine nerve cell death, and the model is able to replicate the slow and progressive development of key PD symptoms. A current literature review of different studies reported a conclusive finding that complex-1 alterations lead to aggravated ROS production and/or defective ROS removal, resulting in oxidative damage to mitochondrial DNA (mtDNA), proteins, and lipids. This, in turn, leads to a viscous cycle of oxidative stress and bioenergetic failure in ageing and PD [49]. Several studies have linked mitochondrial dysfunction with PD, but it is still debatable whether it is the cause or the effect [47].

1.4. Toxin-induced models of PD

The toxin-induced models used to elucidate the pathophysiological mechanism underlying PD have been crucial to the development of therapeutic strategies to treat the motor symptoms [48]. The neurotoxins widely used to study PD are 6-OHDA and MPTP [3]. The 6-OHDA rat model and the MPTP primate model have contributed enormously towards translating animal experimentation into clinical practice [48]. Epidemiological investigations have enhanced studies on the association of pesticides with PD. Besides MPTP analogs, reserpine, paraquat, rotenone, dieldrin, organochlorine, and carbamate derivatives have been extensively studied as potential toxic candidates [61, 62]. The neurotoxin compounds that produce both reversible and irreversible effects such as reserpine, MPTP, 6-OHDA, paraquat, and rotenone have been used effectively. However, recent studies have focused more on the toxins MPTP and 6-OHDA to produce PD-related pathology. These studies have been difficult to interpret unambiguously. To determine the efficacy of these drugs in causing PD, many animal models have been used. These studies have been valuable in proving that these compounds can mimic parkinsonian syndrome and could prove beneficial for drug development [63].

1.4.1. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD

The discovery of MPTP in 1983 as a by-product of the chemical synthesis of a meperidine analog, also called synthetic heroin, provided the first proof that an environmental toxin could produce parkinsonism in humans [64]. MPTP in animal models also produces similar
features of human parkinsonism. Since then, MPTP has indeed been considered as a standard for toxin-based animal models of PD for replicating the hallmark of α-synuclein accumulation [64]. MPTP works by inhibiting mitochondrial function, which is why it provided the first clue that mitochondrial impairment might be important in PD pathogenesis [65]. It is highly lipophilic in nature and rapidly crosses the blood–brain barrier. Within a minute after MPTP injection, levels of the toxin are detectable in the brain [63]. The neurotoxin imparts all the cardinal signs of PD when administered. PD develops gradually with age, but develops in days of MPTP administration. Quite curiously, rats are resistant to MPTP and mouse strains vary widely in their sensitivity to the toxin [66]. MPTP can be administered by systemic injection in all the known animal models, which appears to be a technical limitation at times [3]. Only two features have been lacking in the MPTP model of PD: characteristic Lewy body inclusions and an effect on the TH activity in the locus coeruleus in the brain. The best models to replicate completely the PD-like features of MPTP administration are monkeys [63]. However, other models have been used, which have also replicated human PD pathology and have proven valuable through the generated data in understanding the molecular mechanisms of the disease [17, 59, 61, 63].

1.4.2. Mechanism of action of MPTP

MPTP administered intraperitoneally crosses the blood–brain barrier and is metabolically activated to 1-methyl-4-phenyl-2,3-dihydropyridinium species (MPDP⁺) by the enzyme monoamine oxidase B (MAO-B) in non-dopaminergic cells [67]. MPDP⁺ is then oxidized to 1-methyl-4-phenylpyridinium species (MPP⁺) and released into the extracellular space [68]. MPP⁺ is subsequently taken up into the dopaminergic neurons by the dopamine transporter (DAT) [69]. After MPP⁺ enters the dopaminergic neurons, it is accumulated in the vesicular monoamine transporter (VMAT2). VMAT2 ensures a neuroprotective effect through the sequestration of MPP⁺, so that less free MPP⁺ is available to be accumulated from the cytosol into the mitochondria. This was verified when mice expressing reduced levels of VMAT2 were found more sensitive to MPTP-induced damage at the nigrostriatal DA terminals [70]. Once inside mitochondria, MPP⁺ in turn inhibits the activity of NADH dehydrogenase, which blocks the electron flow in the respiratory chain and impairs the cellular supply of energy in the form of ATP [71]. The impairment in the respiratory chain is mediated by complex-1, which suggests that MPP⁺ would be lethal to any cell type that depends on aerobic respiration [72]. The association of MPP⁺ with complex-1 is capable of generating ROS, which creates
oxidative stress and damages the dopamine homeostasis [48, 63]. Understanding of the mechanism by which MPTP kills cells will aid in finding important cues concerning the molecular events in PD pathology.

2. Modulatory neurotransmitter systems

The basic unit of the brain, the neuron, was discovered by Santiago Ramon y Cajal, who stated that nerve cells were discrete entities that communicate with each other by means of specialized contacts [73]. A variety of chemical messengers in the brain help the neurons communicate and are called neurotransmitters. There are more than 100 different types of neurotransmitters in the brain. Neurotransmitters evoke post-synaptic responses by binding to receptors and activating other neurotransmitter responses. Modulatory neurotransmitters are different from the classical neurotransmitters, because instead of activating fast ion channel receptors, they bind to slowly acting metabotropic receptors, which are most often G-protein-coupled receptors. A modulatory transmitter frequently alters the efficacy of action by other transmitters [74, 75]. The neurotransmitters also belong to distinct categories based on their size, such as neuropeptides and biogenic amines. Neuropeptides are relatively large transmitter molecules composed of 3–36 amino acids, whereas catecholamines and other small transmitters are referred to as small molecule neurotransmitters. The main excitatory neurotransmitter is the amino acid glutamate, and the main inhibitory neurotransmitter is GABA (Y-aminobutyric acid). Neurotransmitter imbalance results in disorders such as PD, AD, and other psychiatric diseases. One of these neurotransmitter dopamine dysfunctions is characteristic of PD. PD treatments that focus on the dopaminergic system alone are unable to alleviate both motor and non-motor symptoms, particularly those that develop in early stages of the disease. Therefore, the development of agents that interact with several of the affected neurotransmission systems could prove invaluable for the treatment of this disease.

2.1. Biogenic amines

The biogenic amines are small molecule neurotransmitters that regulate many brain functions, and are also active in the peripheral nervous system. These amines are formed by enzymatic decarboxylation of naturally occurring amino acids. They are involved in a wide range of behaviors, and defects in their functions are therefore mostly implicated in psychiatric diseases [76]. The five major and well-established biogenic amines, which comprise dopamine, noradrenaline, adrenaline, serotonin and histamine, are associated with a
wide range of behaviors, cognitive functions, and homeostatic functions in the CNS. They even play an important role in brain development [77]. A brief summary of all the modulatory neurotransmitter systems is presented in Table 2 below.

2.1.1. The catecholaminergic system

The catecholaminergic system has received considerable attention, particularly because of its early recognized involvement in neurological disorders such as PD. The catecholaminergic system is comprised of dopamine, noradrenaline and adrenaline, which share the catechol moiety [78]. Adrenaline (ADR) was the first of these to be identified as a neurotransmitter and a hormone by Abel and Crawford in 1897. In 1946, Ulf von Euler discovered that noradrenaline (NA) is the neurotransmitter in sympathetic nerves and the precursor of adrenaline. Dopamine was first synthesized in 1910 by George Barger and James Ewens and was named 3,4-dihydroxyphenylethylamine [77]. Henry Dale proposed the name dopamine (DA) after Peter Holtz discovered the enzyme dopa decarboxylase and showed that it produced dopamine from levodopa (L-DOPA). In 1958, Arvid Carlsson identified the remarkable functionality of DA as a crucial neurotransmitter. That was the discovery of reserpine-induced Parkinsonism with reserpine’s brain dopamine-depleting effect and L-DOPA’s anti-reserpine action [21, 77-79].

The catecholamines DA, NA and ADR are important transmitters in the regulation of physiological processes and the development of neurological, psychiatric, endocrine, and cardiovascular diseases. The pathways for their metabolism are well established and clearly understood. These catecholamines are synthesized from the amino acid precursor L-tyrosine. The primary source of tyrosine is the diet or the hydroxylation of the amino acid phenylalanine in the liver. L-tyrosine is converted to dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase (TH, EC 1.14.16.2) in the adrenal chromaffin cells [80]. TH utilizes molecular oxygen and tetrahydrobiopterin to generate DOPA, dihydrobiopterin and water. TH is a rate-limiting enzyme, and changes in its gene expression hamper major mechanisms in the catecholaminergic system responses. It has been mapped in the brains of many species by antibodies against the enzyme [11, 12]. DOPA is converted into DA by a nonspecific enzyme, aromatic L-amino acid decarboxylase (AAAD; EC 4.1.1.28) [81]. The activity of AAAD depends on the levels of its cofactor, pyridoxal phosphate. Dopamine is taken up from the cytoplasm into storage vesicles and converted into NA by dopamine
hydroxylase (DBH; EC 1.14.17.1), an enzyme found in soluble and membrane-bound forms within storage vesicles [82]. DBH activity utilizes copper, ascorbic acid, and molecular oxygen. NA is then converted into ADR by the soluble cytoplasmic enzyme phenylethanolamine N-methyltransferase (PNMT; EC 2.1.1.28), which uses S-adenosyl methionine as the cofactor [83]. Separate populations of adrenal chromaffin cells contain NA and ADR as the final products of CA biosynthesis. Following the synthesis of catecholamines in the cytoplasm of pre-synaptic terminals, they are loaded into synaptic vesicles via vesicular monoamine transporters (VMAT) and released into the synaptic cleft upon the arrival of an action potential [84]. The process of catecholamine release is similar in the adrenal medulla and sympathetic nerve endings. An increase in membrane permeability to sodium initiates a series of events that lead to an influx of calcium. Via exocytosis, the contents of catecholamines in the storage vesicles are then released. However, the exact mechanism of Ca^{2+}-evoked exocytosis is unclear [78].

Catecholamines are subjected to chemical degradation by catechol-O-methyltransferase (COMT; EC 2.1.1.6) or oxidative deamination by monoamine oxidase (MAO; EC 1.4.3.4) [85]. Both neurons and glia contain mitochondrial MAO and cytoplasmic COMT. MAO is a mitochondrial flavoprotein located in the outer membrane of presynaptic neurons, and COMT is located in the effector cells [83]. In humans, there are two subtypes of MAO localized in the liver. The subtype MAO-A has a higher affinity for NA and ADR and is highly localized in brain neurons, while MAO-B is responsible for the degradation of DA. Dopamine is deaminated to 3,4-dihydroxyphenylacetaldehyde (DOPAL), whereas NA and ADR are both deaminated to 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL) [86]. These aldehyde intermediates exist only transiently and are rapidly metabolized to the corresponding glycols by the enzymes aldehyde reductase (AR) and aldehyde dehydrogenase (AD). The final end products of DA, NA and ADR are homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG) and vanillylmandelic acid (VMA), respectively [83].

The turnover of catecholamines, representing ongoing loss and replenishment by synthesis, is usually considered to be driven by catecholamine release in response to increased nerve impulse activity. Dopamine action in the synaptic cleft is terminated by the reuptake of dopamine by the Na^+-dependent dopamine co-transporter (DAT) [87]. NA is cleared from the synaptic cleft by the Na^+-dependent noradrenaline transporter (NAT) [88]. Although no
specific transporter for ADR has been identified, NAT is capable of transporting ADR. NAT translocates NA about two-fold more effectively than ADR. This explains why sympathetic nerves take up NA more efficiently than ADR [78].

In 1964, catecholaminergic neurons in the CNS were classified into 12 cell groups (A1–A12) [89]. Subsequently, other catecholaminergic cell groups (A13–A16, C1–C3) have been defined [11, 90]. The NA groups in the brain were classified into seven cell groups (A1–A7) and only exist in the pons and medulla oblongata. The ADR nerve fibers and terminals arise in the neuronal perikarya located in three adrenergic cell groups (C1–C3 cell groups) of the medulla oblongata. A1, A2, A4, and A7 NA cell groups, along with the C1 and C2 ADR cell groups, belong to the “ascending catecholaminergic system”. The Na cell groups A5 and A6, together with the C3 ADR group, belong to the “descending catecholaminergic system” [78].

The dopaminergic system in the CNS is organized into three major systems: the mesencephalic (A8–A9), diencephalic (A11, A12, A13, A14, A15), and olfactory (A16) DA systems. The mesencephalic DA groups are comprised of three systems: the nigrostriatal, mesolimbic, and mesocortical systems [91]. Dopaminergic fibers from these cell groups run either in well-organized and target-specific bundles or participate in other neuronal pathways innervating practically the entire CNS.

2.1.2. The serotonergic system

In 1937, a substance found in the enterochromaffin cells of the gut was identified and named enteramine by Vittorio Erspamer. The serum vasoconstrictor factor released from platelets during blood clotting was isolated and identified as 5-hydroxytryptamine (5-HT) by Rapport, Green, and Page [92, 93]. Later on, they coined the term serotonin [94]. About 95% of the total estimated body serotonin exists in the gastrointestinal tract [95]. The existence of serotonin in brain and the discovery of the hallucinergic drug lysergic acid diethylamide (LSD) paved the way for research into the importance of serotonin in mental illness by Woolley [96]. Serotonin is a well-known modulator of cognitive and behavioral functions such as sleep, sexual urge, anxiety, appetite, temperature regulators, learning, memory, and mood. Altered 5-HT signaling results in the etiology of many neurodevelopmental disorders, including depression, anxiety, compulsive disorder, schizophrenia, and autism [94, 97].
The neurotransmitter serotonin is synthesized from the dietary amino acid tryptophan. It is mediated through a two-step metabolic pathway starting with the hydroxylation of L-tryptophan (Trp) by tryptophan hydroxylase (TPH: EC 1.14.16.4) to 5-hydroxytryptophan (5-HTP) [98]. TPH catalyzes the rate-limiting step in the synthesis of serotonin and the initial, uncommitted step in the synthesis of melatonin. It is synthesized in the raphe, pineal, enteric neurons of the gut, mast cells, platelets, retina, and thyroid. TPH-mediated catalysis requires the cofactor ferrous iron, and the co-substrates oxygen and tetrahydrobiopterin (BH4). TPH belongs to a superfamily of aromatic amino acid hydroxylases, together with PAH and TH, and undergoes decarboxylation by an aromatic L-amino acid decarboxylase (AAAD: EC 4.1.1.28) [99]. Two isoforms of TPH exist: TPH1 is peripheral in distribution, while TPH2 is predominantly expressed in the brain [98, 100].

The enzyme AAAD is a shared enzyme in both catecholaminergic and serotonergic synthesis pathways and can be detected in both types of cells [101]. The loading of 5-HT into synaptic vesicles is carried out by VMAT, which is also responsible for other monoamines. The synaptic effects are terminated by transport back into nerve terminals with the aid of serotonin transporter (SERT) [102]. The transport of 5-HT by SERT is inhibited by antidepressants, and these are therefore called selective serotonin re-uptake inhibitors[103]. Serotonin in tissues is rapidly metabolized by the activity of MAO. In the kidney and liver, MAO and aldehyde dehydrogenase convert 5-HT to 5-hydroxyindole acetic acid (5-HIAA), which is excreted in the urine [95].

The first study on the localization of the serotonergic neurons was conducted using histofluorescence techniques, which revealed the preferential location near the midline of the brain stem [89]. The innervation pattern was later confirmed by serotonin antibody staining [104]. The serotonin neuronal clusters are allocated based on their distribution and projection into nine groups, B1–B9. These are further divided into two groups: the rostral group, B5–B9, confined to the mesencephalon and rostral pons, and the caudal group, B1–B4, extending from the caudal pons to the caudal portion of the medulla oblongata [104, 105].

2.1.3. The histaminergic system

One of the first biogenic amines to be found in the brain was histamine. It was first identified by Sir Henry Hallett Dale in 1910 in the smooth muscles of the gut as an imidazolethylamine,
denoting an amine occurring in tissues [106]. Kwiatkowski detected histamine in the grey matter of the brain in 1941 and White demonstrated its formation and catabolic pathway in the brain [107]. The presence of histamine in the brain can be confirmed in two major pools: in neurons and mast cells [108]. Histamine occurs in cells of neuro-epithelial and hematopoietic origin and serves distinct functions, which has important implications for gastrointestinal, immune response, cardiovascular, and reproductive functions [107, 109].

Histamine is synthesized from the amino acid histidine by histidine decarboxylase (HDC; EC 4.1.1.22) through oxidative decarboxylation [110]. HDC is a pyridoxal 5’-phosphate-dependent enzyme found in many species and highly conserved across the animal kingdom [107, 111]. The limiting step in histamine synthesis is the availability of the precursor histidine. Histamine is packed into vesicles and transported by VMAT2 [112]. To date, no other transporter specifically for histamine has been discovered.

Histamine is degraded to an inactive form, tele-methylhistamine (t-MHA), mainly by histamine N-methyltransferase (HNMT, EC 2.1.1.8) in the brain [113]. Tele-methylhistamine is further metabolized to t-methyl-imidazoleacetic acid by monoamine oxidase B and aldehyde dehydrogenase [107]. In non-neuronal tissues, histamine is oxidized by diamine oxidase (DAO; EC 1.4.3.3) [114]. While many biochemical and electrophysiological studies indicating the presence of neuronal histamine had been performed earlier, it was not until the development of antibodies against histamine and HDC by two separate research groups that the exact localization and projection pattern could be determined [115, 116]. These studies demonstrated that the histaminergic neurons are confined to a small region of the posterior hypothalamus called the tuberomammillary nucleus (TMN), with widespread projections to different regions. There are two ascending pathways and one descending from the TMN [115]. All the histaminergic neurons are classified as one group, as they have similar projection patterns. Single neurons may even have both ascending and descending axon projections [117]. Histamine neurons play an intricate role in many neurodegenerative diseases such as AD, PD, schizophrenia, and epilepsy [107, 109]. In AD, the degeneration and tangle formation of histaminergic neurons and decline in HDC activity have been noted [109]. In the case of PD, an increase in the histamine concentration has been observed [109, 118]. The modulator role of the histaminergic system becomes prominent in perturbed states of disease, but the factors that regulate this function are still unclear.
Table 2. Summary of neurotransmitter synthesis, storage, transport, and degradation

<table>
<thead>
<tr>
<th>Neurotransmitter</th>
<th>Precursor</th>
<th>Synthesizing enzymes</th>
<th>Metabolising enzyme</th>
<th>Metabolite</th>
<th>Transporter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noradrenaline (NA) and Adrenaline (A)</td>
<td>L-tyrosine, L-DOPA, Dopamine</td>
<td>Tyrosine hydroxylase, L-aromatic amino-acid decarboxylase, Dopamine-β-hydroxylase, Phenylethanolamine-N-methyl transferase</td>
<td>Monoamine oxidase and Catechol-O-methyl transferase</td>
<td>Vanillylmandelic acid (VMA) and 3-methoxy-4-hydroxy phenylglycol (MHPG)</td>
<td>Noradrenaline transporter</td>
</tr>
<tr>
<td>Dopamine</td>
<td>L-tyrosine, L-DOPA</td>
<td>Tyrosine hydroxylase, Dopamine decarboxylase</td>
<td>Monoamine oxidase and Catechol-O-methyl transferase</td>
<td>3,4-Dihydroxy phenylacetic acid (DOPAC) and homovanillic acid (HVA)</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>5-Hydroxytryptamine (5-HT; serotonin)</td>
<td>5-hydroxytryptophan</td>
<td>Tryptophan hydroxylase, 5HTP decarboxylase</td>
<td>Monoamine oxidase</td>
<td>5-Hydroxyindole acetic acid</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>Histamine (HA)</td>
<td>L-histidine</td>
<td>Histidine decarboxylase</td>
<td>Histamine-N-methyl transferase</td>
<td>Telemethyl histamine, Imidazole acetic acid</td>
<td>Yet to be identified</td>
</tr>
</tbody>
</table>

3. Oxidative stress and hypoxia in PD pathology

A healthy and normal human brain utilizes 20% of the total oxygen taken up by the body, which makes oxygen imperative for life. As a major consumer of energy, the brain is particularly susceptible to changes in the oxygen conditions [119]. The presence of neuromelanin in the dopaminergic cells endures the autoxidation of dopamine, leading to semiquinone formation and polymerization, through which radical species are produced [120]. The degradation of dopamine by MAO also produces hydrogen peroxide ($H_2O_2$), which further emphasizes the involvement of oxidative stress. Enzymatic oxidation of dopamine to $H_2O_2$ has been found to cause the increased formation of oxidized glutathione (GSH), suggesting the occurrence of oxidative stress and impairment of a major antioxidant system [121]. The occurrence of oxidative stress in PD is supported by both post-mortem studies and by studies demonstrating the capacity of oxidative stress and oxidizing toxins to induce nigral cell degeneration [57].

The sensitivity of the nigrostriatal pathway to selective toxins such as MPTP and 6-OHDA demonstrates its vulnerability to free radical attack. Mitochondria are the main producers of ROS and the main targets of oxidative damage. Mitochondrial ROS are putative signaling
molecules between cellular oxygen sensors and hypoxia-inducible factor-1 (HIF-1). HIF-1α, a transcription factor, is a master regulator of cellular oxygen homeostasis. HIF-1α regulates the expression of a wide range of genes involved in vasomotor control, angiogenesis, erythropoiesis, iron metabolism, cell cycle control, cell proliferation and death, and energy metabolism [122]. HIF-1α is impaired in PD, as has been found in an MPTP mouse model and PC12 cell line study [123]. MPP+ effectively inhibits both complex I activity and the hypoxic accumulation of HIF-1α protein in dopaminergic cell lines PC12 and CATH. Promoter activity studies on TH have suggested that the TH gene is under the control of a hypoxia response element [124]. Knockdown mice for HIF-1α exhibited a 40% decline in TH expression in the brain [125].

Severe and prolonged hypoxia contributes to brain damage because of the exacerbated production of reactive oxygen species (ROS), which leads to oxidative stress. Oxidative stress, defined as an imbalance between biochemical processes leading to the production of reactive oxygen species (ROS) and a defect in the cellular antioxidant cascade, causes molecular damage that can lead to the critical failure of biological functions and the death of neurons. The contribution of hypoxia and hypoxia-mediated pathways to neurodegeneration remains unclear. The beneficial effects of HIF-1 can arise mainly from the increased expression of HIF-1 along with its target genes, which can combat oxidative stress, improve the blood oxygen and glucose supply, promote glucose metabolism, regulate iron homeostasis, activate the synthesis of dopamine, and block cell death signaling pathways. Increasing HIF-1 activity may be an important potential strategy for preventing the onset or ameliorating the pathogenesis of PD.

4. Zebrafish as a model

The zebrafish has emerged as an excellent model organism for studies on vertebrate biology. Even though the zebrafish had earlier been used as a model organism, the starting point for the contemporary use of zebrafish was in 1970s, when George Streisinger at Oregon University selected it for studies on genetics and embryology [126].

The zebrafish is a small tropical freshwater fish commonly found in Southeast Asia. It belongs to the order cypriniformes in the class Actinopterygii. A significant step for the use of this organism as a model and in the development of this field was a large mutagenesis
screen carried out in the 1990s [127, 128]. Many mutants identified in this screen are reminiscent of numerous human diseases and have increased our understanding of human biology. The zebrafish has easily distinguishable sexes, as males are torpedo shaped and have gold stripes between the blue stripes, while females have a larger, whitish belly and have silver stripes instead of gold ones (Figure 1). Therefore, it is easy to set up breeding pairs to obtain a large number of progeny. The zebrafish genome has now been completely sequenced and assembled with a sequence length of 1.4 Gb in 4560 scaffolds by the Sanger Center (http://www.sanger.ac.uk/Projects/D_rerio/Zv9_assembly_information.shtml). This is highly beneficial when performing experiments that involve gene manipulations.

Figure 1: Sex determination in zebrafish. The females are larger with silver stripes and males are torpedo shaped with gold stripes between the blue stripes.

4.1. Advantages and disadvantages of zebrafish

The zebrafish is a small vertebrate more closely linked to the human evolutionary lineage than the commonly used Drosophila or C. elegans. Zebrafish are easy to maintain, manipulate and observe. Females lay a large number of eggs, which are externally fertilized. Their high fecundity makes zebrafish an excellent model for genetic screens and analysis. These are very robust fish and can tolerate a wide range of temperatures, lighting, and a reasonable amount of stress. Because of their small size and short generation time, they are cost efficient to maintain in large numbers. The breeding can be timed and easily controlled by changing the light–dark cycle. The large number of embryos from a single batch of fish increases the statistical significance of the sample sizes for any kind of experiment. Moreover, the ability to have a large number of progeny from a single pair makes it easy to map mutations and cloned genes up to 0.1 cM in resolution.

The rapid external development of the embryos facilitates a number of technical manipulations such as microinjections, cell transplantations, microsurgery, and cell ablations. The transparent embryos are an excellent aid to developmental biologists studying the development from a single cell to a larva in 24 hours. The optical clarity and physical accessibility of zebrafish embryos make this an ideal system to exploit the advantages of
transgenic animals expressing fluorescent proteins [129]. By establishing transgenic lines under a tissue-specific promoter, it is also possible to label different cell types and visualize the expression of genes in real time [130].

The zebrafish model also has some pitfalls. The eggs develop outside of the body, and while there are advantages to this for the purpose of observing embryonic development, there are limitations when studying vertebrate maternal behavior or aspects of in utero development. It is a vertebrate, but in comparison to the mammals, it lacks specific organ structures such as the lungs, prostrate, and bladder [131]. This is due to specialized structures unique to the aquatic environment. The biology behind the different aspects of zebrafish behavior has also been insufficiently studied.

4.2. Genetic manipulations

The application of forward and reverse genetic approaches has helped to define and dissect particular pathways or processes without a priori knowledge of genes or their functions (Figure 2). Historically, the first forward genetic approach in microorganisms, bacteriophages and others helped to decipher the triplet genetic code [132]. Subsequently, it has been applied in Caenorhabditis elegans and Drosophila melanogaster, which are invertebrates, and Danio rerio, a vertebrate [133, 134]. Zebrafish genetics gained prominence following two large-scale mutagenesis screens, which provided the basis for the discovery of a multitude of new genes and pathways fundamental to vertebrate development [127, 128]. These studies exploited an enormous number of mutant strains, many of which were relevant for understanding a number of human diseases. The need for complementary reverse genetic approaches to enable relatively the straightforward reverse genetic manipulation of genes of interest arose. Technological advances have mainly occurred in gene knockdown mediated by anti-sense morpholino phosphorodiamidate antisense oligonucleotides (MOs), TILLING (Targeting Induced Local Lesions in Genomes), retroviruses, and transposons. Recently, mutagenesis and the induction of heritable genetic alterations with ZFNs (zinc finger nucleases) and TALENS (transcription activator–like effector nuclease) have opened up a new era in zebrafish reverse genetics [134]. Both the forward and reverse genetic methods facilitate the detailed study of gene function in development and disease. They have a final aim of revolutionizing the way gene function is studied and provide the basis for the
discovery of a multitude of new genes and pathways fundamental to vertebrate development. A comparison of both the strategies is represented below (Figure 2).

**Figure 2:** Comparison of the two different genetic manipulation strategies revealing the current technical advantages of using zebrafish to provide important new insights into vertebrate development and human disease.

### 4.2.1. Forward genetic screens

The forward genetic approach aims to identify genes involved in a biological pathway or process by screening populations of animals that contain random mutations throughout the genome that can alter the gene function. This approach has two key requirements: first, a well-defined genetic background of the organism, and secondly, a reproducible procedure to identify mutants of interest. In zebrafish, several mutagens have been tried and tested for this purpose. The initial research, which involved gamma radiation, resulted in large deletions, translocations and other gross chromosomal aberrations, which were difficult to identify for a mutant phenotype [135]. Alkylation agents, in particular N-ethyl-N-nitrosourea (ENU), were identified that could achieve high mutagenic loads, and the phenotypes could be linked to lesions in one gene [136]. Since then, ENU has been the standard choice for chemical mutagenesis [133]. Insertional mutagenesis by retroviruses has been applied, but with a very much lower mutagenic frequency than ENU [137]. In a few subsequent attempts, small-scale mutagenesis by transposable elements has also been applied [138]. Despite the significant efforts required to perform forward screens in zebrafish, researchers are continuing to develop and apply novel screening strategies to explore vertebrate development in greater breadth and depth.
4.2.2. Reverse genetic screens

In the reverse genetic approach, a known gene is disrupted to analyze the phenotype of the organism to understand the gene function. Due to the large size of the zebrafish genome, and also genome duplication, it is quite difficult to identify all relevant genes using forward screens alone. This can be achieved by four different methods, as described below.

A. Morpholino phosphorodiamidate antisense oligonucleotides (MOs)

The RNAi-mediated knockdown technologies have been problematic in zebrafish, with very few successful efforts [139]. Therefore, knockdown of gene function through the use of antisense morpholino oligonucleotides (MOs) has gained popularity [140]. The resistance to breakdown by nucleases and the ease of binding stably with RNA increases the penetrant gene knockdown effects [141]. MOs sequences are designed so that they function by forming heteroduplexes with the target transcript to interfere with protein synthesis or splicing by binding to the initiation start codon or splice acceptor sequences. MOs are injected into freshly fertilized eggs at the one- to two-cell stage to effectively block the gene function until 5 days post-fertilization (dpf), before becoming diluted to efficiently interfere with gene dysfunction [142]. The ease of use and rapid assessment of gene function are the biggest advantages of MOs, but proper controls are needed. Careful optimization of the dose and observation of the resulting phenotypes for the potential risk of off-target effects are the basis of a good MO design to avoid unspecific phenotypes [143]. With the proper controls and effective downregulation, MO-based knockdown is one of the most advantageous techniques the zebrafish has to offer.

B. TILLING (targeted induced local lesions in genomes)

This is a method combining forward and reverse genetics based on ENU-induced chemical mutagenesis to isolate mutants harboring point mutations in genes of interest. It was the first genetic approach in zebrafish to successfully yield germline mutations in a desired target gene. Mutations in target genes are sought by sequencing the target regions from genomes extracted from the mutagenized individuals [144]. This method involves a large degree of effort and investment. The major drawback of this method is that a mutation of interest that is identified in any given F1 fish is only one of many heterozygous mutations in its genome. To rule out confounding effects from linked mutations, more than one mutant allele should be characterized. Otherwise, mRNA should rescue the phenotype through the expression of a wild-type version of the allele or by a transgene. With the development of massively parallel
sequencing platforms, new avenues have been provided for rapidly screening TILLING libraries, which can dramatically increase the rate of discovery [145].

C. Retroviral and transposon-mediated mutagenesis

Insertional mutagenesis by retroviruses or transposons has been utilized to identify modified alleles of a target gene [137]. Retroviruses appear to cause mutations in zebrafish by several major mechanisms, including exon disruption and gene silencing caused by insertion into an intron. Such insertions either lead to a complete loss of the wild-type gene product or the reduction or complete abrogation of endogenous RNA expression [146]. This method has generated large numbers of genes important for vertebrate development, but the need to use a reporter gene, such as GFP, to visualize living embryos has directly emerged. The Tol2-based transposon system was developed to efficiently generate transposon insertions in the zebrafish genome [147]. Previously, the different methods to create transgenic fish were not as efficient as Tol2. The frequency of obtaining a founder fish following the injection of naked DNA was 5–9%, while the Tc3 transposon system gave a frequency of 7.5%, pseudotyped retrovirus expressing GFP 10%, the I-SceI meganuclease system 30.5%, and the sleeping beauty had an efficiency of 5–31% [147]. Together with gateway technology, co-injection of expression constructs containing transgenes flanked by the Tol2 elements with the transposase mRNAs greatly enhances the efficiency of transgenesis in zebrafish, including germ line transmission up to 50%, a higher frequency than with any other method [148]. This approach is quite useful to study gene function during normal vertebrate development and organogenesis.

D. Zinc finger nucleases (ZFNs)

The need for effective reverse genetic tools for creating targeted knockouts has been growing incessantly. Zinc finger nucleases (ZFNs) showed promise in improving the efficiency of gene targeting. ZFNs are engineered restriction enzymes that can be customized to cut a DNA sequence of interest. These work by introducing DNA double-strand breaks in target genes. This can then stimulate the cell's endogenous homologous recombination machinery and create gene modifications during subsequent repair [149]. The application of ZFNs against zebrafish genes results in an impressive >25% of offspring with germline modification of the exon targeted by the customized gene-specific ZFN [150]. The access to customized ZFNs is the current bottleneck in implementing this technique in zebrafish, and
the production of validated ZFN pairs is technically challenging and costly. This technology needs to be scaled up, and we are still unaware of whether all genes could be targeted by this method due to the complexity of DNA-binding domains. The ZFN-binding domain contains 3 to 4 DNA recognition modules, each of which recognizes 3 DNA bases. When all the modules are assembled as one DNA-binding domain, the specificity of DNA binding can decrease. Therefore, designing specific ZFN pairs that recognize specific loci of the genome is selective, making some genes unavailable for targeted knockout via ZFN [151]. There is a growing list of zebrafish lines produced by this method, but the method also has the potential risk of causing off-target effects [152].

E. Transcription activator-like effector nucleases (TALENS)

TALENs are based on similar principles as ZFNs and are emerging as new and efficient tools for gene-targeted mutagenesis [153]. TALENS use the same functional domains, the restriction enzyme domain and the DNA-binding domain. The transcription activator-like effector (TALE) sequence-specific DNA-binding domain proteins have been used for gene targeting in plant pathogenic bacteria [154]. The mechanism of action is as follows. When fused to the FokI nuclease domain (the same nuclease as used in ZFNs), TALE nucleases (TALENs) recognize specific DNA sequences using a straightforward DNA base recognition cipher. Binding of two TALENs to DNA allows FokI to dimerize and create a targeted chromosome break. TALENs can effectively recognize targeted loci in both somatic and germline cells in the zebrafish. For sites successfully targeted by ZFNs, TALENs appears more effective and readily able to induce cleavage and introduce subsequent mutations [153]. A recent paper reports that TALENs have fewer off-targeting issues than ZFNs [155]. Not all issues encountered by ZFNs have yet been completely investigated by TALENs. A recent advancement in this field has been by the usage of clustered, regularly interspaced, short palindromic repeats (CRISPR) along with the Cas protein system for genome editing in the zebrafish. The CRISPR-Cas system functions in vivo to induce targeted genetic modifications in zebrafish embryos with efficiencies similar to those obtained using zinc finger nucleases and transcription activator-like effector nucleases [156]. The efficacy of mutagenesis varies dramatically, and more characteristics of this system still need to be investigated [157].
4.3. Zebrafish models of human diseases

The large-scale mutagenesis screens identified an enormous number of mutants that resemble human clinical disorders. The challenges in other systems became an advantage for this fish model. Early embryonic study cannot always be re-capitulated in the mice, as they develop in utero. Drosophila and Caenorhabditis elegans can serve as powerful model systems for many biological processes, but they cannot address the development and function of vertebrate-specific features. The rapid progress of the zebrafish genome initiative is bringing this model system to its full potential for the study of vertebrate biology, physiology and human disease.

The zebrafish has been used to study cardiovascular defects, because the fish embryonic heart resembles the heart of the human embryo at three weeks of gestation [158]. Unlike the rodent model, the two-chambered heart does not need blood circulation for survival early during development. Defects in cardiac development or function are more likely to be recoverable and provide the first significant insight into the molecular basis of the defect. The condition ‘bradycardia’ is mimicked in a mutant slow mo, which has a slow heart rate [159]. The zebrafish mutant gridlock resembles the human congenital disorder coarctation of the aorta in both the location and effects of the lesion. Heart rate, oxygen consumption, and blood pressure have all been assayed in developing wild-type embryos. The blood circulation in zebrafish starts as early as 24 hpf, and the number and morphology (of blood vessels?) are easily visible under the microscope. This makes it amenable for the study of hematopoiesis [160]. The defective mutants can provide a better understanding of normal hematopoietic processes, and reveal important clues to the pathophysiology of stem cell disorders and leukemia. Many characteristics of kidney development and function in zebrafish are similar to those of higher vertebrates, thus providing a simple and accessible system for investigation. The zebrafish pronephric kidney is a relatively simple organ, consisting of two nephrons with fused glomeruli and paired, bilateral pronephric ducts [161]. Many mutants are reminiscent of human autosomal dominant polycystic kidney disorders [162]. As the zebrafish homologues of these genes are identified, their function can be dissected. The use of zebrafish can also be extended to study other organs such as the eye and brain, and various other vertebrate-specific and clinically relevant developmental processes. The implications of studies on the brain of zebrafish have been dissected in many neurodegenerative models, including PD (discussed below). Using reverse genetics techniques, transgenic fish have been
successfully generated, allowing for cell lineage tracing experiments, tissue-specific transgene expression, and transplantation studies that will provide invaluable clues to human pathophysiology [163].

4.4. Zebrafish models of Parkinson’s disease

Progress in understanding PD pathophysiology and in the development of treatments would benefit greatly from improved animal models. Due to the simplicity of zebrafish genetics and specific brain regions that are conserved and comparable to human counterparts, it is a good model to study the genetics of PD. Orthologs for genes such as pink1, parkin, lrrk2 and dj-1 responsible for Mendelian PD have been found in zebrafish. These genes have been studied by using methodologies such as MO knockdown or transgenic overexpression of mutants.

Recently, pink1 has been studied using C. elegans, in which a reduction in the length of mitochondrial cristae was observed. This species showed increased sensitivity to paraquat [164]. In the Drosophila pink1 model, mitochondrial deficits leading to the degeneration of flight muscles and DA neuron loss have been found [165-167]. These defects were not observed in the mouse model, and therefore could not reproduce the human symptoms[168]. Some of the observations in mice models also varied due to strain differences. In one of the pink1 knockout mice, impaired mitochondrial respiration and increased sensitivity to H$_2$O$_2$ and MPTP was recorded [169]. This mouse strain also showed heart defects, along with impaired dopamine release in the striatum [170]. In another pink1 deficient strain of mice, abnormalities in the serotonergic system were associated with gait alteration, and olfactory dysfunction was noted [4]. In a zebrafish model for pink1, loss of both the th1 and th2 transcripts was observed [142]. Some researchers have reported patterning differences and projection alteration without a reduction in TH cell numbers [171]. In the pink1 mutant, with a mutation in exon 7, no obvious phenotype was observed, but it had reduced numbers of DA neurons and reduced mitochondrial complex I activity [172]. In the medaka pink1 mutant, there was decrease in dopamine metabolites, but no dopaminergic cell loss was evident. Behavioral abnormalities were also observed in the mutants [173].

In the parkin model, a 20% loss of DA neurons in the vDC with increased susceptibility to MPTP was recorded. Reduced complex I activity similar to parkin patients was noted [54]. In the dj-1 model of the zebrafish morphant, reduced DA neurons were not seen, but they were more sensitive to H$_2$O$_2$ or to proteasome inhibitor MG132. They were also more susceptible
to programmed cell death [50]. The MOs for lrrk2 caused a significant loss of DA neurons in the vDC along with locomotor defects similar to human counterparts [174]. Several other loci have now been identified as PD susceptibility genes, responsible for rare forms of the disease [30]. Two of these have been identified in zebrafish, uchl-1 and GIGYF2 [175, 176]. Thus, the zebrafish is a good alternative model in elucidating the molecular basis of human neurodegenerative diseases.
AIMS OF THE STUDY

The purpose of this study was to understand the organization and involvement of the dopaminergic system in the zebrafish model of PD and use this species to study the mechanism underlying the genetic forms of PD caused by *pink1* dysfunction.

In particular, the study had the following goals:

1. Identification and expression analysis of the two different isoforms of tyrosine hydroxylase (TH) genes in zebrafish and their involvement in different PD models.

2. Characterization of the effects of *pink1* knockdown on larval zebrafish behavior and neural systems.

3. Identification of specific genes and pathways affected by *pink1* dysfunction using microarrays.

4. Detailed functional analysis of the genes and the pathways identified by the microarray analysis following *pink1* knockdown.

5. Creation of a *pink1* transgenic fish model and use of this to study the effects of oxidative stress in larval zebrafish.
MATERIALS AND METHODS

1. Experimental animals

Larval and adult zebrafish from the Turku line were used. The Turku line was originally obtained from a pet source and maintained in the laboratory for over a decade. It has been used in previously published studies [12, 142, 177]. Zebrafish were bred in groups and embryos were collected and staged as described by Kimmel et al. [178]. The permits for the experiments were obtained from the Office of the Regional Government of Southern Finland in agreement with the ethical guidelines of the European Convention.

2. Morpholino oligonucleotides (MOs) knockdown technique and RNA rescue

To knockdown the pink1 gene, two MOs (Gene Tools) were used. One targeted the splice site at the exon 3 and intron 3 boundary (MO1, TCACAACCTACCCGTTCAAAGTCAG) and the other targeted the 5′-UTR site (MO2, GAGAGGAAATCTGAAGGCTTTTACG). A standard control MO with no observed adverse effects (ctrlMO, 5′-CCTCTTACCTCAGTTACAATTTATA 3′) was used in all quantitative RT-PCR experiments. Other controls included the injection of water in place of MO as an injection procedure control and normal uninjected fish. A 4-nl volume of MO solution (25% phenyl red + MO in sterile water) was injected into the yolk at the 1–2-cell stage. Amounts of 8 ng MO1 and 4 ng of MO2 were used. The embryos that were injected with MO at the one-cell stage were screened for fluorescence a few hours post-injection. The embryos that showed fluorescence were selected and raised in petri dishes for further experimentation.

The pink1 full-length cDNA was prepared by RT-PCR. Total RNA was isolated from adult zebrafish brains using the RNAeasy mini kit (Qiagen). The first-strand cDNA was synthesized using 3 µg of total RNA and Superscript III reverse transcriptase (Invitrogen). The cDNA was then amplified with a Phusion High-Fidelity PCR Master mix (Finnzymes) using the forward primer (5′-TTGAATTCTCTATTTGCTC-3′) and the reverse primer (5′-ACATTAGATCTAGACTCTCTTGGGC-3′). The resulting PCR product was cloned into the pGEM-T Easy vector (Promega) and sequenced. The clone with no mutations was digested with EcoRI and the insert was cloned into the pMC expression vector [179]. Capped sense transcripts from the cDNA expression clone were generated by using the mMESSAGE
mMACHINE kit (Ambion). For the RNA rescue experiment, 500 pg of pink1 mRNA with 4 ng of MO2 or 8 ng of MO1 was coinjected into embryos at the one-cell stage.

3. In situ hybridization

The embryos were depigmented in 0.003% phenylthiourea (PTU) in E3 medium at 24 hpf. At 2 dpf, fish were fixed in 4% PFA in phosphate buffered saline (PBS) o/n at +4 °C. The fixative was changed the next day to 100% methanol and the samples were stored at -20 °C until further use [180].

For in situ hybridization, embryos were rehydrated in a 70%, 50%, 25% methanol series and washed twice with PBS with 0.1% Tween 20 (PBST). The specimens were treated with 10 µg/ml of proteinase K for 10 minutes and again treated with the same fixative for 20 min. After washing several times with PBST, the embryos were pre-hybridized in hybridization buffer (HB, 50% formamide, 5x SSC, 5 mg/ml yeast tRNA, 100 µg/ml heparin and 0.1% Tween 20 in DEPC water) at 70 °C for 2 hours followed by hybridization overnight with the riboprobe in the hybridization chamber. The specimens were washed the next day with 75% HB, 50% HB, 25% HB in 2 x SSC series following one wash with 2 x SSC and 2 times 0.2 x SSC for 30 minutes. For probe detection, the embryos were pre-incubated with a blocking buffer (PBST, 1% BSA and 1% sheep serum) for 2 hours at room temperature and then rocked overnight with 1:5000 anti-DIG antibody conjugated with alkaline phosphatase (Roche) in the blocking buffer at +4 °C. After several washes with PBST, hybridized probe was detected with a colorimetric detection method with 450 µg/ml NBT (nitro blue tetrazolium chloride) and 175 µg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) (Roche). The color reaction was stopped by several washes in PBST followed by infiltration with 30%, 50% and 70% glycerol. The samples were mounted in 90% glycerol and imaged with a Leica inverted microscope.

Probes for th1, th2, pax2a, dat, notch1a, and vegfs (vegfaa, vegfab and vegfc) were synthesized from different plasmids with specific restriction enzymes and polymerases, as described in detail in the publications. The digoxigenin (DIG)-labeled probes were generated with the DIG RNA labeling kit according to the manufacturer’s instructions (Roche).

Bright-field images were taken using a Leica DM IRB inverted microscope with a DFC 480 charge-coupled device camera using the multi-focus option, and z-stacks were processed with Leica Application Suite software and CorelDRAW 12 software (Synex).
4. Immunohistochemistry

Embryos of different ages were fixed with 4% PFA overnight at +4 °C. The samples were washed 3 x 30 min with PBST at RT followed by pre-incubation with PBST, 1% DMSO, and 4% normal goat serum (NGS) for 4 hours at RT. Anti-GFP, chicken IgY fraction (Invitrogen), was used for staining the transgenic lines at 1:800 dilution o/n in PBST with 2% NGS at +4 °C. The samples were washed 3 x 30 min in PBST followed by incubation with goat anti-chicken antibody at 1:1000 dilution o/n at +4 °C. The samples were then washed again 3 x 30 min in PBS followed by 2 x 60 min infiltration with 50% glycerol in PBS. Immunofluorescence samples were mounted in 80% glycerol and examined using a Leica TCS SP2 AOBS confocal microscope. For excitation, an argon laser (488 nm) was used. Emission was detected at 500–550 nm, as described earlier [12,17]. Stacks of images taken at 1.2-µm intervals were compiled, and the maximum intensity projection algorithm was used to produce final images with Leica Confocal Software. Pictures were then compiled in Corel Draw 12 software.

Rabbit anti-PINK1 (Cayman Chemicals, 1:1000), mouse anti-TH (1:1000), 5-HT (1:1000) and mouse anti-ZRF-1 ([181]1:500) antibodies were used. These antibodies have been applied in previous studies [12,142]. Anti-GFP, chicken IgY fraction (Invitrogen), was used for staining the transgenic lines at 1:500 dilution. To visualize lateral line neuromasts, 5-dpf larvae were incubated in 25 nM Mitotracker Red (Invitrogen) for 15 min at RT, rinsed, fixed in 4% PFA, and incubated with anti-acetylated tubulin antiserum (Sigma) diluted 1:1000.

5. Microarray and data analysis

RNA was isolated from wildtype and pink1 MO1 using the RNAeasy minikit (Qiagen). The quality of RNA was determined with a bioanalyzer and the RNA integration number (RIN) was determined. A RIN above 8 was considered for further processing. Hybridization of samples was performed on Agilent 4 x 44k microarrays, (Agilent Technologies) at the Biomedicum Biochip Centre, Helsinki, Finland according to the manufacturer’s recommendations. Agilent offers two versions specifically for zebrafish, and we used the two-color chip V1 array version specifically for zebrafish in this study. The V1, referred to as the first version, is the one whose contents are sourced from RefSeq, UniGene, TIGR, and UCSC Zebrafish.
Analyses were carried out with R 2.7.0 and Bioconductor 2.2 packages Limma, Category and GOstats (http://www.bioconductor.org/). First, data were background corrected using the normexp method [182] with an offset of 50, which ensures that all expression value estimates are positive. Before statistical analyses, replicate spots were averaged. Chemical dyes differ in their fluorescence at different excitation levels due to differences in their quenching properties. For a normalization method that can remove such intensity-dependent effects in the log2(ratio) values of the green and red dye used in the array, we carried out normalization using the loess method (locally weighted linear regression curve), as it removes dye unfairness in the two-color array experiment [183]. To find the differentially expressed genes, an empirical Bayes method was used. The Benjamini and Hochberg false discovery rate (FDR) was used to correct the p-values for multiple comparisons [184]. Genes with an FDR value of less than 0.05 were considered as differentially expressed. Hypergeometric tests were used in assessing whether differentially expressed genes were enriched into any KEGG pathways or GO ontology classes [185]. Genes in pathways or classes with a p-value less than 0.05 were considered significantly over-represented.

The raw normalized data set was used in the Ingenuity Pathways Analysis program (Ingenuity® Systems, version 8.7, http://www.ingenuity.com/) to identify significant pathways. Canonical pathway analysis in IPA was used to identify specific genes that are functionally significant and are present within the networks. Genes differentially expressed with a p-value < 0.01 were overlapped in the available networks developed from information contained in the ingenuity pathway knowledge base. In the current study, a score of 10 or higher was used to select highly significant biological networks.

6. RNA isolation and quantitative RT-PCR

All independent RNA samples were isolated from pooled embryos (30 embryos/sample) using the RNAeasy Mini Kit (Qiagen) as described above. RNA was reverse transcribed to produce cDNA from the samples using superscript reverse transcriptase-II and also superscript reverse transcriptase-III (Invitrogen) primed with oligo (dT) primers according to the manufacturer’s instructions for different experiments. The same superscript reverse transcriptase was used throughout for each individual experiment for comparability. SmartCycler instrumentation (Cepheid) was used to amplify the specific gene transcripts. The Q-RT-PCR analysis was performed with the SYBR green mix (Takara) as per the
manufacturer’s instructions in smart cycler tubes in a total volume of 25 µl. The composition of the reaction mixture was the following: SYBR green mix 12.5 µl, diluted cDNA 1–3 µl, primers at a final concentration of 1 µM. Primers for amplification were designed using Primer Express software v3.0 (Applied Biosystems).

7. Microscopy and image analysis

Light microscopic analysis was performed using a Leica DM IRB inverted microscope (Leica Microsystems) connected through a Leica DFC 490 Color Digital Camera to Leica Application Suite 2.7.0 R1 software. Fluorescence microscopy was carried out using a Leica TCS SP2 confocal microscopy system with an argon laser having a 488-nm laser line, a green diode laser with a 561-nm laser line, and a HeliumNeon laser with a 633-nm laser line. All double- or triple-stained samples were scanned sequentially to reduce crosstalk between channels. Images were obtained in 1024 x 1024 pixel format. Stacks of images were taken at 0.2-µm intervals. The maximum intensity projection was compiled from these using Leica confocal software. Figures were compiled with CorelDraw 12 software (Corel Corporation, Ottawa, Canada). Three-dimensional modeling was carried out using Imaris 6.0 software (Bitplane).

8. Reactive oxygen species (ROS) detection and heart rate measurement

The accumulation of ROS inside cells was detected with 2′,7′-dichlorofluorescin diacetate (H2DCFDA) (Sigma-Aldrich). This is a common fluorogenic dye and oxidant-sensitive probe that is oxidized by ROS to produce green fluorescent dichlorofluorescein[186]. It was used at a concentration of 5 nM for 30 min at 28 °C. At 1 dpf, 2 dpf, 3 dpf, and 5 dpf, ten individuals per group were taken and each set of experiments was repeated at least in triplicate. Fluorescence was observed using a Leica confocal microscope under 488-nm wavelength excitation after mounting the fish in 2% methylcellulose. For excitation, an argon laser (488 nm) was used. Emission was detected at 500–550 nm.

The heart rate was obtained from zebrafish larvae at 2 dpf and 6 dpf. It was measured by determining the number of beats per minute for each fish in the group (n = 25) under a stereomicroscope, repeated independently three times. The average value obtained from triplicate measurements was recorded as the number of beats per minute for each individual fish.
9. O-dianisidine staining

Hemoglobin (Hb) activity was detected in whole embryos using the o-dianisidine staining method [187]. Embryos at 1, 3 and 5 dpf were fixed with 4% PFA o/n and washed 3 x 30 min in PBS. They were then placed in freshly prepared o-dianisidine stain solution (40% ethanol with 0.01 M sodium acetate, 0.65% H₂O₂, and 0.6 mg/ml o-dianisidine [D-9143; Sigma, St. Louis, USA]) for 30 min in the dark with regular monitoring of the color development and then washed several times in PBS. The basis of this assay is that Hb catalyzes the H₂O₂-mediated oxidation of o-dianisidine, producing a dark red color in Hb-positive cells. The embryos were stored in 80% glycerol until they were imaged using a Leica inverted light microscope. Bright-field images were taken using a Leica DM IRB inverted microscope with a DFC 480 charge-coupled device camera, and z-stacks were processed with Leica Application Suite and Corel Draw 12 software. Dark red colored Hb-positive cells were counted only in the brain region of the embryos for quantitative purposes.

10. Catalase assay

Catalase activity was assayed by using the CAT 100 kit (Sigma-Aldrich, St. Louis, MO, USA). Larvae at 2 dpf were fresh frozen in liquid nitrogen and homogenized using RIPA buffer (Tris-HCl 50 mM, pH 7.4, 1% NP-40 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF) with one mini-complete protease inhibitor tablet (Roche) added in 10 ml of solution just prior to use. The homogenized sample was centrifuged at 16000 x g in a Spectrafuge 16M Microcentrifuge (Labnet systems, NJ, USA) for 30 min at +4 °C. The supernatant was collected and protein was measured using the BCA assay (Thermo Fisher Scientific, IL, USA). An equal amount of protein for each sample was taken while preparing the samples. H₂O₂ standards and the samples were made according to the manufacturer’s instructions and OD was measured at 540 nM with a Multiskan MS Plate Reader (Labsystems, Midland, Canada). The catalase activity was calculated as:

\[
\text{Activity} = \frac{\Delta \times D \times 100}{V \times t}
\]

where,
\[
\Delta = \text{difference in the amount of H}_2\text{O}_2 \text{ added to the colorimetric reaction between the blank and a given sample}
\]
\[
D = \text{dilution factor}
\]
\[
t = \text{catalase reaction duration}
\]
\[
V = \text{sample volume in the reaction}
\]
\[
100 = \text{dilution of aliquot from the catalase reaction in the colorimetric reaction}
\]
11. Behavioral analysis

The fluorescence-sorted embryos were dechorionated at 24 hpf and transferred to 6-well plates. Each well contained 30 embryos. Larval fish were then raised in the wells of 6-well plate containing 3 ml of E3 medium. MPTP (Sigma, St. Louis, MO) was added to the E3 medium at a final concentration of 50 µM at days 1–4 and replaced daily. Fish were tracked at 5 dpf and 6 dpf using a CCD camera connected to a computer and analyzed using Ethovision 3.1 software (Noldus Information Technologies).

The fish were placed in a 48-well plate with one fish and 1 ml of E3 in each well. The arenas were calibrated in advance, before placing the plate with the fish under the camera. They were analyzed for 10 mins at a sample rate of 5 frames per second. The total distance moved (cm), turn angle (degrees) and movement (percentage of time fish spent moving) were calculated from the coordinates acquired using EthoVision software. Before tracking, system noise was removed using an input filter of 0.2 cm for the minimum distance moved. Tracks were excluded where the sample size was less than 90% of the maximum sample size or if large reflections were detected [188]. All experiments were performed at room temperature and were replicated at least three times.

12. Tol2 mediated transgenesis

The region of interest with minimal promoter activity was designed at -2 kb upstream of the transcription start site of pink1. This was based on Tol2-mediated transgenic technology [148]. The primers used for amplifying the gene promoter were as follows: GGGGACCAAGTTTGTACAAAAAAGCAGGCTAATGATGCATCTCAGTCATTC as the forward primer and GGGGACCACCTTTGTACAAGAAAGCTGGGTCTTTACTGACATTTCACATTTTGAGCCAA as the reverse primer. The gateway cloning kit (Invitrogen) was used in this process due to its cloning efficacy for large inserts. The primers were designed to add attB sites to the final product to be inserted into the donor vector pDONR221 to perform the first reaction step of the gateway cloning kit. The clone was PCR verified and the insert was transferred into the destination vector pXIG-cfos-GW [163].

The transposase cDNA, used for injection, has been cloned in the pCS-TP vector. The mRNA was synthesized in vitro using the mMMESSAGE mMACHINE SP6 Kit (Ambion Inc.). This
mRNA has an SV40 polyA tail in the 3’ region. Next, 25 ng/µl of circular DNA with the gene of interest and 25 ng/µl of transposase mRNA were injected into the one-cell stage of fertilized eggs.

We outcrossed founder fish (F0) with wild-type fish and collected the progeny embryos (F1) with GFP under a fluorescence microscope. The GFP expression was visualized in the injected embryos, and three different founder lines differing in the levels of GFP expression were identified. Out of these founders, only one line was chosen and named Tg(pink1:EGFP) based on the maximum GFP expression pattern, which was crossed and grown up as the stable line of embryos (F2). These fish were grown until adults and experiments were performed on all the fish from the F2 generation onwards.

13. Chemicals

Hydrogen peroxide (H2O2) (Merck, NJ, USA) was used at a concentration of 5 µM in E3 medium for 20 mins to create an oxidative stress environment for the studies on wild-type and transgenic fish. L-Glutathione Reduced (LGR) (G4251) and N-acetyl cysteine (NAC) (A7250) (Sigma-Aldrich, St. Louis, MO, USA) were used at a concentration of 100 µM for 24 hours as the antioxidant treatment after H2O2 treatment [189]. The same dose was also used in the pink1 (MO1) morphant study.

14. Statistical analysis

Statistical analyses for Q-RT-PCR, heart rate determination, red blood cell counts, behavioral analysis, and catalase assays were carried out using GraphPad Prism 4.01 software (GraphPad Software). The unpaired t-test was used for single comparisons, and one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test was applied for multiple comparisons.
RESULTS

1. Gene duplication for tyrosine hydroxylase in the zebrafish: th1 and th2 (I)

The gene encoding tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, is highly conserved among different species and has been duplicated during vertebrate evolution. In mammals there is only one TH, while in teleost fish, two non-allelic genes have been found for TH [14]. We were the first to find two th genes in the zebrafish genome, orthologous to other teleosts: one located on chromosome 25 and the other on chromosome 4. It was, however, unclear whether the gene duplication occurred within the teleost lineage or as a result of whole genome duplication in ancestral vertebrates. A recent study revealed that duplication of the th genes is not only found in teleosts, but also in other non-eutherian vertebrates [190].

Expression levels of the th genes are differentially regulated during development, and expression was detected in multiple brain nuclei. The transcript levels of both the genes remain unchanged during the first 12 hpf, but th1 expression levels gradually increased above those of th2 from 5 dpf. The highest expression of th2 was found in the kidney and liver. The transcript expression of th2 in the adult tissues indicated a more peripheral function than in the brain. All previous studies using TH antibodies have only detected th1 expression and not th2, as has been found in colocalization studies [13]. Based on their immunoreactivity, TH populations have been numbered in an anterior to posterior direction in the brain of the zebrafish to help in identification and labeling. The groups and numbering are as follows: olfactory bulb and telencephalon (1, 2), pre-optic region (3, 4), diencephalon (5, 6, 11), pretectum (7), anterior paraventricular organ (8), intermediate paraventricular organ (9), posterior paraventricular organ (10), posterior tuberculum (12), caudal hypothalamus (13), locus coeruleus (14), internal reticular formation (15), caudal lobe (16) and commissural nucleus of cajal (17) [17]. Expression of th2 mRNA was detected by ISH and the cell groups have been numbered as the anterior (8b), intermediate (9b), and posterior parts of the paraventricular organ (10b) and the preoptic region (3b) (Figure 3b). This numbering of th2 cell groups was adopted in coherence with the th1 cell clusters. Some other research groups have used a different nomenclature for both the th1 and th2 in the zebrafish [190, 191]. The location of th2 cell groups has been verified by combining in situ hybridization for both of the transcripts. Immunohistochemistry for the TH antibody has also been performed in
combination with th2 in situ [13]. The differences that we detected for both forms of TH in expression, timing, pattern, and distribution are in agreement with the concept that TH genes are differentially regulated at the transcriptional and translational levels [14]. The conservation of the dopaminergic system between the mammalian system and the zebrafish has been illustrated below (Figure 3).

Despite the enormous variation in brain morphology and complexity among the vertebrate species, there are six main groups of catecholaminergic cells, out of which groups A9–A16 represent the DA cluster [192]. In zebrafish, the catecholaminergic populations are represented by a combination of groups of th1 and th2. Groups 1–17 represent the th1 group, and the complementary expression of th2 is represented by 3b, 8b, 9b and 10b.

![Figure 3](image)

**Figure 3.** Localization and comparison of discrete dopaminergic cell groups in the sagittal view of (a) the mouse and (b) the zebrafish brain. (a) A9–A16 DA clusters of the mouse brain (modified from [90])(b) Representative th1 and th2 cell groups in the zebrafish brain, color coded according to the expression pattern from dorsal to ventral [13].

2. Zebrafish Pink1 gene structure and expression (II and IV)

The pink1 gene is involved in the early onset form of PD, but its comprehensive function is still not yet understood. There are two specialized regions in the gene essential for PINK1 to function properly. These functional domains are highly conserved across species. The pink1 gene is located on chromosome 23 in the zebrafish genome. It is comprised of eight exons and encodes a 574-amino-acid protein with a predicted mass of 64 kDa. PINK1 shares 58.45% nucleotide and 56.61% amino acid similarity with humans. The mouse knockout
model did not show a critical phenotype, as in the human disease counterpart, which is why there came a greater need for other model organisms.

PINK1 expression has been found to be ubiquitous, but differences in the expression levels nevertheless exist in all tissues. By semi-quantitative RT-PCR, the expression of pink1 mRNA could be detected from as early as 3 hpf until adulthood. With a thorough analysis by real-time PCR, we found that the expression increases significantly more at 5 dpf onwards. At the tissue level, the transcript was found to exist in all organs. In the brain, the highest levels were recorded in the forebrain and midbrain. In situ results revealed pink1 gene expression in the brain, eyes, heart, liver, muscle and lateral line.

The PINK1-ir results demonstrated widespread expression in the brain gray matter. It was detected in comparable regions to the mRNA distribution. In the morphant discussed in detail below, PINK1-ir was abolished. It could be rescued by pink1 mRNA injection, proving that the polyclonal antibody was able to detect PINK1. As an additional means to examine pink1 expression, we created a transgenic reporter line -2 kb upstream of the transcription start site of the gene by using the tol2 transposition method. This line revealed a more specific promoter-driven expression pattern of PINK1. The prominent GFP expression showed the highest levels in the telencephalon, midbrain and rhombencephalon, continuing to the spinal cord. GFP-ir was also found in the heart, liver and muscle at 5 dpf. The transgene expression colocalized with the PINK1 antibody.

3. Characterization of Pink1 knockdown fish (II)

To study gene function in the zebrafish, a sequence-specific knockdown of gene expression is mediated by morpholino oligonucleotides (MOs). While this study was being carried out, another paper was published on the zebrafish model in which pink1 was knocked down. The fish showed greater variability and a severe phenotype, a series of observations surrounding unanticipated effects that were independent of the intended gene target. Gene knockdown by MOs is known to trigger off-targeting effects by p53 activation [143]. We therefore made two different MOs to check for the specificity and authenticity of the gene knockdown. By validating the data with controls for tp53 and Δ113 transcripts, we established that pink1 knockdown did not show highly deleterious phenotypic effects such as those previously reported [16]. One of our translational blocking MOs (MO2) showed a dismorphic external
phenotype that could be partially rescued by \textit{pink1} mRNA injection. However, the splice blocking MO (MO1) did not show any obvious external phenotype, as has been observed in mice knockout models and also in human disease counterparts. The fish were indistinguishable from the wildtype counterparts in their external morphology. Therefore, we proceeded with MO1, the efficacy of which could be quantified by RT-PCR [193] (Figure 4a). The tp53 and \(\Delta113\) transcripts were highly upregulated in MO2- but not in MO1-injected fish (Figure 4b and 4c). Therefore, only MO1 was used for all further experimentation.

\textbf{Figure 4.} Validating the efficiency of \textit{pink1} knockdown by MO. (a) Effective knockdown by MO1 as detected by RT-PCR. (b) Comparison of MO1 and MO2 by checking the tp53 transcript by Q-RT-PCR. \textit{Pink1} mRNA could not rescue the tp53 effect. Injecting p53 MO reduced the transcript, but not up to the control group level. (c) Only the MO2 group showed extensive up-regulation of the \(\Delta113\) transcript by Q-RT-PCR. All statistical comparisons with the control uninjected (UI) group were performed by one-way ANOVA with the Bonferroni post hoc test (* \(p < 0.05\) ** \(p < 0.01\), ***\(p < 0.001\)).

In \textit{pink1} morphants, we observed a clear reduction in \textit{th1}and \textit{th2} transcripts in the absence of any striking external phenotype. This was also evident in the \textit{in situ} results, and both of the phenotypes could be rescued by \textit{pink1} mRNA co-injection (Figure 5). The TH-ir, which mostly detects th1, showed that there was a reduction in cell groups of the pre-tectum (group 7) and in the diencephalic region (5,6,11) of the morphant as compared to the control. The \textit{th1} transcript also revealed a decline in the reaction in the ventral diencephalic cluster (Figure 5 A–C). For \textit{th2}, no antibody is currently available that could determine the protein expression pattern of all the groups. Therefore, we used only \textit{in situ} hybridization to locate the decline in the \textit{th2} transcript in the \textit{pink1} morphant. The decline was observed in groups 8b, 9b and the large 10b group in the hypothalamus. All the loss of \textit{th2} expression was rescued by \textit{pink1} mRNA (Figure D–F).
While the dopaminergic populations were exacerbated, we also analyzed possible changes in the other neurotransmitter systems. No significant change in *hdca* and *dat* transcripts was detected in the morphants. There was also no change in the immunoreactivity patterns for orexin, calretinin, ZRF-1, or NPFF (Neuropeptide FF). Histamine IHC could not be performed because the ir is better detected at 7 dpf, and by that time the MO effect becomes diluted. In summary, knockdown of *pink1* leads to specific effects in the dopaminergic neurons, while other neuronal subtypes remain unaffected.

**Figure 5:** *In situ* hybridization with probes for *th1* and *th2* in *pink1* morphants at 3 dpf. A–C: The decline in the *th1* transcript was recovered by *pink1* mRNA injection. D–F: The *th2* transcript could also be rescued by *pink1* mRNA.

### 4. Effects of Pink1 knockdown on MPTP toxicity (II)

A combination of environmental and genetic factors influences the etiology of PD, and we therefore examined whether *pink1* morphants are sensitized to MPTP. Loss of *pink1* generated significant sensitivity to damage induced by systemic MPTP treatment. The MPTP concentration to treat the morphants was selected after a careful dose-dependency analysis. MPTP was administered to the morphants at 50 µM from 1 dpf until 5 dpf. At this concentration there were no detectable alterations in the gross morphology, spontaneous locomotor activity, or TH-ir in the brain of normal zebrafish. Therefore, this was regarded as a sub-effective dose. After exposure of the *pink1* MO1 at 5 dpf to this concentration of MPTP, there was a significant decline in the TH-ir cell neurons in the pretectal population, but no significant decline in the cell group 5, 6, 11. For the other MO2, the decline in TH populations in the pre-tectal population and 5, 6, 11 were significant. Thus, TH-ir was sensitized by further addition of MPTP in *pink1* morphants.
5. Effects of MPTP along with Pink1 knockdown on fish behavior (II)

We examined the collective effect of *pink1* knockdown and MPTP treatment on larval motility. The *pink1* MO1-injected fish were subjected to MPTP from 1 dpf until 4 dpf. After one day of recovery, the swimming pattern was tracked and the total distance moved was calculated for each group. No changes were observed for MO1-injected fish, fish treated with MPTP alone, or the control group. Following MO1 injection and exposure to MPTP, the swimming pattern was disturbed and the total distance moved was considerably reduced. There was no alteration in the other parameters such as the turn angle, meander, or angular velocity measured during the behavior acquisition. This led us to conclude that a lack of *pink1* makes the fish highly vulnerable to sub-effective concentrations of MPTP that affect the locomotor activity, which was observed in the altered swimming pattern.

6. Pink1 function analysis and novel pathways (III)

A microarray study was carried out using the *pink1* morphants to identify the roles of key genes and pathways affected by the loss of *pink1* function. This resulted in identifying 177 genes that were significantly altered in *pink1* morphants compared to wild-type fish at a cut-off p-value < 0.05 using the program R version 2.7.0. The microarray data were filtered quite stringently for the fold-change and p-value, which are the most critical factors for successful data analysis. We validated the array results with qPCR on many independent samples and compared the fold change values between the two methods. The altered genes showed similar trends with both methods. Each gene expression alteration could also be rescued by *pink1* mRNA injection. This demonstrates the efficiency of the microarray results.

To find potential pathways affected by *pink1* knockdown, we used the commercially available Ingenuity pathway analysis program. This helped us identify a novel pathway that was affected by *pink1* dysfunction. The HIF signaling pathway was carefully scrutinized by using different independent assays and methods to determine whether only the transcription factor *hif1a* was affected or other targets of the same pathway were also altered. We have previously found that TH transcripts decline in *pink1* morphants. Experimental evidence from primary neuronal precursor cells suggested that HIF1 inactivation in the substantia nigra leads to a 41% reduction in the TH marker in the dopaminergic neurons [125]. While there was a decline in antioxidants such as *cat* and *sod2*, increases in erythropoiesis, ROS, *notch1a*, and VEGFs (*vegfaa, vegfab* and *vegfb*) were also observed. In the zebrafish genome, there are
still many gaps in the annotation that need to be curated. Following regressive curation, we could also be able to potentially map all other transcripts that were altered. The effect of pink1 knockdown on erythropoiesis and both TH transcripts could be rescued by the addition of antioxidants such as LGR and NAC. This was, to our knowledge, the first time that the phenomenon had been reported, and provided an important link to identify HIF signaling in the context of pink1 dysfunction. The HIF signaling pathway may now prove useful for drug development towards the prevention or cure of Parkinson’s disease. This suggests pathway-specific therapies may need to be developed for PD patients with diverse molecular etiologies.

7. Pink1 and neuroprotection (IV)

Many studies have suggested that PINK1 can protect neurons from apoptosis induced by oxidative stress and neurotoxins [194, 195]. To carefully monitor the in vivo expression of pink1, we generated a transgenic fish line using the Tol2 transposon, which expressed GFP under the pink1 promoter. This line was named Tg(pink1:EGFP) and was used to study the effect of oxidative stress on pink1 expression. The expression pattern of the pink1 GFP was similar to the in situ pattern in the zebrafish larvae. A very low concentration of H2O2 was chosen to mimic oxidative stress as in the environment in vivo. This concentration did not affect the survival rates or normal external phenotype of the larval fish. Addition of H2O2 increased the transgene GFP levels, which were detected in the brain of larval fish by GFP-ir. No change was observed in TH-ir in the same fish by double immunostaining. Another set from the same batch of fish was treated with the antioxidant LGR to observe whether any change occurred in the transgene levels. The increased transgene effect was rescued by the addition of the antioxidant LGR. The transgene expression level was almost comparable to the untreated samples. This experimental evidence suggests that pink1 is highly receptive to oxidative stress-mediated challenges, and this can be easily visualized in vivo by using this model.
DISCUSSION

Model organisms provide an important platform for the analysis of human disease processes and the development of therapies. The use of model organisms for the analysis of disease relies on the underlying conservation of pathways and processes between the organism and humans. Through the development of gene targeting via homologous recombination, the knockout mouse has become a paradigm of disease modeling. Many studies have highlighted the difficulty of recapitulating the pathology of human diseases in mice, for example[168,196]. *Drosophila* and *C. elegans*, being invertebrates, also have major drawbacks for this purpose. This problem has prompted the use of multiple model organisms to study disease in an effort to highlight conserved vs. organism-specific processes.

Understanding of the neurobiology behind the monogenic variant, Pink1, as a cause of PD was the main objective of this study. We reasoned that the zebrafish offers an economical and rapid alternative for testing and designing novel therapies for PD. Although they do not substitute the other mammalian models, zebrafish offer complementary benefits such as convenient genetic manipulation to assess the role of different genes and their regulation for better understanding of the disease. The work described here supports the use of zebrafish as a critical vertebrate species for the modeling of human PD mediated by Pink1.

1. Zebrafish genome and gene structure

Analysis of the zebrafish genome suggests that the zebrafish and human lineages have shared two rounds of whole genome duplication, and a third whole genome duplication occurred just prior to the teleost radiation. Therefore, for many genes in humans, there maybe two copies in zebrafish [197]. Analysis of a group of genes that is syntenic in humans tends to have orthologs that are syntenic in zebrafish. The evolutionarily distant human and zebrafish genomes share the shortest synteny blocks, which correspond to ancient essential *cis*-regulatory regions. The developmental genes in humans represent the largest blocks of synteny, and these are conserved all the way to teleosts. This establishes that these conserved blocks of synteny in the genomes of vertebrates are kept intact as a general principle of development and gene regulation [198]. The study of regulatory elements is possible with the help of enhancer screening and transgenic technology [130]. These regulatory elements of a gene, which are not regulated by the gene itself but by a target further away from the gene, can be studied by taking the conserved synteny into account.
Taking into consideration the conserved syntenies between zebrafish and mammals, candidate genes can be selected for disruption and studied by a myriad of genetic manipulations in zebrafish. Reciprocally, if the phenotype of a zebrafish mutation can suggest functions for a human gene, this can also be studied with cellular precision. This may provide an efficient way of assigning functions to essential orthologous human genes, which are only known by their sequence from the human genome project. In this way, the molecular and formal genetics of the zebrafish can contribute to functional human genomics.

2. Molecular and genetic characterization of zebrafish Pink1

2.1. Distribution of Pink1 in the larval and adult brain of zebrafish

The distribution of the gene in larval fish was studied by in situ hybridization with a pink1 full-length probe. The regions of strong pink1 expression during development at 5 dpf included the developing heart, liver, lateral line, and brain. These are areas that are particularly energy consuming. The mitochondria, which are also referred to as the powerhouses of the cell, support the cell with the requisite amount of energy via the electron transport chain [199]. PINK1 was shown to localize in mitochondria, thus suggesting an essential role of PINK1 in mitochondrial function [200]. Other areas of strong pink1 expression in the brain during embryonic development were found to be the telencephalon, thalamus, hypothalamus, midbrain, and rhombencephalon. Using PINK1-ir, we detected the expression of pink1 in almost all brain areas, but mostly in the gray matter. The white matter was devoid of any staining. This is consistent with the findings in rodents [201, 202]. In one study, PINK1 has been found in glial and endothelial cells [59], but the lack colocalization with the zebrafish zrf-1 antibody suggested that zebrafish PINK1 is absent in glial cells. In the larval brain, we detected mRNA as well as protein expression in the lateral line, which mainly functions in mechanosensation and the maintenance of sensory stimuli. Sensory abnormalities have been found in PINK1 heterozygote patients [45]. In summary, expression of pink1 in zebrafish closely resembles the expression reported in mammals.

2.2. Pink1 knockdown and knockout models

The main impact of Pink1 dysfunction in humans is Parkinson’s disease and the associated symptoms. Different model systems have been studied to recapitulate the disease progression in order to devise drugs that could cure PD or prevent disease progression. The common animal models that have been used to provide insights into the molecular pathology of
PINK1 mutations in PD include *C. elegans, Drosophila*, the mouse, and zebrafish. The findings have been summarized in a Table 2 below.

**Table 3.** PINK1 knockout and knockdown in different animal models

<table>
<thead>
<tr>
<th>Model Animal</th>
<th>Phenotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. elegans</td>
<td>Reduced mitochondrial cristae length</td>
<td>Sämann et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>Increased paraquat sensitivity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Axon pathfinding defects</td>
<td></td>
</tr>
<tr>
<td>Drosophila</td>
<td>Complete male sterility</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Downturned wing phenotype with rigidity and crushed thorax</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe locomotive defects (i.e., slower climbing speed and complete loss of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fitness ability)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduced dopamine levels in brain tissues</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Specific loss of DA neurons in 30-day-old flies</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased sensitivity to paraquat and rotenone</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitochondrial swelling in sperm, indirect flight muscles, and DA neurons</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased mitochondrial content and ATP levels in indirect flight muscles</td>
<td>Clark et al. (2006); Park et al. (2006); Yang et al. (2006)</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>Movement disorders (i.e., reduced swimming activity and impaired response to</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tactile stimuli)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No significant alteration in the number of DA neurons</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Disorganized patterning of DA neurons</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased susceptibility to MPTP</td>
<td></td>
</tr>
<tr>
<td>Medaka</td>
<td>Decrease in dopamine metabolite</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Locomotory defects</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No change in DA neuron numbers or dopamine levels in the striatum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased dopamine release under electrical stimulation in the striatum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impaired mitochondrial respiration in the striatum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Left ventricular dysfunction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pathological cardiac hypertrophy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impaired olfaction, gait and serotonergic innervation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased susceptibility to MPTP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Age dependent decrease in the DA content in the striatum and reduced</td>
<td></td>
</tr>
<tr>
<td></td>
<td>spontaneous voluntary activities</td>
<td></td>
</tr>
</tbody>
</table>

The main goal of all the models is to recapitulate the key symptoms of the disease. All these animal models help us to obtain insights into the mechanisms underlying particular symptoms of PD, allowing us to investigate new therapeutic strategies and, in addition, provide an indispensable tool for basic research. As PD does not arise spontaneously in animals, characteristic and specific functional changes have to be induced by the administration of toxins or by genetic manipulation. The toxin models along with the genetic models are used because the etiology and progression of the disease can result from a combination of genetic factors and environmental exposures.
2.3. Pink1 knockdown and MPTP toxicity

The discovery of MPTP essentially demonstrated that environmental agents could reproduce most, if not all, of the selective parkinsonian symptoms [64]. The relationship between neurotoxins and PD represents, in our view, an important step towards a better understanding of how genetic and environmental interactions may ultimately contribute to the pathogenesis of the disease. Thus, similarly to mammals, MPTP can produce significant dopaminergic neuronal loss in zebrafish [17]. Most toxins used in PD animal models inhibit mitochondrial function and reveal a greater susceptibility of dopamine neurons to mitochondrial dysfunction and ROS production. Many genes implicated in PD are directly or indirectly involved in mitochondrial function: PINK1, DJ-1, and possibly Parkin and LRRK2 are at least partly localized in mitochondria [3, 48].

In our study, a lack of pink1 increased the susceptibility of zebrafish to sub-effective doses of MPTP that affected the ventral diencephalic cluster of TH-ir. Therefore, it could be stated that the neurons in the ventral diencephalon of zebrafish are comparable to the mammalian nigrostriatal pathway [12, 15, 203]. This was the most vulnerable group to MPTP exposure as well as pink1 knockdown. While our study was being published, another report of MPTP in the pink1 -/- mouse model was identified. The pink1 -/- and knockdown mice were susceptible to MPTP and the effect could be rescued by the downstream genes parkin and dj-1[204]. This result showed that pink1 -/- mice were more susceptible to MPTP, and this was not due to developmental compensation, because the transient knockdown of pink1 mice also produced similar results. The loss of pink1 together with exposure to MPTP has similar effects on DA populations in both the rodent model and zebrafish. These findings in two different models clearly demonstrate that endogenous pink1 is an essential component that prevents DA neuronal loss in response to environmental stress. The mechanism by which pink1 regulates survival has not yet been identified. The zebrafish toxin-induced models and mutants generated by advancements in knockout techniques such as TALEN, TILLING, and ZFNs could enhance current knowledge of disease progression in PD. Together, these studies might greatly facilitate the screening of small molecules or drugs for therapeutic purposes.

3. Pink1, PD, and HIF-1 signaling

Microarray technology has been successfully applied to the simultaneous expression of many genes and to large-scale gene discovery. These advancements and technological innovations
have resulted in massive mining of biological data[205]. The quality of gene expression data obtained from microarrays can vary greatly depending on the platform and procedures used. Variability in both technical and biological procedures can have a great impact on the analyzed results. Precautionary measures must be taken in the experimental design to minimize irregularities and ensure replicability. The quality of RNA is essential to obtain accurate results. The RNA integrity number (RIN) should always be checked for purity, and RNA with a RIN value below 8 should be discarded, because carry-over contaminants can affect the qPCR and also the array results. Quantitative RT-PCR is a commonly used validation tool for confirming the gene expression results obtained from microarray analysis. Both methods have many pitfalls, but for reliable data, rigorous normalization and robust experimental design would be useful. Our generated data were comparable using both of the methods, demonstrating the careful filtering, normalization, and a good experimental design.

Current experimental and literature evidence has proved oxidative stress and impaired oxygen supply to be major players in the neurodegeneration process. This type of data has for the first time linked a genetic model of PD with HIF signaling. Such impartial high-throughput analysis of *pink1* knockout or knockdown has not yet been published. HIF-1 activity is impaired by MPTP treatment in the PC12 cell line and also in mice[206,207]. Addition of the iron chelators clioquinol and desferrioxamine increases HIF-1α and protects against nigral degeneration by MPTP [207, 208]. Previous studies on neurotoxin-induced PD models have been used to test for HIF-1 association and iron chelators for neuroprotection. Many lines of evidence have demonstrated that HIF-1 is involved in the pathogenesis of AD, amyotrophic lateral sclerosis (ALS), Huntington’s disease (HD), and a neurotoxin-based PD model [206]. Antioxidants such as LGR and NAC were found to be successful in providing neuroprotection in our *pink1* knockdown study. The increase in HIF-1 activity following the addition of antioxidants such as LGR and NAC could combat oxidative stress, increase the blood oxygen supply, increase the synthesis of dopamine, and block cell death signaling pathways. This raises the possibility that HIF-1 is a potential therapeutic target for neurodegenerative diseases such as AD, ALS, HD, and PD.

HIF-dependent gene expression can provide resistance to oxidative stress by regulating genes such as VEGF, erythropoietin, and MnSOD, which by themselves prevent oxidative stress-induced cell death [209]. Many different PD models have been linked to the downstream
effector genes of HIF signaling. Abnormal regulation of VEGF has been linked to neurodegenerative disorders such as AD and PD [210]. These findings have come from both in vitro and in vivo toxin models. In the toxin-based PD rat model with rotenone and 6-OHDA, VEGF-B is upregulated [211]. The neuroprotective effects of VEGFs should be considered as a potential disease modifying therapy for PD. Similarly, erythropoietin (EPO) neuroprotective effects have also been studied in the MPTP mice model [212]. While most of the genes were affected, some of the genes were also found to be unaffected by pink1 knockdown. The hdc and dat transcripts remain unaltered throughout the morpholino activity, as detected by Q-RT-PCR [142].

All the previous PD models have interpreted their results based either on toxin models or genetic models, but not in combination. Our results for the first time link a genetic model of PD to the HIF signaling cascade. Before these new results can be fully interpreted, and before a specific hypothesis can be formulated to guide future experiments, it will be essential to obtain a more complete picture of the changes in gene expression under multiple experimental conditions. Microarray technology must be associated with functional studies in an effort to identify specific and selective biomarkers and druggable targets, thus allowing the successful discovery of disease-modifying therapeutic treatments.

4. The significance of the Pink1 transgenic model

It is interesting that despite all the evidence for a role of oxidative stress in PD, relatively few studies have extensively characterized oxidative stress in animal models of PD. The transgenic models vary in the ease of manipulation and phylogenetic relatedness to humans, but they are still highly useful for research, especially into neurodegeneration [213]. Among the mouse models, the α-syn transgenic mouse is the best so far, because it can recapitulate the pathologies of synucleinopathy with protein aggregation and neurodegeneration [214]. Advances in mouse technologies provide unparalleled opportunities to refine or reinvent novel PD genetic mouse models, such as the Tet-off conditional transgenic approaches combined with the development of appropriate driver lines, and tamoxifen-sensitive Cre-dependent gene deletion with temporal and regional control [215].

The ease of creating transgenic fish in the zebrafish model keeps it on par with other known vertebrate and even mammalian models. Experiments can be performed on zebrafish in a
noninvasive way at much higher spatial and temporal resolution. Utilizing the advantages of the fish system, we have characterized the Pink1 promoter region and identified a region upstream of the translation start site where minimal promoter activity was found when injected into the zebrafish embryos. The transgenic line was created using the Tol2 transposon, a versatile and autonomously active transposon. Tol2 was identified in medaka fish, but has a greater transpositional activity in other vertebrates than medaka itself due to an extranuclear localization signal in the transposase protein [216]. This model could be used to study the endogenous function of pink1 in greater detail.

The transgenic systems have immense importance for understanding the pathophysiological basis of disorders. These genetically modified animals have been instrumental in understanding the molecular disease mechanism and to screen for therapeutic compounds [213]. The TAU transgenic fish has been a valuable tool in studying potent GSK3β inhibitors [217]. The GAL4 driver lines are being generated to mirror the expression patterns of endogenous genes in order to target desired temporal and tissue-specific transgenic expression [218]. Thus, the development of PD genetic models holds tremendous potential for testing novel approaches to treatment. In the case of genetic disorders, once the chromosomal loci are determined along with the pattern of inheritance, a transgenic system can be generated to model the different aspects of the disease. The transgenic systems have immense importance for understanding the pathophysiological basis of disorders that could mimic the human counterpart. These transgenic model organisms aid in identifying the cellular and molecular mechanisms responsible for disease phenotypes. They also provide a platform on which the efficacy and side effects of potential drug treatments can be evaluated. The development of PD genetic models holds tremendous potential for the testing of novel approaches.
CONCLUSIONS

A better understanding of the pathophysiology of PD is only possible with the development of reliable experimental models that can mimic disease processes with good reliability. A large number of animal models of PD have now been developed, and they have revolutionized PD research. Understanding of the etiology, pathogenesis, and molecular mechanisms of PD has improved, and new potential therapeutics have emerged. While cellular models have helped to identify specific events and signaling cascades, animal models, both toxin-based and genetic, have replicated almost all of the hallmarks of PD and are useful for testing new neuroprotective or neurorestorative strategies. The zebrafish, with a vertebrate biology and with easy methods of genetic manipulation, has become more feasible for research and comparable to mammals than invertebrate models.

Overall, the results in this work provided novel insights into the function of pink1. The main conclusions that can be drawn from this study are as follows:

1. The development of the catecholaminergic system in zebrafish has now been well characterized. The identification in zebrafish of a second non-allelic gene, th2, which has a complementary expression to th1, adds a new dimension to the future analysis of brain functions in this species. A lack of pink1 significantly affects both the th1 and th2 transcripts. This is consistent with human PD patients, in whom nigral dopaminergic cell loss occurs.

2. A lack of pink1 sensitizes the fish to sub-effective doses of MPTP. The loss of TH-ir was observed in the pre-tectum and vDC group (5,6,11). Major changes were observed in the two th transcripts. Both th1 and th2 were significantly downregulated. No significant change in the dat or hdc transcripts was observed in the pink1 morphants. The effects of both forms of th could be rescued by injection with pink1 mRNA, suggesting specific roles for pink1 in the development of these neurons. Pink1-ir was detected in most parts of the brain, and the data were in agreement with the in situ pink1 mRNA.

3. The gene list obtained by the microarray data analysis was validated by Q-RT-PCR. Using a commercial pathway analysis program, potentially important pathways were mapped. This revealed that the most important pathway was HIF signaling.

4. Using independent assays, the HIF targets such as VEGF, erythropoiesis, ROS, and notch were analyzed in the pink1 morphants. VEGF transcripts were altered in the pink1 morphants.
*Vegfaa* and *vegfab*, which are important for angiogenesis, were upregulated, while the other forms of vegfs were unaltered. Erythropoiesis was increased in the morphants, which could be rescued by the addition of antioxidants such as LGR and NAC. An increase in ROS and *notch1a* was also observed, and these were rescued by the injection of *pink1* mRNA. The observed decrease in *cat*, *sod2*, and *prdx3* transcript levels together with catalase activity suggests that due to the lack of *pink1*, the essential antioxidative enzyme systems were also compromised.

5. The *pink1* transgenic fish line was created using a promoter construct at -2 kb upstream of the translation start site. The GFP expression completely colocalized with the PINK1-ir. When the *Tg(pink1:EGFP)* fish were subjected to a very low dose of H$_2$O$_2$, an increase in the expression of the transgene was observed in the brain of the larval fish. This effect could be reversed by the addition of the antioxidant LGR. These findings indicate the development of a new tool to analyze *pink1* regulation under conditions relevant for disease mechanisms.

The mechanisms of these novel-signaling pathways would help in improvement of potential therapeutic targets for attenuation of the cardinal symptoms and motor complications in patients with Pink1 deficiency or PD as whole in the future.

![Figure 6](image.png)

**Figure 6.** A schematic diagram representing the data generated from the *pink1* knockdown studies using MO in zebrafish. Pink1 dysfunction affects the function of HIF. The targets of HIF are also altered in the morphants, concluding that HIF-mediated neuroprotective strategies could be important for the development of effective therapies to mitigate or prevent PD.
ACKNOWLEDGEMENTS

This work was carried out at the Institute of Biomedicine, Anatomy and Neuroscience Center at the University of Helsinki. The Finnish Graduate School of Neuroscience, Academy of Finland, Sigrid Juselius Foundation and grants from Finska Läkaresällskapet, Magnus Ehrnrooth Foundation, University of Helsinki Funds, Finnish Parkinson Foundation and University of Helsinki Chancellor Funds are warmly acknowledged for supporting this study.

I would like to extend my sincere thanks to my supervisor, Prof. Pertti Panula, M.D., Ph.D. I am fortunate to have him as a mentor who always had the time for listening to the little problems and obstacles that crop up during the course of performing research. Pertti taught me how to question thoughts, express ideas and build hypotheses. His positive outlook and confidence in my research inspired me and gave me confidence to explore new ideas. He has taught me innumerable lessons and insights on the workings of academic research in general.

I am grateful to the pre-examiners of my thesis, Dr Robert Cornell, Ph.D. and Dr Mikko Airavaara, Ph.D. for their constructive comments that improved the presentation and contents of this thesis. The thesis committee members, Prof. Heikki Rauvala, M.D., Ph.D. and Prof. Pentti Tienari, M.D., PhD., are sincerely thanked for their critical input and suggestions during the course of the study. I would like to thank my co-authors, Dr Yu-Chia Chen, Ph.D., Dr Jarno Tuimala, Ph.D., Dr Ville Sallinen, M.D., Ph.D., Dr Juha Kohlemainen, Ph.D., Dr Lori Orosco, Ph.D. and Gabiya Toleikyte, M.Sc., for their productive collaboration. I acknowledge my gratitude to Prof. Eero Castren, M.D., Ph.D. for his encouragement and support to walk through the last mile of my thesis. Dr Roy Sidall, Ph.D. is thanked for the English language revision of my manuscripts and the thesis in the nick of time.

During my thesis, I had the privilege to visit the lab of Prof. Marnie Halpern, Ph.D., Carnergie Institute of Science, John Hopkins University, Baltimore, USA. Marnie inculcated in me the passion and the beauty of zebrafish research. Mrs. Christine Pratt, whose hospitality was really commendable, made me feel at home away from home.

I would like to thank Henri Koivula and Reeta Huhtala for their tremendous technical help. I am deeply grateful to Yu-Chia for the collaboration, critical comments and vigorous long discussions that helped me sort out the technical details of my work. I have had a good time working in the lab with Saara, Maria, Jenni, Stanislav, Svetlana, Henri, and Shamsi and also with other past members of the lab Veera, Anna, Jenny, Susanna, Ville, Juha, Piotr, Hiroshi,
Luisa and Hisaaki. Special thanks go to Saara, Maria and Veera for their lovely company, trips and parties outside the lab. Katri and Outi are highly thanked for their timely help in administrative issues. I am grateful to Mikko for his help with the confocal issues.

I am sincerely thankful to all my teachers for their valuable contribution during the budding years of my life. I am indebted to Mr A.K. Pati, Mr. C.C. Das, Dr S.C. Das and Prof. A.C. Saklani for their continuous encouragement and guidance that had a major contribution during my study years. My childhood buddies Jigyansa, Monita, Himaja, Madhulita and Lipika are thanked for their wonderful friendship, which I will always treasure. I am grateful to my friends from India in Finland for the get-togethers and social functions that have always made things more jovial here. Deepti bhabhi and Preeti are specially thanked for their beautiful company and for all the big laughs and good times. A big thanks goes to Sinu bhai for his admirable spirits and never-ending enthusiasm. He made all my US trips wonderful and great fun.

My pillars of inspiration, Mummy and Bapuli, receive my deepest gratitude and love for their dedication and their invaluable support during my studies that provided the foundation for this work. My li’l sibling Bapu is cherished for being a loving and caring brother. It was a great adventure growing up with him. For the unending pampering and teaching the core values, I owe it to my loving grandparents, Aja and Aei. My Uncles (Kuna and Tuna) and Aunts (Mami, Chattu and Kunmuni) and their extended families are warmly thanked for showering their care, blessings and affection time and again. I am thankful to my sister-in-laws, Julie and Urmy, for many fun-filled moments. I warmly appreciate the love and affection of Baba and Ma.

I reserve my warmest and sincere gratitude to Julu for being my best buddy and a loving husband. His meticulous and critical assessment, tremendous support and encouragement were in the end what made this thesis possible. My bundle of joy Tanvi enriched the cheerfulness and happiness of our family. She is the one who has brought out the best in me.

I owe my gratitude to everyone and I will cherish this experience forever. Thank you from the bottom of my heart!!

Helsinki, 2013
REFERENCES


204. Haque ME, Mount MP, Safarpour F, Abdel-Messih E, Callaghan S, et al. (2012) Inactivation of Pink1 gene in vivo sensitizes dopamine-producing neurons to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and can be rescued by autosomal recessive parkinson disease genes, park or DJ-1. J Biol Chem 287: 23162-23170.


