Zebrafish as a model for the study of Parkinson's disease

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Preface

The work presented in this thesis is focusing on the study of Parkinson's disease using zebrafish as a model system, which has lead to the following manuscript:

- Chapter 2 is modified from the manuscript published as: Xi, Y., Ryan, J., Noble, S., Yu, M., Yilbas, A. E., Ekker, M., 2010. Impaired dopaminergic neuron development and locomotor function in zebrafish with loss of *pink1* function. Eur J Neurosci. 31, 623-33.

Statement of Contributions

The thesis, “Zebrafish as a model for the study of Parkinson’s disease” is composed of three related studies, all of which were collaborative efforts.

Chapter 2 is the result of experiments carried out primary by myself. Joel Ryan designed the swimming tracking system, and helped in performing touch stimuli and swimming tracking experiments and analyzing the data. Sandra Noble helped in Western blotting. Man Yu did the statistical analysis. Ayse Yilbas helped in immunostaining and confocal microscopy. The experiments were conceived by Dr. Marc Ekker and myself. I wrote the manuscript. Dr. Marc Ekker and Sandra Noble helped in revising the manuscript.

Chapter 3 is the result of experiments carried out primary by myself. I injected the morpholino into embryos and collected them at appropriate stages. Cindy Leggiadro helped in sample preparation for transmission electronic microscopy (TEM). Junhui Tan made cryosections for TEM and imaged them. Alborz Jooya helped in citrate synthase assay. The experiments were conceived by Dr. Marc Ekker and myself.

Chapter 4 is the result of experiments carried out primary by myself. Man Yu did immunostaining on sections. The experiments were conceived by Dr. Marc Ekker and myself.

Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder that is characterized by the degeneration of dopaminergic (DA) neurons in the substantia nigra and motor deficits. Although the majority of PD cases are sporadic, several genetic defects in rare familial cases have been identified. Animal models of these genetic defects have been created and have provided unique insights into the molecular mechanisms of the pathogenesis of PD. However, the etiology of PD is still not well understood.

Here, taking advantage of the unique features offered by zebrafish, I characterized the functions of *PINK1* (*PTEN-induced kinase 1*) gene, which is associated with recessive familial PD, in the development and survival of DA neurons. In zebrafish, antisense morpholino knockdown of *pink1* did not cause a large loss of DA neurons in the ventral diencephalon (vDC), but the patterning of these neurons and their projections were perturbed. The *pink1* morphants also showed impaired response to touch stimuli and reduced swimming behaviour. Moreover, the *pink1* knockdown caused a significant reduction in the number of mitochondria, as well as mitochondrial morphological defects such as smaller size or loss of cristae, thus affecting mitochondrial function. These results suggest that zebrafish *pink1* plays conserved important roles in the development of DA neurons and in the mitochondrial morphology and function.

To better follow DA neurons after injury or administration of toxins, I generated a transgenic zebrafish line, Tg(*dat:EGFP*), in which the green fluorescent protein (GFP) is expressed under the control of *cis*-regulatory elements of *dopamine transporter* (*dat*). In Tg(*dat:EGFP*) fish, all major groups of DA neurons are correctly labeled with GFP,

especially the ones in the vDC, which are analogous to the ascending midbrain DA neurons in mammals. In addition, we observed that the DA neurons in the vDC could partially be replaced after severe laser cell ablation. This suggests that zebrafish may have the unique capacity of regenerating DA neurons after injury.

Taken together, my studies suggested that zebrafish could be a useful alternative animal model for the study of the molecular mechanisms underlying PD and for the screening of potential therapeutic compounds for PD.

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List of Abbreviations

6-OHDA: 6-hydroxydopamine

CA: catecholaminergic

CNS: central nervous system

CS: citrate synthase

DA: dopaminergic

DAT: dopamine transporter

dpf: days post-fertilization

EGFP: enhanced green fluorescent protein

EM: electron microscopy

ENU: *N*-ethyl-*N*-nitrosourea

EOPD: early-onset Parkinson's disease

GFP: green fluorescent protein

hpf: hours post-fertilization

kb: Kilobases

kDa: Kilodaltons

LB: Lewy body

L-DOPA: L-dihydroxyphenylalanine

MAO-B: monoamine oxidase B

mis: mis-matched

MO: morpholino oligonucleotide

MPP+: 1-methy-4-phenylpyridinium

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MTS: mitochondrial targeting sequence

PAC: P1 artificial chromosome

PD: Parkinson’s disease

PINK1: PTEN-induced kinase 1

ROS: reactive oxygen species

SNc: substantia nigra pars compacta

TH: tyrosine hydroxylase

TILLING: target induced local lesions in genome

UPS: ubiquitin–proteasomal system

vDC: ventral diencephalon

VMAT2: vesicular monoamine transporter 2

ZFN: zinc-finger nucleases

Acknowledgements

I would first and foremost like to thank my supervisor, Dr. Marc Ekker, who gave me a great opportunity to mature scientifically. His constant support for my interests, the freedom to design experiments and always ready for my questions and discussions have gave me an incredible experience of graduate studies in his lab. I learned from him not only critical thinking on science but also positive attitude on everything. I truly appreciate everything he gave me in the last five years. I would also like to thank my thesis advisory committee members, Dr. David Park, Dr. Steve Perry and Dr. Shelley Hepworth for their invaluable advice and guidance during my study.

I would like to express my thanks to all the past and current members of Ekker lab for the pleasant memories and frequent helps. I thank Luc for his brilliant ideas and helpful discussions all the time. I thank Gary for his training and constant helpful advices on my experiments. I thank Vishal for his hard work to take care of my baby fish. I thank my "Parkinson" buddy, Sandra, for her always help and discussion on everything. I thank Joel for his help in behaviour experiments and now trying to save my precious fish in Montreal. I thank Stephen for his help on immunostaining experiments. I sincerely thank all my collaborators, Dr. Marie-Andrée Akimenko, Dr. Charles Darveau, Dr. M. Emdadul Haque, Dr. Kelly Soanes and Junhui Tan etc.

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Chapter 1

General introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is one of the most common neurodegenerative disorders and affects around 1% of humans over the age of 65 years (de Lau and Breteler, 2006). It was first described by a British physician named James Parkinson in 1817 (Kempster et al., 2007). The main clinical features of PD are motor disorders, including tremor at rest, bradykinesia, rigidity and postural instability. Non-motor symptoms, such as dementia, autonomic dysfunction and sleep disorders, also develop in many cases, especially in the late stages (Chaudhuri et al., 2006). The pathological hallmark of PD is the predominant loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) in human midbrain, which causes the neurotransmitter dopamine depletion in the striatum. Deficiency of dopamine causes decreased stimulation of the motor cortex by basal ganglia, and eventually results in motor disabilities. In more severe or late-stage PD, neuronal loss is found not only in the SNc but also in locus coeruleus, raphe nucleus, olfactory bulb and cerebral cortex. The widespread neurodegeneration in the central nervous system (CNS) might be responsible for the various non-motor symptoms of PD (Mizuno et al., 2008). Another hallmark is the presence of intracellular proteinaceous inclusions, known as Lewy bodies (LBs), in the remaining neurons. Whether the LBs are toxic or protective to neurons is still under debate (Lotharius and Brundin, 2002; Dauer and Przedborski, 2003).

The onset of PD is gradual. The earliest symptoms, such as the impaired dexterity, might go unnoticed for a long time. However, by the time clinical manifestations appear, about 60% of the DA neurons in the substantia nigra (SN) are already lost and the striatal

dopamine levels reduced to about 80% of wildtype (Dawson and Dawson, 2003). The median age of onset is 60 years and the mean duration of the disease from diagnosis to death is 15 years, with a mortality ratio of 2 to 1 (Katzenschlager et al., 2008). In North America, PD affects over 1.6 million individuals.

The current most effective PD drug is L-DOPA (L-dihydroxyphenylalanine), the biochemical precursor to dopamine. Treatment with L-DOPA helps relieve the motor symptoms by restoring striatal dopamine levels. However, it does not prevent or retard the degeneration of neurons in the brain (Dauer and Przedborski, 2003). Moreover, in the peripheral nervous system, L-DOPA is also converted into dopamine, which results in adverse side effects, such as dyskinesia (involuntary movements), particularly after several years of L-DOPA treatment (Dawson and Dawson, 2003). Recently, new treatments, such as cell replacement therapy, have emerged. Successful replacement of the degenerating DA neurons with dopamine-producing cell transplants has been explored (Wijeyekoon and Barker, 2009). The use of stem cells, especially induced pluripotent stem cells, might be promising in the future (Redmond et al., 2007).

1.1.1 Genetics of PD

The causes for most PD cases are unknown or not specific, and as such they are referred to as “sporadic” PD (Chade et al., 2006). A combination of aging, environmental and genetic factors is commonly postulated as the cause of PD. However, the detailed etiology of PD is still not well understood. 5-10% of PD patients exhibit a typical recessive or dominant Mendelian inheritance pattern. Over the last decade, linkage

analysis has identified 15 genetic loci that are associated to inheritable PD, designated as PARK 1-15 (Table 1.1). Eleven genes have been identified to carry mutations that are linked to familial PD patients. Some of these mutations are also implicated in sporadic PD. Functional characterization of these genes may provide critical clues to our understanding of the mechanisms of DA neuron death in PD. Following is a brief review of recent progress in analysis of the genes linked to familial PD.

α-Synuclein

Our understanding of the genetics of PD started with the mutations identified in α-synuclein. α-Synuclein is a small protein of 140 amino acid residues, and was originally identified as a presynaptic protein and involved in neuronal plasticity (Maroteaux et al., 1988). In 1997, Polymeropoulos and colleagues isolated an α-synuclein A53T mutation from an Italian kindred and three unrelated families of Greek origin with autosomal dominant inherited PD (Polymeropoulos et al., 1997). Similar clinical symptoms were also observed in sporadic PD patients with an A53T mutation (Spira et al., 2001). After that, other pathogenic point mutations in α-synuclein, such as A30P and E46K, were discovered (Kruger et al., 1998; Zarranz et al., 2004). PD patients with α-synuclein mutations showed widespread LBs pathology in the brain (Spira et al. 2001; Zarranz et al. 2004). Moreover, the genomic duplication and triplication of *SNCA* (the gene encoding α-synuclein) have been also identified to cause autosomal dominant familial PD (Chartier-Harlin et al., 2004; Ibanez et al., 2004; Nishioka et al., 2006; Ahn et al., 2008). Thus, α-synuclein might lead to PD pathology through both

Table 1.1 Summary of Parkinson's disease-associated genes.

Acronym	Locus	Inheritance	Gene	Function
PARK1/4	4q21.3-q22	Dominant	*SNCA*	Membrane trafficking
PARK2	6q25.2-q27	Recessive	*Parkin*	UPS, Mitochondria
PARK3	2p13	Dominant	Unknown	Unknown
PARK5	4p14	Dominant	*UCH-L1*	UPS
PARK6	1p35-36	Recessive	*PINK1*	Mitochondria
PARK7	1p36	Recessive	*DJ-1*	Oxidative stress
PARK8	12q12	Dominant	*LRRK2*	Membrane trafficking
PARK9	1p36	Recessive	*ATP13A2*	Lysosome
PARK10	1p32	Dominant	Unknown	Unknown
PARK11	2p37.1	Dominant	*GIGYF2*	IGF-1 signaling
PARK12	Xq21-q25	X-linked	Unknown	Unknown
PARK13	2p13.1	Dominant	*HTRA2/OMI*	Mitochondria
PARK14	22q13.1	Recessive	*PLA2G6*	Phospholipids hydrolysis
PARK15	22q11.2	Recessive	*FBXO7*	UPS

gain-of-function of mutant protein and over-expression of wildtype protein. In addition, nucleotide polymorphisms in the promoter region and in the 3'-region of *SNCA* have also been associated with risk for development of PD, suggesting that transcriptional regulation might also play a role in the pathogenesis of *SNCA*-associated PD (Maraganore et al., 2006; Mueller et al., 2005; Mizuta et al., 2006).

The major filamentous component of Lewy bodies in both familial and sporadic *SNCA*-linked PD is α-synuclein, suggesting that the aggregation of α-synuclein might be crucial for the pathogenesis of PD (Lee and Trojanowski, 2006). Wildtype α-synuclein has a high propensity to form insoluble fibrillar aggregates (Cookson, 2005). Pathogenic mutations seem to result in protein misfolding and accelerate fibril formation (Narhi et al. 1999; Conway et al., 1998, 2000). Fibrils have been shown to be toxic by increasing cell membrane permeability (Volles and Lansbury, 2002).

α-Synuclein is suggested to be involved in protein degradation through the ubiquitin–proteasomal system (UPS) (Rideout et al., 2001; Zhang et al., 2008). Several studies suggest that α-synuclein could also participate in the PD pathogenesis of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, a PD-inducing environmental toxin). MPTP-treated mice exhibited high levels of α-synuclein and promoted its aggregation (Vila et al., 2000; Gibrat et al., 2009). *SNCA*-null mice exhibited marked resistance to MPTP-induced DA neuron death (Dauer et al., 2002). In addition, several lines of *SNCA* transgenic mice have showed increased sensitivity to MPTP (Nieto et al., 2006; Yu et al., 2008).

The physiological functions of α-synuclein have not yet been fully understood.

α-Synuclein was shown to inhibit dopamine release by affecting exocytosis (Larsen et al., 2006). α-Synuclein was also shown to participate in modulation of tyrosine hydroxylase (TH) activation (Perez et al., 2002) and regulation of vesicular monoamine transporter-2 (VMAT2) activity (Guo et al., 2008). Recently, α-synuclein was found to be involved in blocking endoplasmic reticulum (ER)-to-Golgi vesicular trafficking and inducing ER stress followed by cell death (Cooper et al., 2006). Thus, these studies suggested that α-synuclein might play important roles in synaptic vesicle trafficking and modulate neurotransmitter release (Jensen et al., 1998; Li et al., 2004).

LRRK2

Mutations in *LRRK2* seem to be the most frequent cause of autosomal-dominantly inherited familial PD (Belin and Westerlund, 2008). LRRK2 mutations were discovered not only in familial PD but also in sporadic PD (Cookson et al., 2005). So far, more than 20 pathogenic mutations have been identified in the *LRRK2* gene, among which, the G2019S mutation is the most common one (Lu and Tan, 2008). PD patients with *LRRK2* mutations display neuronal cell loss, as well as Lewy bodies and good response to L-DOPA as sporadic PD; though some cases display pleomorphic pathologies including synuclein pathology, tau pathology and symptoms of motor neuron disorders (Khan et al., 2005; Giasson et al., 2006; Hasegawa et al., 2008; Santpere and Ferrer, 2009).

The *LRRK2* gene encodes a very large protein of 2527 amino acids with multiple functional domains, including ankyrin repeats, two Ser/Thr kinase and Roc GTPase domains, COR and WD40 domains, indicating that LRRK2 has complex multi-functions.

LRRK2 is widely expressed in the brain, including the SN, striatum and other regions related with PD (Higashi et al., 2007). Thus, LRRK2 seems to play important roles not only in the nigrostriatal pathway but also in other regions of the brain. LRRK2 has been shown to be present in membranous organelles and may play roles in membrane trafficking (Mandemakers et al., 2007). Some studies showed that LRRK2 regulates neurite processes and may function in axon guidance (Hatano et al., 2007). PD-associated LRRK2 mutations, including G2019S, I2020T, R1441C and T1348N, may alter its kinase activity, which may eventually contribute to PD pathology (Cookson et al., 2007). However, the detailed mechanisms and substrates for LRRK2 remain to be determined. Interestingly, LRRK2 seems to interact with *Parkin*, another PD-associated gene (Smith et al., 2005). Further studies may focus on how LRRK2 and other PD genes together result in DA neuron death.

Parkin

In 1998, Kitada and colleagues first found homozygous mutations in the *Parkin* gene in Japanese familial PD (Kitada et al., 1998). Further studies indicated that mutations in *Parkin* are the most common cause in autosomal-recessively inherited early-onset PD (EOPD). Around 50% of familial EOPD (< 40 years), as well as a significant proportion of sporadic PD, are due to homozygous or heterozygous *Parkin* mutations. Interestingly, Lewy bodies are generally absent in some of *Parkin*-associated PD cases (Belin and Westerlund, 2008).

Parkin encodes a 465 amino acid polypeptide with an N-terminal ubiquitin-like

domain and a C-terminal “Really Interesting New Gene” (RING) box domain, suggesting an important role for Parkin in the ubiquitin-proteasome degradation system (UPS). The UPS is a major pathway that is responsible for the removal of damaged or misfolded proteins in the cell. It was reported that Parkin has E3 ligase activity, which is responsible for the addition of ubiquitin to specific substrates and targeting them for degradation by the proteasome (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). It is hypothesized that protein degradation and accumulation are critical in the pathogenesis of the *Parkin*-associated PD (Hattori and Mizuno, 2004). In addition, Parkin is also suggested to be involved in the autophagic clearance of misfolded proteins (Olzmann and Chin, 2008; Tan et al., 2008). Parkin could be covalently modified by dopamine, resulting in the inactivation of its activity. This suggests a specific vulnerability of DA neuron to pathologic *Parkin* mutations (Farrer, 2006).

Recently, Parkin was shown to be critical in maintaining mitochondrial integrity and physiology. *Parkin*-deficient *Drosophila* have severely altered mitochondrial structure, as well as flight muscle degeneration and sensitivity to oxidative stress (Greene et al., 2003). Although *parkin*-null mice do not exhibit PD-like pathological features, some lines showed decreased mitochondrial respiratory capacity in the midbrain and increased vulnerability to oxidative damage (Palacino et al., 2004). Moreover, Parkin was shown to be selectively recruited to damaged or uncoupled mitochondria and mediate the clearance of dysfunctional mitochondria through autophagy, suggesting a major role for Parkin in the regulation of mitochondrial turnover (Narendra et al., 2008). Therefore, these studies support a critical role for Parkin in mitochondrial morphology and function.

PINK1 (PINK1 will be the object of a separate section 1.1.2 PINK1 and PD)

DJ-1

Mutations in *DJ-1* lead to very rare autosomal recessive EOPD (Abou-Sleiman et al., 2003). Pathogenic mutations include exon deletions and duplications, as well as homozygous or heterozygous missense mutations. These mutations are thought to cause the disease by a loss of DJ-1 function (Kubo et al., 2006). The *DJ-1* gene is comprised of 7 exons, and encodes a 189 amino acid protein, which is ubiquitously expressed in the brain and peripheral tissues. In the brain, DJ-1 is expressed in both neurons and glia, suggesting its important role in the cross-talk between glia and neurons (Bandopadhyay et al., 2004). DJ-1 is distributed mainly in the cell cytosol, and can be redistributed to the nuclei and mitochondria under stress (Belin and Westerlund, 2008).

Based on its structural features, DJ-1 belongs to the ThiJ/Pfpl protein family, which is always involved in protection against oxidative stress (Canet-Aviles et al., 2004). In fact, DJ-1 is known to protect against a variety of toxic oxidative agents such as MPTP, rotenone, 6-OHDA (6-hydroxydopamine) and paraquat. DJ-1 may exert its protection through up-regulation of the PI(3)K/Akt signaling pathway (Yang et al., 2005; Kim et al., 2005a, 2005b), or through the stabilization of the antioxidant transcriptional master regulator Nrf2 (Clements et al., 2006). In addition, DJ-1 can also act as a sensor of the cellular reactive oxygen species (ROS) level since it is known that oxidative stress can up-regulate the *DJ-1* promoter (Keyser et al., 2009).

Other genes

Mutations in *ATP13A2* were detected from families with autosomal recessive EOPD known as Kufor-Rakeb disease (Ramirez et al., 2006). These patients exhibit early-onset parkinsonism with additional features such as pyramidal signs and dementia (Di Fonzo et al., 2007). The function of ATP13A2 remains relatively unknown. The *ATP13A2* gene encodes a multi-transmembrane lysosomal ATPase. However, the pathogenic mutant proteins are largely localized in the ER instead of the lysosome. The mis-located proteins are degraded by the proteasome (Ramirez et al., 2006). Thus, it seems that the lysosomal dysfunction is the main cause for neuronal degeneration in *ATP13A2*-linked EOPD.

Mutations and polymorphisms in *HTRA2/OMI* are found both in inherited and sporadic PD (Strauss et al., 2005). HTRA2/OMI is a serine protease, located in the inter-membranous space of the mitochondria. It can be released into the cytoplasm during apoptosis (Suzuki et al., 2001; Martins et al., 2002). Some studies suggested that HTRA2/OMI is involved in the PINK1/Parkin pathway to protect neuronal cells from apoptosis when under oxidative stress (Plun-Favreau et al., 2007; Whitworth et al., 2008; Tain et al., 2009). However, other studies argued that HTRA2/OMI seems unlikely to be in the same pathway as PINK1 and Parkin (Yun et al., 2008).

A missense mutation (I93M) in the gene encoding ubiquitin carboxyl hydrolase L1 (UCH-L1) was found in a family with autosomal dominant inherited PD (Leroy et al., 1998). The clinical symptoms of *UCH-L1*-linked PD are similar to sporadic PD. UCH-L1 is mainly expressed in neuronal cells in the brain with de-ubiquitinating enzyme activity

(Wilkinson et al., 1989; Larsen et al., 1996, 1998). The I93M mutant of UCH-L1 has reduced enzymatic activity (Liu et al., 2002). Although the *UCH-L1*-null mice do not exhibit any PD phenotype, axonal degeneration was observed (Saigoh et al., 1999).

1.1.2 PINK1 and PD

Mutations in *PTEN-induced kinase 1* (*PINK1*) were discovered to cause PARK6 familial PD in 2004 (Valante et al., 2004). So far, more than 50 homozygous or heterozygous *PINKl* mutations have been identified and linked to familial PD (Mills et al., 2008). PINKl mutations are the second most common cause for autosomal recessive EOPD after Parkin. It was estimated that 1–8% of familial or EOPD was due to PINK1 mutations (Klein and Schlossmacher, 2007). In addition, PINKl mutations are responsible for a small but still considerable percentage of sporadic cases of parkinsonism. The clinical symptoms of *PINK1*-linked PD resemble sporadic PD, including slow progression, early onset and good response to L-DOPA. However, some patients showed atypical clinical symptoms such as dementia and dystonia (Ibanez et al., 2006).

Expression and subcellular localization of PINK1

PINK1 gene was originally cloned as a transcript activated by the expression of the tumor suppressor gene *PTEN* in cancer cells (Unoki and Nakamura, 2001). *PINK1* encodes a 581 amino acid protein with an N-terminal mitochondrial targeting sequence (MTS) and a highly conserved serine/threonine kinase domain (Mills et al., 2008). *PINKl* mRNA was expressed in all adult tissues with more abundance in the brain, heart, skeletal

muscles and testis (Unoki and Nakamura, 2001). In the human brain, *PINK1* mRNA is present in the hippocampus, substantia nigra and cerebellar Purkinje cells (Taymans et al., 2006; Blackinton et al., 2007). *PINKl* was found in all cell types, including neurons, glia cells, endothelial cells and smooth muscle cells of blood vessels (Gandhi et al., 2006). In addition, the PINK1 protein was detected in a small proportion (5-10%) of LBs in sporadic PD brain. However, the small percentage of PINKl-positive LBs suggests that PINKl is not a prerequisite to their formation (Gandhi et al., 2006). By Western blotting, at least two bands were detected using PINK1 antibodies: one band at around 63 kD presumably corresponds to the full-length PINKl, and another band at 53 kD is likely to represent PINKl protein without the N-terminal MTS (see below) (Muqit et al., 2006). The detail mechanisms of mitochondrial import and processing of PINK1 remain unknown.

The PINK1 protein contains an N-terminal putative MTS (Beilina et al., 2005; Muqit et al., 2006), which was confirmed to be sufficient for mitochondrial targeting. It was reported that PINK1 was localized in the mitochondrial intermembrane space in tissue culture, with the MTS already cleaved (Silvestri et al., 2005). A chimeric protein with a fluorescent protein fused to the N-terminus of PINK1 localizes to the mitochondria as well (Silvestri et al., 2005). The endogenous PINKl protein was initially reported to be colocalized with MitoTracker, a mitochondria-specific dye (Valante et al., 2004). Later, subcellular fractionations confirmed that endogenous PINKl is present in the mitochondria-enriched fraction from human and rat brains, while submitochondrial fractions and immunogold electron microscopy further revealed that PINKl proteins are

localized on mitochondrial cristae, predominantly the inner mitochondrial membrane (IM) (Gandhi et al., 2006).

PINK1 function

So far, the functions of PINK1 are still not well known, although some studies suggested the importance of its kinase activity for its mitochondrial function. In SH-SY5Y cells, over-expression of wild-type PINK1 seems to decrease staurosporine-induced apoptosis and siRNA knockdown of endogenous PINK1 increases vulnerability to rotenone and MPP+ (1-methy-4-phenylpyridinium, the toxic metabolite of MPTP) toxicity (Deng et al, 2005). It was also reported that over-expression of wild-type PINK1 protects the loss of mouse dopaminergic neuron in the striatum from MPP+ toxicity (Haque et al., 2008). However, the endogenous PINK1 present in the cytosol, rather than that in the mitochondria seems to be essential for its protective effects (Haque et al., 2008). In addition, over-expression of wild-type PINK1 blocks the release of cytochrome *c* during oxidative stress, which then inhibits the activation of downstream caspases (Petit et al., 2005). Recently, PINK1 was found to provide protection against oxidative stress by phosphorylation of the TNF receptor-associated protein 1 (TRAP1) (Pridgeon et al., 2007).

PINK1-null mice did not develop any dopaminergic neuron death or behavioral abnormality although decreased dopamine release and impaired mitochondrial functions were noticed (Kitada et al., 2007; Gautier et al., 2008). However, *PINK1*-mutant flies exhibit flight muscle defects, dopaminergic neuron degeneration and mitochondrial

dysfunctions (Clark et al., 2006; Park et al., 2006; Yang et al, 2006; Wang et al., 2006). The phenotypic differences between *PINK1* null flies and mice could be explained by species difference or compensation. Additional experiments are necessary for determining the compensating mechanisms in *PINK1*-null mouse and the evolutionarily conserved PINK1 function.

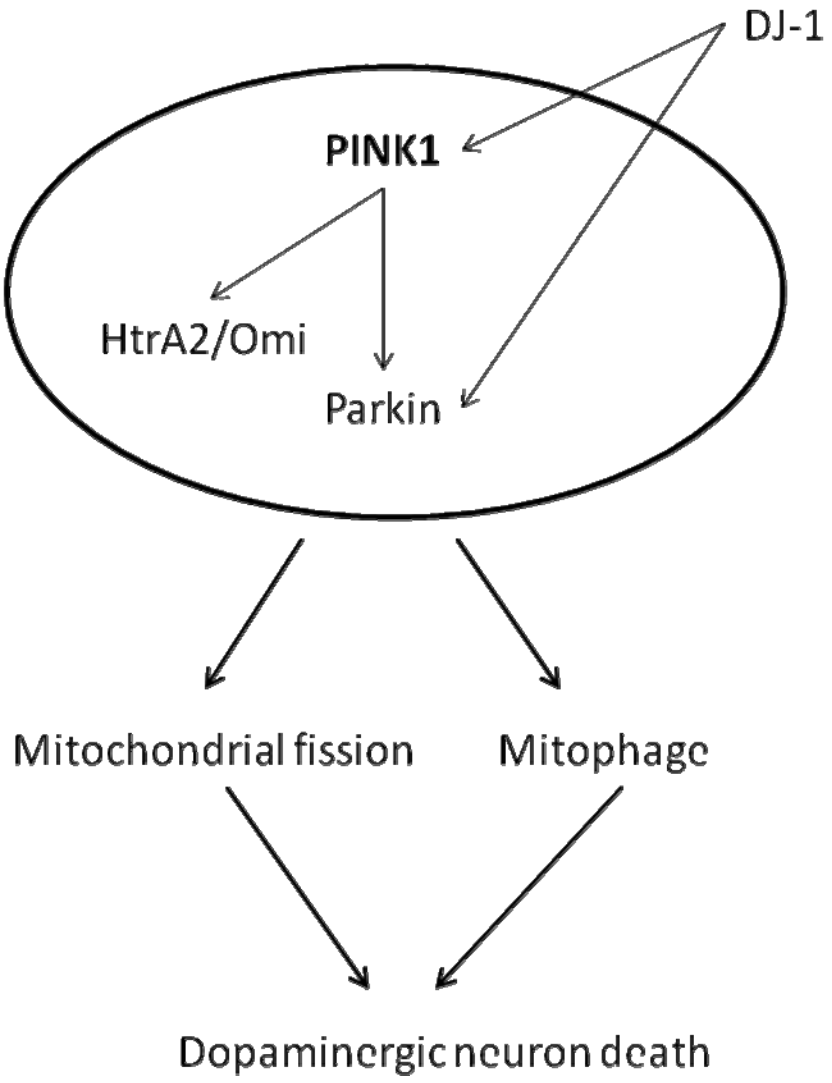
Potential mechanisms of PINK1 in PD pathogenesis

The kinase domain in PINK1 is homologous to the Calcium/Calmodulin serine/threonine kinase family. Most of the conserved mutations of PINK1 are within its protein kinase domain, resulting in truncated or mutated proteins possibly with loss or reduced kinase activity (Silvestri et al., 2005; Sim et al., 2006). The C-terminal region also seems to be critical for its kinase activity. Mutations in the PINK1 C-terminal region would impair its substrate selectivity (Mills et al., 2008). The interference of its kinase function is very likely to be the major cause of *PINK1*-linked PD. It was shown that PINK1 interacts and phosphorylates TRAP1, a mitochondrial chaperone, to protect against oxidative stress (Pridgeon et al., 2007). The phosphorylation of HtrA2, a mitochondrial serine protease, is also found to be modulated by PINK1 (Plun-Favreau et al., 2007). The potential mechanisms of PINK1 in PD pathogenesis are described in Figure 1.1. Further studies need to focus on the regulatory mechanisms of PINK1 kinase activity and the activities of its substrates, and how these correlate with DA neuronal degeneration.

The mitochondrial localization of PINK1 suggests an important role for PINK1 in the regulation of mitochondrial functions, the abnormality of which might be involved in

Figure 1.1 Potential mechanisms of PINK1 in PD pathogenesis.

PINK1 is a mitochondrial kinase in which mutations cause recessively-inherited PD. PINK1 and Parkin share a common pathway in regulating mitochondrial fission and mitophagy with Parkin at downstream of PINK1. PINK1 also modulates the phosphorylation of HtrA2. DJ-1 seems to regulate the stability of PINK1 and the E3-ligase activity of Parkin. Mutations in these PD-associated genes eventually result in DA neuron death.


DJ-1
PINK1
HtrA2/Omi
Parkin
Mitochondrial fission
Mitophage
Dopaminergic neuron death

PD pathogenesis. *PINK1*-deficient *Drosophila* exhibited mitochondrial defects leading to degeneration of flight muscle and mild to severe loss of DA neurons (Clark et al., 2006; Park et al., 2006). *PINK1*-null mice also exhibited impaired mitochondrial functions and a higher sensitivity to oxidative stress (Gautier et al., 2008). Interestingly, the phenotypes of *PINK1*-deficient *Drosophila* closely resemble that of *parkin*-deficient *Drosophila*. Over-expression of Parkin rescued the phenotypes in *PINK1*-deficient *Drosophila*, but PINK1 over-expression could not rescue *parkin*-deficient phenotypes (Clark et al., 2006; Park et al., 2006). This suggests that PINK1 and Parkin share a common pathway in regulating mitochondrial morphology and functions with Parkin at downstream of PINK1. Similarly, the abnormalities resulted from PINK1 depletion in HeLa cells can be rescued by over-expression of Parkin as well (Exner et al., 2007).

One possible mechanism to modulate mitochondrial function is through the dynamic regulation of mitochondrial morphology by fission and fusion. Several studies suggested that PINK1/Parkin regulate mitochondrial morphogenesis through promoting mitochondrial fission and/or inhibiting fusion. The mitochondrial abnormalities result from mutations in *Drosophila PINK1* and *parkin* were rescued by knockdown of *Mitofusin* (*Mfn*), *Optic atrophy 1* (*Opa1*) or over-expression of Dynamin-related protein1 (Drp1), which are associated with mitochondrial fusion and fission (Poole et al., 2008; Deng et al., 2008; Yang et al., 2008). Thus, PINK1/Parkin might be linked to PD pathogenesis by regulation of mitochondrial fission and fusion.

Recently, Narendra and colleagues demonstrated that Parkin is selectively recruited to dysfunctional mitochondria in mammalian cells, and then mediates the clearance of

mitochondria by autophagosomes (Narendra et al., 2008). These results suggest that Parkin may play an important role in the regulation of mitochondrial turnover by mitophagy (autophagy of mitochondria). In addition, the recruitment of Parkin to mitochondria is mediated by PINK1. PINK1 is accumulated to the damaged mitochondria and then signals Parkin to promote their elimination (Geisler et al., 2010; Narendra et al., 2010). Thus, the impaired quality control of mitochondria by PINK1/Parkin mutations might cause the pathogenesis of PD.

PINK1 is also linked to axonal outgrowth with critical roles in mitochondrial function. *PINK1* mutants of *C. elegans* exhibited defects in mitochondrial structure, ATP production and axonal outgrowth (Marongiu et al., 2009; Samann et al., 2009). PINK1 might also be implicated in transportation of mitochondria to the growth cones together with other mitochondrial proteins such as Miro and Milton (Weihofen et al., 2009). Poor axonal transport and mitochondrial dysfunction might trigger axon degeneration and eventually DA neuron death.

PINK1 has also been shown to associate with DJ-1, which seems to regulate the stability of PINK1 (Tang et al., 2006). It was suggested also that PINK1, Parkin and DJ-1 form a complex to promote the ubiquitination of the Parkin substrate. In this complex, PINK1 and DJ-1 might play a role in regulating the E3-ligase activity of Parkin (Xiong et al., 2009).

1.1.3 MPTP and PD

In most cases, the etiology of PD probably cannot be explained by a single cause, but by

a combination of both genetic and environmental factors. Exposure to agricultural chemicals, including pesticides (e.g. rotenone) and herbicides (e.g. paraquat), has been associated with an increased risk of developing PD (Tanner and Aston, 2000). Individuals who were exposed to pesticides had a 70% higher incidence of developing PD than individuals who were not exposed (Ascherio et al., 2006). An environmental hypothesis for the pathogenesis of PD was dominant during the late 20th century, especially due to the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism (Langston et al., 1983). The pathogenic rationale behind this hypothesis is the disruption of mitochondrial electron transport chain and increased ROS level, which eventually lead to the degeneration of DA neurons.

In 1982, after the use of synthetic heroin, a group of young drug users developed an acute and severe parkinsonism, which was due to MPTP produced during their illegal synthesis process (Langston et al., 1983). MPTP is highly lipophilic, and can be metabolized into MPP+, the active toxic molecule, by brain monoamine oxidase B (MAO-B). MPP+ is a high-affinity substrate for the dopamine transporter (DAT). The function of DAT, a specific marker for DA neurons, is to re-uptake excess released dopamine from synaptic cleft. MPP+ enters DA neurons via binding to DAT (Gainetdinov et al., 1997). Genetic deletion or pharmacological inhibition of DAT prevents MPTP-induced DA neuron degeneration, implicating the importance for DAT in relation to MPTP toxicity (Gainetdinov et al., 1997; Storch et al., 2004). Once in the neuron, MPP+ can remain in the cytosol, enter the mitochondria or be sequestered into vesicles by the vesicular monoamine transporter 2 (VMAT2) (Ramsay and Singer, 1986;

Liu et al., 1992; Klaidman et al., 1993). The diffusion of MPTP into mitochondria is driven by the transmembrane electrical gradient (Abou-Sleiman et al., 2006). Once inside the mitochondria, MPP+ binds to and inhibits complex I (NADH:ubiquinone oxidoreductase) of the electron transport chain (Nicklas et al., 1987), resulting in a decrease of ATP production and an elevated level of ROS. ROS, especially superoxide and hydroxyl radicals, result in oxidative stress and eventually DA neuron death (Nakamura et al., 2000). Over-expression of copper-zinc superoxide dismutase (SOD) in mice can prevent the MPTP-induced neuronal damage (Przedborski et al., 1992).

MPTP-induced parkinsonism is highly similar to PD with a preferential loss of DA nerve terminals at putamen and DA neuron loss at SN. MPTP-exposed humans and non-human primates showed successful response to L-DOPA treatment, and developed long-term motor defects (Beal, 2001). Studies using MPTP-treated animals have revealed many pathways that may play a role in PD pathogenesis. However, MPTP-induced parkinsonism is acute instead of chronic, and does not present the classic LBs although an increase in α-synuclein was observed (McCormack et al., 2008; Shimoji et al., 2005). In addition, MPTP toxicity is selective for DA neurons. The loss of neurons in MPTP-treated animals is not as widespread as the neuronal degeneration in human PD patients. Thus, the underlying pathways may not all be shared with those in PD patients (McCormack et al., 2008). So far, no therapeutic trials of chemicals based on MPTP models have been successful.

1.1.4 Animal models of PD

Studies of PD-linked genes and related neurotoxins have shed great light on the etiology of this disorder, revealing complex pathogenic mechanisms such as abnormal protein aggregation, disturbed kinase activity, impairment of ubiquitin-proteasome system, oxidative stress, mitochondrial dysfunction and mitophagy, etc. The overall effect of these various pathways is to produce the degeneration of DA neurons in SNc and cause motor defects. In this process, various animal models of PD have made tremendous contribution to our understanding of the pathogenesis of PD, primarily including mice, fruitfly (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*).

However, modeling PD in mice seems to be difficult. A number of *SNCA* (WT or mutants) transgenic mice have been created using different driven promoters, but none of these models accurately represent PD. Although functional abnormalities in the nigrostriatal system were noticed, there was no progressive loss of DA neurons (Chesselet, 2008; van der Putten et al., 2000; Giasson et al., 2002; Lee et al., 2002; Neumann et al., 2002; Rockenstein et al., 2002; Martin et al., 2006; Norris et al., 2007; Freichel et al., 2007). *LRRK2* (WT or mutants) transgenic mice also have minimal evidence of neurodegeneration (Li et al., 2009; Li et al., 2010). Neither *parkin*-null, *PINK1*-null, *DJ-1*-null, nor even double or triple-knockout mice exhibited any DA neuron degeneration and behavioral abnormalities (Itier et al., 2003; Von Coelln et al., 2004; Perez and Palmiter, 2005; Kitada et al., 2007; Gautier et al., 2008; Gispert et al., 2009; Goldberg et al., 2005; Kim et al., 2005b; Palacino et al., 2004; Kitada et al., 2009). The reason for the lack of DA neuron degeneration in mice PD models might be that there are compensatory mechanisms preventing the loss of DA neuron in the mouse.

Understanding these compensatory mechanisms would provide important clues for therapeutic treatments. Future studies may utilize conditional over-expression or conditional knockout techniques to bypass the compensatory mechanisms.

Drosophila and *C. elegans* seem to be powerful models for PD, but also have their limitations. Over-expression of α-synuclein (WT or mutants) or LRRK2 in *Drosophila* and *C. elegans* both resulted in selective DA neuron loss (Feany and Bender, 2000; Pesah et al., 2005; Lakso et al., 2003; Kuwahara et al., 2006; Liu et al., 2008; Ng et al., 2009; Venderova et al., 2009; Saha et al., 2009). *Parkin*-deficient or *PINK1*-deficient *Drosophila* exhibited similar phenotypes with a mild loss of DA neurons and mitochondrial defects (Greene et al., 2003; Whitworth et al., 2005; Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Transgenic RNAi-knockdown of *DJ-1* also led to the loss of DA neurons (Menzies et al., 2005; Meulener et al., 2005; Park et al., 2005; Yang et al., 2005). However, the only abnormality shared by PD patients and *Drosophila PINK1* and *parkin* mutants is the loss of DA neurons. *Drosophila* and *C. elegans* are evolutionarily too far away from human, and lack the complexity of vertebrates. Thus, their value mainly lies in identifying evolutionarily conserved genetic pathways and screening for pharmacologic modifiers of the key factors in these pathways. So it is necessary to model PD using new vertebrate models, for which zebrafish (*Danio rerio*) is a good candidate.

1.2 Zebrafish as a model for PD

The zebrafish has been widely used as a model for the study of developmental biology.

One major reason for its popularity is that, not only it is a vertebrate and thus closer to human than invertebrate models, but also it has several advantages over other vertebrate models. These include high fecundity (a few hundred eggs per spawning) and transparent and externally developing embryos. Zebrafish can be easily visualized and experimentally manipulated. Their generation time is short (2-3 months). The zebrafish genome is being sequenced and genomic data readily accessible. Various new biological tools are being created for zebrafish researchers, including forward and reverse genetics techniques. All these features, previously found mainly in invertebrate models, facilitate genetic and high-throughput functional studies.

Zebrafish have also proven to be a particularly relevant vertebrate model for the study of human diseases. Compared to other vertebrate models, screens for zebrafish mutants are less costly and easier to perform. Large-scale mutagenesis screens have been performed in zebrafish (Driever et al., 1996; Haffter et al., 1996; Amsterdam et al., 1999). Some of the mutants identified in these screens are reminiscent of human diseases and made great contributions to our understanding of human biology. For example, a gene responsible for human skin color was recently revealed from the study on a zebrafish ”*golden*” mutant (Lamason et al., 2005). It is estimated that more than 30,000 genes exist in zebrafish genome. This implies that more mutants remain to be discovered. Zebrafish mutants have allowed a better understanding of various human conditions including blood disorders, aging, muscular dystrophy and autism, etc (Paffett-Lugassy and Zon, 2005; Keller and Murtha, 2004; Guyon et al., 2007; Tropepe and Sive, 2003).

1.2.1 Methodologies for zebrafish research

Gene knockdown/knockout

The straightforward and yet powerful experimental approaches based on microinjection of antisense morpholino oligonucleotides (MOs) into one-cell stage embryos is commonly used to temporarily silence zebrafish genes during the first few days of embryonic development. MOs are chemically modified oligonucleotides that can specifically bind to their target mRNAs with more stability and resistence to endogenous degradations. MOs are normally 25 nucleotide-long with morpholine rings in their backbones instead of the deoxyribose rings in DNA or ribose rings in RNA. MOs carry A, C, G or T bases positioned according to Watson-Crick base pairing. MOs contain uncharged phosphorodiamidate intersubunit linkages instead of the anionic phosphodiester linkage found in DNA or RNA. Their hybridization to mRNA can block translation when they are targeted to sequences near the translation initiation codon. Alternatively, they can block splicing when targeted to exon-intron boundaries. This method has proven to be simple, rapid, specific and effective (Eisen and Smith, 2008). The effectiveness of MOs can be evaluated using Western blotting (for translation-blocking MO) with specific antibodies against the protein of interest or Reverse Transcription-PCR (for splice-blocking MO) with primers spanning the altered splicing region. There are some limitions to the use of MOs. Firstly, the effectiveness of MO can only last 3-5 days post-fertilization (dpf). Due to the quick cell proliferation during embryogenesis, the injected MO is too diffused to effectively bind to their cellular targets after 5 dpf. Secondly, MOs can induce some phenotypes resulting from their

toxicity and off-target effects. Cell death is one of the typical phenotypes from non-specific MO effects. Commonly, two different MOs (either translation-blocking MO or splice-blocking MO targeting different regions of the mRNA interest) are used to define the shared phenotypes, which may be the MO-specific effects. Standard control MO and custom control MOs are routinely used to isolate the MO off-target effects. Standard control MO targets a region on a mutated human *β-globin* pre-mRNA, and have no target and no significant biological activity in zebrafish. Custom control MOs inculdes invert-antisense MO, sense sequence MO and mis-pair control MO, which have nearly the same sequence as the antisense MO sequence but incorporates five mispairs along the length of the oligo. Rescue of MO-induced phenotypes by injection of mRNA (with silent mutations in the target region of the antisense MO) encoding the protein of interest can also help to establish whether the phenotypes are MO-specific. Rescue with the corresponding human wildtype or mutant mRNA can address the evolutionarily conserved functions of the gene of interest and help understand the functional effects of mutations implicated in disease pathogenesis.

Due to the limitations of MO strategy, various other approaches to induce stable loss of function have been developed. So far, the conventional gene targeting method, used in mouse embryonic stem cell lines, is not suitable for zebrafish since there are no proper embryonic stem cell lines available for zebrafish. The alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) has been used in several large-scale mutagenesis screens (Mullins et al., 1994; Driever et al., 1996; Haffter et al. 1996). ENU can result in point mutations on a single DNA strand. Therefore, if induced in premeiotic sperm cells, the

ENU-induced mutations become fixed in both strands through subsequent DNA replication, and result in non-mosaic progeny. Then the mutated site for the phenotype of interest can be identified by positional cloning. Alternatively, mutations in a specific gene can be isolated by TILLING (target induced local lesions in genome), which was first used in *Arabidopsis* (Wienholds et al., 2003; Moens et al., 2008). Although the ENU and TILLING stategies have yielded some extremely useful stable mutant lines, some genomic sequences seem to be more susceptible to ENU than others, and TILLING seems to be more laborious and less efficient than initially hoped.

Recently, engineered zinc-finger nucleases (ZFN) have emerged as another promising technique for generating loss of function mutations in zebrafish genes (Doyon et al., 2008; Meng et al., 2008b; Foley et al., 2009a). ZFNs are artificial restriction endonucleases with a non-specific nuclease domain fused to zinc-finger arrays which can be engineered to recognize specific DNA sequences of interest. ZFNs are able to bind to a particular target sequence and induce a double-strand break (DSB) in the genome. The DSB is later repaired by nonhomologous end joining (NHEJ) which is known to generate small insertions and deletions. Studies showed that ZFN can induce mutations in the zebrafish genome with high efficiency.

Transgenesis

Due to its features of transparency and external development, another widely used technique in zebrafish is transgenesis, with a fluorescent reporter expressed under the control of a tissue- or cell type-specific promoter. Transgenesis can also be used to

over-express specific genes or their mutated versions in specific tissues or cell types to evaluate their pathological gain-of-function or dominant-negative effects. Microinjection of plasmid DNA into the cytoplasm of one-cell embryos is traditionally used for transgenesis in zebrafish (Stuart et al., 1988). However, the efficiency of this method is low due to the fact that transgenes are prone to form concatemers prior to integration. The integrated concatemers are unstable, and easy to be lost in subsequent cell divisions. The recent introduction of *I-SceI* meganuclease or of *Tol2* transposons greatly increased the efficiency of transgenesis in zebrafish (Thermes et al., 2002; Kawakami et al., 2000). *Tol2* is a transposable element that was identified in the genome of the freshwater fish medaka (Koga et al., 1996). The *Tol2* element is 4.7 kilobases (kb) in length, consists of the left and right ends as well as a middle region encoding a fully functional transposase, which can autonomously catalyze the transposition of the *Tol2* element. Deleting the transposase-coding region while retaining the *Tol2* left and right ends yields a non-autonomous element, which could efficiently insert into the genome of zebrafish when the transposase is present (Kawakami et al., 2000). A relatively high efficiency of transgenesis can be obtained by cloning a transgene expression cassette between the *Tol2* ends and co-injecting it with the transposase mRNA into one-cell embryos. Thus, only a small number of founder fish are needed to screen for stable transgenic lines. *Tol2* integrates as a single copy through a cut-and-paste mechanism, and it does not cause any rearrangement or modification at the target site (Kawakami, 2004).

1.2.2 Dopaminergic system in zebrafish

Anatomical similarity between zebrafish brain and human brain

Although there are some notable differences in structure and scale between the zebrafish brain and that of human, the overall organization shows similarities. Specific key regions of the zebrafish brain can be related to their human counterparts and are often strikingly conserved. For example, the zebrafish ventral telencephalon is suggested to be homologous to the striatum in human (Rink and Wullimann, 2001, 2002). In fact, this area is rich in dopaminergic nerve terminals. Retrograde tracing experiments in adult zebrafish brain found that DA neurons projecting to the ventral telencephalon are located in the posterior tuberculum of the ventral diencephalon (vDC). Thus, zebrafish DA neuron clusters in the vDC are analogous to the ascending midbrain DA neurons of the mammalian nigrostratial pathway (Rink and Wullimann, 2002), which was strongly confirmed in subsequent studies (refer to 1.2.3 Current progress in zebrafish PD models).

Development of the zebrafish DA system

Zebrafish embryos develop rapidly and the CNS is well established by 3 dpf (Kimmel et al., 1995; Kuwada et al., 1995). The DA system has been characterized during embryogenesis in zebrafish based on the expression patterns of tyrosine hydroxylase (TH, the first and rate-limiting enzyme in dopamine biosynthesis, and marker for all catecholaminergic neurons) or dopamine transporter (DAT, specifc marker for DA neurons) (Holzschuh et al., 2001; Rink and Wullimann, 2002; Ma, 2003). DA neurons are first detected at about 18 hours post-fertilization (hpf) in a cluster of cells in the vDC. At 72 hpf, development of the zebrafish central nervous system (CNS) is well advanced. DA

neurons are found mainly in the olfactory bulb, preoptic region, pretectum, retina and vDC (Fig. 1.1). DA neurons do not develop in the zebrafish mesencephalon, which is a major difference between fish and mammals. Study showed that this might be resulted from a caudal-ward shift in dopaminergic activity during evolution from fish to mammals (Smeets et al., 2000). Recently, another *th* gene (*th2*) was discovered and characterized in zebrafish (Candy and Collet, 2005; Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010). The appearence of *th2* in zebrafish genome may be due to the whole genome duplication event during evolution. The *th2* gene starts to be expressed from late larval stages and appears to be expressed in the posterior preoptic area, the paraventricular area, and the caudal hypothalamus. However, further studies are necessary to determine whether the commonly used TH antibodies can recognize both THs.

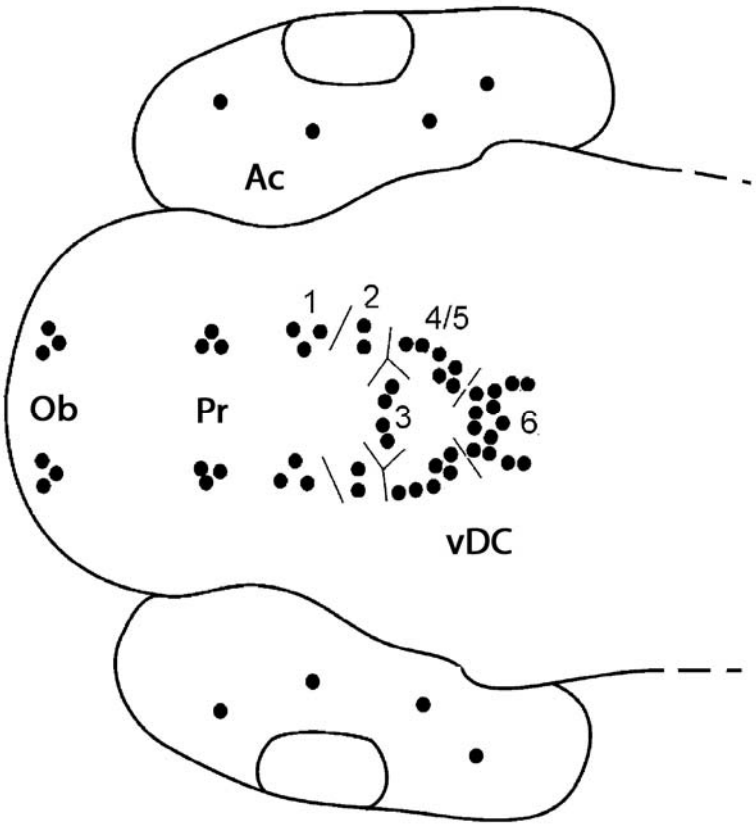
Several mutants with abnormal patternings of DA neurons in the vDC have been identified in large-scale mutant screens. Characterization of these mutants suggests that the Shh and Nodal signaling pathways play key roles in DA neuron differentiation in zebrafish as they do in mammals (Guo et al., 1999; Holzschuh et al., 2003; Ye et al., 1998). Transcription factors, including Nurr1/Nr4a2, Pitx3 and Lmx1b, have been shown to play evolutionarily conserved roles in the specification of zebrafish DA neurons (Filippi et al., 2007; Blin et al., 2008).

1.2.3 Current progress in zebrafish PD models

To date, several orthologs of major PD-associated genes have been found in zebrafish, including *parkin*, *pink1*, *dj-1* and *lrrk2*. MO knockdown and/or transgenic overexpression

Figure 1.2 Dopaminergic neuron groups in the zebrafish brain at 3 dpf.

Schematic drawing of the dorsal view of 3 dpf zebrafish with locations of major dopaminergic groups in the brain. Anterior to the left. Abbreviations: Numbers 1-6 indicate DA subgroups in the ventral diencephalon according to the numbering of (Rink and Wullimann, 2002); Ob: olfactory bulb; Pr: pretectum; vDC: ventral diencephalon; AC: amacrine cells.


Ac
Ob
Pr
1
2
4/5
3
6
vDC

of mutants of these genes suggest that they have conserved functions in the development and/or survival of DA neurons.

parkin

The zebrafish *parkin* gene encodes a 458 amino acid protein, which is highly conserved between zebrafish and human with overall 62% identity and up to 92% similarity in its functional domains (Flinn et al., 2009). Similar to human *Parkin*, zebrafish *parkin* is ubiquitously expressed throughout embryonic development and in adult tissues. MO knockdown of *parkin* resulted in ~20% loss of DA neurons in the vDC with increased susceptibility to the PD-inducing neurotoxin 1-methyl-4-phenylpyridinium (MPP+). Zebrafish with *parkin* deficiencies did not show any abnormal mitochondrial morphology but activity of the mitochondrial respiratory chain complex I was reduced by 45%. The impairment of the mitochondrial respiratory chain is also found in in the tissues of PD patient with *Parkin* mutations. In contrast, a different study showed no significant loss in diencephalic DA neurons after *parkin* knockdown although DA neurons of the morphant fish showed an increased vulnerability to stress-induced cell death (Fett et al., 2010). This discrepancy could result from different knockdown effciencies of MOs targeted to different sequences in the zebrafish *parkin* gene.

Similar to human *Parkin*, zebrafish *parkin* is transcriptionally up-regulated in response to mitochondrial stress. In addition, stable over-expression of *parkin* in transgenic zebrafish lines protect fish from cell death induced by proteotoxic stress, suggesting a protective capacity of parkin *in vivo*. This implies a potential therapy for PD

by a transcriptional up-regulation of endogenous Parkin (Fett et al., 2010).

pink1

In zebrafish, *pink1* is expressed ubiquitously and the predicted protein has 54% amino acid sequence identity to human PINK1. An initial study reported that MO knockdown of *pink1* resulted in a ~40% reduction in the number of DA neurons in the vDC (Anichtchik et al., 2008). We also performed functional studies of zebrafish *pink1*, and these are described in Chapter 2 of this thesis.

dj-1

The zebrafish Dj-1 protein shows 83% overall amino acid identity to human DJ-1 (Bai et al., 2006). The amino acids affected by pathogenic mutations in PD patients are especially well conserved in zebrafish Dj-1. The gene is expressed through embryogenesis and transcripts are ubiquitously found in all adult tissues with a relatively higher abundance in the brain (Bretaud et al., 2007). MO knockdown of *dj-1* did not cause a decrease in number of DA neurons. However, DA neurons in *dj-1* morphants were more sensitive to hydrogen peroxide or to the proteasome inhibitor MG132. They were also more susceptible to programmed cell death (Bretaud et al., 2007; Baulac et al., 2009). Up-regulation of *dj-1* was reported in the brain of zebrafish subjected to oxidative stress (Baulac et al., 2009). These findings suggest that DJ-1 has conserved functions in zebrafish and humans. Mutations in DJ-1 may impair the response of DA neurons to environmental stress and eventually lead to cell death.

lrrk2

Zebrafish and human LRRK2 have highly conserved amino acid sequences and predicted structures (Sheng et al., 2010). The zebrafish *lrrk2* gene is ubiquitously expressed during embryogenesis with more abundance in the brain, and transcripts are found in multiple tissues and organs of adult fish. The deletion of the WD40 domain of lrrk2 by splice-blocking MOs caused a significant loss of DA neurons in the vDC as well as locomotor defects. This also resulted in reduced and disorganized axon tracts, mainly in the midbrain. These results implicate the WD40 domain of LRRK2 in DA neuron development consistent with its known functions in mediating protein-protein interactions and regulation of LRRK2 kinase activity. Future studies should investigate the effects of over-expressing *lrrk2* (WT or mutant forms) in transgenic zebrafish.

Zebrafish orthologs of other PD-related genes such as *UCH-L1* and *GIGYF2* have also been identified (Son et al., 2003; Guella et al., 2010). Although several members of the *synuclein* family have been found in the zebrafish genome, the zebrafish ortholog of *α-synuclein* has yet to be found, suggesting that the *α-synuclein* gene may have been lost in zebrafish perhaps due to the functional redundancy among *synuclein* family members.

MPTP-induced zebrafish PD model

In adult zebrafish, MPTP induced a transient decrease in dopamine levels as well as behavioural defects (Anichtchik et al., 2004). Earlier studies in larval zebrafish showed a significant reduction of DA neurons in the vDC following MPTP treatment (Bretaud et

al., 2004; Lam et al., 2005; McKinley et al., 2005). More extensive studies suggested that different groups of DA neurons in the vDC show different sensitivities to MPTP, with those neurons with ascending projections being more sensitive (Wen et al., 2008; Sallinen et al., 2009). Dopamine levels also declined after MPTP treatment in larval zebrafish, which is consistent with findings in humans. Thus, similar to mammals, MPTP can induce a significantly functional deficit of DA neurons in zebrafish.

Together, these studies strongly indicated that the DA neurons in the vDC of zebrafish are analogous to those in the mammalian nigrostriatal pathway. Zebrafish orthologs of PD-related genes have highly conserved functions in the development and survival of DA neurons and in motor behaviour. Toxin-induced loss of DA neurons in zebrafish is also a relevant model of human PD. In the future, more PD-related mutants will likely be obtained using methods such as TILLING or ZFN. These mutants might greatly facilitate the screen for small molecules with therapeutic effects.

Generation of a transgenic zebrafish line with DA neurons specifically labeled with GFP

Successful generation of a transgenic zebrafish line expressing GFP specifically in DA neurons will remarkably facilitate the study of PD. Especially, the low cost of zebrafish husbandry allows to use this transgenic line for screening potential genes related to the pathogenesis of PD by forward genetics, and also for screening small molecules with therapeutical potential for PD. Several attempts at generating such transgenic fish have been only partilly successful. A 4.5 kb rat *th* promoter fragment drives GFP reporter

expression mainly in retinal cells (Gao et al., 2005). Similar results were obtained using a 12 kb zebrafish *th* promoter fragment (Meng et al., 2008a). Neither a 7 kb nor a 11 kb promoter fragment of *dat* can drive GFP expression in DA neurons of the vDC (Bai and Burton, 2009). Recently, an enhancer trap transgenic line, *ETvmat2:GFP*, showed only some DA neuron groups in the vDC that were labeled with GFP (Wen et al., 2008). I generated a transgenic zebrafish line, Tg(*dat:EGFP*), using a reporter construct with *EGFP* inserted in frame into the first exon of *dat*. Characterization of this transgenic zebrafish line is described in Chapter 4 of this thesis. Since zebrafish have been shown to regenerate several regions of the brain after injury (Becker and Becker, 2008), we also examined the capacity of zebrafish to regenerate specific DA neurons from neural stem cell populations using this transgenic line (Chapter 4).

1.3 Statement of objectives

Human Parkinson's disease is one of the most common neurodegenerative diseases. Genetic animal models of PD contributed remarkably to our understanding of the molecular and cellular mechanisms underlying this disease. However the detailed etiology of PD is still not well understood, partially due to the limitations of current PD models. In addition, current treatments can only alleviate the symptomatic motor dysfunction but not prevent or slow the progression of the disease. Thus, there is a need to establish a new vertebrate model for PD and to discover new therapies for this disorder.

Given its unique features, the zebrafish has emerged as a most useful vertebrate

model organism to study mechanisms controlling development and human diseases, especially in developmental neuroscience. The central objectives of this thesis are to characterize the functions of PD-related genes in the development and pathogenesis of PD by taking the advantages of zebrafish, and to establish a transgenic zebrafish line that will be used in future studies to discover novel PD-linked factors and to screen for potential therapeutic compounds, and therefore establish zebrafish as a model for PD.

Mutations in the human *PINK1* gene are associated with recessive familial EOPD. Animal models with altered *PINK1* function vary greatly in their phenotypic characteristics. *pink1*-deficient *Drosophila* exhibit mild loss of DA neurons and locomotor dysfunctions. Such defects are not observed in *pink1*-null mice, although these mice exhibit impaired dopamine release and abnormal mitochondrial functions. In my PhD research, we aim to identify the *pink1* orthologue in the zebrafish genome, and examine its expression during embryonic development and in adult by RT-PCR. Using MO knockdown strategy, we intent to characterize the functions of the *pink1* gene in DA neuron development especially in their patterning and axonal projections, and also in locomotor behavior. Furthermore, *pink1* function in mitochondria will also be examined in zebrafish by electron microscopy (EM). Through these studies, we wish to correlate the genetic alteration in *pink1* with the development of DA neurons and the pathogenesis of PD.

Several attempts to target transgene expression to DA neurons have been tried but none was truly successful (Gao et al., 2005; Meng et al., 2008; Bai et al., 2009; Wen et al., 2008). We aim to generate a transgenic line, in which all major groups of DA neurons in

the zebrafish brain are correctly labeled with GFP, especially the ones in the vDC. MPTP toxicity in GFP-positive neurons will be examined in the transgenic animals. Finally, a laser ablation approach will be adopted for the first time to verify the regenerative potential of DA neurons, especially those in the vDC. We hope this research will be instrumental in developing novel medical treatments for PD patients.

Chapter 2

Impaired dopaminergic neuron development and locomotor function in zebrafish with loss of *pink1* function

2.1 Abstract

Mutations in *PTEN-induced kinase 1 (PINK1)* are linked to recessive familial Parkinson's disease. Animal models of altered PINK1 function vary greatly in their phenotypic characteristics. *Drosophila pink1* mutants exhibit mild dopaminergic neuron degeneration and locomotion defects. Such defects are not observed in mice with targeted null mutations in *pink1*, although these mice exhibit impaired dopamine release and synaptic plasticity. Here, we report that in zebrafish, morpholino-mediated knockdown of *pink1* function did not cause large alterations in the number of dopaminergic neurons in the ventral diencephalon. However, the patterning of these neurons and their projections are perturbed. This is accompanied by locomotor dysfunction, notably impaired response to tactile stimuli and reduced swimming behaviour. All these defects can be rescued by expression of an exogenous *pink1* that is not a target of the morpholinos used in our study. These results indicate that normal PINK1 function during development is necessary for the proper positioning of populations of dopaminergic neurons and for the establishment of neuronal circuits in which they are implicated.

2.2 Introduction

Parkinson's disease (PD) is a common neurodegenerative disease, characterized by the loss of dopaminergic (DA) neurons in the substantia nigra of the midbrain and by movement disorders. Although most cases of Parkinson's disease are sporadic and could be linked to environmental factors, a number of genes have been identified that may be potentially associated with this disease, including *α-synuclein, parkin, DJ-1, UCHL1,*

PINK1, *LRRK2*, *HtrA2* and *ATP13A2* (Cookson, 2005; Farrer, 2006). The detailed etiology of PD is still not well understood.

Mutations in *PTEN-induced kinase 1 (PINK1)* are associated with early-onset PD (Valente et al., 2004; Beilina et al., 2005). *PINK1* encodes a ubiquitously expressed Ser/Thr kinase protein with an N-terminal mitochondrial targeting motif and a conserved kinase domain. PINK1 was originally shown to have anti-apoptotic properties in cancer cells (Unoki & Nakamura, 2001). Over-expression of PINK1 in cell culture seems to protect neurons against mitochondrial dysfunction and apoptosis induced by stress (Plun-Favreau et al., 2007; Pridgeon et al., 2007). However, evidence was also recently obtained that a cytoplasmic role for Pink1 may also contribute to its protective effects (Haque et al., 2008). In *Drosophila*, *pink1*-deficient mutants have mitochondrial defects leading to degeneration of flight muscles and mild loss of dopaminergic neurons (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Such defects are not observed in mice with targeted null mutations in *pink1*. However, defects are seen in dopamine release and impairment in synaptic plasticity (Kitada et al., 2007). Since the phenotype in mice is much milder than that seen in human patients with *PINK1* mutations, additional vertebrate models for *pink1* loss of function are needed.

In this study, we have examined the impact of the morpholino-mediated loss of function of the zebrafish *pink1* gene (Anichtchik et al., 2008) on the development of DA neurons in the ventral diencephalon and on locomotor behaviour. In zebrafish as in other teleosts, no DA neurons are found in the midbrain but clusters of DA neurons in the ventral diencephalon have ascending projections to the subpallium of the telencephalon,

suggesting that these neurons are homologous to the ascending midbrain DA neurons of the mammalian nigrostratial pathway (Rink & Wullimann, 2001, 2002). These neurons are sensitive to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treatment and their loss is associated with locomotor dysfunction (Lam et al., 2005; McKinley et al., 2005). We also observed altered patterning of the DA neurons in the ventral diencephalon including altered axonal projections as well as impaired locomotor behaviour following loss of *pink1* function.

2.3 Materials and methods

2.3.1 Animal maintenance

Zebrafish and embryos were maintained according to methods described in Nüsslein-Volhard and Dahm (2002). Adult zebrafish were kept in circulating fish water at 28.5 °C with a controlled light cycle. Newly laid eggs were collected and sorted. One-cell stage embryos were used for microinjection, which was performed using a Narishige IM300 microinjector. Injected and wildtype (non-injected) embryos were raised at similar densities in an incubator at 28.5 °C. Embryos for whole-mount *in situ* hybridization and immunostaining were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), then dehydrated in 100% methanol, and stored at -20 °C in 100% methanol. All experiments were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the University of Ottawa animal care committee.

2.3.2 *pink1* mRNA expression

Total RNA was extracted at different stages of embryonic development with the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada), and reverse transcribed into cDNA using the SuperScript II RT kit (Invitrogen, Burlington, ON, Canada), according to the manufacturer's instructions. The following primers were used for PCR reactions: *pink1* forward, 5'-GGCAATGAAGATGATGTGGAAC-3'; *pink1* reverse, 5'-GGTCGGCAGGACATCAGGA-3'; *β-actin* forward, 5'-TGGATGAGGAAATCGCTGCC-3'; *β-actin* reverse, 5'-TCTTCTCTCTGTTGGCTTTGGG-3'. The identity of the amplicons was verified by sequencing.

2.3.3 Morpholino knockdown and rescue of *pink1*

Two antisense morpholino oligonucleotides (MO) were used in this study: *pink1*-MO1 and *pink1*-MO2, specifically designed to be complementary to the translation initiation site and 5'-UTR of zebrafish *pink1* mRNA (positions -28 to -53 relative to the initiator codon), respectively (Table 2.1). These, as well as a standard control morpholino (5′-CCTCTTACCTCAGTTACAATTTATA- 3′) were purchased from Gene Tools, LLC (Philomath, OR, USA) and were diluted with nuclease-free H_2O into different concentrations. Phenol Red was added to the MO injection solution at a final concentration of 0.05%. The morphology of *pink1* morphants was examined under a Nikon SMZ1500 dissection microscope and pictures were taken using a Nikon DXM 1200C camera (Nikon, Mississauga, ON, Canada).

A zebrafish *pink1* cDNA clone (GenBank: BC086729) was purchased from Open

Biosystems (Huntsville, AL, USA). The primers for making synthetic mRNA encoding constructs were: *pink1*-EGFP (forward, 5'-AAGGATCCAAATGTCAGTAAAGCATGTTCTCAGCATGGTGAGCAAGGGCGA G-3'; reverse, 5'-TTACTTGTACAGCTCGTCC-3'), *mis-pink1*-EGFP (forward, 5'-AAGGATCCATATGTCTGTAAAACATGTTTTGAGCATGGTGAGCAAGGGCGA G-3'; reverse, 5'-TTACTTGTACAGCTCGTCC-3') and *mis-pink1* (forward, 5'-GGGAATTCTATGTCTGTAAAACATGTTTTGAGCCGAGGGCTGGAGCTGGGC-3'; reverse, 5'-GGGAATTCACTATGGCTGAGTGTTTGACATC-3'). DNA fragments obtained by PCR were subcloned into the pCS2+ vector. For the *mis-pink1* construct, the PCR amplified fragment was subcloned into the pT7TS vector. *In vitro* mRNA synthesis was carried out according to the instructions provided by mMessage-mMachine kit (Ambion, Streetsville, ON, Canada). After assessment by agarose gel electrophoresis, synthetic mRNA was microinjected alone or together with *pink1*-MOs into one-cell-stage zebrafish embryos.

2.3.4 Western blotting

Zebrafish embryos of 24–72 hpf were manually dechorionated and deyolked in cold Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.2) containing 0.3 mM PMSF and 1 mM EDTA. Embryos were homogenized in ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0) containing 5 µg/ml leupeptin, 1 µg/ml pepstatin, 100 mM PMSF, 0.5 mM dithiothreitol and 5 mM sodium fluoride, at the concentration of 1 µl buffer per

embryo, followed by briefly centrifugation (10,000 X g, 5 min at 4 °C). The supernatant of embryo lysates was separated by SDS-PAGE using 10% polyacrylamide gels. Then proteins were electrophoreticly transferred onto nitrocellulose membranes, and detected by immunoblotting using primary and secondary antibodies and a chemiluminescence detection system (Bio-Rad, Mississauga, ON, Canada). The antibodies used were anti-PINK1 polyclonal antibody (Cayman Chemicals, catalog # 10006283, Burlington, ON, Canada) and anti-actin antibody (Sigma-Aldrich, catalog # A2066, Oakville, ON, Canada). The antigen used to produce anti-PINK1 antibody has high identity compared with its zebrafish corresponding sequence, 13 out of 21 amino acids are identical. At least three separate experiments were performed.

2.3.5 Dopaminergic neuron examination

Dopaminergic neurons in zebrafish embryos and larvae were analyzed by whole-mount *in situ* hybridization using a probe against a zebrafish *tyrosine hydroxylase (th)* gene, that we generated as follows. In brief, total RNA was isolated and reverse transcribed into cDNA (Nüsslein-Volhard and Dahm, 2002). A partial *th* cDNA fragment was amplified by PCR from the reverse transcribed cDNA preparation (forward primer, 5'-TCAAGCAGCTCCACATCTTC-3'; reverse primer, 5'-AGGGATGCCAGACCAATAC-3'), and cloned into the pDrive plasmid vector (Qiagen, Mississauga, ON, Canada). The *th*-containing plasmid was linearized and a digoxigenin-labeled antisense riboprobe was synthesized by *in vitro* transcription (Roche, Mississauga, ON, Canada). Whole-mount *in situ* hybridization was performed as

described by Akimenko et al. (1994). Stained larvae were digitally photographed with a Nikon DXM 1200C camera mounted on a Nikon SMZ1500 dissecting microscope (Nikon, Mississauga, ON, Canada). Images were further analyzed for distance of DA neurons from the midline using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Abnormal DA neuron patterning was evaluated with the following parameters: the DA neurons in ventral diencephalon (vDC) are asymmetrically distributed, with more than 20% of the total number of DA neurons located further away from midline (by 50 μm) and have shorter or no axons projecting anteriorly (see below), compared to the corresponding neurons in wild-type embryos.

Dopaminergic neurons were also examined by whole-mount immunostaining followed by confocal microscopy. The method used was modified from Kay et al. (2001). Briefly, embryos were stored in 100% methanol, rehydrated in PBS, permeablized in acetone at -20 °C for 5 min, and then incubated with 3% H_2O_2 in PBS for 10 min to quench endogenous peroxidase activity. After washing in PBS, the embryos were blocked with 10% newborn calf serum (NCS) in PBSDTx (PBS + 1% DMSO + 0.3% Triton X-100) for 45 min and incubated in NCS-PBSDTx with anti-TH antibody (1:100; Chemicon, AB152, Ottawa, ON, Canada) overnight. An AlexaFluor 488-conjugated goat anti-rabbit secondary antibody (1:200; Molecular Probes, A-11034, Burlington, ON, Canada) was used for staining. The TH-labeled embryos were decapitated and mounted on glass slides with Vectashield mounting media (Vector Lab, Burlington, ON, Canada), and then observed by confocal microscopy using an Olympus BX50WI confocal microscope (Olympus, Markham, ON, Canada) with an Argon laser (488 nm). Stacks of

images were taken at 0.8-1.2 µm intervals, and compiled to produce maximum intensity with the Olympus Fluoview software (Olympus, Markham, ON, Canada). The number of TH-positive neurons was calculated from whole stacks. Images were further analyzed for length of axons using the ImageJ software.

2.3.6 Locomotor behaviour examination

The response of 72-hpf embryos to tactile stimulation was examined using a method modified from Granato et al. (1996). Larvae were placed in a petri dish (6 cm in diameter) with fish embryo media for 10 min. Using forceps, a gentle tactile stimulus was applied to the tail of the larvae and the response was recorded by a high-speed digital video camera (FASTCAM, Photoron, San Diego, CA, USA), and analyzed later using ImageJ software. At least three stimuli were applied to each larva. At 72 hpf, wild-type larvae are not very active and spend the majority of their time resting. They showed strong response to a gentle touch and escaped rapidly from the stimulus with high frequency lateral undulations of the tail. It normally took less than 0.05 sec for them to escape out of the field of view. A weak response to a tactile stimulus was defined as a low frequency lateral undulation of the tail compared to the normal response and a longer time for the larvae to swim out of the field of view (normally more than 0.5 sec). Absence of response was noted when the larvae did not move their tails and stayed motionless. The dopamine D1 receptor agonist SKF-38393 (Sigma-Aldrich, Oakville, ON, Canada) was diluted in 0.1% dimethylsulfoxide (DMSO) prior to a final dilution of 10 µM in fish embryo media. Tactile stimulation was applied to larvae 30 min after treatment with SKF-38393.

Controls received 0.1% DMSO and this did not have any effect on the response of embryos to the tactile stimulus (data not shown).

A simple tracking system for zebrafish swimming was designed as follows. At 5 dpf, each zebrafish larva (wild-type or *pink1* morphant) was placed in a 9-mm diameter dish containing embryo media. To avoid recording a stress response caused by the transfer of the larva from the culture dish to the swim tracking dish, the larva was allowed to acclimate to its new environment for 10 min prior to video capture. The zebrafish swimming behaviour was recorded at 12 frames per second using the Nikon ACT-1C software and a Nikon DXM 1200C digital camera mounted to a Nikon SMZ1500 microscope. Live capture was then recorded on-screen for 90 sec with the Quick Screen Recorder software (Etrusoft, Kaysville, UT, USA). Tracking was performed manually, using Transparentizer (Fridgesoft, Konstanz, Germany) with Microsoft Paint (Microsoft, Redmond, WA, USA), in order to trace the motion over the video in an image file. The swimming distance of each fish was calculated using the Mousotron (Blacksun Software, Turnhout, Belgium). Swimming distances were calculated for at least 15 larvae per group and averaged.

2.3.7 Statistical analysis

All data quantification and statistical analysis were performed with Microsoft Excel 2003. The percentage of abnormal DA neuron patterning among various *pink1* MO treatment groups was compared using Student's *t*-test or one-way ANOVA when required. Difference in the proportion of response to tactile stimulation after *pink1* MO or rescue

treatment was examined with Student's *t*-test. Comparison of different swimming distances between the *pink1* morphants and uninjected zebrafish larvae was also done by using Student's *t*-test. Data were presented as mean ± standard deviation (SD) and all experiments were independently repeated at least three times. The number of fish used in each experiment was indicated in the corresponding section of text or figure legend. *P*-values < 0.05 (two-sided) were considered as statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

2.4 Results

2.4.1 Genomic localization and expression of the zebrafish *pink1* gene

We assigned the zebrafish *pink1* gene (Anichtchik et al., 2008) to chromosome 23 (Fig. 2.1B) by radiation hybrid mapping with the LN54 panel (Hukriede et al., 1999). This was necessary as BLAST searches of the zebrafish genome, up to Ensembl version Zv7, had suggested the existence of two zebrafish *pink1* genes located on chromosome 17 and on chromosome 6. However, the coding sequences of these two *pink1* genes were almost identical, suggesting the possibility of sequencing error and/or mis-assembly in the database. Our ability to identify a single chromosomal location by radiation hybrid mapping with high LOD values >16 using *pink1* sequences argued for the existence of a single *pink1* gene in zebrafish. Furthermore, conserved synteny near *pink1* in human and zebrafish (Fig. 2.1B) strongly supported the mapping of zebrafish *pink1* to chromosome 23, instead of 6 or 17. A BLAST search of the latest release of the zebrafish genome sequence (http://vega.sanger.ac.uk/Danio_rerio/index.html) also supported our findings.

The temporal and spatial expression of *pink1* mRNA was analyzed by RT-PCR (Fig. 2.1C). The *pink1* mRNA is maternally expressed and was detected throughout embryonic development. Whole-mount *in situ* hybridization also confirmed ubiquitous expression of *pink1* mRNA throughout embryogenesis (data not shown). *pink1* was also expressed in adult zebrafish tissues that were tested (Fig. 2.1C), with a higher abundance in the brain.

2.4.2 Specificity and effectiveness of zebrafish *pink1* antisense morpholinos

We employed antisense morpholino (MO) to analyze the role of *pink1* in zebrafish embryo development. In a dose-response analysis of the *pink1* MOs, injection of less than 6 ng of *pink1*-MO1 per embryo did not produce any apparent defects compared to the uninjected control embryos. Embryos injected with more than 8 ng of *pink1*-MO1 showed an increased percentage of embryonic mortality. However, 88.8 ± 4.2% (n = 74) of embryos injected with 6 ng *pink1*-MO1 showed specific abnormal patterning of dopaminergic neurons in the ventral diencephalon. Based on the above observations, 6 ng of *pink1*-MO1 per embryo was determined to be the lowest effective concentration, which was, therefore, used in the subsequent experiments in this study. The lowest effective concentration for the *pink1*-MO2 was also determined to be 6 ng per embryo (data not shown).

The *pink1*-MO1 was designed to target the translational start site of *pink1* mRNA (Table 2.1). To confirm the specificity of *pink1*-MO1 targeting sequence, two EGFP fusion constructs were made (Table 2.1, Fig. 2.2). In the *pink1*-EGFP construct, the *pink1*-MO1 target sequence was introduced upstream of and in frame with the EGFP

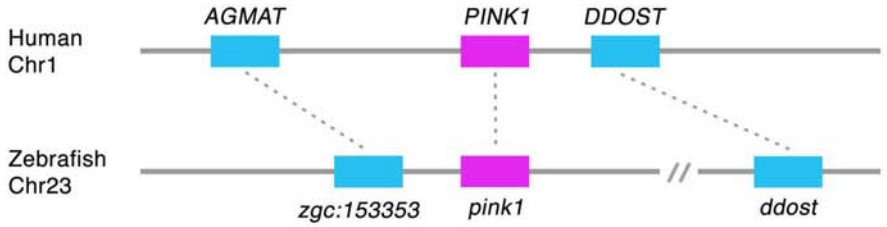
Figure 2.1 Characterization of zebrafish *pink1*.

(A) Amino acid sequence alignment of zebrafish, human, mouse, and *Drosophila* Pink1. Sequence alignments were performed using the ClustalW software. The mitochondria targeting sequence (MTS) and the kinase domain are shaded in green and blue, respectively. The conserved residues mutated in homozygous *PINK1*-associated PD patients are boxed in red. The GenBank accession numbers for the zebrafish, human, mouse, and fly sequences are BC086729, NM_032409, NM_026880, and CG4523, respectively. (B) Synteny comparisons of *PINK1*/*pink1* gene regions in human and zebrafish. The human *PINK1* gene is located on chromosome 1p36. The human *DDOST* sequence is found near *PINK1* on human chromosome 1, and its zebrafish ortholog is found on chromosome 23. Similarly, the zebrafish *zgc153353* sequence, found near *pink1* on zebrafish chromosome 23 (and on the *pink1* "regions" in ENSEMBL), is related to *AGMAT* which in human maps to chromosome 1p36. (C) *pink1* mRNA expression during embryonic development and in adult tissues. RT-PCR analysis of *pink1* was performed on total RNA isolated from different embryonic stages or adult tissues. RT-PCR for *β-actin* was used as a positive loading control, and RT-PCR in the absence of cDNA was used as a negative control. The following abbreviations are used: brain (B), heart (H), muscle (M) and liver (L).

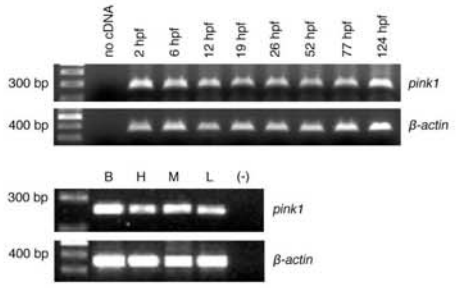
A

```
Zebrafish-Pink1  MSVKHVLSRGLELGR-------------SVFQLGLLKP----------AGRVAAKFR-----------------------GER--LRVSRPTRTVQPQTFLPGRYRFFRLSVSGLAAQLQSG  74
Human-PINK1      MAVRQALGRGLQLGR-------------ALLLRFTGKPGRAYGLGRPGPAAGCVR-----------------------GERPGWAAGPGAEPRRVGLGLPNRLRFFRQSVAGLAARLQRQ  84
Mouse-Pink1      MAVRQALGRGLQLGR-------------ALLLRFAPKPGPLFGWGKPGPAAAWGR-----------------------GERPGQVVSPGAQPRPVGLPLPDRYRFFRQSVAGLAARIQRQ  84
Fly-Pink1        MSVRLLTVRLIKHGRYILRSYCKRDIHANILDQNQLKTRSKRGFPLPSTAANVLRTTPQQAAKSVVNVVPRTINSPSGSSPFNGSGSSPTSSSGIFRVGQHARKLFIDNILSRVTTTYSED 120
                 *:*:    * :: **              ::     *.        .. .*  *                        .       .  : .         *  *:   :: :::  .

Zebrafish-Pink1  AFRRVIGGGSARNRAVFLAFG-VGLGLIEQEQEEDRTSAAICQEIQAVFRKKKFQSLPKPFTS----GYRLEDYVIGKQIGKGCNAAVYEAAAP-------------------------  163
Human-PINK1      FVVRAWGCAGPCGRAVFLAFG-LGLGLIEEKQAESRRAVSACQEIQAIFTQK-SKPGPDPLDTRRLQGFRLEEYLIGQSIGKGCSAAVYEATMP-------------------------  176
Mouse-Pink1      FMVRARGGAGPCGRAVFLAFG-LGLGLIEEKQAEGRRAASACQEIQAIFTQK-TKRVSDPLDTRCWQGFRLEDYLIGQAIGKGCNAAVYEATMP-------------------------  176
Fly-Pink1        LRQRATRKLFFGDSAPFFALIGVSLASGSGVLSKEDELEGVCWEIREAASRLQNAWNHDEISDTLDSKFTIDDLEIGPPIAKGCAAVVYAADFKKDVASDGASLHTDAQPQATPAFAPNS  240
                    *.       . * *:*:  :.*.  .    :     .*  **:    :         . :       : :::  **  *.*** *.** *

Zebrafish-Pink1  ------------------------------------------------------FAPPVESKKCSLVELNQKEAEDDNKKEEPLRLSASPSFPLAMKMMWNIGAGSSSDAILRSMSMELV  229
Human-PINK1      ------------------------------------------------------TLPQNLEVTKSTGLLPGRGPGTSAPGEGQERAPGAPAFPLAIKMMWNISAGSSSEAILNTMSQELV  242
Mouse-Pink1      ------------------------------------------------------TLPQHLEKAKHLGLI-GKGPDVVLKGADGEQAPGTPTFPFAIKMMWNISAGSSSEAILSKMSQELV  241
Fly-Pink1        WSTHEMMSPLQNMSRFVHNFGGSVDNVFHYSQPSAASDFVGAQSREQDQRHHEQQQHQNQEQEQHQNQEPSSSAFNVTSPANSNINSSVDSYPLALKMMFNYDIQSNALSILRAMYKETV  360
                                                                        .              .           ..  ::*:*:***:* .  *.: :**  *  * * *

Zebrafish-Pink1  PSCPQALRKEQGELTLNGHFGAVPKRLSAHPNVITVYRAFTAEVPLLPGAREEYPDVLPTRLNPHGLGSNRTLFLVMKNYPCTLRQYLEVCVPKRTQASLMFLQLLEGVDHLCRQNIAHR  349
Human-PINK1      PASRVALAGEYGAVTYR-KSKRGPKQLAPHPNIIRVLRAFTSSVPLLPGALVDYPDVLPSRLHPEGLGHGRTLFLVMKNYPCTLRQYLCVNTPSPRLAAMMLLQLLEGVDHLVQQGIAHR  361
Mouse-Pink1      PASRVALAGEYGAVTYR-RSRDGPKQLAPHPNIIRVFRAFTSSVPLLPGALADYPDMLPPHYYPEGLGHGRTLFLVMKNYPCTLRQYLEEQTPSSRLATMMTLQLLEGVDHLVQQGIAHR  360
Fly-Pink1        PARQRGMN--EAADEWERLLQNQTVHLPRHPNIVCMFGFFCDEVRNFPDGHLLYPVAQPQRINPQGYGRNMSLYLLMKRYDHSLRGLLDSQDLSTRNRILLLAQMLEAVNHLSRHGVAHR  478
                 *:   .:     .    .      . :*. ***:: :   *  .*  :*..   **   * :  *.* *  . :*:*:**.*  :**  *     .      ::  *:**.*:** ::.:***

Zebrafish-Pink1  DLKSDNILLEFDNTGCPRLVITDFGCCLAEDS-GLKLPFSSWWVNRGGNSCLMAPEVSTAVPGPGVVIDYSKADVWAVGAIAYELFGQPNPFY--------------TLESRSYQEKQL  453
Human-PINK1      DLKSDNILVELDPDGCPWLVIADFGCCLADESIGLQLPFSSWYVDRGGNGCLMAPEVSTARPGPRAVIDYSKADAWAVGAIAYEIFGLVNPFYGQG---------KAHLESRSYQEAQL  471
Mouse-Pink1      DLKSDNILVEWDSDGCPWLVISDFGCCLADQHVGLRLPFNSSSVERGGNGSLMAPEVSTAHSGPSAVIDYSKADTWAVGAIAYEIFGLANPFYGQG---------SAHLESRSYQEAQL  470
Fly-Pink1        DLKSDNVLIELQDDAAPVLVLSDFGCCLADKVHGLRLPYVSHDVDKGGNAALMAPEIFNTMPGPFAVLNYGKADLWACGALAYEIFGNRNPFYSSSGGMARERGEMTLSLRNSDYRQDQL  598
                 ******:*:* :  ..* **::**:****:.  **:**: *  *::**..****: .: .** .*::*.*** ** **:***:**  ****                *..*:: **

Zebrafish-Pink1  PALPAAAPDDVQLVVKLLLRKNPHKRPSARVAANILHISLWGR-RVLMGLDKVQMAEMMAWLQCQSAVVLLKGR-------------GRDQSSVEAELQRSFLANIELEDLRTAVSFMTY  559
Human-PINK1      PALPESVPPDVRQLVRALLQREASKRPSARVAANVLHLSLWG--EHILALKNLKLDKMVGWLLQQSAATLLANR-------------LTEKCCVETKMKMLFLANLECETLCQAALLLCS  576
Mouse-Pink1      PEMPESVPPEARRLVRSLLQREASKRPSARLAANVLHLSLWG--EHLLALKNLKLDKMIAWLLQQSAATLLADR-------------LREKSCVETKLQMLFLANLECEALCQAALLLSS  575
Fly-Pink1        PPMSDACPPLLQQLVYNILNPNPSKRVSPDIAANVVQLFLWAPSNWLKAGGMPNSPEILQWLLSLTTKIMCEGRPQMGAGLMPVASCGNRRAYVEYLLICSFLARARLRRIRGALNWIQN  718
                 * :. : *   : :*  .:*. :. ** *. :***:::: **.  . : .    :  ::: **   ::  :  .*               :. **  :   ***. . . :  *   :

Zebrafish-Pink1  ERKQWRSLLMSNTQP 574
Human-PINK1      ----WRAAL------ 581
Mouse-Pink1      ----WRAAP------ 580
Fly-Pink1        ------VVA------ 721
```

B



C



coding sequence. Embryos were injected with *in vitro* synthesized *pink1*-EGFP mRNA alone or together with *pink1*-MO1 (Fig. 2.2G, H). The embryos injected with *pink1*-EGFP alone exhibited ubiquitous expression of GFP (Fig. 2.2G). However, no GFP expression was observed when *pink1*-MO1 was injected in the embryos along with or after *pink1*-EGFP injection (Fig. 2.2H and data not shown), indicating that the *pink1*-MO1 can effectively block the translation of *pink1*-EGFP. We then synthesized a mis-matched (mis) *pink1*-EGFP construct, which contained 5 synonymous point mutations (encoding the same amino acids) out of a total of 25 nucleotides in the *pink1*-MO1 targeting sequence. The embryos injected with *mis-pink1*-EGFP alone exhibited ubiquitous expression of GFP (Fig. 2.2I). Notably, ubiquitous GFP expression was also observed when *pink1*-MO1 was injected into the embryos along with or after the *mis-pink1*-EGFP injection (Fig. 2.2J and data not shown), indicating that the *pink1*-MO1 cannot inhibit the expression of *mis-pink1*-EGFP mRNA when the target sequence is mutated. Therefore, one can predict that *pink1*-MO1 will specifically and effectively block the expression of endogenous *pink1* mRNA in zebrafish embryos, as opposed to other mRNAs with sequences highly similar to that of *pink1*. Furthermore, these results indicate that a synthetic *pink1* mRNA containing the same mis-matches as *mis-pink1*-EGFP should be efficient at rescuing a *pink1* morphant phenotype without titrating out the morpholinos.

A polyclonal antibody against human PINK1 peptide (Cayman Chemicals, catalog # 10006283) was used to detect the endogenous zebrafish Pink1 protein. Two bands corresponding to Pink1 were detected in whole zebrafish embryo lysates: a larger one at

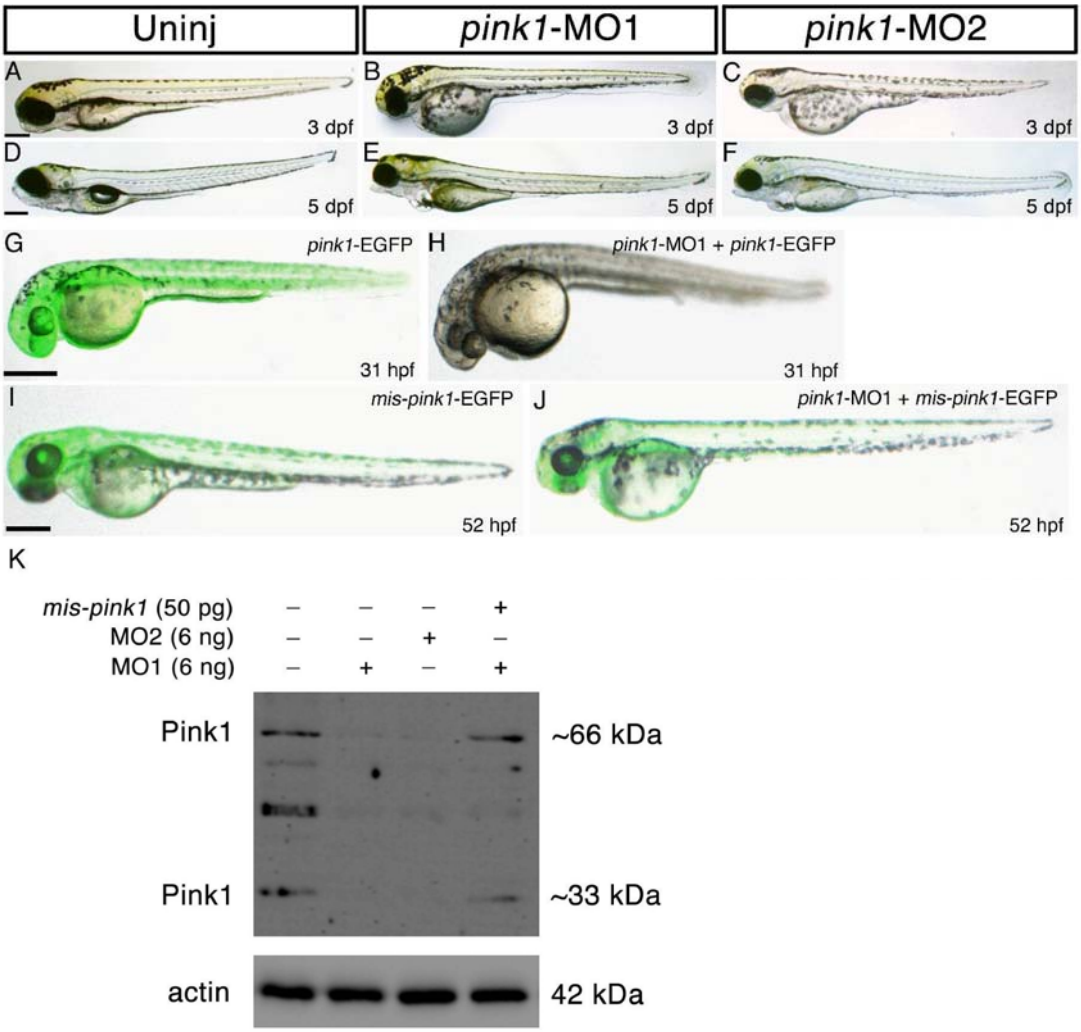
Table 2.1 Sequences of zebrafish *pink1* mRNA and the corresponding morpholinos used in this study.

pink1 mRNA		a	AUG	UCA	GUA	AAG	CAU	GUU	CUC	AGC	CGA	GGG	CUG
pink1-MO1	3'-	t	TAC	AGT	CAT	TTC	GTA	CAA	GAG	TCG	-5'		
pink1-EGFP		a	ATG	TCA	GTA	AAG	CAT	GTT	CTC	AGC	ATG	GTG	AGC
mis-pink1-EGFP		**t**	ATG	TC**T**	GTA	AA**A**	CAT	GTT	**TT**G	AGC	ATG	GTG	AGC
mis-pink1		**t**	ATG	TC**T**	GTA	AA**A**	CAT	GTT	**TT**G	AGC	CGA	GGG	CTG
pink1 5'-UTR			AAG	CCU	UCA	GAU	UUC	CUC	UCA	UAG	U		
pink1-MO2		3'-	TTC	GGA	AGT	CTA	AAG	GAG	AGT	ATC	A-5'		

The translation initiation codon is underlined. The mis-matched *pink1* (*mis-pink1*) was used in the rescue experiment. For *mis-pink1* and *mis-pink1*-EGFP sequences, the mis-matched nucleotides are in bold letters. For *pink1*-EGFP and *mis-pink1*-EGFP fusion sequences, the EGFP sequence is shaded in grey.

Figure 2.2 Efficiency and specificity of zebrafish *pink1* morpholinos.

Translation-blocking MO, *pink1*-MO1 (6 ng/nl) or *pink1*-MO2 (6 ng/nl), was microinjected into one-cell-stage zebrafish embryos at a volume of 1 nl. The morphology of morphants was examined at 3 dpf (A-C) and 5 dpf (D-F). The *pink1*-EGFP fusion mRNA (100 pg/µl, G and H) or mis-matched (mis) *pink1*-EGFP mRNA (100 pg/µl, I and J) were microinjected into one-cell-stage zebrafish embryos alone or together with *pink1*-MO1. Identical results were obtained when the two reagents were injected consecutively. The zebrafish larvae were checked by fluorescence microscopy at 31 hpf or 52 hpf. All animals are shown as lateral view, anterior to the left. Scale bars represent 250 µm. (K) A Western blot showing that the synthesis of zebrafish Pink1 protein (~66 kDa and ~33 kDa) can be significantly inhibited by *pink1*-MO1 (or *pink1*-MO2). Coinjection of mis-matched (mis) *pink1* mRNA can specifically restore Pink1 levels in embryos. At least three independent experiments were performed each involving a minimum of 50 embryos.


Uninj
pink1-MO1
pink1-MO2
A
3 dpf
B
3 dpf
C
3 dpf
D
5 dpf
E
5 dpf
F
5 dpf
G
pink1-EGFP
31 hpf
H
pink1-MO1 + pink1-EGFP
31 hpf
I
mis-pink1-EGFP
52 hpf
J
pink1-MO1 + mis-pink1-EGFP
52 hpf
K
mis-pink1 (50 pg) – – – +
MO2 (6 ng) – – + –
MO1 (6 ng) – + – +
Pink1
~66 kDa
Pink1
~33 kDa
actin
42 kDa

the size of about 66 kDa and a smaller one at the size of about 33 kDa (Anichtchik et al., 2008). The intensity of both bands was significantly decreased when 6 ng of *pink1*-MO1 (or *pink1*-MO2) were injected into embryos. Coinjection of zebrafish *mis-pink1* full length mRNA (50 pg) can restore decreased levels of Pink1 protein (Fig. 2.2K), which confirmed that the *pink1*-MOs used in this study can specifically and effectively knockdown the endogenous Pink1 in zebrafish.

2.4.3 MO-mediated loss of *pink1* function specifically perturbs dopaminergic neuron development in zebrafish

Less than 20% of the *pink1* morphants survive past 10 days (13.1 ± 5.8%, n = 255). In contrast, embryos injected with the control morpholino have 87.0 ± 3.5% (n = 343) survivals, comparable to uninjected embryos. Several morphological defects were observed in *pink1* morphants (Fig. 2.2A-F), as independently reported by Anichtchik and collaborators (2008). We concentrated our analysis on the patterning of dopaminergic (DA) neurons and on the behaviour of the MO-injected zebrafish (next section).

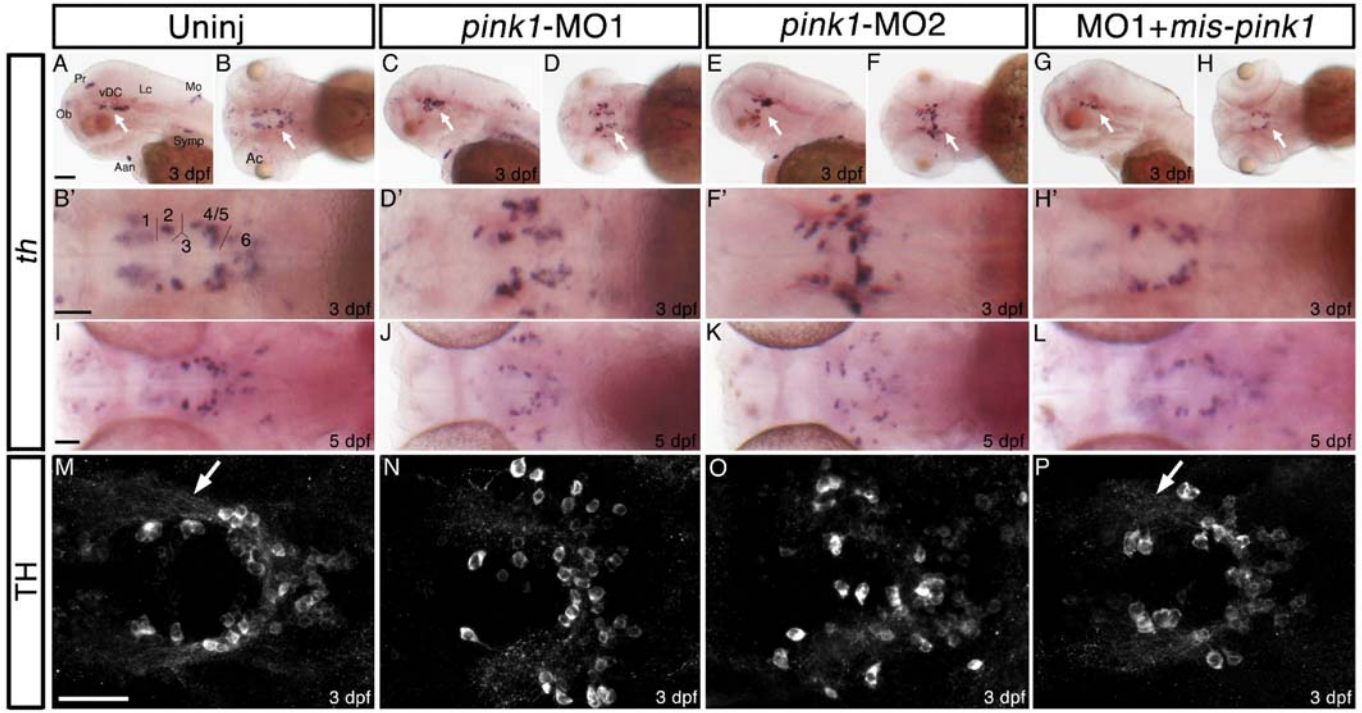
At 3 dpf and 5 dpf, the DA neurons were first examined by whole-mount *in situ* hybridization with a *tyrosine hydroxylase* (*th*) probe. Compared with control embryos, the number of DA neurons in *pink1* morphants did not seem to be decreased in the ventral diencephalon (vDC), in contrast to what was reported by Anichtchik and collaborators (2008). In comparison to controls (69.5 ± 6.5), the total number of TH-positive neurons (63.7 ± 7.1) in the vDC was only reduced by 8.3 ± 3.9% (Student's *t*-test, n = 7, P = 0.128). However, patterning of DA neurons in the vDC was remarkably

disorganized in *pink1* morphants at 3 dpf and 5 dpf. The DA neurons in the vDC of *pink1* morphants were located more ventrally and more scattered (Fig. 2.3C-F, J, K, N and O). This phenotype was largely rescued by mis-matched (*mis*) *pink1* mRNA (Fig. 2.3G-H, L and P). In 3 dpf control embryos, the DA neurons in the vDC (groups 1-6) were symmetrically patterned along the midline (Fig. 2.3B', Rink & Wullimann, 2002). However, in *pink1* morphants, the DA neurons found in groups 2 and 4/5 were asymmetrically located further away from the midline (Fig. 2.3D', F'). Despite the fact that the total number of DA neurons seemed to remain unchanged, the number of group 1 DA neurons in *pink1* morphants (4.3 ± 2.9) was significantly reduced by 53.0 ± 12.1% (versus controls: 9.1 ± 2.4, Student's *t*-test, $n = 7$, $P = 0.005$). When 3 ng of *pink1*-MO1 (or of *pink1*-MO2) were co-injected with 3 ng of the control (Ctrl) MO, less than 10% of embryos showed abnormal DA neuron patterning (Fig. 2.3R). In contrast, when 3 ng *pink1*-MO1 and 3 ng *pink1*-MO2 were co-injected, more than 75% of embryos showed abnormal DA neuron patterning (Fig. 2.3Q, R), suggesting that *pink1*-MO1 and *pink1*-MO2 were equally efficient in inhibiting endogenous Pink1 protein synthesis. When full-length *mis-pink1* mRNA was co-injected with *pink1*-MO1, the phenotype of abnormal DA neuron patterning was largely rescued (Fig. 2.3G, H and H'), and the located further than 50 μm away from midline and have shorter or no axons projecting anteriorly, compared to the corresponding neurons in wild-type embryos. Compared to uninjected controls (3.8 ± 3.8%, $n = 276$), treatments with 6 ng *pink1*-MO1, 6 ng *pink1*-MO2, or 3 ng MO1 and 3ng MO2 all significantly elevated the rate of abnormal DA neuron patterning in zebrafish larvae (6 ng MO1: 88.8 ± 4.2%, $n = 224$; 6 ng MO2:

Figure 2.3 Altered dopaminergic neuron patterning in the ventral diencephalon of *pink1* morphants.

(A-L) *In situ* hybridization with a *tyrosine hydroxylase* (*th*) cRNA probe. (A, C, E, G) are lateral views, (B, D, F, H-L) are dorsal views, anterior is always to the left. The dopaminergic neurons in the vDC are indicated by white arrows in panels A-H. (B', D', F', H') are higher magnification of panels (B, D, F, H) showing the ventral diencephalic area. The different DA neuron groups in vDC are labeled 1 to 6 based on morphology and position according to Rink and Wullimann (2002). The rescue experiment was performed by co-injecting mis-matched (mis) *pink1* mRNA with *pink1*-MO1 into one-cell-stage zebrafish embryos. The following abbreviations are used: olfactory bulb (Ob), pretectum (Pr), ventral diencephalon (vDC), locus coeruleus (Lc), arch-associated neurons (Aan), medulla oblongata (Mo), sympathetic neurons (Symp), and amacrine cells (Ac). (M-P) Immunolocalization of tyrosine hydroxylase (TH) in 3 dpf larvae. M-P are dorsal views, anterior is to the left. The forebrain axonal projections of DA neurons in vDC are indicated by white arrows in panel M and P. (Q and R) Quantification of the abnormal DA neuron patterning phenotype shown in panels A-L. Abnormal DA neuron patterning was determined using the following parameters: the DA neurons in the vDC are asymmetrically distributed, with more than 20% of the total number of DA neurons 90.1

± 5.1%, *n* = 122; 3 ng MO1 + 3ng MO2: 74.2 ± 4.5%, *n* = 176; Student's *t*-test, *** *P* < 0.001 for all comparisons). When full-length *mis-pink1* mRNA was co-injected with *pink1*-MO1, the phenotype of abnormal DA neuron patterning was significantly rescued (versus 6 ng MO1, 20.0 ± 6.9%, Student's *t*-test, *n* = 195, *** *P* < 0.001). In addition, when 3 ng *pink1*-MO1 or 3 ng *pink1*-MO2 were co-injected with 3 ng of the control (Ctrl) MO, less than 10% of embryos showed abnormal DA neuron patterning (MO1 + Ctrl MO: 11.7 ± 4.7%, one-way ANOVA, *F* = 311.1, *d.f.* = 2, *n* = 189, *** *P* < 0.001; MO2 + Ctrl MO: 5.4 ± 4.5%, one-way ANOVA, *F* = 337.9, *d.f.* = 2, *n* = 175, *** *P* < 0.001), which were significantly lowered as compared to that in 3 ng MO1 and 3 ng MO2 treatment group. Data were presented as mean ± SD and all experiments were repeated at least three times. Scale bars represent 100 μm (A-H) and 50 μm (B'-H' and I-P).


Uninj
pink1-MO1
pink1-MO2
MO1+mis-pink1
th
TH
A
B
C
D
E
F
G
H
B'
D'
F'
H'
I
J
K
L
M
N
O
P
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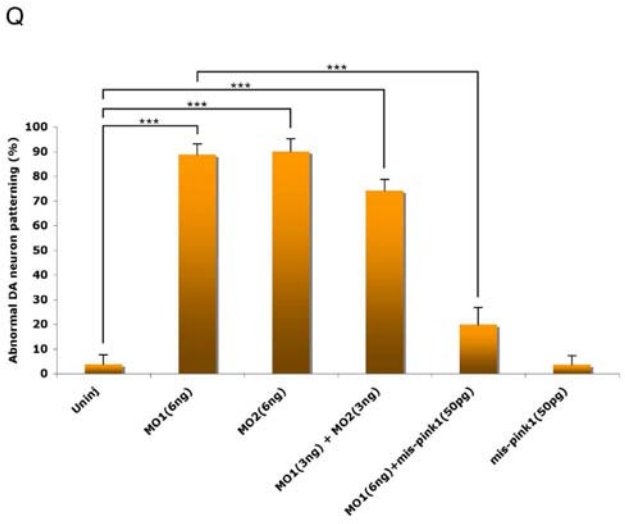
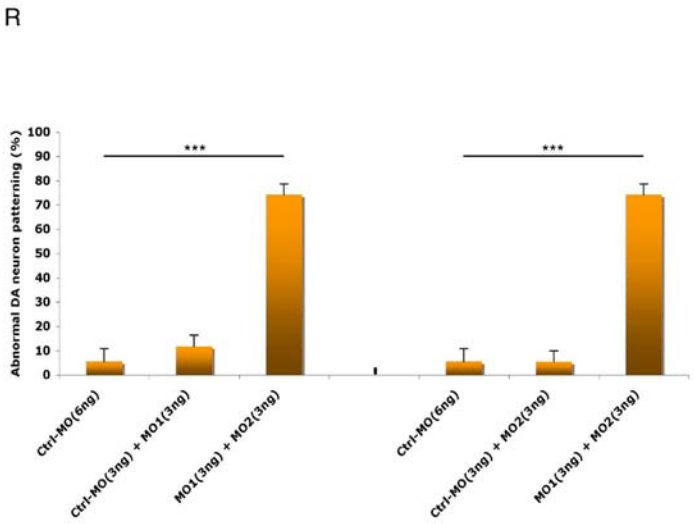
4/5
6
3 dpf
5 dpf


Q
Abnormal DA neuron patterning (%)
***
Uninj
MO1(6ng)
MO2(6ng)
MO1(3ng) + MO2(3ng)
MO1(6ng)+mis-pink1(50pg)
mis-pink1(50pg)


R
Abnormal DA neuron patterning (%)
***
Ctrl-MO(6ng)
Ctrl-MO(3ng) + MO1(3ng)
MO1(3ng) + MO2(3ng)
Ctrl-MO(6ng)
Ctrl-MO(3ng) + MO2(3ng)
MO1(3ng) + MO2(3ng)

number of group 1 neurons (6.6 ± 1.9) was partially rescued to 72.0 ± 7.8% of control values (Student's *t*-test, $n = 7$, $P = 0.039$).

In order to test whether these phenotypes persist during development after 3 dpf, we also examined the DA neuron patterning in *pink1* morphants at 5 dpf. At 5 dpf, a disrupted patterning of diencephalic DA neurons was still observed in *pink1* morphants (Fig. 2.3J, K), and this was rescued by injection of *mis-pink1* mRNA (Fig. 2.3L). The DA neurons in the vDC of morphants were located more ventrally and more scattered, similar to what was observed at 3 dpf (Fig. 2.3C-F).

To more closely examine the disrupted patterning of diencephalic DA neurons, we used confocal microscopy after whole-mount immunolocalization of TH. In control embryos, the DA neurons of groups 1, 2 and 4/5 in the diencephalic posterior tuberculum sent projections towards the forebrain (Fig. 2.3M, Rink & Wullimann, 2002). However, in *pink1* morphants, the mis-located DA neurons of group 2 and 4/5 had shortened axonal projections or had no projections to the forebrain (Fig. 2.3N, O). Axons appeared to be fragmented at the position where we normally see axonal projections. This phenotype was largely rescued by co-injection of the *mis-pink1* mRNA (Fig. 2.3P).

It is important to note that *th*-positive neurons include not only dopaminergic neurons but also noradrenergic neurons. The *pink1* morphants showed normal patterning of noradrenergic neurons in the locus coeruleus (Lc), medulla oblongata (Mo) and other places (Fig. 2.3 C-F and data not shown). Moreover, it was also shown that serotonergic neurons were not affected by *pink1*-MOs (Anichtchik et al., 2008). This suggests that the *pink1*-MOs can specifically perturb the development of DA neurons in the ventral

diencephalon of zebrafish.

Overall, our observations suggest that normal *pink1* function is necessary for the proper development of DA neurons in the zebrafish ventral diencephalon.

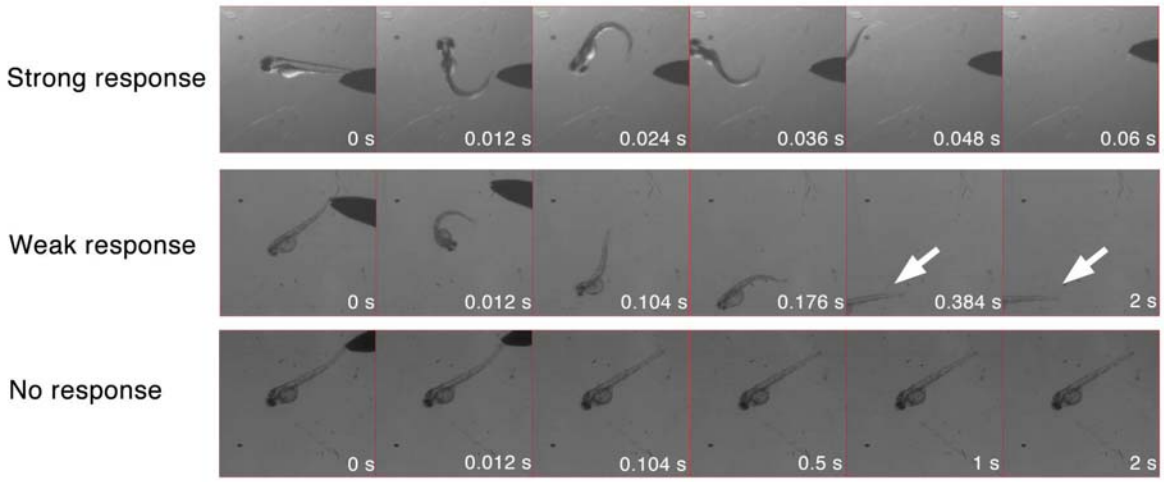
2.4.4 Loss of *pink1* function results in impaired response to tactile stimulation and hypoactivity in zebrafish

To determine whether the loss of *pink1* function also affects behaviour of the zebrafish larvae, we examined their response to tactile stimulation at 3 dpf (Fig. 2.4). We divided the responses of the larvae into three categories (Fig. 2.4A): 1) a strong response, in which the embryos escape rapidly (in less than 0.5 sec although most of these escape in less that 0.05 sec) from the gentle tactile stimulus with high frequency tail movements; 2) a weak response, in which the embryos escape from a tactile stimulus with low frequency tail movements and take more than 0.5 sec to disappear from the field of view; and 3) no response after three consecutive tactile stimuli. More than 90% of larvae injected with 6 ng *pink1*-MO1 (93.5 ± 4.2%, Student's *t*-test, $n = 310$, $P < 0.001$) or 6 ng *pink1*-MO2 (96.5 ± 4.2%, Student's *t*-test, $n = 221$, $P < 0.001$) showed weak or no response, either of which was significantly higher than the percentage identified in control larvae (21.7% ± 11.8%, $n = 189$; Fig. 2.4B). This loss of response to a tactile stimulus was partially rescued by co-injection of 50 pg mis-matched *pink1* mRNA (MO1 + mis-*pink1*: 46.8 ± 22.2%, Student's *t*-test, $n = 163$; MO2 + mis-*pink1*: 69.9 ± 13.8%, Student's *t*-test, $n = 154$; $P < 0.05$ for both comparisons; Fig. 2.4B). It was also rescued by administration of 10 μM dopamine D1 receptor agonist, SKF-38393 (MO1 + D1 agonist: 41.7 ± 13.7%,

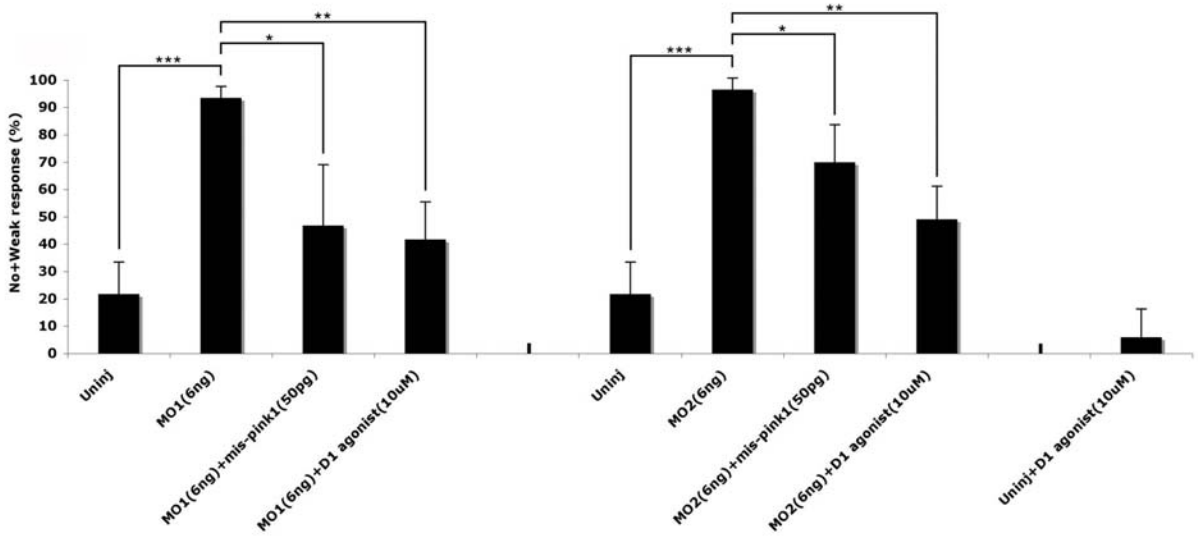
Figure 2.4 Reduced response to tactile stimulation in *pink1* morphants.

(A) Categories of response observed upon tactile stimulation. The pictures shown are sequential frames taken from videos captured with a high-speed camera. (B) Quantitative measurement of the responses observed in *pink1* morphants. As compared to uninjected controls (21.7% ± 11.8%), zebrafish larvae treated with 6 ng *pink1*-MO1 (93.5 ± 4.2%, Student's *t*-test, $n = 310$, *** $P < 0.001$) or 6 ng *pink1*-MO2 (96.5 ± 4.2%, Student's *t*-test, $n = 221$, *** $P < 0.001$) showed a significantly increased rate of weak or no response to tactile stimulation. This reduced response was significantly rescued in larvae that were injected with a *pink1* MO and the mis-matched (mis) *pink1* mRNA (MO1 + mis-*pink1*: 46.8 ± 22.2%, Student's *t*-test, $n = 163$, * $P < 0.05$; MO2 + mis-*pink1*: 69.9 ± 13.8%; Student's *t*-test, $n = 154$, * $P < 0.05$). Likewise, administration of 10 μM of the dopamine D1 receptor agonist SKF-38393 (Sigma) 30 min before tactile stimulation also significantly rescued the morphant phenotype (MO1 + D1 agonist: 41.7 ± 13.7%, Student's *t*-test, $n = 121$, ** $P < 0.01$; MO2 + D1 agonist: 49.0 ± 12.2%, Student's *t*-test, $n = 122$, ** $P < 0.01$). Data were presented as mean ± SD and all experiments were repeated at least three times.

A


Strong response
0 s
0.012 s
0.024 s
0.036 s
0.048 s
0.06 s
Weak response
0 s
0.012 s
0.104 s
0.176 s
0.384 s
2 s
No response
0 s
0.012 s
0.104 s
0.5 s
1 s
2 s

B


No+Weak response (%)
100
90
80
70
60
50
40
30
20
10
0
***
*
**
***
*
**
Uninj
MO1(6ng)
MO1(6ng)+mis-pink1(50pg)
MO1(6ng)+D1 agonist(10uM)
Uninj
MO2(6ng)
MO2(6ng)+mis-pink1(50pg)
MO2(6ng)+D1 agonist(10uM)
Uninj+D1 agonist(10uM)

Student's *t*-test, *n* = 121; MO2 + D1 agonist: 49.0 ± 12.2%; Student's *t*-test, *n* = 122; *P* < 0.01 for both comparisons; Fig. 2.4B).

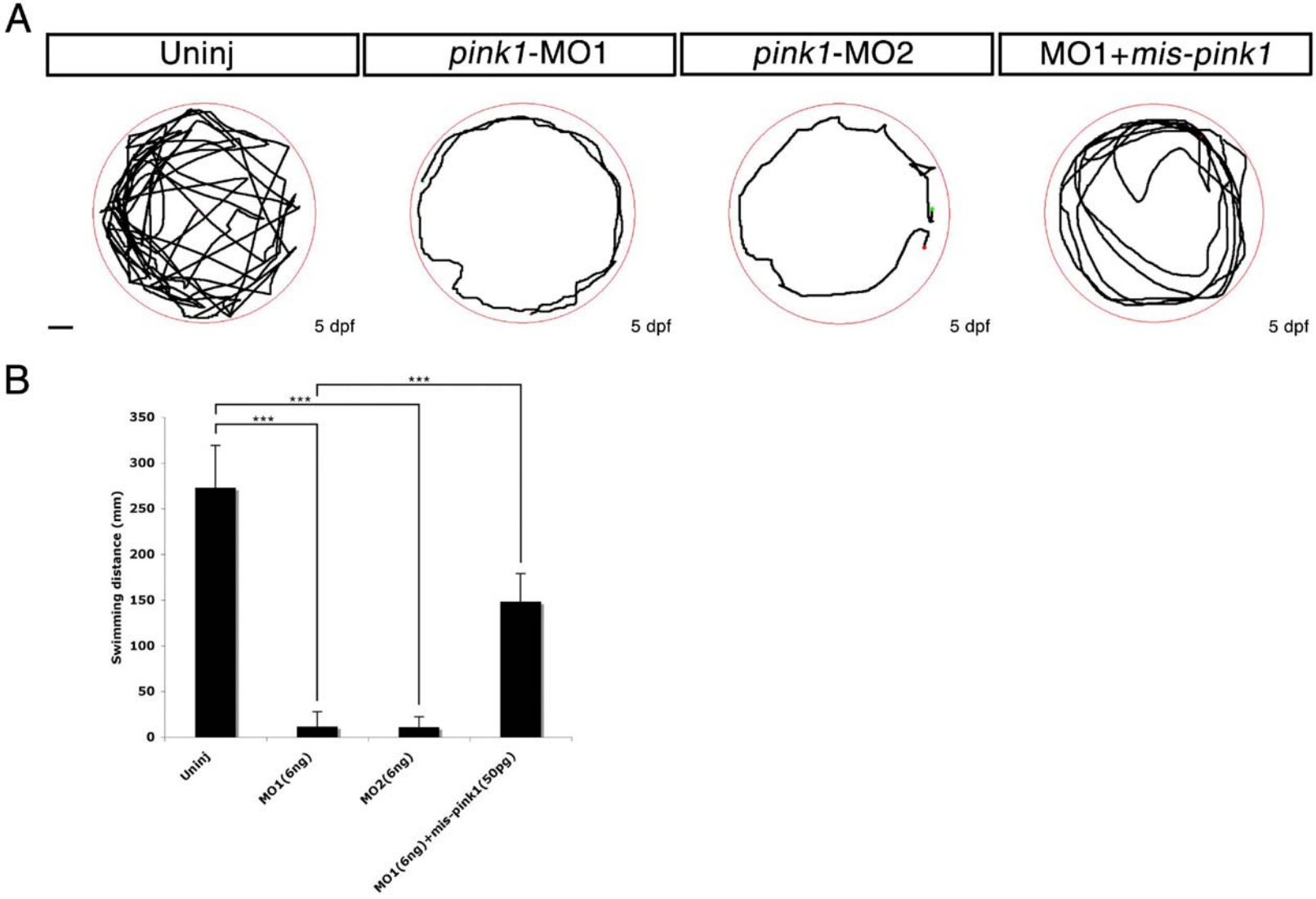
We also compared the locomotor activity of zebrafish larvae injected with the *pink1*-MOs to that of uninjected zebrafish larvae. A simple test was designed to track the swimming of larvae at 5 dpf, a time at which we observe abnormal DA neuron patterning in the vDC. Compared to uninjected wild-type larvae (272.8 ± 46.5 mm, *n* = 16), those injected with the *pink1*-MOs showed a marked reduction in swimming distance and speed (6 ng MO1: 11.7 ± 16.3 mm, Student's *t*-test, *n* = 12, *P* < 0.001; 6 ng MO2: 10.9 ± 11.6 mm, Student's *t*-test, *n* = 12, *P* < 0.001; Fig. 2.5). The *pink1* morphants exhibited an increased turning angle, tended to swim along the periphery of the observation dish and seldom swam across the open field in the middle of the dish (shown schematically in Fig. 2.5A). Moreover, the *pink1* morphants showed difficulties in balancing themselves while swimming (data not shown). In addition, the *pink1* morphants also showed slow or no response to tactile stimuli versus uninjected embryos (data not shown). Finally, the total swimming distance of *pink1* morphants was significantly rescued by 50 pg *mis-pink1* mRNA (MO1 + mis-*pink1*: 148.4 ± 30.8 mm, Student's *t*-test, *n* = 17, *P* < 0.001; Fig. 2.5B), demonstrating that these phenotypes are also specifically attributable to loss of *pink1* function.

2.5 Discussion

Morpholino-mediated loss of *pink1* function in zebrafish profoundly affects the development of dopaminergic neurons in the ventral diencephalon and affects behaviour

Figure 2.5 Reduced swimming in *pink1* morphants.

(A) Video traces of swimming behaviour of uninjected larvae and *pink1* morphants at 5 dpf for 90 sec. Scale bar represents 1 mm. (B) When compared to uninjected controls, the average swimming distance (mm) of 5 dpf zebrafish larvae treated with 6 ng *pink1*-MO1 or MO2 was significantly decreased (Student's *t*-test, $n = 12$ per experiment set, *** $P < 0.001$). Phenotype of reduced swimming ability was significantly rescued in larvae injected with both *pink1*-MO1 and the mis-matched (mis) *pink1* mRNA (Student's *t*-test, $n = 17$, *** $P < 0.001$). Data were presented as mean ± SD.


A
Uninj
pink1-MO1
pink1-MO2
MO1+mis-pink1
5 dpf
5 dpf
5 dpf
5 dpf
B
***
***
***
Swimming distance (mm)
350
300
250
200
150
100
50
0
Uninj
MO1(6ng)
MO2(6ng)
MO1(6ng)+mis-pink1(50pg)

of the zebrafish larvae, namely, their response to a tactile stimulus and their locomotor behaviour. All these effects can be rescued by co-injection of a synthetic *pink1* mRNA that is unable to bind the morpholinos, demonstrating the specificity of the MO effects.

PINK1 mutations are found in inheritable human PD and are associated with significant DA neuron loss in the substantia nigra and motor behaviour impairment. However, the mouse *pink1*-deficient model of PD does not exhibit such defects (Kitada et al., 2007). In *Drosophila*, *pink1* mutants only have mild loss of DA neurons with subtle locomotion defects (Park et al., 2006). In our study, we report that the patterning of zebrafish DA neurons in the vDC is perturbed following *pink1* deficiency (Fig. 2.3). The mis-located DA neurons seem to have inappropriate projections to the striatum of *pink1* morphants, and this might explain the impaired response to tactile stimulation (Fig. 2.4) and hypoactivity of larvae (Fig. 2.5). In zebrafish, MO-mediated loss of *pink1* function was recently shown to cause a moderate decrease in the number of DA neurons in the ventral diencephalon, altered mitochondrial function and increased reactive oxygen species levels (Anichtchik et al., 2008). However, DA neuron patterning and locomotor behaviour were not examined.

In zebrafish, the development of the catecholaminergic system has been examined by morphological and genetic approaches (Holzschuh et al., 2003; Ma, 2003). DA neurons are first detected at 1 dpf in a cluster of cells in the ventral diencephalon area. At 3 dpf, the zebrafish central nervous system (CNS) development is well advanced (Kimmel et al., 1995). DA neurons are found mainly in the olfactory bulb (Ob), pretectum (Pr), retina and ventral diencephalon (vDC). It has been previously suggested

that the DA neurons of groups 1, 2 and 4/5 located in the vDC of zebrafish represent the equivalent of the substantia nigra neurons in humans (Rink & Wullimann, 2001, 2002). In *pink1* morphants, reduced number of group 1 neurons and abnormal patterning of groups 2 and 4/5 neurons further relate these neurons to the nigrostratial DA neurons in mammals.

PINK1 mutations cause early-onset PD, which are more related with genetic background than to environmental factors. In our study, we found abnormal DA neuron expression patterning in Pink1-deficient zebrafish, which indicates that Pink1 is also involved in DA neuron development in zebrafish. Thus, we suggest that the altered development of DA neurons, resulting from *PINK1* mutations, may contribute to the pathogenesis of PD. However, the severity of the mutations in terms of Pink1 function must be taken into account and the zebrafish MO-model probably resembles a null mutant phenotype more than the mutations found in human PD patients. Developmental defects in DA neurons, resulting from *PINK1* mutations, may also render DA neurons more susceptible to environmental stress. In a recent study, morpholino knockdown of *Lmxb1* or *Nurr1/Nr4a2*, which are important transcription factors in DA neuron specification, resulted in abnormal patterning of DA neurons in the vDC of zebrafish (Filippi et al., 2007), a phenotype that resembles what we observed in the *pink1* morphants. In addition, another transcription factor, FOXO3a (Forkhead box, subgroup O), protected neurons from oxidative stress by transcriptional regulation of *pink1* activity (Mei et al., 2009). These studies suggest a role for *pink1* in DA neuron development.

In our study, we show that weak or absent response of *pink1* morphants to tactile

stimuli can be significantly rescued by treatment with the dopamine D1 receptor agonist SKF-38393. This may imply that the ability of the fish to respond to dopaminergic stimulation remains intact; however, the dopaminergic neuronal projections towards the striatum are probably abnormal in *pink1* morphants. This is consistent with our observation that the mis-located diencephalic DA neurons in zebrafish *pink1* morphants have improper projections towards the forebrain. Abnormal connectivity would explain why hypoactivity and weak response to tactile stimuli are observed in *pink1* morphants, even though the total number of DA neurons did not seem to be largely affected (except for the possible reduction in the number of group 1 neurons).

Although several genes have been found to be associated with familial PD, the mechanisms underlying neurodegeneration are still not well understood. One of the prevailing hypotheses is that mutations in genes such as *PINK1* result in mitochondrial dysfunction. *Drosophila* models of PD support this hypothesis with evidence such as mitochondrial defects due to the loss of function of PD-related genes. Moreover, these DA neurons are more susceptible to mitochondrial toxins (Greene et al., 2003; Clark et al., 2006; Park et al., 2006). Pink1 is a mitochondrial protein kinase that is suggested to regulate mitochondrial function. *Drosophila pink1* mutants display mitochondrial defects such as loss of cristae and reduced production of ATP (Clark et al., 2006; Park et al., 2006). Pink1 is also found to interact with HtrA2, a mitochondrial protease (Plun-Favreau et al., 2007). Defects in mitochondrial function have been identified in patients with *PINK1* mutations (Hoepken et al., 2007). Although the number and morphology of mitochondria in *pink1* null mice seem normal, moderate mitochondrial

defects were displayed under intrinsic or environmental stress (Kitada et al., 2007; Gautier et al., 2008). Further animal models of PD in which mitochondria function is affected by abnormal function of PD-related genes will contribute to our understanding of the pathogenesis of human Parkinson's disease.

In human PD patients, it has been suggested that the loss of axons and synapses happen long before the loss of DA neurons during the early pathogenic process. Such axonal degeneration is associated with mitochondrial dysfunction (Sajadi et al., 2004; Coleman, 2005; Hasbani and O'Malley, 2006; Saxena and Caroni, 2007). During neural development, axonal growth cones have a relatively higher density of mitochondria compared to the rest of the cell. Mitochondria are recruited to growth cones to support the increased demand in energy that they require (Saxena and Caroni, 2007). It is possible that impaired mitochondrial function in *pink1* morphants may hinder axonal outgrowth and lead to fragmented axons (Fig. 2.3M-P). Two recent studies also suggest a link between Pink1 and axonal outgrowth with an important role in mitochondrial function. *Pink1* mutants displayed defects in axonal outgrowths together with abnormal mitochondrial structure and reduced ATP levels (Marongiu et al., 2009; Samann et al., 2009). Pink1 might function in mitochondria through two possible mechanisms during axonal growth. Pink1 may control mitochondrial quality together with Parkin, encoded by another PD-related gene (Deng et al., 2008; Narendra et al., 2008; Yang et al., 2008). Pink1 may also be implicated in mitochondria transport to the growth cones together with other mitochondrial proteins such as Miro and Milton (Weihofen et al., 2009). Poor axonal transport and mitochondrial dysfunction will eventually trigger axon degeneration.

The relationship between altered mitochondrial function and neuronal properties are, thus, likely to contribute to our understanding of the role of altered PINK1 activity in the etiology of Parkinson's disease.

2.6 Acknowledgements

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Chapter 3

Pink1 is involved in the maintenance of mitochondrial morphology and function in zebrafish

3.1 Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder and thought to be related to mitochondrial dysfunction. Mutations in *PINK1* (*PTEN-induced kinase 1*), encoding a putative mitochondrial protein, have been linked to familial early-onset PD. However, the relation of PINK1 to mitochondrial function in terms of the pathogenesis of PD remains to be fully understood. Here, we report that the loss-of-function of *pink1* gene in zebrafish by antisense morpholino oligonucleotides significantly impairs mitochondrial morphology and function. Transmission electron microscopy shows that, in *pink1*-deficient zebrafish, the numbers of mitochondria in brain and muscle are both significantly reduced, and the structure of some mitochondria is disrupted, with fragmented cristae. Moreover, the citrate synthase activity in *pink1*-knockdown zebrafish is also significantly reduced. Together, our findings demonstrate that PINK1 plays important roles in the maintenance of mitochondrial integrity and function, which may be involved in the pathogenesis of PD.

3.2 Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder and characterized by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc). Recent studies on the rare inherited forms of PD have provided insight into the mechanisms of PD pathogenesis. One of the prevailing hypotheses is that it could be caused by mitochondrial dysfunction and oxidative stress. Previous study has shown reduced complex I activity of mitochondria in PD patients (Schapira et al., 1989).

Moreover, exposure to mitochondrial toxins causes parkinsonian symptoms with selective loss of DA neurons (Betarbet et al., 2000).

Mutations in *PINK1* (*PTEN-induced kinase 1*) are linked to recessively inherited familial PD. *PINK1* encodes a ubiquitously expressed serine/threonine kinase with a putative mitochondrial targeting sequence at its N-terminal end (Mills et al., 2008). Upon cellular fractionation, PINK1 is present in the mitochondria-enriched fractions. Further studies confirmed that PINKl protein molecules are localized on mitochondrial cristae (Gandhi et al., 2006). These observations suggest PINK1 might play a role in the normal biology of mitochondria. *Drosophila pink1* mutants showed dramatic mitochondrial defects, including loss of cristae and decreased production of ATP along with mild loss of DA neurons (Clark et al., 2006; Park et al., 2006). However, in *pink1*-null mice, there was no DA neuron loss, and the number and structure of mitochondria seemed to be normal although moderate mitochondrial dysfunctions were displayed under environmental stress (Kitada et al., 2007; Gautier et al., 2008). Thus, further animal models with *pink1* loss-of-function are needed.

Zebrafish has been shown to be a good model for PD. DA neurons in the ventral diencephalon of zebrafish are equivalent to those at mammalian midbrain, and are sensitive to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treatment (Rink & Wullimann, 2002; Lam et al., 2005). Here, we examined *pink1* function in mitochondria in zebrafish using a morpholino knockdown strategy. We found that *pink1*-deficient zebrafish embryos displayed a significantly reduced activity of citrate synthase (CS) and decreased number of mitochondria, with a disrupted structure such as loss of cristae.

3.3 Materials and methods

3.3.1 Animal maintenance

Adult zebrafish and embryos were raised according to standard procedures described in Nüsslein-Volhard and Dahm (2002). All experiments were performed according to animal care guidelines provided by the Canadian Council on Animal Care and the University of Ottawa animal care committee approved all protocols.

3.3.2 Morpholino knockdown of *pink1*

Morpholino oligonucleotide (MO) was purchased from Gene Tools, LLC (Philomath, OR, USA) and was diluted with nuclease-free H_2O at 6 ng/nl and with 0.05% Phenol Red at final concentration. The *pink1*-MO1 described in Xi et al. 2010 was used in this study. One-cell stage embryos were microinjected with approximately 1 nl *pink1*-MO1 solution at 6 ng/nl. The *pink1* morphants were indistinguishable from wildtype embryos except the presence of a small bump on its head at 2 days post-fertilization (dpf). *pink1* morphants were identified by morphological examination under a Nikon SMZ1500 dissection microscope (Nikon, Mississauga, ON, Canada). 3 dpf wildtype embryos or *pink1* morphants were fixed in 1G4F solution (1% glutaraldehyde, 4% formaldehyde in PBS, pH 7.4) at 4 °C for at least overnight for transmission electron microscopy. Some embryos were collected immediately for citrate synthase assay.

3.3.3 Transmission electron microscopy

Fixed zebrafish embryos were post-fixed in 1.5 % osmium tetroxide in PBS at pH 7.4 for 1 hr at room temperature (RT). Embryos were then dehydrated in an ethanol/H_2O series, then in acetone at RT. Embryos were taken through an Epon 812/acetone series and embedded at 60 °C in pure Epon 812. Thin sections of 70 nm thickness were made on a Reichert Ultracut E ultramicrotome and mounted on formvar-coated copper slot grids. Poststaining was done with 2% aqueous uranyl acetate at pH 3.5 and Venable and Cogglesall's lead citrate. Grids were analyzed on a JEOL 1230 electron microscope at 60 kV adapted with a 2000 by 2000 pixel bottom mount CCD digital camera (Hamamatsu, Japan).

3.3.4 Citrate synthase assay

About 50 wildtype embryos or *pink1* morphants were collected and weighed. Samples were then homogenized on ice in 39 vol of homogenization buffer (75 mM Tris-HCl, 5 mM EDTA and 0.5% Triton-X 100, pH 7.5), centrifuged and the supernatant was collected for the assay. The V_{max} was measured by a Thermomax 96-well microplate reader (Molecular Devices, Sunnyvale, CA, USA). Citrate synthase was assayed in 100 mM Tris (pH 8.0) with 0.1 mM 5,5-Vdithiobis (2-nitrobenzoic acid, DTNB), 0.3 mM acetyl-CoA, and 1 mM oxaloacetate (omitted from the control) at 412 nm for 5 min at 25 °C. All assays were run in triplicate.

3.3.5 Statistical analysis

All experiments were independently performed at least three times. All data

quantification and statistical analysis were performed with Microsoft Excel 2003. Student's *t*-tests were used when applicable. Data were expressed as mean ± SD. P-values of < 0.05 (two-sided) were considered as statistically significant.

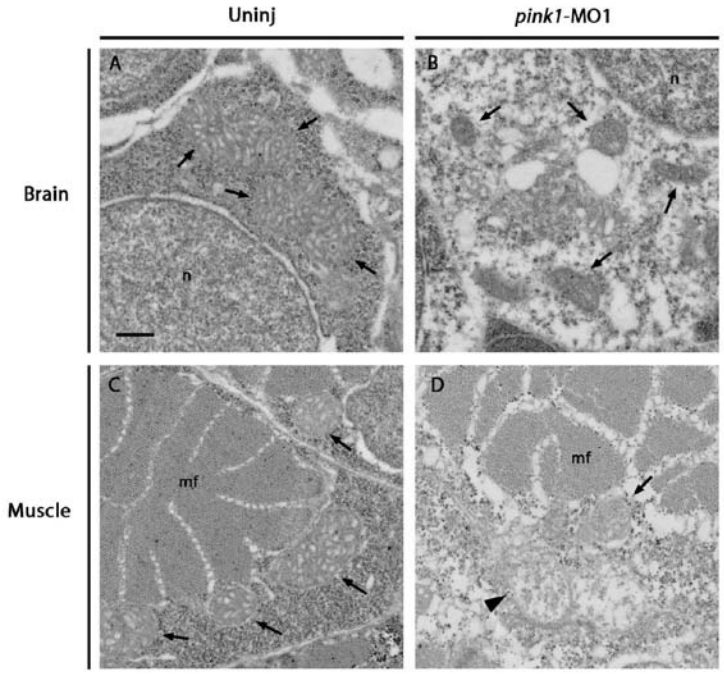
3.4 Results

3.4.1 Abnormal mitochondrial structure and reduced number of mitochondria in *pink1* morphants.

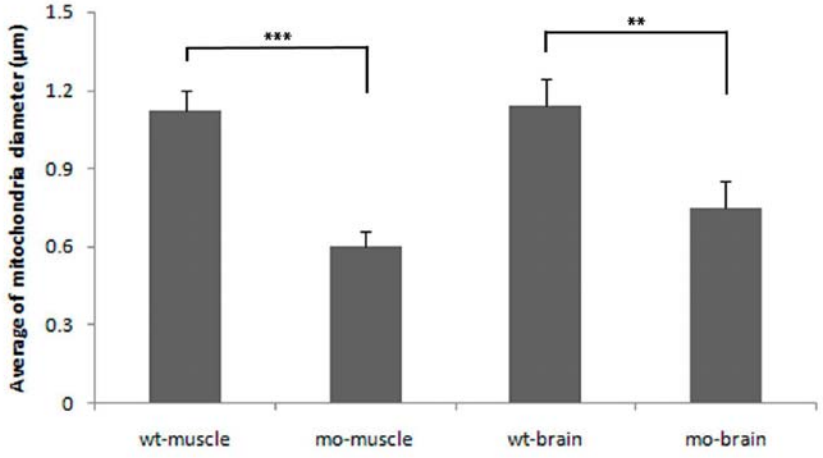
Loss of *pink1* function in *Drosophila* resulted in striking morphological defects, such as fragmented cristae, in mitochondria derived from indirect flight muscles (Clark et al., 2006; Park et al., 2006). In contrast, such defects were not observed in *pink1*-null mice (Gautier et al., 2008). We therefore examined whether such defects would be seen in zebrafish, another vertebrate model for PD. Wildtype embryos or *pink1*-morphants at 3 dpf were subjected to transmission electron microscopy. Transverse sections were made in the brain and trunk of the embryos. We examined mitochondria in the ventral diencephalon (vDC) area, where the zebrafish equivalent to DA neurons of the mammalian nigrostriatal pathway are localized (Rink & Wullimann, 2002). Mitochondria in muscle tissue were also examined since Pink1 is ubiquitously expressed (Xi et al., 2010) and muscle also has high-energy demands and requires robust mitochondrial function. Compared with un-injected wildtype embryos, *pink1* morphants displayed severely damaged mitochondrial structure in the vDC and muscle (Fig. 3.1A-D). Some

Figure 3.1 Abnormal mitochondrial morphology, reduced number of mitochondria and reduced citrate synthase activity in *pink1*-knockdown zebrafish embryos.

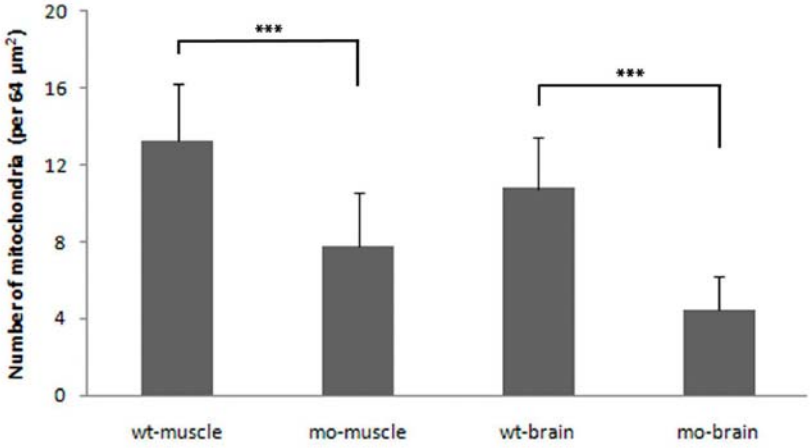
(A-D) Representative electron microscopy photographs of un-injected wildtype embryos and *pink1* morphants. Arrows and arrowhead indicate the presence of mitochondria. Arrowhead indicates a typical, enlarged mitochondria with severely fragmented cristae. n: nucleus; mf: muscle fibre. Scale bar represents 500 nm. Quantitative analysis of mitochondrial diameter (E), the number of mitochondria (F) and citrate synthase activity (G) in *pink1* morphants. ** $P < 0.01$; *** $P < 0.001$. All experiments were repeated at least three times.


Uninj
pink1-MO1
Brain
Muscle
A
B
C
D
n
n
mf
mf

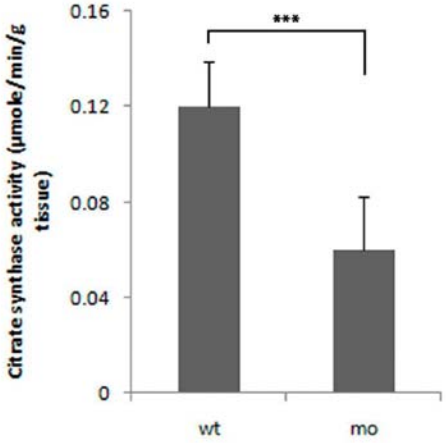
E


Average of mitochondria diameter (μm)
1.5
1.2
0.9
0.6
0.3
0
***
**
wt-muscle
mo-muscle
wt-brain
mo-brain

F


Number of mitochondria (per 64 μm²)
20
16
12
8
4
0
***
***
wt-muscle
mo-muscle
wt-brain
mo-brain

G


Citrate synthase activity (μmole/min/g tissue)
0.16
0.12
0.08
0.04
0
***
wt
mo

mitochondria were enlarged with severely fragmented cristae (Fig. 3.1D, arrowhead). Most mitochondria seem smaller than those in wildtype embryos (Fig. 3.1B, D). Quantitative analysis revealed that the average diameter of mitochondria in the vDC or muscle of *pink1* morphants was 65.9 ± 2.7 % (Student's *t*-test, $n = 12$, $P < 0.01$) or 54.1 ± 7.7 % (Student's *t*-test, $n = 29$, $P < 0.001$) of that in wildtype embryos, respectively (Fig. 3.1E). The number of mitochondria in the vDC or muscle was also reduced to 41.0 ± 3.8 % (Student's *t*-test, $n = 16$, $P < 0.001$) or 51.3 ± 3.1 % (Student's *t*-test, $n = 13$, $P < 0.001$) of that in wildtype embryos, respectively (Fig. 3.1F). Consistent with the above results, the activity of citrate synthase, a common marker for mitochondrial density in tissue, was reduced to 47.7 ± 1.3 % (Student's *t*-test, $n = 6$, $P < 0.05$) of that in wildtype embryos (Fig. 3.1G).

3.5 Discussion

In the present study, we report that loss of *pink1* function in zebrafish results in severe mitochondrial morphological defects such as a smaller size and fragmented cristae, and in a significantly reduced number of mitochondria and reduced citrate synthase activity both in the vDC area of brain and in muscle tissue. These observations are in consistent with the phenotypes found in *Drosophila pink1* mutants (Clark et al., 2006; Park et al., 2006). These findings strongly suggest a key role for Pink1 in the maintenance of mitochondrial integrity and function.

Interestingly, our results showed that mitochondria in *pink1* morphants have a smaller size, while there is an increase in the abundance of larger mitochondria in

pink1-null mice (Gautier et al., 2008). PINK1 is a mitochondrial kinase, which has been shown to be involved in the control of mitochondrial dynamics by promoting fission (Deng et al., 2008; Narendra et al., 2008; Yang et al., 2008). PINK1 also accumulates to damaged mitochondria and then signals Parkin, encoded by another PD-linked gene, to promote their elimination (Geisler et al., 2010; Narendra et al., 2010). Therefore, in *pink1* morphants, we speculate endogenous Parkin cannot be recruited to and eliminate the damaged mitochondria due to the lack of Pink1. In addition, the damaged mitochondria might not be able to get rid of the damaged parts themselves through fission due to the lack of Pink1. These result in the dysfunction of the large, damaged mitochondria and, eventually, in their death. It is possible that only the small healthy ones can survive. However, in *pink1*-null mice, there is perhaps a more elaborate compensatory mechanism to protect the mitochondria from damage although the mitochondria become larger due to lack of fission.

PINK1 and Parkin were suggested to be in the same pathway involved in regulating mitochondrial function. It was reported that *parkin*-knockdown zebrafish embryos developed specific reductions in the activity of the mitochondrial respiratory chain complex I (Flinn et al., 2009). It will be interesting to examine whether Pink1 deficiency will cause the same defects in mitochondrial respiratory chain. Due to the technical difficulties, we could not compare the oxygen consumption rate of *pink1* morphants with that of wildtype embryos. An adult zebrafish with *pink1* mutations would be helpful to provide enough tissue for this assay.

Citrate synthase (CS) is an enzyme localized in the mitochondrial matrix and

catalyzes the condensation of oxaloacetate and acetyl coenzyme-A (acetyl-CoA), an important step of the Krebs cycle. Nicotine adeninedinucleotide (NADH) and succinate are generated in the Krebs cycle, and then oxidized in the mitochondrial electron transport chain to produce ATP. Reduced CS activity may reflect a mitochondrial dysfunction in *pink1* morphants, confirming that mitochondria have a central role in the pathogenesis of PD. It remains to be determined how the reduction in brain energy metabolism might be related to the pathophysiology of PD.

3.6 Acknowledgements

We thank Dr. Kelly Soanes and Cindy Leggiadro (NRC Institute for Marine Biosciences), and Peter Rippstein and Junhui Tan (University of Ottawa, Heart Institute) for helping with the preparation and analysis of electron microscopy examinations, and Dr. Charles Darveau and Alborz Jooya for the performance and analysis of citrate synthase assay. This work was supported by the Canadian Institute of Health Research #MOP93803 to M.E.

Chapter 4

Regeneration of dopaminergic neurons labeled with green fluorescent protein in transgenic zebrafish

4.1 Abstract

We have generated a line of transgenic zebrafish, Tg(*dat:EGFP*), in which the green fluorescent protein (GFP) is expressed under the control of *cis*-regulatory elements of the *dopamine transporter* (*dat*) gene. In Tg(*dat:EGFP*) fish, all major clusters of dopaminergic (DA) neuron are labeled with GFP, including those in the ventral diencephalon (vDC), amacrine cells in the retina, in the olfactory bulb, pretectum, and caudal hypothalamus. In the vDC, DA neurons of groups 2-6, homologous to the ones in the human midbrain, are correctly labeled with GFP. MPTP treatments induced a significant but modest loss of DA neurons in the vDC (groups 2-6). Moreover, we observed that DA neurons in the vDC (groups 2-6) could partially be replaced after severe laser ablation. This suggests that zebrafish may have the capacity of regenerating DA neurons after injury. This transgenic line would be very useful for studies of DA neuron development and its relation with Parkinson's disease.

4.2 Introduction

Dopaminergic (DA) neurons are a class of neurons that synthesize and release the neurotransmitter dopamine. In the central nervous system (CNS), dopamine plays important roles in a variety of physiological and behavioral processes such as voluntary movement, cognition, memory and reward (Bjorklund and Dunnett, 2007). Dopamine is biosynthesized from tyrosine. Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme that converts tyrosine to L-DOPA. Then L-DOPA is decarboxylated into dopamine by DOPA decarboxylase. After release, the excessive dopamine in the synaptic

cleft can be re-uptaken back into DA neurons via the action of the dopamine transporter (DAT), which is an inter-membrane protein exclusively expressed in DA neurons (Ciliax et al., 1995, 1999).

Deficiency of dopamine neurotransmission is implicated in several neurological diseases including Parkinson's disease (PD). The main pathological hallmark of PD is the progressive loss of DA neurons in the substantia nigra pars compacta (SNc) of the midbrain, which results in a deficiency in released dopamine in the striatum and causes movement disorders. Although several genes have been found to be related with familial inheritable PD, the detailed etiology of PD is still not well understood (Cookson, 2005). The majority of PD cases are sporadic, which may result from environmental factors. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin known to induce PD in human, has been used to induce PD symptoms and DA neuron degeneration in various animal models (Langston et al., 1983).

The zebrafish has proved to be an excellent vertebrate model for development biology and the study of human diseases (Driever et al., 1994, 1996; Zon, 1999). Transgenesis is widely used in zebrafish due to its transparent and externally developing embryos (Lin, 2000). Despite notable differences, the overall organization of the zebrafish brain shows similarity to that of the human brain. In zebrafish, the development of DA neurons has been examined by morphological and genetic approaches (Ma, 1994, 2003; Rink and Wullimann, 2002). Although no DA neurons have been found in midbrain of zebrafish, several groups of DA neurons in the posterior tuberculum of ventral diencephalon send projections to the subpallium of telencephalon and are

suggested to be the zebrafish homologues of DA neurons in SNc of human (Rink and Wullimann, 2001). Several genes, including PD-associated genes, have been suggested to be related with the development of these DA neurons and with motor behavior in larvae (Guo et al., 1999; Ryu et al., 2007; Xi et al., 2010).

In this study, we generated a transgenic zebrafish line, in which all major clusters of DA neurons are correctly labeled with green fluorescent protein (GFP) during embryogenesis, especially for the ones in the vDC. We took advantage of the fact that DAT expression distinguishes DA neurons from other catecholaminergic (CA) neurons in the developing embryo (Holzschuh et al., 2001) and produced a line of transgenic fish in which GFP is expressed under the control of regulatory elements of the zebrafish *dat* gene. MPTP treatment caused a significant but not large number of GFP-positive DA neuron death in the vDC of these fish. In addition, the GFP-positive DA neurons in the vDC were found to partially recover after severe laser ablation. This suggests that DA neurons in the vDC may have regenerative capacity after injury.

4.3 Materials and methods

4.3.1 Animal maintenance

Zebrafish and embryos were maintained according to methods described by Nüsslein-Volhard and Dahm (2002). Embryos were staged as hours post-fertilization (hpf) according to specific morphological features outlined by Kimmel et al. (1995). All experiments were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the University of Ottawa animal care committee.

4.3.2 Construct for transgenesis

A partial sequence of exon 1 of zebrafish *dat* gene was PCR amplified with oligonucleotides 5'-ATGCTGAGAGGCAGACCGG-3' and 5'-GTAGCACAGGTATGGGAACC-3', and radioactively labeled. This probe was used to screen a zebrafish genomic PAC library (RZPD, Berlin, Germany) according to the protocol recommended by the manufacturer. A PAC clone (BUSMP706K0187Q9) was identified to contain the *dat* gene. The *EGFP* coding sequence was cloned in frame into *dat* exon 1 using an *E. coli* strain (DY380) with temperature inducible RecET homologous recombination system according to procedures described by Liu et al. (2003). A fragment of 27 kb from this PAC-EGFP construct, containing the whole genomic sequence of *dat* (including approximately 13 kb of its 5'-flanking region) except for its last coding exon and 3'-flanking region, was cloned into a modified pGEM vector with *Tol2* arms at both ends by homologous recombination. This Tol2-PAC-EGFP construct was utilized for producing transgenic zebrafish lines.

4.3.3 Zebrafish transgenesis

The Tol2-PAC-EGFP plasmid DNA (54 ng/μl) and 35 ng/μl transposase mRNA were co-injected into one-cell stage zebrafish embryos according to established procedures (Xi et al., 2010). Injected embryos were screened for GFP expression in the desired area between 48-72 hpf. GFP-positive individuals were raised to sexual maturity and outcrossed with wildtype adult fish to identify transgenic carriers.

4.3.4 Whole-mount *in situ* hybridization and immunostaining

Embryos for whole-mount *in situ* hybridization and immunostaining were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), then dehydrated in 100% methanol, and stored at -20 °C in 100% methanol until use. Whole-mount *in situ* hybridization, immunostaining and confocal microscopy observation were performed as described by Xi et al. (2010). A *th* antisense probe was used as previously described (Xi et al., 2010). The following primary antibodies were used: Mouse anti-GFP (1:300, Invitrogen, A-11120, Burlington, ON, Canada); Rabbit anti-TH (1:100; Chemicon, AB152, Ottawa, ON, Canada). The following secondary antibodies were used: Goat anti-mouse AlexaFluor 488 (1:200, Invitrogen, A-10667, Burlington, ON, Canada); Goat anti-rabbit AlexaFluor 594 (1:200, Invitrogen, A-11012, Burlington, ON, Canada).

4.3.5 Double immunohistochemistry on forebrain cryosections

Embryos for double immunohistochemistry were fixed in 4% PFA/1xPBS overnight at 4 °C, rinsed in 1xPBS and placed in 30% sucrose/1xPBS to equilibrate. Embryos were then embedded in Shandon cryomatrix (ThermoFisher Scientific, 2860015, Ottawa, ON, Canada), sectioned on a Leica CM1850 (Leica Microsystems, Weltzar, Germany) cryostat at a thickness of 10-20 μm and stored at -20 °C until use. After 3X washes in 1xPBS for 10 min, the sections were preincubated with a blocking solution containing 10% new calf serum and 0.1% Tween 20 for 2 hr at room temperature, and then incubated with the same blocking solution containing the primary antibodies (see below)

overnight in a humid chamber at 4 °C. After removal of the primary antibodies with 3X PBS washes, the appropriate secondary antibodies were incubated on the slides for 2 hr at room temperature. After the final washes, the slides were mounted using Vectashield mounting medium (Vector labs, H-1000, Burlington, ON, Canada). Signals were visualized on a Nikon Eclipse E3600 stereomicroscope with filters for both fluorescent stains. The following primary and secondary antibodies were used: Mouse anti-GFP (1:1000, Invitrogen, A-11120); Rabbit anti-TH (1:500; Chemicon, AB152); Goat anti-mouse AlexaFluor 488 (1:300, Invitrogen, A-10667); Goat anti-rabbit AlexaFluor 594 (1:300, Invitrogen, A-11012).

4.3.6 MPTP treatment

MPTP (Sigma-Aldrich, Oakville, ON, Canada) was dissolved in distilled water to 10 mM as a stock solution. Embryos were obtained from an outcross between the *dat:EGFP* transgenic fish and wildtype individuals. At 24 hpf, GFP-positive embryos were collected, manually dechorionated, and transferred into a six-well plate containing 4 ml E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) with 0.003% phenylthiourea (PTU, Sigma-Aldrich, Oakville, ON, Canada) and MPTP at the following concentrations (100 μm, 500 μm and 1 mM). The MPTP-containing buffer was changed daily. The embryos were examined under a fluorescence microscope, and then fixed in 4% PFA (paraformaldehyde) in PBS for immunostaining. Control groups received no MPTP. MPTP exposures were performed and toxins were disposed according to appropriate safety protocols (Przedborski et al., 2001).

4.3.7 Laser cell ablation

The procedure of laser cell ablation was modified from the protocol described by Roeser and Baier (2003). Briefly, 3 dpf embryos were immobilized in 0.1% low-melting agarose gel with dorsal up in a petri dish with fresh embryo medium just covering the head of the fish. Laser cell ablations were performed using a VSL-337ND-S laser system (Spectra-Physics) installed on a Zeiss Axioskop compound microscope. A laser beam of 435 nm wavelength was generated and aimed via a 40x water immersion lens under GFP fluorescence. 30 Hz laser pulses were applied for about 5 seconds on each cell. It took from 5 to 10 min to completely ablate GFP-positive DA neurons in the vDC. GFP fluorescence disappeared after laser ablation with no sign of damage to the tissues surrounding the targeted area. The efficiency of laser cell ablation was later confirmed by immunostaining with anti-GFP antibody on ablated embryos.

4.3.8 Statistical analysis

All data quantification and statistical analysis were performed with Microsoft Excel 2003. Student's *t*-tests were used when applicable. Data were expressed as mean ± SD. P-values of < 0.05 (two-sided) were considered as statistically significant. All experiments were independently repeated for at least three times.

4.4 Results

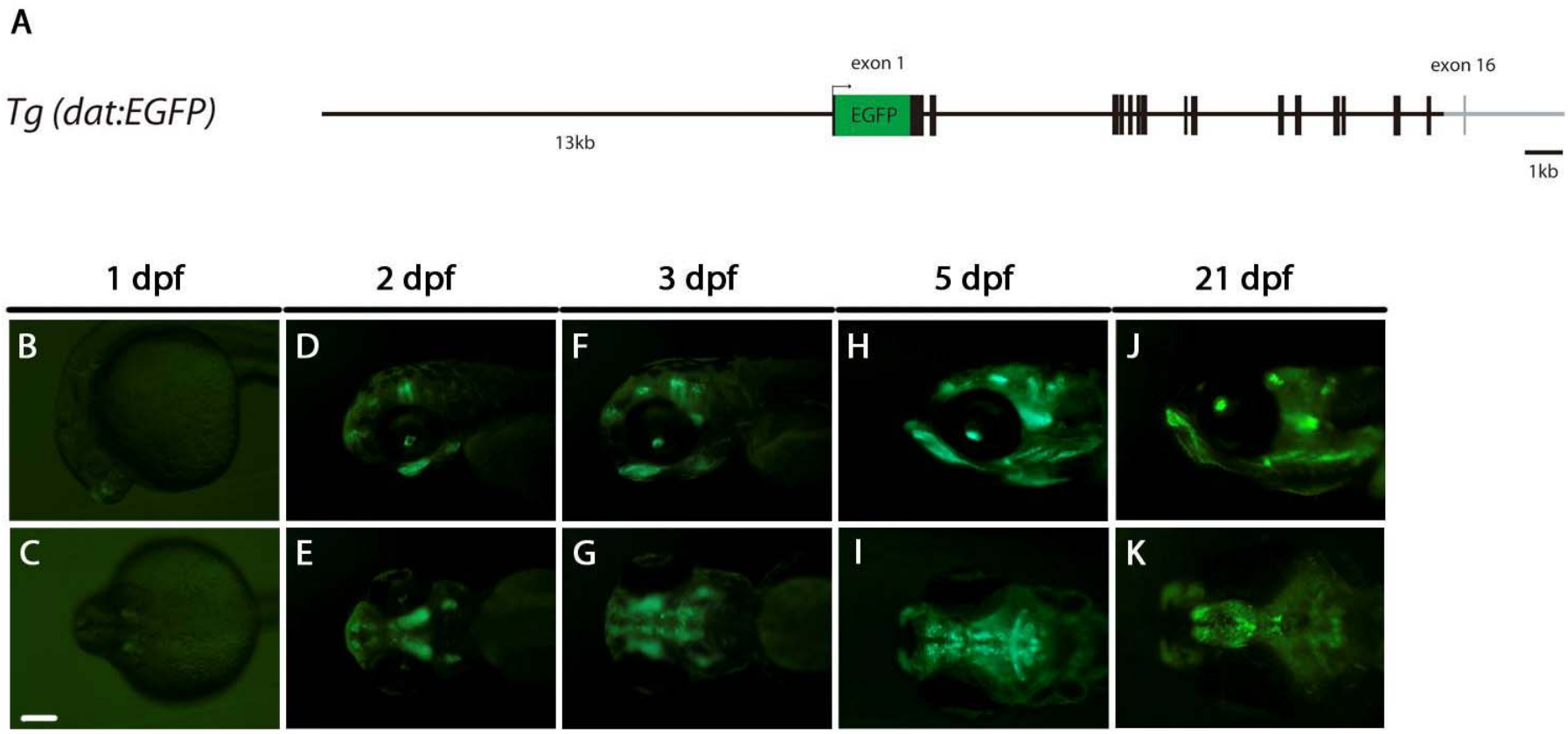
4.4.1 Generation of Tg(*dat:EGFP*) lines

In order to screen for clones containing the *dopamine transporter* (*dat*) gene from a zebrafish genomic PAC library (P1 artificial chromosome; RZPD, Berlin, Germany), a partial sequence of exon 1 of zebrafish *dat* gene was amplified and radioactively labeled by PCR, and later used as a probe for DNA hybridization. A PAC clone (BUSMP706K0187Q9) was identified and confirmed to contain the whole genomic sequence of *dat* except for its last coding exon and 3'-flanking region. The construct contained approximately 13 kb of *dat* 5'-flanking region (Fig. 4.1A). The *enhanced green fluorescent protein* (*EGFP*) was inserted in frame into *dat* exon 1 by homologous recombination (Fig. 4.1A). This 27kb *dat-EGFP* construct was then cloned into a pGEM-Tol2 vector by homologous recombination. The transgene DNA and *Tol2* mRNA were injected simultaneously into one-cell stage embryos. More than 90% of injected embryos showed GFP expression in the brain area roughly between the two eyes at 48 hpf. These embryos were raised until adulthood, and then crossed with wildtype fish and screened for germ-line transmission of the transgene. Among 42 founder fish, 6 fish were identified to have germ-line transmission and 4 of them gave similar GFP expression patterns in transgenic F1 fish.

To determine GFP expression in Tg(*dat:EGFP*) fish, we crossed transgenic F1 fish with wildtype fish and examined their progeny under a fluorescence microscope (Fig. 4.1B-K). GFP starts to be expressed at approximately 20 hpf in the telencephalon. GFP expression persists until adulthood, mainly in the telencephalon, diencephalon, midbrain and jaw area.

Figure 4.1 The Tg(*dat:EGFP*) line.

(A) A schematic map of the DNA fragment used in *Tol2*-based *dat* transgenesis. The inserted DNA is total 27kb, containing the whole *dat* genomic sequence except for the last coding exon and 3'-flanking region, with *EGFP* (in green shade) inserted at the beginning of exon 1 and maintaining the same reading frame. (B-K) Time course of GFP expression in Tg(*dat:EGFP*) embryos. GFP was detected starting at 1 dpf, and was expressed mainly in the brain until adulthood. Panels B, D, F, H, J are lateral views, anterior to the left. Panels C, I, K are dorsal views. Panels E, G are ventral views. Scale bar represents 100 μm.


A
Tg (dat:EGFP)
exon 1
exon 16
EGFP
13kb
1kb
1 dpf
2 dpf
3 dpf
5 dpf
21 dpf
B
C
D
E
F
G
H
I
J
K

4.4.2 The expression of *tyrosine hydroxylase* (*th*) compared with that of GFP in Tg(*dat:EGFP*)

Tyrosine hydroxylase and dopamine transporter are commonly used as markers for mature DA neurons. We examined their expression in zebrafish by whole-mount *in situ* hybridization (Fig. 4.2). During embryogenesis, both *th* and *dat* are expressed in the DA neurons, based on their position in the vDC, that send projections anteriorly and were suggested to be equivalent to DA neurons in the mammalian nigrostriatal pathway (Rink and Wullimann, 2001).

In order to verify that GFP expression in Tg(*dat:EGFP*) coincides with DA neurons, we performed whole-mount *in situ* hybridization with a *th* probe on 3dpf embryos and compared that with GFP expression in Tg(*dat:EGFP*) embryos (Fig. 4.3). At 3 dpf, similar to *th* expression, GFP is expressed mainly in the olfactory bulb (Ob), pretectum (Pr) and vDC. Although there was no obvious *th* expression observed in the caudal hypothalamus (Hc), GFP expression was seen there (Fig. 4.3), and their co-localization could be confirmed by double immunostaining for Th and GFP in Tg(*dat:EGFP*) embryos. The GFP expression in the jaw and other areas is most likely to be unspecific and attributable to insertion effects.

4.4.3 The majority of DA neurons are correctly labeled with GFP.

To further determine whether the various clusters of DA neuron in zebrafish brain are labeled by GFP, double immunofluorescence staining for Th and GFP was performed in Tg(*dat:EGFP*) embryos. At 3dpf, DA neurons are mainly found in the vDC, Ob, Pr, Hc

Figure 4.2 Whole-mount *in situ* hybridizations of *tyrosine hydroxylase* (*th*) and *dopamine transporter* (*dat*) in zebrafish embryos.

Whole-mount *in situ* hybridization with a *tyrosine hydroxylase (th)* cRNA probe (A-F) and a *dopamine transporter* (*dat*) cRNA probe (G-L). The DA neurons in the ventral diencephalon are shown by arrowheads. Panels A, C, E, G, I, K are lateral views with anterior to the left; panels B, D, F, H, J, L are ventral views. Scale bar represents 100 μm.

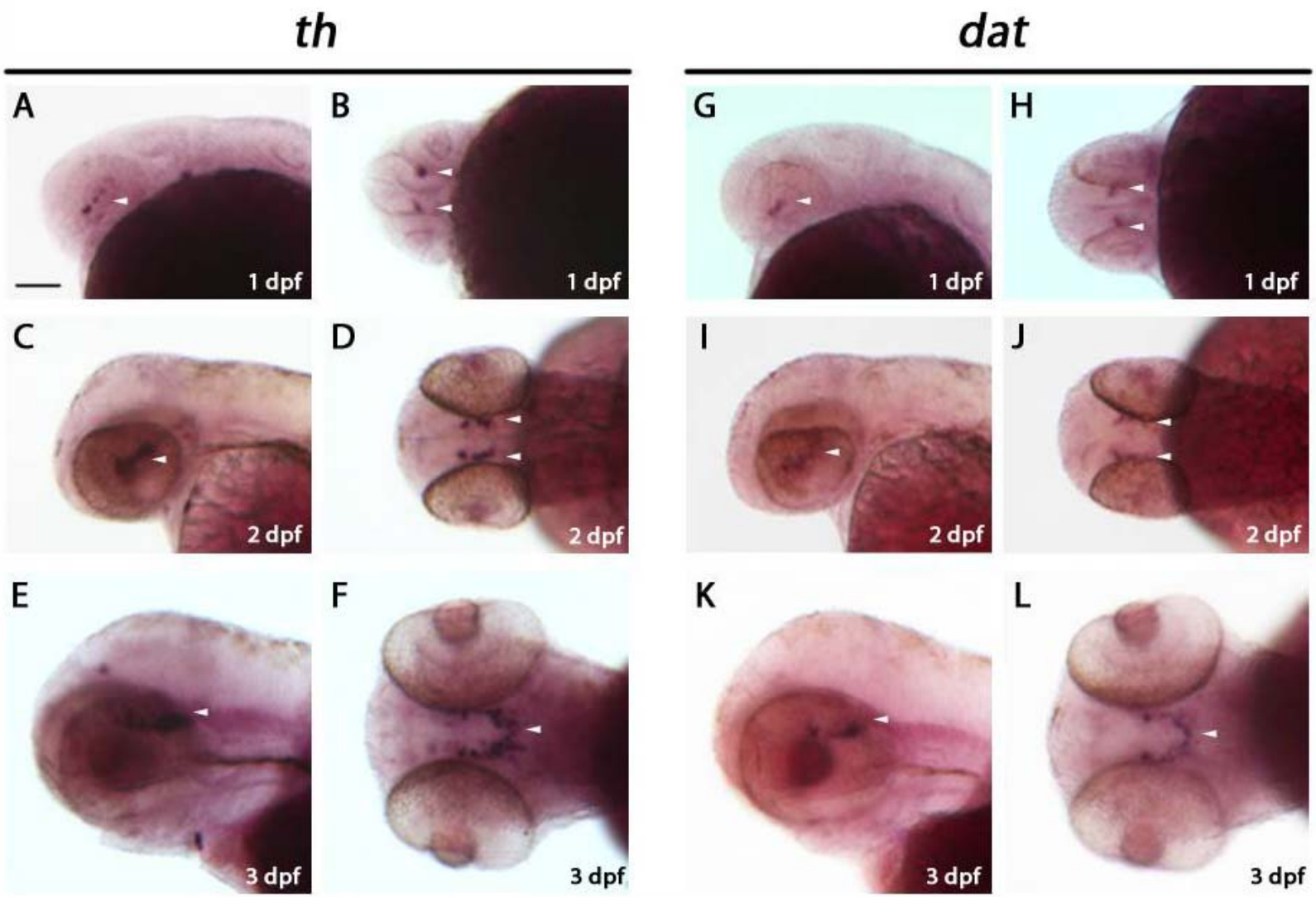
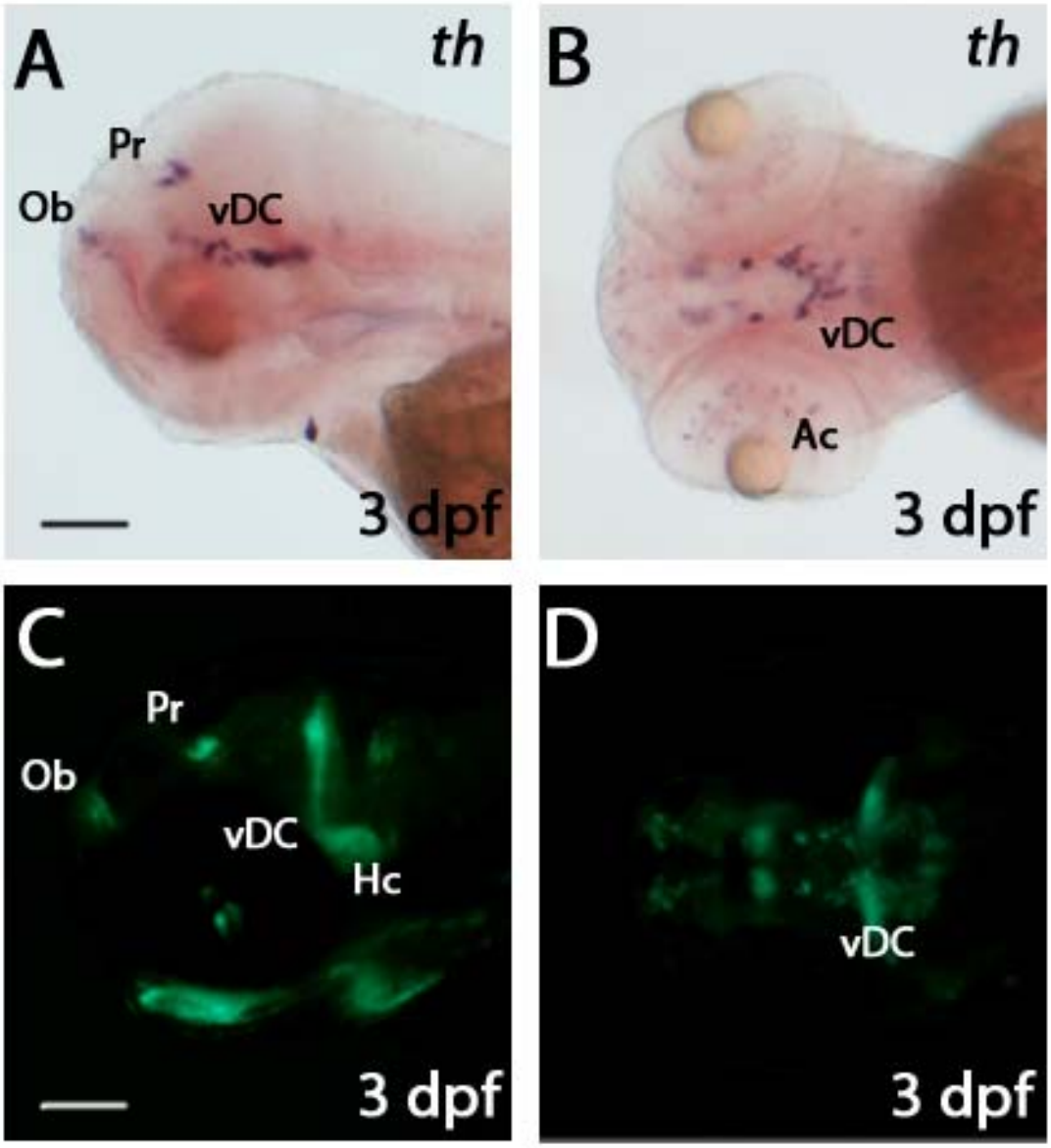

th
dat
A
B
G
H
1 dpf
1 dpf
1 dpf
1 dpf
C
D
I
J
2 dpf
2 dpf
2 dpf
2 dpf
E
F
K
L
3 dpf
3 dpf
3 dpf
3 dpf

Figure 4.3 Comparison of *th* and GFP expression patterns in Tg(*dat:EGFP*) embryos.

(A-B) whole-mount *in situ* hybridization showing *tyrosine hydroxylase (th)* expression in 3 dpf embryos. (C-D) Live images showing GFP expression in 3 dpf Tg(*dat:EGFP*) embryos. Panels A, C are lateral views with anterior to the left; panels B, D are ventral views. The following abbreviations are used: olfactory bulb (Ob), pretectum (Pr), ventral diencephalon (vDC), amacrine cells (Ac) and caudal hypothalamus (Hc). Scale bars represent 100 μm.


A
th
Pr
Ob
vDC
3 dpf
B
th
vDC
Ac
3 dpf
C
Pr
Ob
vDC
Hc
3 dpf
D
vDC
3 dpf

and Ac in the retina based on the immunostaining with Th antibodies. In the vDC, DA neurons are normally divided into 6 different groups based on their location, morphology and function (Rink and Wullimann, 2002). In the vDC of 3 dpf Tg(*dat:EGFP*) embryos, the Th-positive DA neurons of groups 2, 3, 4/5 and most of those in group 6 are correctly labeled with GFP although those of group 1 are not (Fig. 4.4). Furthermore, the GFP-positive neurons of groups 2, 3, 4/5 and most of those in group 6 are Th-positive (Fig. 4.4A-B'''). This was also confirmed by confocal miscroscopy on double immunostaining for GFP and Th on whole-mount 3 dpf Tg(*dat:EGFP*) embryos, by double immunostaining on transverse cryosections of 3dpf Tg(*dat:EGFP*) embryos and on horizontal cryosections of 5 dpf Tg(*dat:EGFP*) embryos (Fig. 4.5, 4.6 and 4.7). It seems that all Th-positive DA neurons in the Ac, Ob and Pr are GFP-positive, and *vice-versa* (Fig. 4.4 B-D'''). The Th-positive DA neurons in the Hc are all GFP-positive, but not all GFP-positive neurons are Th-positive (Fig. 4.4E-E'''). The reason for this might be the anti-Th antibody used here can only recognize a fraction of Th-positive neurons in the Hc, which are mainly *th2*-expressing cells but not *th1*-expressing cells (Chen et al., 2009; Fillip et al., 2010; Yamamoto et al., 2010).

4.4.4 MPTP affects the survival of DA neurons in the ventral diencephalon.

In zebrafish, the DA neurons in the vDC, especially the ones of groups 2 and 4/5 that send projections towards the forebrain, are suggested to be homologous to those in the substantia nigra of midbrain in humans (Rink and Wullimann, 2001). Since these DA neurons are perfectly labeled with GFP in Tg(*dat:EGFP*) fish, we used them as a live

Figure 4.4 Double immunostaining for GFP and Th in 3 dpf Tg(*dat:EGFP*) embryos. Horizontal cryosections of 3 dpf Tg(*dat:EGFP*) embryos were stained with anti-GFP and anti-Th antibodies. GFP-positive cells, Th-positive cells and GFP/Th-positive cells are shown in green, red and yellow, respectively. The following abbreviations are used: olfactory bulb (Ob), pretectum (Pr), ventral diencephalon (vDC), amacrine cells (Ac) and caudal hypothalamus (Hc). Numbers in A''' and B''' indicate different groups (1-6) of DA neurons in the vDC. Arrows show GFP/Th-positive cells in the retina. Arrowheads show GFP/Th-positive cells in the Ob, Pr and Hc. Section (A-A''') and section (B-B''') are the same section, focusing on different cell groups when imaging. All sections are shown as anterior to the left. Scale bars represent 100 μm (A-A'', B-B'', C-C'', D-D'' and E-E'') and 25 μm (A''', B''', C''', D''' and E''').

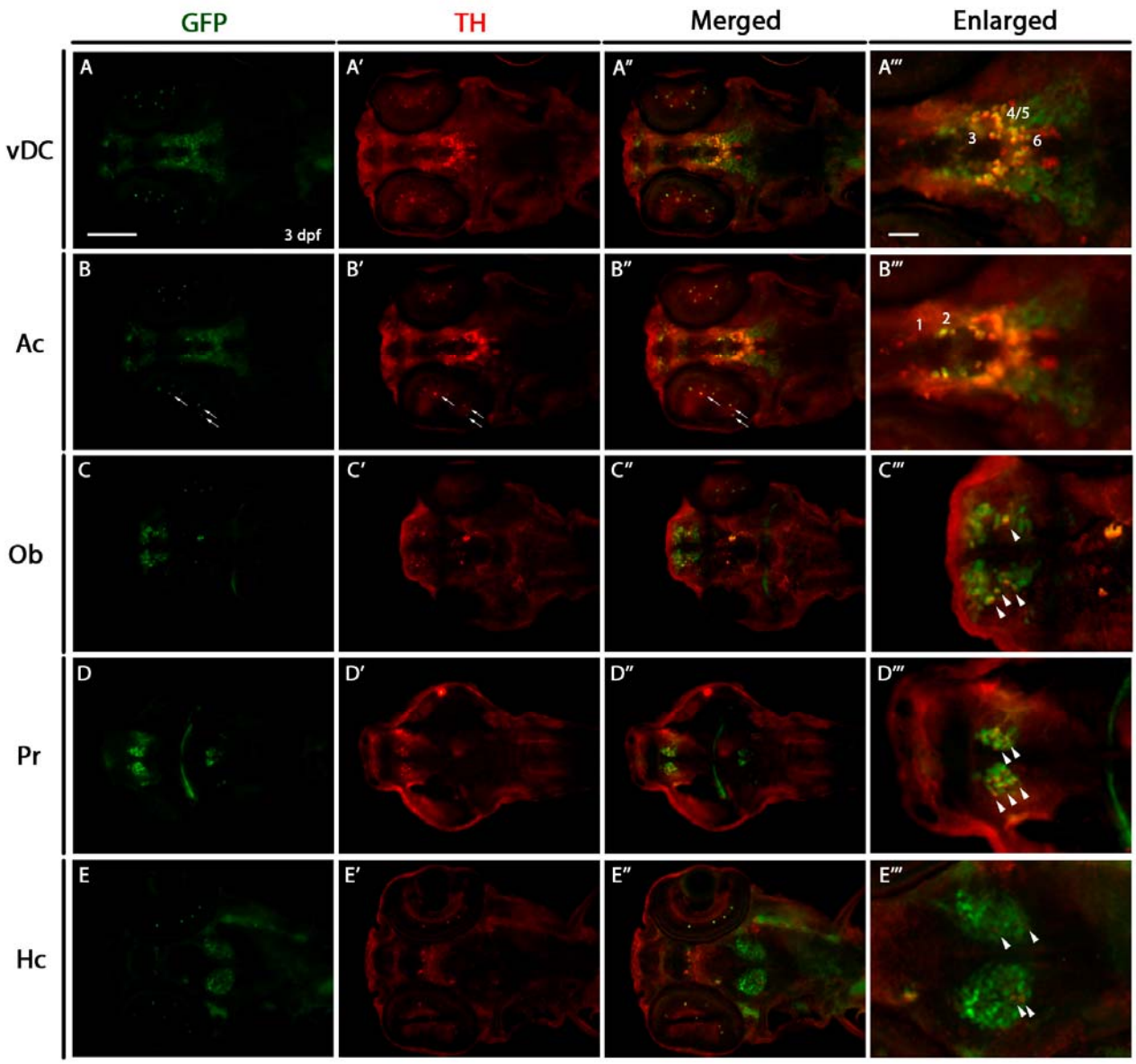

GFP
TH
Merged
Enlarged
vDC
Ac
Ob
Pr
Hc
A
A′
A″
A‴
B
B′
B″
B‴
C
C′
C″
C‴
D
D′
D″
D‴
E
E′
E″
E‴
3 dpf
4/5
3
6
1
2

Figure 4.5 Whole-mount immunostaining for GFP and Th in 3 dpf Tg(*dat:EGFP*) embryos.

3 dpf Tg(*dat:EGFP*) embryos were stained with anti-GFP and anti-Th antibodies, followed by confocal microscopy. GFP-positive cells and Th-positive cells are shown in green and red, respectively. (A-H) A series of confocal images focusing at different levels in the ventral diencephalon (vDC). (I) Projection of confocal images A-H. Numbers in I indicate different DA neuron groups (1-6) in vDC. The animal was shown as dorsal views with anterior to the left. Scale bars represent 25 μm.

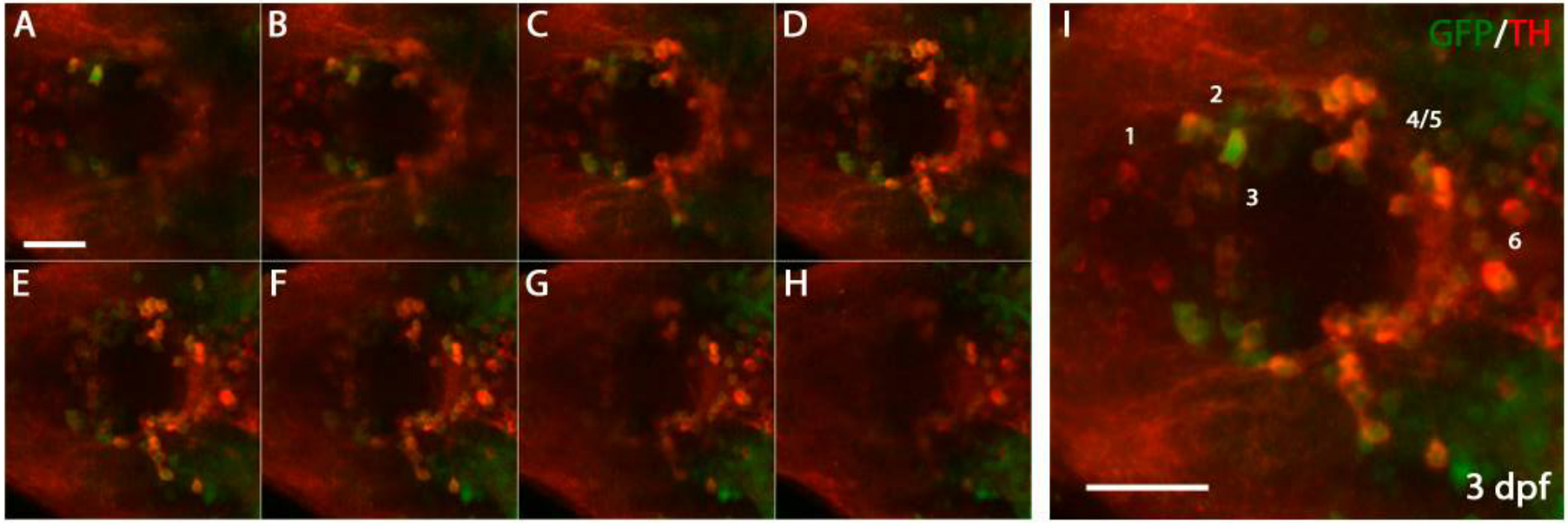

A
B
C
D
E
F
G
H
I
GFP/TH
1
2
3
4/5
6
3 dpf

Figure 4.6 Double immunostaining for GFP and Th on transverse cryosections of Tg(*dat:EGFP*) embryos at 3 dpf.

3 dpf Tg(*dat:EGFP*) embryos were transversely cryosectioned and stained with anti-GFP and anti-Th antibodies. GFP-positive cells and Th-positive cells are shown in green and red respectively. Numbers (1-6) indicate different groups of DA neurons in the ventral diencephalon. Pr: pretectum. All sections are shown as dorsal to the top. Scale bar represents 100 μm.

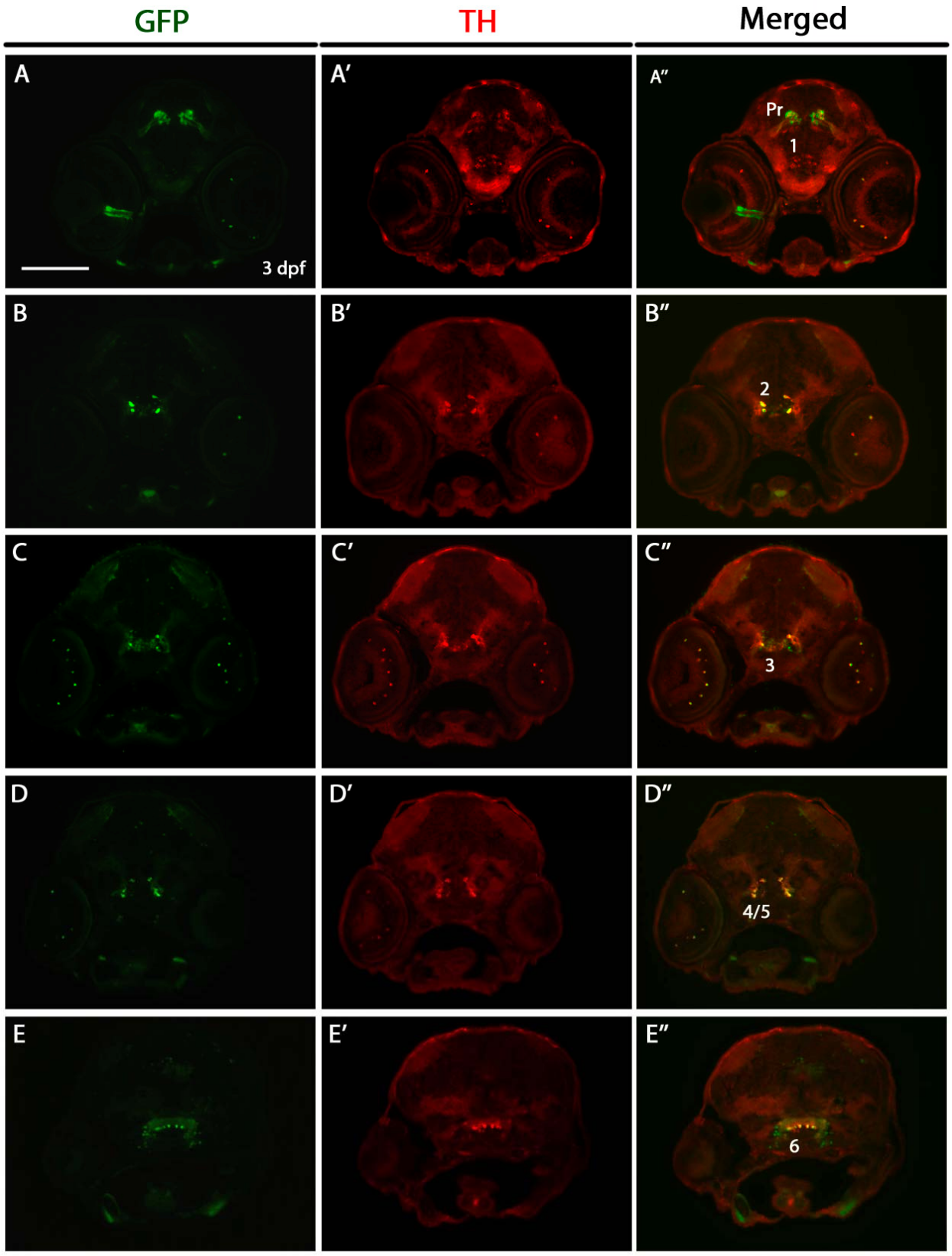
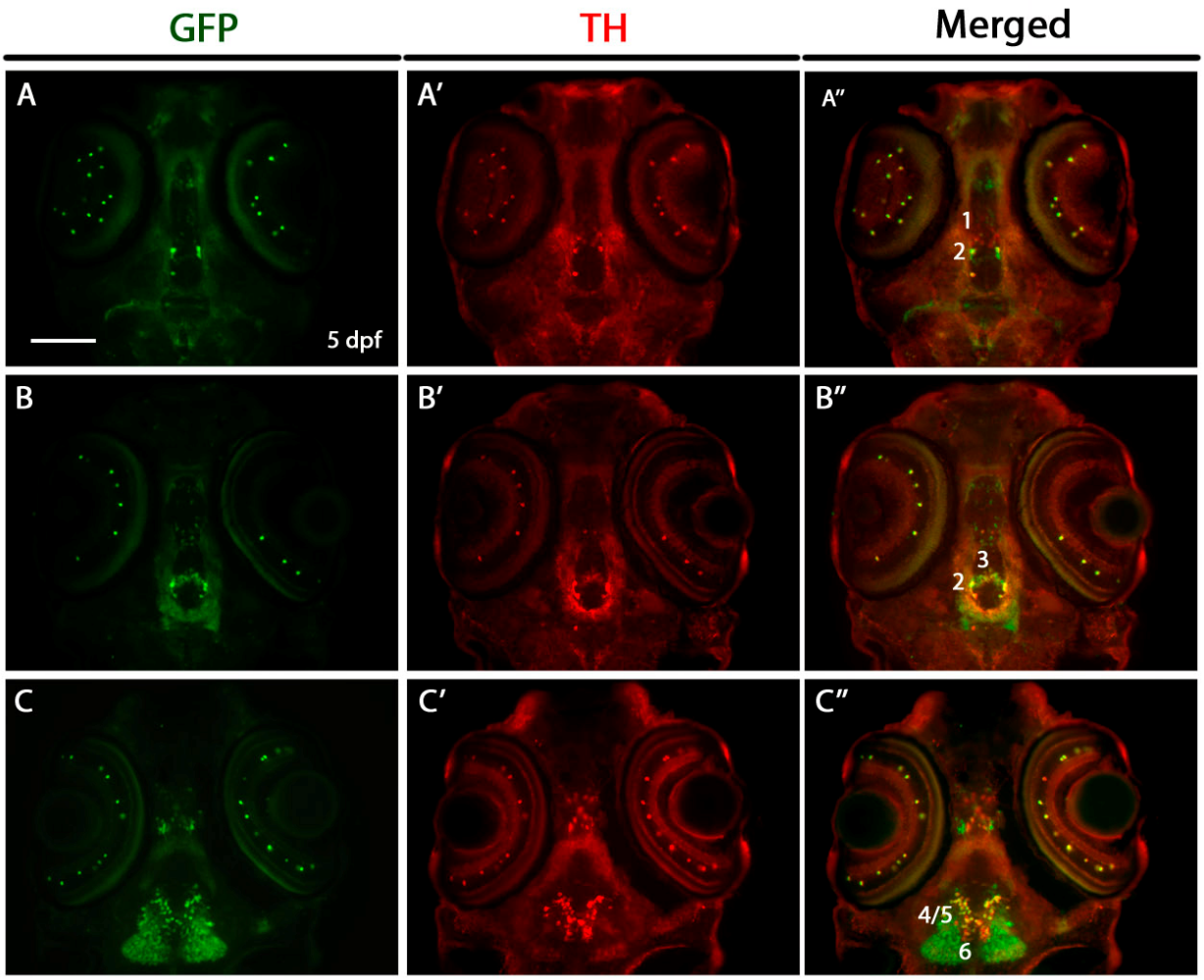

GFP
TH
Merged
A
A′
A″
Pr
1
3 dpf
B
B′
B″
2
C
C′
C″
3
D
D′
D″
4/5
E
E′
E″
6

Figure 4.7 Double immunostaining for GFP and Th on horizontal cryosections of Tg(*dat:EGFP*) embryos at 5 dpf.

5 dpf Tg(*dat:EGFP*) embryos were horizontally cryosectioned and stained with anti-GFP and anti-Th antibodies. GFP-positive cells and Th-positive cells are shown in green and red respectively. Numbers (1-6) indicate different groups of DA neurons in the ventral diencephalon. All sections are shown as anterior to the top. Scale bar represents 100 μm.


GFP
TH
Merged
A
A′
A″
5 dpf
1
2
B
B′
B″
3
2
C
C′
C″
4/5
6

model to examine the effects of MPTP on DA neuron survival. MPTP is a neurotoxin that causes PD-like symptoms in humans. The MPTP mouse model has been popularly used for the study of PD. It is suggested that MPTP can interrupt the electron transfer chain in mitochondria at complex I, and this eventually causes cell death (Nicklas et al., 1987). Conflicting results were obtained from previous studies as to the effects of MPTP on DA neuron survival in zebrafish. Earlier studies in larval zebrafish showed an almost complete loss of DA neurons in the vDC following MPTP treatment (Bretaud et al., 2004; Lam et al., 2005; McKinley et al., 2005). However, recent studies reported that not all DA neurons in the vDC but only those with ascending projections are susceptible to MPTP treatment (Wen et al., 2008; Sallinen et al., 2009). Here we re-examined this issue using Tg(*dat:EGFP*) fish.

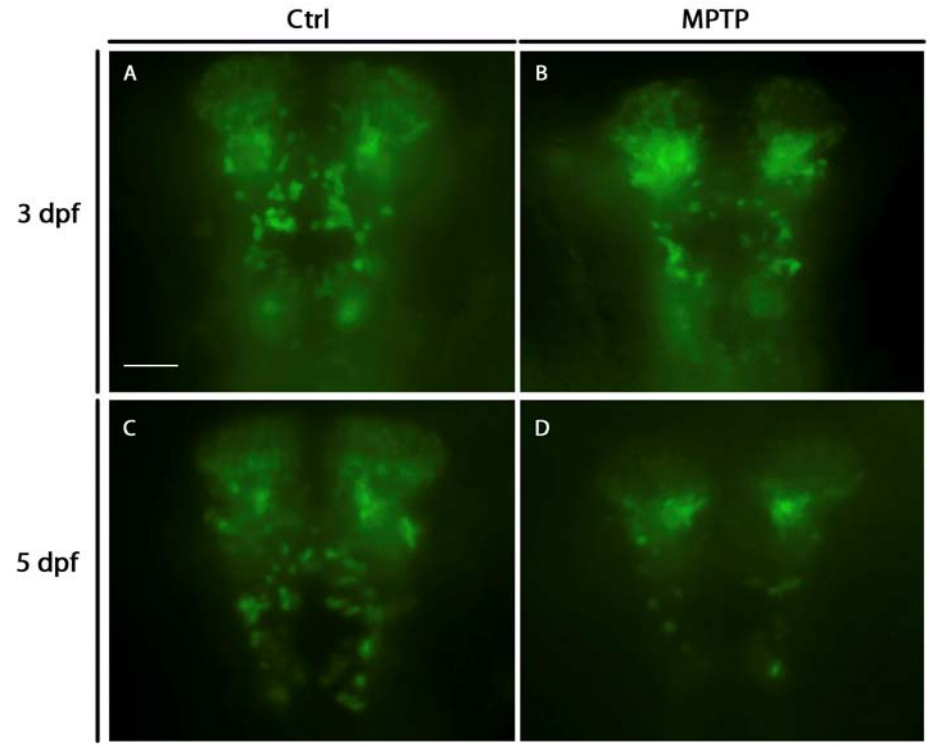
GFP starts being expressed at around 22 hpf in Tg(*dat:EGFP*) embryos. Therefore, we exposed embryos to MPTP from 1 dpf. Different concentrations of MPTP (100 μM, 500 μM and 1 mM) were used. MPTP at 1 mM was used as the optimal dose since it caused the most severe reductions in DA neuron numbers in the vDC. After 2 days of MPTP treatment, the number of GFP-positive neurons in the vDC (groups 2-6) is significantly reduced from 49.8 ± 3.9 (n = 19) to 38.9 ± 6.1 (n = 18, Student's t-test, $P < 0.01$; Fig. 4.8A, B and E). After 4 days of MPTP treatment, the number of GFP-positive neurons (groups 2-6) is also significantly reduced from 74.0 ± 2.4 (n = 14) to 59.8 ± 4.8 (n = 16, Student's t-test, $P < 0.001$; Fig. 4.8C, D and E). The DA neurons of groups 4/5 died first after MPTP treatment, and seemed more sensitive to MPTP than those of other groups (Fig. 4.8A-D). This was also confirmed by double immunostaining for GFP and

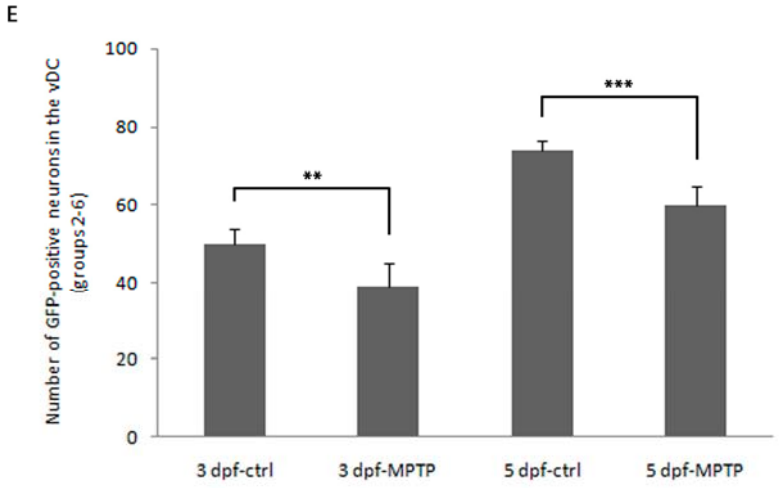
Th on cryosections of control and MPTP-treated Tg(*dat:EGFP*) embryos (Fig. 4.8F-G’’’).

4.4.5 Regeneration of DA neurons in the ventral diencephalon after laser ablation.

Zebrafish is known to have the capacity to regenerate injured or lost tissues in various organs and parts of its body, including some areas in the brain (Becker and Becker, 2008). The optical clarity of zebrafish embryos makes the procedure of laser cell ablation feasible. By aiming a laser beam of a specific wavelength at the GFP-expressing cells, these cells can be selectively destroyed without damaging the other cells. The technique of laser cell ablation has been shown to be efficient in determining the function of specific cell types in zebrafish embryos and larvae (Greenspoon et al., 1995; Li et al., 2003; Roeser and Baier, 2003). Here, utilizing the advantage of live observation of DA neurons in Tg(*dat:EGFP*) fish, we examined the regenerative ability of DA neurons in the vDC. GFP-positive DA neurons in the vDC of 3 dpf Tg(*dat:EGFP*) larvae were laser-ablated on only one side (Fig. 4.9B), on both sides (Fig. 4.9E) and on both sides plus in the Hc (Fig. 4.9H). Double immunostaining for GFP and Th was performed on cryosections of hemi-ablated Tg(*dat:EGFP*) embryos immediately after laser ablation, and showed a total loss of GFP-positive cells on the laser-ablated side (Fig. 4.9N-N’’). The laser-ablated larvae were raised until 5 dpf and examined again by fluorescence microscopy. A significant number of new GFP-positive cells were apparent in the ablated area (Fig. 4.9C, F and I). The number of recovered GFP-positive neurons were counted (59.4 ± 7.6, n = 18) and compared with that of un-ablated larvae (75.8 ± 5.1, n = 22,

Figure 4.8 Effects of MPTP on GFP-positive neurons in the ventral diencephalon. Tg(*dat:EGFP*) embryos were untreated (Ctrl) or treated with MPTP at 1000 μM from 24 hpf, and examined under fluorescence microscope at 3 dpf (A-B) and 5 dpf (C-D). The numbers of GFP-positive neurons in the ventral diencephalon (DA neuron groups 2-6) were counted and statistically analyzed (E; ** $P < 0.01$, *** $P < 0.001$). (F-G’’’) double immunostaining for GFP and Th on Ctrl or MPTP-treated embryos at 3 dpf. (A-D) anterior is to the bottom; (F-G’’’) anterior is to the left. More than 30 larvae were examined in each group. Scale bars represent 25 μm.


Ctrl
MPTP
A
B
C
D
3 dpf
5 dpf


E
Number of GFP-positive neurons in the vDC (groups 2-6)
100
80
60
40
20
0
**
***
3 dpf-ctrl
3 dpf-MPTP
5 dpf-ctrl
5 dpf-MPTP

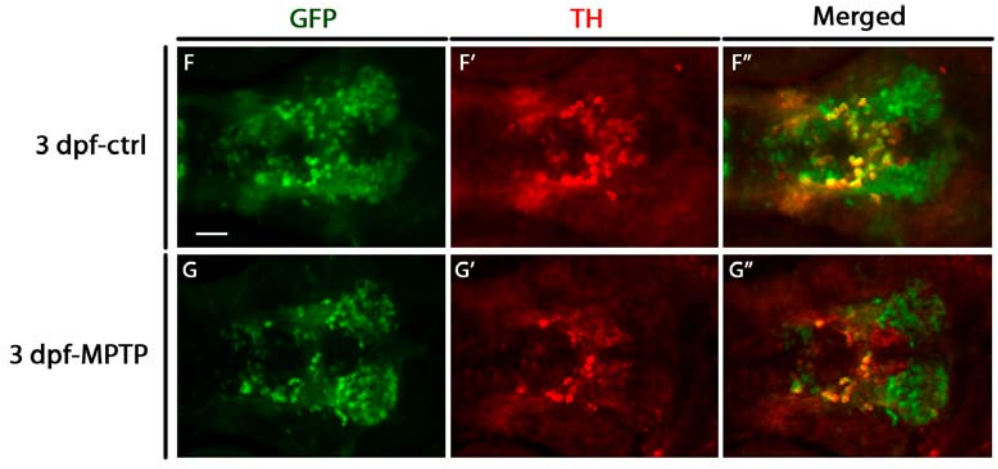
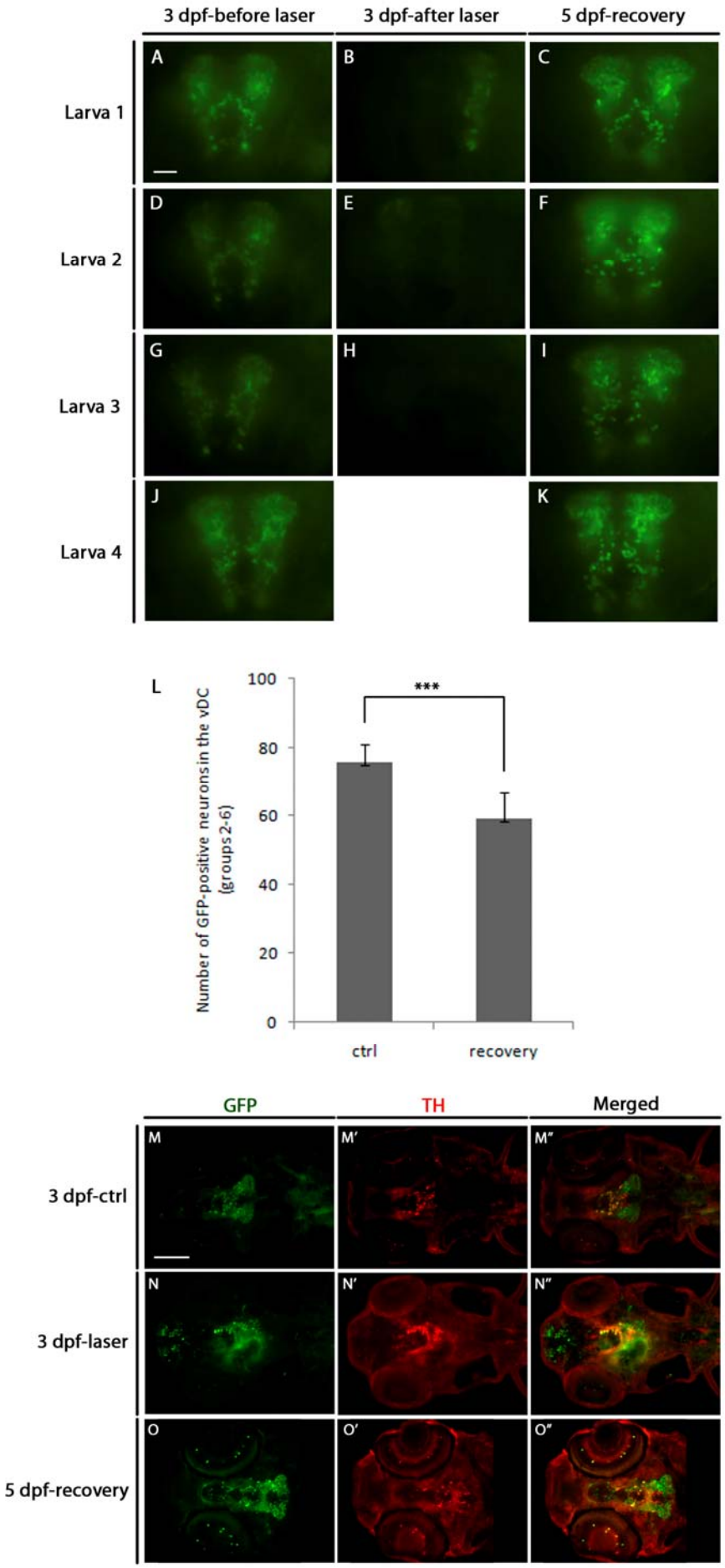

GFP
TH
Merged
F
F′
F″
G
G′
G″
3 dpf-ctrl
3 dpf-MPTP

Figure 4.9 Regeneration of GFP-positive neurons in the ventral diencephalon after laser ablation.

3 dpf Tg(*dat:EGFP*) larvae were subjected to laser ablation (at 60 Hz for 5 sec on each cell) aiming at DA neurons in the ventral diencephalon (B, E and H), and examined by fluorescence microscopy at 5 dpf (C, F and I). The numbers of GFP-positive neurons in the vDC (DA neuron groups 2-6) at 2 days after laser ablation were counted and numbers were compared with those of un-ablated embryos (L; ** $P < 0.01$, *** $P < 0.001$). (M-O’’’) double immunostaining for GFP and Th on control or laser-ablated embryos at 3 dpf and 5 dpf. (A-K) anterior to the bottom; (M-O’’’) anterior to the left. More than 30 larvae were examined in each group. Scale bars represent 25 μm (A-K) and 100 μm (M-O’’).


3 dpf-before laser
3 dpf-after laser
5 dpf-recovery
Larva 1
Larva 2
Larva 3
Larva 4
A
B
C
D
E
F
G
H
I
J
K
L
Number of GFP-positive neurons in the vDC (groups 2-6)
100
80
60
40
20
0
***
ctrl
recovery
GFP
TH
Merged
3 dpf-ctrl
3 dpf-laser
5 dpf-recovery
M
M′
M″
N
N′
N″
O
O′
O″

Student's *t*-test, $P < 0.001$; Fig. 4.9J, K and L). These observations strongly suggest that the DA neurons in the vDC (groups 2-6) have the capacity to regenerate after damage. Moreover, almost all the DA neurons of group 4/5 seemed to have been replaced, which suggested that they might have higher regenerative ability than those of other groups.

4.5 Discussion

4.5.1 Transgene expression in zebrafish DA neurons

In order to express a reporter transgene in zebrafish DA neurons, previous studies have been done with different approaches. A 4.5 kb rat *th* promoter fragment could only drive GFP expression in retina cells (Gao et al., 2005). A 12 kb zebrafish *th* promoter could not drive GFP expression in DA neurons either (Meng et al., 2008). Either a 7 kb or a 11 kb promoter fragment from the *dat* gene was shown to only drive GFP expression in DA neurons of the pretectal region (Bai et al., 2009). An enhancer trap transgenic line, *ETvmat2:GFP*, can label most monoaminergic neurons with GFP but was not specific for DA neurons (Wen et al., 2008). Here we introduced *EGFP* in frame within the first exon of *dat* in a PAC that contains almost the entire *dat* transcription unit. Using this reporter gene, we generated, for the first time, a transgenic zebrafish line Tg(*dat:EGFP*) that specifically recapitulates most of the endogenous DA neuron patterning. This includes the DA neurons in the vDC (groups 2-6), suggested to represent the equivalent of the substantia nigra neurons in human (Rink and Wullimann, 2001). These vDC neurons are correctly labeled with GFP. Consistent with previous studies (Wen et al., 2008), the DA neurons of group 1 and some neurons in group 6 are not labeled with GFP possibly due

to the absence of Dat expression in those neurons.

It is suggested that there are two *tyrosine hydroxylase* genes in zebrafish (*th1* and *th2*) due to the teleost whole genome duplication (Candy and Collet, 2005). Most of *th2*-positive neurons are located in the caudal hypothalamus (Hc) during brain development (Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010). We showed that GFP expression is detected in most of neurons in the Hc in transgenic line Tg(*dat:EGFP*). These GFP-positive neurons are probably *th2*-positive neurons. However, not all GFP-positive neurons are Th-positive due to the fact that the anti-Th antibody used in our study can detect all the Th1-expressing cells but not the majority of the Th2-expressing cells (Yamamoto et al., 2010). Non-specific GFP expression is observed in the jaw and other areas, but these cells are not neurons (data not shown).

4.5.2 MPTP toxicity on zebrafish DA neurons

Previous studies showed conflicting results after MPTP treatment on zebrafish larvae. It was shown that almost all the DA neurons in the vDC seemed to be lost after MPTP treatment (Bretaud et al., 2004; Lam et al., 2005). However, some studies showed only a partial loss of DA neurons in the vDC after MPTP (McKinley et al., 2005; Wen et al., 2008; Sallinen et al., 2009). In all these previous studies, DA neurons were identified based on the Th/*th*-immunoreactivity and on their locations. The Th/*th* expression pattern is different from that of Dat/*dat* (Holzschuh et al., 2001), which is more specifically found in DA neurons. In zebrafish, MPTP is metabolized into MPP+, which is toxic and will be taken into DA neurons through Dat. Therefore, expression of *dat*, but not

necessarily *th*, is a good predictor of locations of MPTP toxicity. Here, we re-examined this issue by observing MPTP-treated Tg(*dat:EGFP*) embryos alive under a fluorescence microscope. We tried different concentrations of MPTP on embryos and chose a 1 mM MPTP concentration, which is the highest concentration used in previous studies. Previous studies showed that DA neurons of different groups in the vDC showed different sensitivity to MPTP (Wen et al., 2008; Sallinen et al., 2009). We showed here that the DA neurons of group 4/5, that send anterior projections to subpallium, seem to be more sensitive to MPTP than those of other groups (Fig. 4.8A-D). However, we didn't observe an overall severe DA neuron loss in the vDC (approximately 20%), which is consistent with some of the previously reported observations (Sallinen et al., 2009).

4.5.3 Regeneration of zebrafish DA neurons

Zebrafish have been shown to be a good model for the study of regeneration since it has the ability of regenerating its fin, heart, scale and other tissues after injury, while mammals retain little regenerative capacity. Studies on zebrafish regeneration have yielded important insights into regenerative processes. Many of the molecular mechanisms of regeneration in zebrafish are remarkably similar to those in the rare instances of mammalian regeneration (Anderson et al., 2007). Recently, zebrafish have also been shown to have the regenerative capacity in the nervous system, including axonal regeneration and various neuronal or tissue regeneration. Zebrafish have been shown to regenerate in several regions of the brain including the telencephalon, tectum and cerebellum (Senut et al., 2007; Zupanc et al., 2005; Adolf et al., 2006; Chapouton et

al., 2006). Although previous studies have shown that DA neurons might regenerate after MPTP treatment in other teleost fish (Pollard et al., 1992), here, for the first time, we showed that DA neurons in the vDC of zebrafish also have the ability to regenerate after injury. DA neurons in the vDC can be replaced by approximately 75% in 2 days after laser ablation.

DA neurons in the vDC groups 2-6 are located in the paraventricular area, where cell proliferation is still active after embryogenesis (Becker and Becker, 2008). This indicates the potential capacity to regenerate DA neurons in adult zebrafish after injury as well. The regenerative capacity of DA neurons in the mammalian midbrain is still controversial (Frielingsdorf et al., 2004; Lie et al., 2002; Zhao et al., 2003). The Tg(*dat:EGFP*) transgenic zebrafish provides a useful vertebrate model for the study of DA neuron regeneration, which might contribute to the initiation of DA regeneration in mammalian models in the future.

Overall, the Tg(*dat:EGFP*) line can be used as a live animal model for understanding the mechanisms of DA neuron development and the pathology of Parkinson's disease. By mutagenesis, it can also be used to screen for potential PD-related factors. Further studies on the regenerative mechanisms of DA neurons in zebrafish might potentially shed light on the inducible regeneration in adult zebrafish and other animals.

4.6 Acknowledgements

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Chapter 5

Conclusions and future directions

Animal model systems have made tremendous contributions to our understanding of the biological basis of complex diseases. The zebrafish, *Danio rerio*, has been established as an excellent vertebrate model for the study of developmental biology and gene function. It also has proven to be a valuable model to study human diseases, along with the mouse, rat and invertebrate models. In my graduate research, I took advantage of the possibilities offered by the zebrafish to test the conserved functions of a gene associated with early-onset Parkinson's disease in humans, specially the *PINK1* gene. An earlier study showed a ~40% reduction in the number of DA neurons in the vDC of *pink1*-deficient zebrafish embryos (Anichtchik et al., 2008). However, we and others have been unable to replicate such a severe phenotype (Chapter 2; Sallinen et al., 2010). The phenotypes reported in the earlier study may have resulted from MO-induced effects unrelated to *pink1* gene silencing. Although *pink1* knockdown did not cause large alterations in the number of DA neurons in the vDC, we observed that the patterning of these neurons and their projections were perturbed. The *pink1* morphants also showed impaired response to touch stimuli and reduced swimming behaviour (Chapter 2). The DA neuron clusters expressing *th2* were also affected by *pink1* loss-of-function at the later stages of 5 dpf and 7 dpf (Sallinen et al., 2010). These results indicate a role for *pink1* in DA neuron development and function in zebrafish. In addition, the DA neuron clusters in *pink1*-deficient zebrafish were more sensitive to 1-methyl-4-phenyl-1,2,3,6-tetrapyradine (MPTP) toxicity (Sallinen et al., 2010), which suggests that the developmental defects in DA neurons, resulting from PINK1 mutations, may also render DA neurons more susceptible to environmental stress in humans.

The *pink1* knockdown caused morphological defects in zebrafish mitochondria, such as a smaller size or loss of cristae, as well as a reduced number of mitochondria, thus affecting mitochondrial function (Chapter 3). These results are consistent with the fact that the impairment of mitochondrial respiratory function interferes with mitochondrial morphology and trafficking (Miller and Sheetz, 2004; Benard et al., 2007; Parone et al., 2008). A *pink1* mutant zebrafish line with a nonsense mutation in exon 7 (Y431*) was found in an ENU-mutagenesis library (Bandmann et al., 2010). This mutation is predicted to result in a partial Pink1 protein with loss of both its C terminus and of a small part of its kinase domain. Although there were no obvious behavioural abnormalities, the larvae (5 dpf) of this line showed a significant decrease in the number of DA neurons and a reduction in mitochondrial complex I activity. Impaired complex I activity was also observed in the striatum of *pink1*-null mice and in fibroblasts derived from PD patients bearing PINK1 mutations (Gautier et al., 2008; Hoepken et al., 2007). It remains unclear how impairment of complex I occurs when PINKl is depleted. PINKl might modulate the activities of complex I subunits, directly and/or indirectly, via protein phosphorylation. Genetic screens could be performed to identify PINK1 substrates, especially the subunits of mitochondrial respiratory complexes.

The mitochondrial phenotypes resulting from loss of *pink1* function are similar to those observed in *parkin*-deficient zebrafish (Flinn et al., 2009). These latter observations further supported the notion that *PINK1* and *Parkin* are in the same pathway in regulating DA neuron development and mitochondrial functions, as was previously suggested by *Drosophila* PD models. Other genes with potential mitochondrial functions are also

identified in PD patients, including *HTRA2* and *PARL* (the *presenilin-associated rhomboid-like* gene, a member of rhomboid proteases family and localized to the inner mitochondrial membrane, McQuibban et al., 2003, 2006). *PINK1*, *Parkin*, *HTRA2* and *PARL* might be part of a common genetic pathway that is involved in the development and survival of DA neurons. Thus, studying how abnormal functions of these genes affects mitochondrial structure and function in zebrafish will be crucial to our understanding of how mutations in these genes cause/may cause PD in humans. Morpholino knockdown of these genes and rescue experiments could be performed to examine the interactions between them. Zebrafish lines with functional mutations in these genes, generated through TILLING or ZFN, would be an important complement to the morpholino studies. Immunogold electron microscopy could be used to examine the morphology of mitochondria in DA neurons with an antibody dectecting DA neurons specifically. In addition, trangenic fish expressing a targeted, mitochondrial GFP would be helpful to monitor mitochondrial dynamics in real time and *in vivo* (Kim et al., 2008).

The structure and expression patterns of zebrafish Pink1 are highly similar to those of human PINK1. Given the mild phenotype in the mouse and the severe phenotype in *Drosophila*, the phenotype of *pink1* knockdown in zebrafish, described by us and others, is more similar to the phenotype in human. Therefore, zebrafish could be a good alternative vertebrate model to study the functions of PD-related genes and reveal their basic mechanisms relevant to PD pathogenesis. Although the listed experiments above do not fully represent the scope of PINK1 research, the zebrafish could be helpful in answering some of the questions due to its unique features.

The elucidation of the pathological mechanisms resulting in the preferential loss of dopaminergic neurons in the midbrain and the identification of effective therapeutical drugs blocking the progressive loss of DA neurons are the two key goals of PD-related research. Due to their unique features, zebrafish could be used as a good platform to find the clues for achieving these two goals. The transparency of the embryos/larvae allows *in vivo* visualization of the development and changes of DA neurons; their small size and easy husbandry make zebrafish amenable to large genetic or drug screens, which are virtually impossible to perform with rodent models. In my graduate research, I generated a transgenic zebrafish line Tg(*dat:EGFP*), which might be a good starting point in such endavours (Chapter 4).

In the Tg(*dat:EGFP*) zebrafish brain, all major groups of DA neurons are correctly labeled with GFP, especially the groups in the vDC, which are analogous to the ascending midbrain DA neurons in mammals (Rink and Wullimann, 2002). The development of DA neurons in zebrafish can be followed at high resolution by four-dimensional live imaging on embryos of this line using multiphoton microscopy (Carvalho et al., 2009; Supatto et al., 2009; Olivier et al., 2010). The dopaminergic circuitry could be precisely described as well by confocal microscopy on live embryos (Graeden and Sive, 2009; O'Brien et al., 2009). This transgenic line could be used to perform an ENU (*N*-ethyl-*N*-nitrosourea)-based mutagenesis screen. Mutations that affect the development and survival of DA neurons could potentially be identified, and later be characterized at the molecular level. This transgenic line will facilitate live observation of DA neurons under various conditions such as:

1) over-expression by mRNA injection or transgenic expression of dominantly inherited PD-linked genes including *α-synuclein* and *lrrk2*. The dominant inheritance pattern suggests a gain-of-function of these genes in the pathogenesis of PD. The zebrafish ortholog of *α-synuclein* seems to be lost during evolution probably due to to the functional redundancy among *synuclein* family members. A number of *α-synuclein* transgenic mice have been generated, but none of these mice show any loss of DA neurons (Chesselet, 2008). Human α-synuclein (WT, A53T, A30P and E46K) could be transiently over-expressed in zebrafish embryos by mRNA injection, or stablely over-expressed by *Tol2*-mediated transgenesis, or conditionally over-expressed by the binary Gal4-UAS transgenesis system (Paquet et al., 2009). By using Tg(*dat:EGFP*) fish, the effects on DA neurons by these over-expressions of α-synuclein, including the possible DA neuron number reduction and increased sensitivity to neurotoxins (MPTP and paraquat), can be observed *in vivo*. The presence of Lewy bodies and mitochondrial abnormalities could also be examined. Similar procedures could also be applied the zebrafish *lrrk2* gene.

2) morpholino-knockdown or knockout/mutant of recessively inherited PD-linked genes including *parkin, pink1, dj-1,* etc. The recessive inheritance pattern suggests a loss-of-function of these genes in the pathogenesis of PD. Morpholino-knockdown provides a quick and efficient way to evaluate the functions of these genes in zebrafish. Most of current genetic zebrafish models of PD are based on MO-knockdown, and seem to recapitulate some PD-specific symptoms such as loss of DA neurons and behaviour defects. However, these MO-models may resemble a null mutant phenotype other than

the mutations found in human PD patients. TILLING or ZFN can be applied on Tg(*dat:EGFP*) fish to screen mutations of these genes in their various putative functional domains. The changes of DA neurons due to these mutations can be followed in real time and *in vivo*. Whether the DA neurons are more vulnerable to environmental factors such as MPTP, due to the developmental defects in DA neurons resulting from these mutations, can also be examined *in vivo* using these transgenic fish.

3) screening for chemical compounds with therapeutic potential under the experimental conditions listed in the preceding paragraph. One of the most advantages of zebrafish over other vertebrates is its suitability for high throughput chemical library screening. Zebrafish larvae can be housed in 96-well plates, and their behaviour can be automatically traced by computer software. The Tg(*dat:EGFP*) fish enables live observation of DA neurons, which will greatly facilitate chemical screenings performed on the zebrafish larvae generated by over-expressions or mutants of PD-linked genes discussed above.

In chapter 4 of this thesis, I found that the DA neurons in the vDC have the capacity to regenerate after a severe laser cell ablation on 3 dpf Tg(*dat:EGFP*) larvae. PCNA/Nestin staining could be performed on regenerating brain to determine whether new DA neurons come from proliferating cells. It was reported that new TH-expressing neurons are added to all major DA regions in adult zebrafish brain including posterior tuberculum in the vDC (Grandel et al., 2006), which strongly suggested the possibility of regeneration of DA neurons in adult fish after injury. Neurotoxins, such as MPTP and 6-OHDA, could be applied to adult Tg(*dat:EGFP*) fish to induce DA neuron loss. After

treatment, the process of DA neuron regeneration could be examined by staining with developmental markers such as Msx1, Nurr1 and TH. Microarray analyses could also be carried out on sorted GFP-positive cells to screen for differentially expressed genes during the process of DA neuron regeneration.

In conclusion, the characteristics of the zebrafish and the experimental approaches to which it is amenable make this species a useful complement to other animal models for the study of pathological mechanisms of Parkinson's diseases and for the screening of compounds with therapeutic potential.

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