ELUCIDATING THE CELLULAR AND MOLECULAR CHANGES OF DOPAMINERGIC NEURONS BY ROTENONE-INDUCED NEURODEGENERATION IN ZEBRAFISH

by

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Abstract

Chemical-induced models have revealed the crucial role of oxidative stress and mitochondrial dysfunction in the development of Parkinson's Disease. In this project, firstly, we investigated the mechanism of action of rotenone, a commercialized pesticide that was previously described to reproduce the bradykinetic dopaminergic neurodegeneration symptoms of Parkinson's Disease in zebrafish by inhibition of the mitochondrial complex I. We found out that rotenone caused change in the morphology of the zebrafish dopaminergic mitochondrial network. We also observed the altered expression of various genes involves in mitochondrial fusion and fission in response to rotenone exposure. Secondly, to develop the use of adult zebrafish as a toxin-based model for Parkinson's Disease, we sought to minimize any off-target effects by exposure of rotenone specifically to the brain. We demonstrated that microinjection of rotenone into the forebrain ventricular zone of adult zebrafish decreases the number of dopaminergic neurons. However, behavioural tests suggested that did not translate into locomotor impairment in these fish.

Taken together, these results gave us more information about the potential use of zebrafish to study the physiological mechanism leading to dopaminergic degeneration and allow for the development of therapeutic strategies for Parkinson's Disease.

Key words: *rotenone; complex I inhibitor; Parkinson's disease; dopaminergic neurons*

Résumé

Les modèles animaux exposés à des produits chimiques ont révélé le rôle essentiel du stress oxydatif et de la dysfonction des mitochondries dans le développement de la maladie de Parkinson. Dans ce projet, nous avons, en premier lieu, examiné le mécanisme d'action de la roténone, un pesticide communément utilisé qui a été précédemment décrit comme pouvant reproduire les symptômes neurodégénératifs des systèmes dopaminergiques et la bradykinésie chez les poissons-zèbres par l'inhibition du complexe I de la mitochondrie. Nous avons montré que la roténone cause une transformation de la morphologie mitochondriale dans le système dopaminergique du poisson-zèbre. Nous avons aussi observé un changement du niveau d'expression de différents gènes impliqués dans la fusion et la fission des mitochondries suite à l'exposition à la roténone. Deuxièmement, afin de développer un modèle de la maladie de Parkinson basé sur une toxine en utilisant des poissons-zèbres adultes, nous avons voulu réduire les effets non-spécifiques des drogues en administrant la roténone directement au cerveau. Nous avons montré que la microinjection de roténone dans la zone ventriculaire du cerveau antérieur du poisson-zèbre adulte réduit le nombre de neurones dopaminergiques. Cependant, les tests de comportement suggèrent que cette réduction ne se traduit pas par des déficits locomoteurs chez ces poissons.

Pris ensemble, mes résultats ont donné plus d'information quant à la possibilité d'utiliser le poisson-zèbre comme outil pour étudier les mécanismes physiologiques qui causent la dégénérescence dopaminergique et contribueront au développement de stratégies thérapeutiques pour la maladie de Parkinson.

Mots clé: *Roténone; inhibiteur de complexe I; maladie de Parkinson; neurones dopaminergiques*

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Symbol	*Definition*
6-OHDA	6-hydroxydopamine
CNS	central nervous system
CVF	cerebroventricular fluid
CVMI	cerebroventricular microinjection
DA	dopaminergic
DAPI	4',6-diamidino-2-phenylindole
dat	dopamine transporter
DMSO	dimethyl sulfoxide
Dnm1l	dynamin-1-like
dpf	day(s) post-fertilization
Drp1	dynamin-related protein 1
(e)GFP	(enhanced) green fluorescent protein
ETC	electron transport chain
FACS	fluorescent-activated cell sorting
GABA	gamma-aminobutyric acid
hpf	hour(s) post-fertilization
IM	intramuscular
IMM	inner mitochondrial membrane
IP	intraperitoneal
LRRK2	leucine-rich repeat kinase 2

mfn	mitofusin
MLS	mitochondrial localizing signal
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	mitochondrial DNA
NA	numerical aperture
OMM	outer mitochondrial membrane
PARL	presenilin-associated rhomboid-like
PD	Parkinson's Disease
PINK1	PTEN-induced kinase 1
qRT-PCR	quantitative Reverse Transcription-Polymerase Chain Reaction
ROS	reactive oxygen species
SNc	substantia nigra pars compacta
TH	tyrosine hydroxylase
tom20	translocase of outer membrane- 20
(v)DC	(ventral) diencephalon
VTA	ventral tegmental area
WT	wild type

Epigraph

"Mitochondria is the powerhouse of the cell."

– Every high school Science teacher ever

CHAPTER 1: INTRODUCTION

1.1 Dopaminergic neurons

Dopaminergic (DA) neurons of the midbrain, although corresponding to less than 1% of the number of brain cells, are one of the most intensively studied group of neurons in the mammalian central nervous system (CNS). Their product, the neurotransmitter dopamine, plays a key role in a lot of pathways in the brain such as motivation, reward, learning and most importantly the control of voluntary motor movement. The DA neurons in the CNS are usually identified and localized as clusters and projections. Essentially, they can be considered totally unrelated neurons in charge of various pathways to control certain cellular functions (Chinta and Andersen 2005; Huot and Parent 2007). In the entire length of the pathway, DA neurons transmit dopamine to their destination through their axons. In the human brain, there are four major pathways for the DA system:

The mesolimbic pathway, where the dopaminergic neurons originate in the ventral tegmental area (VTA), and then project into the nucleus accumbens and the olfactory tubercle of the ventral striatum. This pathway is believed to play important roles in motivation, pleasure and reward-seeking feelings. The direct proof for this is that dopamine is found to be released during pleasurable activities such as food searching, sex, and several drug abuses (Ikemoto 2010; Robison and Nestler 2011).

The DA mesocortical pathway connects the VTA to the prefrontal cortex. Mesocortical dopamine takes part of cognitive control and emotional behaviour, as well as memory and motivation. Because of the intermixing of DA neurons in the VTA of the two pathways, along with

the similarities in functions, the two systems are often collectively referred to as the mesocorticolimbic system (Wise 2004).

The tuberoinfundibular pathway, which originates in the arcuate nucleus of the hypothalamus, is mainly a hormonal control system. Dopamine synthesized in the arcuate nucleus is released in the hypothalamo-hypophysial blood vessels of the median eminence, which supply the pituitary gland (Low 2012). In the presence of dopamine, production of prolactin in lactotrope cells is inhibited.

Lastly, the best known dopaminergic pathway is the nigrostriatal system. This pathway includes DA populations in the substantia nigra pars compacta (SNc) to supply dopamine to the dorsal striatum. This pathway is part of the basal ganglia motor loop and thus controls movements. The basal ganglia is a multi-origin group of nuclei including the dorsal striatum (caudate nucleus and putamen) and ventral striatum (nucleus accumbens and olfactory tubercle), globus pallidus, ventral pallidum, substantia nigra, and subthalamic nucleus (Graybiel 2000). It participates in various segregated loops that involve specific thalamic and cortical areas. In the basal ganglia, the SNc controls the "motor" loop through either direct or indirect pathway which either promotes or decreases the inhibitory signals released by the striatum to the thalamus. DA neurons synapse onto GABAergic neurons in the globus pallidus. This makes dopamine in a position to regulate the production of gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter. In the case of direct movement, GABAergic neurons carry the dopamine D1-receptors which downregulates GABA secretion. GABAergic neurons in the indirect pathway carry D2-receptors which do the opposite (Tepper 2010). Therefore, dopamine's net action refines voluntary movements and at the same time suppresses unwanted movements. Abnormalities in the ba-

sal ganglia function is considered the main reason for the typical motor deficits of Parkinson's Disease (Figure 1.1).

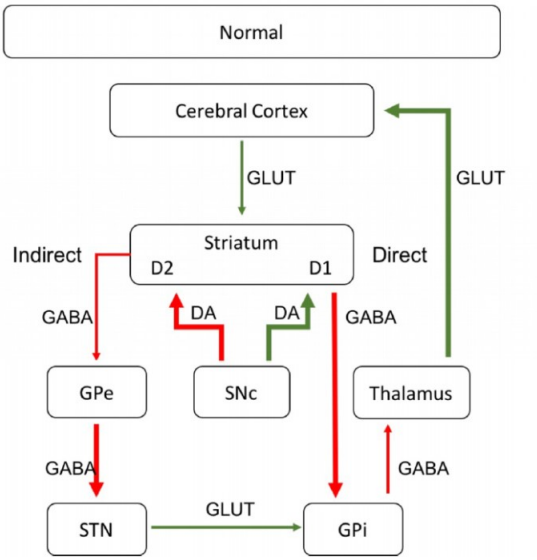
1.2 Parkinson's Disease

Parkinson's Disease (PD) one of the most prevalent neurodegenerative disorders. First described by Doctor James Parkinson in 1817, PD is clinically classified as a movement disorder such as tremor at rest, bradykinesia (slow movement), rigidity and postural instability (Parkinson 2002). In most cases, PD is clearly age-related, typically at the age of 65 and its incidence significantly increases at the age of 75 (de Lau et al. 2004). Gender seems to be also a factor. The male-to-female ratio has been estimated to be 3:2 (Kalia and Lang 2015). Although this is not always the case, PD patients may also suffer from several neuropsychiatric complications such as depression or anxiety. In addition, PD can impair other functions such as decreased sensory responsiveness, sleeping disorder, and sexual dysfunction (Sveinbjornsdottir 2016). Some of these "non-motor" symptoms even manifest before the motor deficits by several years.

The hallmark of the disease is the progressive, irreversible loss of DA neurons in the SNc which depletes the dopamine input into the striatum. The lack of nigrostriatal DA neurons impairs the basal ganglia motor cortex, skews the balance of excitability and inhibitory responses of areas of the cerebral cortex that are involved in the planning and execution of movement. Another abnormality that is also found specifically in PD is the presence of structures called Lewy bodies inside the remaining neurons. Lewy bodies contain an abundant amount of the protein alpha-synuclein. Alpha-synuclein is a small 140 amino acid protein localizing to the neuronal presynaptic compartment. It is thought to oversee the regulation of vesicles, molecular trafficking and

Figure 1.1: Simplified schematic diagram of the basal ganglia motor circuits in the normal condition and in Parkinson's Disease.

Input from the cerebral cortex projects to specific regions of the basal ganglia. In the basal ganglia, the SNc transmits dopamine to specific regions of the striatum which either stimulate or inhibits the transmission of GABA to form direct and indirect pathways through the GP. The GP in response projects to specific regions in the thalamus, which then projects back to the same regions of the Cerebral Cortex to form the motor loop. In Parkinson's Disease, the degeneration of dopaminergic neurons in the SNc leads to dopamine deficiency in the striatum which ultimately leads to decreased input into the cerebral cortex. Abbreviations: GPe – external globus pallidus, GPi – internal globus pallidus, STN – subthalamic nucleus. Image adapted from Jung et al., unpublished, used with permission.


Normal
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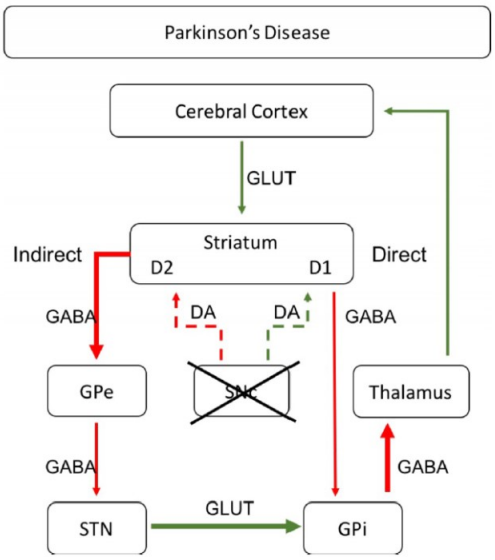
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neuronal plasticity (Lotharius and Brundin 2002). Some believe α-synuclein accumulation can impair synaptic dopamine release and induces the death of nigrostriatal neurons. The toxicity of Lewy bodies is still matter of debate. Despite several assumptions that Lewy bodies caused neurodegeneration, there is a lot of research in the past years claiming that Lewy bodies in fact act as a cytoprotective mechanism of the brain (Wakabayashi et al. 2007). Several abnormalities were also found including mitochondrial dysfunction, reactive oxygen species damage, inflammation and proteasomal and lysosomal dysfunctions from the autopsy of patients with sporadic PD (Schapira and Jenner 2011)

Unfortunately, there is no cure for PD. Usually, treatments for the disease can only provide symptoms relief, not long-term improvement. Most commonly the patients are prescribed with dopamine precursor or dopamine receptor agonists. However, these eventually become ineffective as the on-going cell death cannot be stopped. Deep brain stimulation is the most common neurosurgical option after symptoms can no longer be managed with medication (reviewed by Bot et al. 2018).

1.3 Causes of Parkinson's Disease

Even though the pathophysiology of PD is well documented, the etiology of the disease has yet to be found. It is believed to be caused by a combination of several environmental and genetics factors. In this section, some the most potent elements in each factor were reviewed:

1.3.1 Genetics factors of Parkinson's Disease

Although the most cases of PD are sporadic, several mutations in specific genes have been identified to be associated with the disease, suggesting hereditary factors involved (Table 1.1).

The first gene identified to be related to PD is *SNCA*. The accumulation of alpha-synuclein in the pathological of PD made *SNCA*, the gene encoding it, a point of interest. Point mutation in the *SNCA* gene was found to be responsible for some cases of inherited PD (Polymeropoulos et al. 1997; Spillantini et al. 1997). Moreover, Chartier-Harlin and colleagues identified inherited PD cases with duplication and triplication of the *SNCA* gene (Chartier-Harlin et al. 2004). Knockout of *SNCA* in mice did not exhibit any signs of neurodegeneration while overexpression of *SNCA* resulted in alpha-synuclein aggregation followed by cell death (Hallett et al. 2012). This suggests alpha-synuclein in PD is likely to be linked to a toxic gain-of-function mechanism. Mutation of the *LRRK2* gene is the most commonly known cause of autosomal dominant case of inherited PD. *LRRK2* encodes the 2527-amino-acid-large leucine-rich repeat kinase 2 (LRRK2) protein. It is involved in numerous cellular functions and processes such as axonal growth, synaptic regulation and protein synthesis (reviewed by Xiong et al. 2012). However, how *LRRK2* mutations contribute to PD pathology remain unknown. Mutations of *Parkin*, *PINK1* and *DJ-1* are the cause of autosomal recessive form of PD. They are frequently associated with early on-set of the disease (patients with less than 40 years of age). PINK1 (PTEN-induced kinase 1) and Parkin are both a mitochondrial-targeting protein. PINK1 and Parkin has been found to work together to be neuroprotective by attenuating oxidative stress, decreasing cytochrome c release (Clark et al. 2006; Jin et al. 2010; X. Wang et al. 2011). DJ-1 is also known

Table 1.1: Summary of genes implicated to the development of Parkinson's Disease

Gene	***Product***	***Potential pathophysiology***	***Remarks***
SNCA	Alpha-synuclein	Lewy bodies aggregation	• Autosomal dominant • Early on-set
Parkin	Parkin	Mitochondrial dysfunction	• Autosomal recessive • Early on-set
PINK1	PTEN-induced putative kinase 1	Mitochondrial dysfunction	• Autosomal recessive • Early on-set
PARL	Presenilin-associated rhomboid-like	Mitochondrial dysfunction	• Autosomal recessive
DJ-1	DJ-1	Oxidative stress	• Autosomal recessive • Early on-set
LRRK2	leucine-rich repeat kinase 2	*Unknown*	• Autosomal dominant in all variations and mutations
ATP13A2	ATP13A2	*Unknown*	• Autosomal recessive; • Parkinsonism with dementia

to be part of the oxidative stress response mechanism of the cell. Recently, a new PD-associated gene, the *PARL* gene was discovered. *PARL* encodes the presenilin-associated rhomboid-like protein which affects PINK1 processing and Parkin recruitment (Jin et al. 2010; Shi et al. 2011).

All of these implicate that a disturbance in mitochondrial function seems to play a crucial role in the progression of PD.

1.3.2 Environmental factors in Parkinson's Disease

Although no direct causations have yet to be associated with PD, exposure to a number of toxic compounds may increase the risk for the disease (Table 1.2).

The most notable example of chemical related to PD is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a by-product during the synthesis of an illegal drug 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), an analog of meperidine. In 1982, a group of drug addicts accidentally injected the compound and developed severe parkinsonism motor symptoms along with decreased DA levels (reviewed by Langston 2017). MPTP is highly lipophilic and can easily cross the blood-brain barrier. In the brain, MPTP is metabolized into the active compound 1-methyl-4-phenylpyridinium (MPP+) by the enzyme monoamine oxidase B (MAO B). MPP+ gets uptake by dopamine transporter (DAT), a specific marker for DA neurons. Inside DA neurons, MPP+ exhibits its toxicity by inhibits the complex I of the electron transport chain in the mitochondria, building up reactive oxygen species (ROS) and eventually cell destruction. Recent studies have found that DA neurons in the SNc seem to be more vulnerable to MPTP. In animal models, MPTP has been shown to exhibit DA neuron toxicity in primates and

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Table 1.2: Summary of environmental toxins implicated to the development of Parkinson's Disease

Chemical	***Proposed mechanism***	***Origins***
MPTP	Cross blood-brain barrier → metabolized by MAO B into MPP+ → Enter the cell through DAT → mitochondrial complex I inhibition → neuronal damage	By-product of a synthetic drug
6-OHDA	Enter DA neurons through DAT → Oxidation by MAO A → production of ROS	Synthesized neurotoxin
rotenone	Cross blood-brain barrier → enter the cell by diffusion → mitochondrial complex I inhibition → neuronal damage	Commercial pesticide and piscicide
Paraquat	Cross blood-brain barrier → enter the cell (unknown mechanism) → production of ROS → neuronal damage	Commercial pesticide
Heavy metals	Variable or unknown	Lead, Manganese, Mercury

mice but it lacks an important characteristic of PD which is the formation of Lewy bodies (Langston et al. 1984; Varastet et al. 1994).

Oxidopamine, also known as 6-hydroxydopamine (6-OHDA) is a neurotoxin that also exhibits selective degeneration in DA neurons. It enters the neuron via DAT, accumulates in the cytosol and promotes the formation of free radicals. It is also a potent mitochondrial complex I and IV inhibitor (Glinka et al. 1997). Since 6-OHDA cannot cross the blood-brain barrier, delivery of the drug was frequently performed by stereotaxic injections. Striatal injection of 6-OHDA in rats led to a vast depletion of nigrostriatal neurons (Simola et al. 2007). Injection into the SNc and the VTA of rats also caused progressive decrease in DA levels (Ungerstedt and Arbuthnott 1970; Costall et al. 1976). Locomotion deficits were also reported. However, one may reason that the risk of mechanical damage due to the injection procedure might be involved. Another limitation of the use of 6-OHDA is, also similar to MPTP, the failure of Lewy bodies induction.

Paraquat or N,N′-dimethyl-4,4′-bipyridinium dichloride is one of the most commonly used herbicide. Thanks to its structural similarity to MPP+ and the elevated risk of PD in people with farming lifestyle, the compound is usually associated with the disease. However, reports on testing of paraquat on several rodent strains showed variable results. Paraquat was showed to ablate SNc DA neurons as well as triggered Lewy body formation in mice models (McCormack et al. 2002; Peng et al. 2005). In contrast, Miller and colleagues demonstrated that paraquat did not alter DA distribution (Miller 2007).

Along with paraquat, many of the PD-linked chemicals are in fact ingredients of commonly used pesticides, herbicides and piscicides. Although they have been banned long ago, organochlorine pesticides such as DDT (dichlorodiphenyltrichloroethane), lindane or dieldrin have been assessed to have an increased risk in certain communities' population (Goldman 2014). Di-

othiocarbamate fungicides such as maneb (manganese ethylenebisdithiocarbamate), ziram (zinc dimethylbisdithiocarbamate) and other analogs have been reported to cause DA neuron damage (Zhang et al. 2003; Zhou et al. 2004; Gatto et al. 2009; A. Wang et al. 2011).

Rotenone is another potent insecticide and piscicide (fish poison). Similar to MPTP, the highly lipophilic alkaloid rotenone is a mitochondrial complex I inhibitor that impairs ATP production, generates and accumulates free radicals and ultimately apoptosis (Sherer et al. 2003). Exposure of rotenone to Lewis rats reproduces most of the characteristics of PD: Lewy bodies were observed, DA neuron loss followed by several symptoms of parkinsonism (Cannon et al. 2009). Recently, Martel and Keow demonstrated that rotenone caused severe decrease in DA neurons in larval zebrafish. Larval motor deficits were also observed (Martel et al. 2015). Thus, the use of zebrafish and rotenone as a model for environmentally-acquired PD is the central research interest in this thesis.

1.4 Parkinson's Disease and the mitochondria network

Research of PD nowadays is to find out the mechanism that are likely to participate in DA cell death in both sporadic and inherited from of PD. As briefly discussed in the previous sections, it is found out that DA neurons death can be caused by a number of autosomal pathways and mitochondrial dysfunction is one of the common factor contributing to the pathogenic development of all forms and variants of PD.

1.4.1 Mitochondrial structure is directly linked to their function and cellular health

Over the time it has been revealed that mitochondria also hold a number of key activities being calcium homeostasis, apoptosis and ROS generation. However, their classical role in producing cellular energy is still the chief function in determining cell fate. Different type of cell in the body requires different energy level to function and therefore their mitochondria may have different morphology. Neurons are an energy-demanding type of cell. Therefore, the balance of the mitochondria network in neurons is particularly important for the integrity of the CNS.

In contrast to being generically viewed as a rod-shaped organelle, mitochondria in the cell are in fact a dynamic network of involving fusion and fission. These processes regulate the length and the shape of the mitochondria, thus making them able to adapt to changes in energy requirements, communication with other organelles and self-quality control. Therefore, a lot of studies have allowed mitochondrial shape to emerge as a key regulator of respiratory efficiency that can enforce the bioenergetic status of cells and thereby determine cell fate (Suen et al. 2008). Mitochondrial fusion and fission are mediated by specific proteins, located in either the outer mitochondrial membrane (OMM), or the inner mitochondrial membrane (IMM). For mitochondrial fusion, three key proteins are required: Mitofusin 1, Mitofusin 2 (Mfn1, Mfn2) and the Optic Atrophy 1 (OPA1). Mfn1 and Mfn2 are in the OMM and facilitate OMM fusion by interacting with each other to form homo- or hetero-oligomeric complexes to attach two mitochondria (Chen et al. 2003; Detmer and Chan 2007). Meanwhile, OPA1 is localized in the IMM and directly fuses IMM. In addition to its pro-fusion role, OPA1 is also a regulator in respiration, cristae structure, mitochondrial DNA (mtDNA) maintenance and apoptosis (Frezza et al. 2006; Song et al. 2007; Varanita et al. 2015). For mitochondrial fission, dynamin-1-like protein (Dnm1l), or

sometimes referred to as dynamin-related protein 1 (Drp1), is the most essential element. It localizes in the cytosol and needs to be recruited by the OMM protein Fis1 to the mitochondria to facilitate division (Smirnova et al. 2001; Shen et al. 2014). The activity of Dnm1l is also regulated by phosphorylation by different kinases. As evidence for this, is mitochondria appear to be elongated after mutations in several GTPase effector domains(Chang and Blackstone 2007; Cribbs and Strack 2007; Han et al. 2008). In addition to Dnm1l and Fis1, more proteins have been shown to be associated with mitochondrial fission like Mff, MiD49 and MiD51. The role of these proteins remains to be evaluated, but some studies suggested that they seem to be independent on the Fis1-mediated fission. Indeed, the balance of mitochondrial fusion and fission is critical for cellular homeostasis and stress survival. Fusion may promote mitochondrial activity by allowing them to repair their damaged compartments and mtDNA (Ono et al. 2001). Fission may help isolating damaged mitochondrial segments for autophagy. In addition, extensive fission may also promote apoptosis by activating the pro-apoptotic protein BAX to release cytochrome c (Arnoult et al. 2005). In neurons, the mitochondria are transported from the cell body to the axons and dendrites, promoting synapse formation, disruption in fission processes also play a role in synaptic depletion (Saxton and Hollenbeck 2012). Furthermore, many studies involving knockdowns of fission pathways or overexpressing fusion have consistent results to be protective against different stress conditions and promote neuronal survival (Gautier et al. 2008; Grünewald et al. 2009; Kathrin Lutz et al. 2009).

1.4.2 Evidence for mitochondrial disruption as a potential pathway for Parkinson's Disease

As reviewed in the previous sections, the discovery of PD-related genes supports the role of mitochondrial dynamics in the development of PD. Namely, five proteins implicated in PD including PINK1, Parkin, DJ-1 and PARL play an important role in mitochondrial structure and function. One particularly relevant pathway is the mitochondrial quality control mechanism by PINK1 and Parkin. In *Drosophila*, it has been proposed that PINK1 accumulates at the OMM of damaged mitochondria and recruits Parkin. Parkin, in response, translocates to the mitochondria and promotes the degradation of Mfn1 and Mfn2 by the ubiquitin-dependent pathway, thus facilitating the isolation of damaged mitochondria segment from the healthy network. Mutations in *pink1* or *parkin* genes both altered mitochondrial morphology and lead to DA degeneration and can be rescued by overexpression of *dnm1l*. In mammals, the role of PINK1/Parkin pathway appears to be similar, but not as crucial as in *Drosophila*. Loss of PINK1 in mice caused no gross changes in mitochondrial structure, but increased sensitivity to oxidative stress (Gautier et al. 2008). Overexpression of *PINK1* or *Parkin* in rat hippocampal axons decreased mitochondrial motility, which is believed to quarantine damaged mitochondria prior to their degradation (X. Wang et al. 2011). Silencing of *PINK1* in several cell lines caused mitochondrial fragmentation and respiratory deficiency (Park et al. 2009). Together, these results illustrated that the PINK1/Parkin pathway serves as a protective system by removing dysfunctional mitochondria but might act differently depending on different cell types and different conditions, from inhibiting fusion, decreasing mitochondrial motility or promoting mitophagy. In addition, PINK1 needs to be cleaved to perform its protective function. Interestingly, one of the potential protease involved in PINK1 cleavage is the IMM protease PARL, a recently identified PD-linked protein.

Overexpression of *PARL*'s paralog in *Drosophila*, *Rhomboid-7*, increases the activity of PINK1 (Rahman and Kylsten 2011).

The main source of mitochondrial dysfunction is oxidative stress. As the main respiratory process in the mitochondria is by the electron transport chain (ETC), it is inevitable that a certain amount of oxygen is converted to ROS and damages the cell. Researchers hypothesized that ETC malfunction leading to the accumulation of ROS is one of the pathways for DA neuron death. The most direct evidence is the mitochondrial complex I deficiency observed in the post-mortem brains of PD patients (Schapira et al. 1990). A number of neurotoxins that cause Parkinsonism seem to target the mitochondria complex I such as MPTP, paraquat and rotenone. These toxins are believed to inhibit the transportation of electron from complex I to ubiquinone and promote ROS production, resulting in oxidative damage to the mitochondria. Another support for this pathway is the altered function of the PD-linked gene DJ-1. Even though the actual function of DJ-1 is unknown, Taira and colleagues have showed that overexpression of *DJ-1* is protective against oxidative damages (Taira et al. 2004). In line with this, it is also hypothesized that DA neurons are subjected to oxidative stress due to the conversion of tyrosine to Dopamine which involves oxygen as a substrate as well as the process of re-uptake of excess dopamine. Therefore, it is implied that DA neurons are more susceptible to ROS damages than other type of cells.

1.5 Zebrafish as a model for human disease

Experimental models are of paramount importance for researchers to study the pathological progression and the etiology of a disease, as well as for the design of future clinical trials. A broad spectrum of models is being used in the field of science, but none is considered perfect.

Zebrafish (*Danio rerio*) are small fish originating from tropical lakes and rivers in the regions of India and South East Asia. They can be easily found in any pet stores as they are considered beginner level aquarium fish. The zebrafish were introduced as a vertebrate model organism for biomedical science by George Streisinger. Since then, the zebrafish has been widely used among researchers in the field of developmental biology and medicine thanks to numerous ideal properties.

1.5.1 The advantages of the zebrafish model

As mentioned earlier, the zebrafish are relatively small (about 3cm long as an adult) so they can be easily maintained in large number for a comparatively low cost. They have short generation time (3 months) and a high fecundity. A healthy pair of zebrafish can produce hundreds of offspring every week. Zebrafish embryos develop *ex utero* and are transparent, allowing various means of manipulation: transgenesis, morpholino knockdowns, transplantation and drug screening. Transparency allows organogenesis to be observed non-disturbingly in a living organism. In the field of biomedicine and pharmaceutics, the high fertility rate gives rise to high throughput drug screening to identify hit compounds. Embryos can be treated with thousands of small molecules for *in vivo* effects as well as behavioural changes. Genetically, the zebrafish genome is completely sequenced and readily accessible (Howe et al. 2013). Genetic manipulation methods are well developed for zebrafish, most frequently involving the microinjection of certain compound during early embryogenesis. The morpholino technique is a rapid and easy way to transiently reduce expression of specific genes. It utilizes oligonucleotides that bind to the target messenger RNA (mRNA) sequence, blocking their translation or their splicing. This method

has some disadvantages such as off-target effects and low efficacy as the oligonucleotides only stay in the embryo for only 2 to 4 days (Eisen and Smith 2008).

Recently, the rise of CRISPR/Cas9 techniques made targeted gene knockout in zebrafish relatively easy. This system includes the DNA-cleaving Cas9 protein and a single guide RNA. The guide RNA is designed to bind to a specific sequence of the genome and Cas9 follows the guide RNA to the targeted location and cuts both strands of the DNA. At this stage the damage-repair mechanism of the cell may introduce various mutations (Hwang et al. 2013).

Transgenesis is another powerful tool on zebrafish. The most common way of generating a transgenic line in zebrafish is microinjecting predesigned plasmid or bacterial artificial chromosome at the one cell stage of development (Yang et al. 2006; Suster et al. 2009). Transgenic zebrafish expressing fluorescent proteins under the control of specific regulatory elements allows gene expression studies. It also helps in live imaging of certain process of interest at cellular level especially in larval zebrafish.

In disease modeling, zebrafish have the advantages of invertebrate models such as easy maintenance and cost effectiveness. They are, in terms of basic vertebrate plan, evolutionally, developmentally and anatomically similar to mammals (review in Wullimann et al. 1996). Human and zebrafish genes share a high degree of similarity as well as certain biological pathways. A variety of diseases have developed models in zebrafish such as cardiovascular diseases, genetic syndromes or neurodegeneration (Lieschke and Currie 2007; Sullivan and Kim 2008; Seth et al. 2013).

1.5.2 Zebrafish as a model for Parkinson's Disease

So far, zebrafish models for PD have been categorized into two major types: toxin models and genetic models. The goal of each model is to replicate different aspects of the PD phenotype to find out the mechanisms that are likely to contribute to the progression of the disease. The zebrafish CNS has been thoroughly studied and similarities with the human brain in terms of patterning and signaling system were also established. Most human PD-linked genes have their homologs in zebrafish (reviewed in Xi, Noble, et al. 2011). Locomotor phenotypes such as swimming velocity and total distance traveled, can also be measured, although some of them are being questioned as to their relevancy. Furthermore, the biggest advantage of zebrafish compared to other mammalian models is their ability to regenerate their neurons throughout their life via the process of adult neurogenesis, allowing research on applicable regenerative strategies in humans. However, a downside of the zebrafish model for PD is the lack of alpha-synuclein. Therefore, the pathophysiological aspect of Lewy body formation can be difficult to elucidate.

Most PD-related genes have been investigated in zebrafish, either by morpholino knockdown or complete knockout. Knockdown of *LRRK2* caused severe developmental defects, showed signs of DA loss, synuclein aggregation and locomotion phenotypes (Prabhudesai et al. 2016). However, another group reported that *LRRK2* knockdown did not have any effects (Ren et al. 2011). *Parkin* knockdown also showed inconsistent results in DA patterning in different studies (Flinn et al. 2009; Fett et al. 2010). *PINK1* and *PARL* morphants have decreased locomotion as well as increased DA neuron mispatterning (Anichtchik et al. 2008; Sallinen et al. 2010; Noble et al. 2012). Morpholino knockdown of *dj-1* did not cause any decrease in DA neurons but they are more susceptible to oxidative stress (Bretaud et al. 2007).

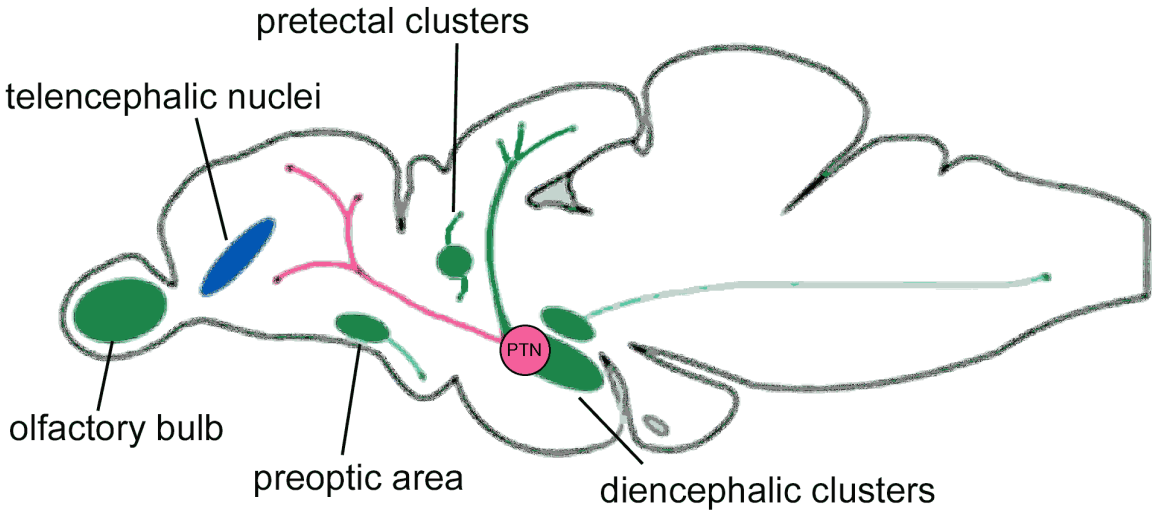
Exposure of zebrafish embryos to various PD-associated toxins have been performed the in the past several years. Few of these common toxins appeared to succeed in inducing DA neuron loss. The only toxin for which there are consistent reports of inducing loss in DA neurons is MPTP. The phenotype can be rescued by either knockdown of *dat* or the use of anti-oxidants (McKinley et al. 2005; Díaz-Casado et al. 2016). This further confirmed the mechanism of action of MPTP toxicity specifically to DA neurons. Recently, Martel and colleagues discovered a method of exposure of the pesticide rotenone into larval zebrafish that reproduced bradykinetic DA neuron loss phenotype (Martel et al. 2015). This give rise to the development of a new toxin-based model of PD for high throughput drug screening.

1.5.3 The dopaminergic system in zebrafish and its functional homolog of human nigrostriatal dopaminergic pathway

The anatomy of the DA system in zebrafish has been thoroughly investigated by *in situ* hybridization and immunohistochemistry of DA markers such as TH, dat and dopamine (Figure 1.2). Zebrafish start to develop DA neurons as soon as 20 hours post-fertilization (hpf). By 5 days post-fertilization (dpf), DA populations have been fully established throughout the brain with distinct clusters in the olfactory bulb, pretectum, telencephalon, preoptic nuclei and diencephalon. The DA groups in the olfactory bulb have been proposed to be homologous to the mammalian A16 group (Panula et al. 2010; Yamamoto et al. 2011). No obvious homology between the DA pretectal groups in zebrafish and mammals was found. Based on the location and their projections towards the pituitary, the three DA clusters in the preoptic area and rostral diencephalon

Figure 1.2: Schematic drawing of major dopaminergic clusters and their projections in the zebrafish brain.

DA neurons in the zebrafish brain are marked in green. Two candidates for the functional homolog of the mammalian nigrostriatal pathway are marked in different colors: the intrinsic projection of DA neurons within the telencephalon (marked in blue) and the projection of the DA clusters in the posterior tuberculum (PTN) to the telencephalon (marked in pink).


pretectal clusters
telencephalic nuclei
PTN
olfactory bulb
preoptic area
diencephalic clusters

are very likely homologous to some parts of the tuberoinfundibular system (Björklund and Dunnett 2007).

The identification of functional homolog of midbrain dopaminergic system in zebrafish has been difficult. Zebrafish lack midbrain DA neuron populations and a significant DA population is present in the subpallium of the telencephalon. Immunohistochemical and tracer studies have suggested that part of the DA neuron population in the posterior tuberculum that project to the DA population in the pallium as the strongest candidate for zebrafish homolog of the substantia nigra and the striatum (Rink and Wullimann 2001; Panula et al. 2010; Du et al. 2016). Another hypothesis is that the nigrostriatal pathway in zebrafish is the intrinsic projection of DA neurons within the telencephalon itself. There have been several lines of reason supporting both hypotheses (Tay et al. 2011). However, there are several noticeable points favoring the posterior tuberal hypothesis instead of intrinsic telencephalic one. Firstly, the telencephalic DA group in zebrafish is highly similar to the olfactory A16 group in mammals in terms of morphology as well as development, suggesting that it may be a part of the sensory system. Secondly, the zebrafish posterior tuberculum contains high density of other neurons such as histaminergic, GABAergic and serotonergic neurons which is similar to the SNc in mammals. Furthermore, in some reports of locomotor deficits secondary to MPTP and MPP+ treatments in zebrafish, the ventral diencephalon seems to be more susceptible to neuronal loss even though some DA loss was also observed in the subpallium (Sallinen et al. 2009; Xi, Yu, et al. 2011).

1.5.4 Rotenone as a neurotoxin model for Parkinson's Disease in zebrafish

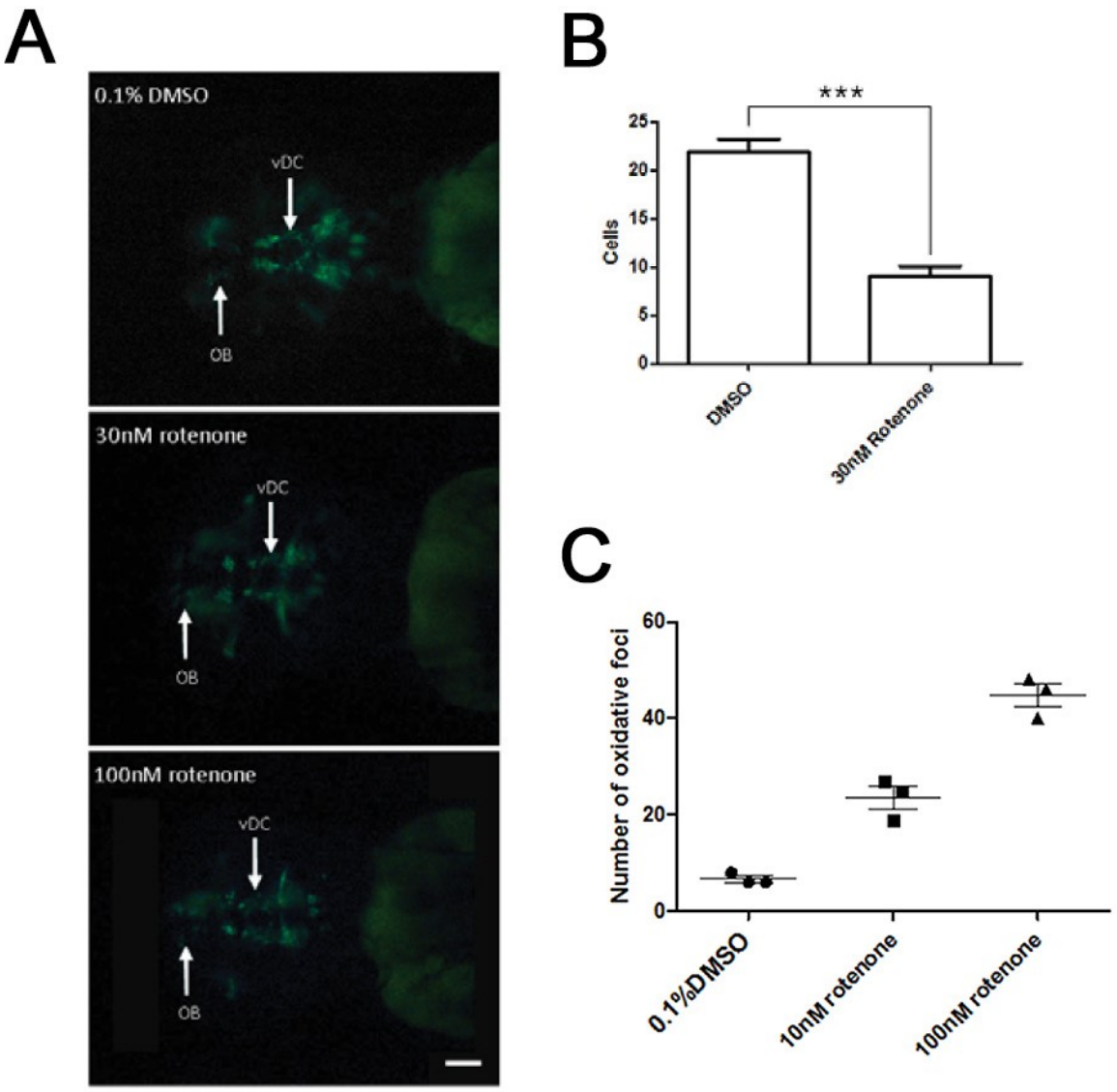
In contrast with previous studies, it was demonstrated that rotenone displays DA neurotoxicity when fish were raised at 22ºC instead of 28.5ºC. We hypothesized that the zebrafish are less sensitive to rotenone thanks to the lowered metabolic rate at lower temperature. This allows rotenone to have enough time to generate sufficient oxidative damage to be lethal to DA neurons. The DA neuron deficits were observed in most regions of the brain and were most pronounced in the ventral diencephalon and the olfactory bulb at a dose of 100nM. The mechanism of action of rotenone is similar to MPTP. It damages the mitochondria by accumulation of ROS and subsequently leads to apoptosis (Sherer et al. 2003). This was confirmed by measurement of oxidative foci using MitoSOX Red (Figure 1.3). Pro-apoptosis markers are also upregulated. The degeneration can be partially rescued by co-treatment with ascorbic acid, an anti-oxidant. Embryos exposed to rotenone also displayed motor deficits namely decreased distance traveled and insensitivity to touch. On the other hand, staining on a few other major neuron groups showed no difference between 100nM rotenone-treated and non-treated zebrafish, suggesting high selectivity of the drug to DA neurons. In this study, the toxic effect of rotenone in the control of mitochondrial integrity in DA neurons will be investigated.

1.5.5 Transgenic approach for zebrafish dopaminergic labelling and regeneration

In order to label DA neurons of the zebrafish, Xi and colleagues have generated a transgenic zebrafish line known as *Tg(dat:eGFP)* using the cis-regulatory elements of the dopamine transporter (*dat*) gene to drive the expression of the enhanced green fluorescent protein (eGFP)

Figure 1.3: Rotenone causes dopaminergic neurodegeneration and increased oxidative foci in zebrafish

DA neurons of 5dpf *Tg(dat:eGFP)* zebrafish exposed to rotenone became mispatterned (A) and reduced by more than 50% (B). MitoSOX Red assay showed rotenone is associated with oxidative stress (C). Results taken from Keow et al. 2015, unpublished. Used with permission.


A
0.1% DMSO
vDC
OB
30nM rotenone
vDC
OB
100nM rotenone
vDC
OB
B
***
Cells
25
20
15
10
5
0
DMSO
30nM Rotenone
C
Number of oxidative foci
60
40
20
0
0.1%DMSO
10nM rotenone
100nM rotenone

(Xi, Yu, et al. 2011). This line labelled most of the endogenous DA neurons, including clusters in the olfactory bulbs, telencephalon, diencephalon hypothalamus and the pretectum, as well as the amacrine interneurons in the retina. GFP expression mostly colocalizes with tyrosine hydroxylase (TH), the common DA neuron marker. GFP expression can also be observed in the jaws. However, this is due to non-specific expression as these cells are not identified to be neurons. MPTP treatment reduced GFP expression along with decreased locomotion which is consistent with the DA ablation effect of MPTP in previous studies (Langston et al. 1984; Varastet et al. 1994).

Using the same regulatory elements of the *Tg(dat:eGFP)*, Godoy and colleagues generated the *Tg(dat:CFP-NTR)* line to conditionally and specifically kill DA neurons using the nitroreductase-metronidazole (NTR-MTZ) system. In the *Tg(dat:CFP-NTR)* zebrafish, NTR fused with cyan fluorescent protein (CFP) are only produced in the *dat*-expressing cells. Upon treatment with the pro-drug MTZ, NTR reduces MTZ into a cytotoxic compound that induces apoptosis. Overall, treatment with MTZ in larval *Tg(dat:CFP-NTR)* decreased CFP levels in most regions and effects were more pronounced in the olfactory bulb and the subpallium of the telencephalon (Godoy et al. 2015). This transgenic line has been used as a DA regeneration model in our lab secondary to ablation.

In parallel, Noble and colleagues replaced the eGFP cassette and designed a new construct to express the red fluorescent protein mCherry to the OMM of DA neurons. In this *Tg(dat:tom20 MLS-mCherry)* line, mCherry was fused with the mitochondrial localizing signal (MLS) peptide of the zebrafish tom20 (Translocase of outer membrane), a receptor localizing in the OMM responsible for the mitochondrial translocation system (Noble et al. 2015). The expression patterns of mCherry closely colocalize with the GFP patterns of *Tg(dat:eGFP)* zebrafish

and the CFP patterns of the *Tg(dat:CFP-NTR)* line. MPTP treatment also reduced mCherry expression in *Tg(dat:tom20 MLS-mCherry)* fish. mCherry is properly targeted to the mitochondria as double staining of mCherry and tom20 antibodies perfectly co-localized (our own unpublished observations). We have been using this transgenic line to follow the mitochondrial morphology of DA neurons, as described in later chapters.

1.6 The use of adult zebrafish as a model for Parkinson's Disease

Even though larval zebrafish have been an excellent tool for PD research, as PD is more relevant to older patients, efforts have been made to use environmental toxins to model PD in adult zebrafish. However, in the literature, neurotoxin treatment was limited to conventional methods such as dissolution of the drug into the tank water or systemic injections. None of them were able to reproduce signs of a bradykinetic DA degeneration (Table 1.3). Bretaud and colleagues, in several approaches, exposed the adult zebrafish to three neurotoxins: rotenone, paraquat and MPTP/MPP+. The only noteworthy effect was a decreased locomotion after a single intraperitoneal (IP) injection of MPTP at the concentration of 225 mg/kg (Bretaud et al. 2004). Anichtchik and colleagues performed a single intramuscular (IM) injection of MPTP and 6-OHDA in adult zebrafish. Even though no gross change in the number of DA neurons was observed, behavioural tests showed a decrease in locomotion. Interestingly, decreases in the concentration of cerebral catecholamines was also noticed. However, no clear explanation for these alternations was given (Anichtchik et al. 2003). The likely reasons for these failures were due to the possibilities that these toxins are either metabolized, trapped in the adipose tissues or become lethal the fish before they have any effect on the neurons. The marked locomotor impairments

Table 1.3: Summary of neurotoxin treatment in adult zebrafish

Drug	*Dose*	*Method*	*Time of administration*	*DA neurons*	*locomotion*	*Reference*
rotenone	2µg/L	Fish water	4 weeks	No effects	No effects	Bretaud et al. 2004
rotenone	10µg/L	Fish water	Fish died after 48 hours			
MPTP	225mg/kg	IP injection	Once	No effects	Decreased total distance	
MPP+	75mg/kg	IP injection	Once	No effects	No effects	
MPTP	75mg/kg	IP injection	3 times for one day	No effects	No effects	
Paraquat	5mg/L	Fish water	4 weeks	No effects	No effects	
MPTP	20 mg/kg	IM injection	Once	No effects	Decreased for 6 days	Anichtchik et al. 2003
6-OHDA	25mg/kg	IM injection	Once	No effects	Decreased total distance	
Paraquat	20mg/kg	IP injection	1 injection every 3 days, 6 times total	No effects	Decreased total distance and velocity	Bortolotto et al. 2014

reported in these studies could be due to the transient inhibition of catecholamine neurotransmitter or could simply be an off-target effect of the peripheral motor and/or sensory system.

1.7 Aim of the study

Previous work has shown that rotenone ablates the DA neurons of the larval zebrafish in a dose-dependent manner. The loss of DA neurons translates into locomotor phenotypes which resembles the bradykinetic symptoms of PD patients. We hypothesized that rotenone causes DA neural death by impairing the mitochondrial ETC, inducing oxidative stress- mediated apoptosis. Therefore, my first aim is to investigate whether rotenone caused dopaminergic neuron death by mitochondrial dysfunction. Our objective is to follow the dopaminergic mitochondria morphology secondary to rotenone exposure using the *Tg(dat:tom20 MLS-mCherry)* transgenic line. We also demonstrated the changes in the regulation of mitochondrial fusion and fission machinery in zebrafish.

Secondly, we aimed to develop an adult zebrafish toxin-based model for PD by finding a way to expose neurotoxins specifically to the brain of the adult zebrafish and find out whether it could ablate DA neurons and reproduce locomotor phenotype. To reach this goal, we used a Cerebroventricular Microinjection (CVMI) approach to deliver rotenone and MPTP into the ventricular zone of the *Tg(dat:eGFP)* transgenic line. We have been able to assess the sensitivity of the adult zebrafish following the injection of rotenone and MPTP. We were able to quantify changes in the DA neurons in the telencephalon as well as some behavioural assays of the adult zebrafish rotenone post-injection.

We also sought to develop a method to isolate and culture primary zebrafish DA neurons using fluorescent-activated cell sorting (FACS). Apart from PD studies, DA cells can be co-cultured with other cell types to provide insights in cell-cell interaction and neurogenesis by either looking at their morphological changes or their signal transduction.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animal care and husbandry

All procedures and protocols were approved by the University of Ottawa Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care. Adult zebrafish were maintained on a 14-hour light/10-hour dark cycle at 28.5ºC and fed a diet of fish pellets and Artemia. Zebrafish embryos were obtained from natural spawning from adults. Embryos were raised in petri-dishes with embryo media (13mM NaCl, 0.5mM KCl, 0.02mM Na_2HPO_4, 0.04mM KH_2PO_4, 1.3mM $CaCl_2$, 1.0mM $MgSO_4$, and 4.2mM $NaHCO_3$). Two transgenic lines were primarily used in these experiments: *Tg(dat:eGFP)* and *Tg(dat:tom20 MLS-mCherry)*. Both lines have the reporter gene placed under the control of cis-regulatory elements of the dopamine transporter (*dat*) gene that drive enhanced green fluorescent protein (eGFP) and the mitochondrial localization signal (MLS) peptide of tom20 fused with mCherry, respectively.

2.2 Rotenone preparation and larval zebrafish exposure

Rotenone powder (Sigma-Aldrich) was dissolved in 100% DMSO (dimethyl sulfoxide) (Fisher Scientific) at a concentration of 100mM, then serially diluted up to 1,000,000-fold in 10% water-diluted DMSO to half-log working concentrations between 10nM and 100nM. Only freshly made rotenone solutions were used. Embryonic zebrafish were collected and raised in 6-well plates or petridishes (Corning) with no more than 20 embryos per well. Embryos were exposed to a single dose of rotenone at 24hpf without water changes until 7dpf. Controls were

zebrafish embryos exposed to a 0.01% DMSO solution, the same DMSO concentration used for the highest dose of rotenone. Afterwards, any rotenone or DMSO-containing solution were bleached and disposed in organic waste.

2.3 Cerebroventricular microinjections of neurotoxins in adult zebrafish

Adult *Tg(dat:eGFP)* 10-month-old zebrafish were anaesthetized in 0.24mg/mL MS-222 (ethyl 3-aminobenzoate methanosulgonate) (Sigma-Aldrich) in a petri dish until no noticeable operculum movement. Under a dissecting microscope, a sharp 30-gauge needle (BD Ultra-Fine II, BD Biosciences) was used to make an incision with a diameter of approximately 200 μm on the cranial bone close to the midline of the skull above the optic tectum without damaging the brain underneath. Using thin glass capillary microinjection needles (Sutter Instrument), the solution was injected through the slit to deliver compounds to the cerebroventricular fluid (CVF). The IM-300 vacuum pump microinjector station (Narishige) was used for the injection with the inject pressure of 50 psi. To make sure the injected liquid disperses through the CVF of the telencephalon, Phenol Red (Sigma) was mixed with the toxin solution.

Rotenone powder was dissolved in 100% DMSO (Fisher Scientific) at a concentration of 100mM, then diluted to a final working concentration of 2mM, 1.5mM or 1mM in 20% DMSO. MPTP powder (Sigma) was dissolved in distilled water to a stock concentration of 100mM and then diluted to a final working concentration of 50mM, 35mM 20mM and 10mM. The 100mM stock solution was aliquot into 1.5mL microcentrifuge tubes and stored at -80ºC and were thawed only once. After injection, the fish were put to a recovery tank and recovery time was recorded until their swimming pattern returned to normal.

Injections were performed a total of four times, once every two days. The fish were maintained in the dark during the whole treatment process. Four days after the last injection, the fish were then monitored for behavioural assays and brain dissected for immunohistochemistry.

2.4 Immunohistochemistry on zebrafish cryosections

Zebrafish embryos were euthanized with an overdose of MS-222 after reaching the desired developmental stage. They were then fixed in a 4% solution of paraformaldehyde (PFA) (ThermoFisher Scientific) dissolved in 1x phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 4.3mM Na2HPO4, 1.47mM KH2PO4) overnight at 4°C, then rinsed thrice in PBS for 10 minutes. The fish were incubated in 30% Sucrose/PBS at 4ºC overnight and were then flash frozen in Tissue-tek OCT compound (VWR Canada) and sectioned onto the Leica CM 1850 cryostat (Leica Microsystems) at a thickness of 12µm on to Superfrost class slides and stored at -20°C until use.

For immunohistochemistry on adult zebrafish, the fish were euthanized with 2-4ºC ice-cold water for a minimum of 5 minutes. The fish were then fixed in 4% PFA (ThermoFisher Scientific) overnight at 4°C and then brain-dissected in 1x PBS solution. After dissection, the brains were equilibrated in 30% Sucrose/PBS and flash frozen in Tissue-tek OCT compound (VWR Canada) and sectioned at a thickness of 20µm on to Superfrost class slides and stored at -20°C until use.

Zebrafish cryosections were then blocked for 2 hours at room temperature (RT) in a blocking solution consisted of 10% heat-inactivated calf serum solution (Ambion) in PBS containing 0.1% tween-20 (Sigma-Aldrich) (PBS-Tw), followed by an incubation overnight at 4°C

in primary antibody solution [rabbit polyclonal anti-DsRed (No. 632496, Clontech) diluted to a 1:100 concentration, rabbit anti-mouse polyclonal anti-Tom20 (FL-145, Santa Cruz Biotechnology) diluted to a 1:100 concentration or mouse monoclonal anti-GFP (A11120, Invitrogen) diluted to a 1:350 concentration, in the previously mentioned blocking solution]. Samples were then washed with PBS-Tw thrice for 10 minutes, and then incubated for 2.5 hours at 4°C in secondary antibody solution [goat anti-rabbit Alexa 488 conjugate (A11008, Invitrogen) diluted to a 1:500 concentration, goat anti-rabbit Alexa 594 conjugate (A11012, Invitrogen) diluted to a 1:500 concentration or goat anti-mouse Alexa 488 conjugate (A11019, Invitrogen) diluted to a 1:1000 concentration, in blocking solution] and were washed thrice for 10 minutes at room temperature in PBS. Samples were then incubated in 1µg/mL DAPI (4',6-diamidino-2-phenylindole) solution for 20 minutes to stain the nucleus and were washed twice for 10 minutes. The slides were then mounted with the Immu-Mount mounting media (Shandon). All immunohistochemistry experiments were performed in biological quadruplicates at minimum and the most representative sample images were chosen.

2.5 Microscopy and image analysis

Light microscopic analysis was performed using three different microscope systems: For live embryo imaging, the SMZ1500 fluorescent dissecting microscope (Nikon) connected through an Olympus DP70 color digital camera to the NIS Elements 3.22 imaging software (Nikon) was used.

For larval zebrafish mitochondrial imaging, the LSM 880 Confocal Laser Scanning Microscope with AiryScan Processing (Zeiss) was used. Samples were scanned using lasers with

wavelengths of 401 nm (DAPI), 488 nm (green) and 561 nm (red) with coverslip-corrected Plan-Apochromat DIC M27 63x objective (1.4 NA, glycerol immersion). Images were obtained in a z-stack at 0.18μm intervals. They were then processed with AiryScan Processing and z-stack slices were compiled to produce maximum-intensity projection images using ZEN Black software (Zeiss). Cell count was performed using the ZEN Blue software (Zeiss). Cells were ranked by their mitochondria morphology:

Normal: mitochondria are uniformly distributed and linked to each other around the nucleus.

Fragmented: mitochondria appear in less volume and are apart from each other.

Partially fragmented: Fragmentations were only observed in less than 50% portion of the network.

Immunostained slides of adult *Tg(dat:eGFP)* zebrafish brains were imaged on the Axiophot upright epifluorescence microscope (Zeiss) with Plan-Neofluar 40x objective (0.75 NA) connected through an Olympus DP70 color digital Camera to the Image-Pro Plus software (MediaCybernetics). Fluorophores were excited by a 100W mercury arc lamp filtered for the wavelengths of 395nm (DAPI), 510 (green) and 580 (red). Images were assembled using Image-Pro Plus and Adobe Photoshop CS6. Four sections of the telencephalic region on each fish were used to count the number of GFP-positive cells.

2.6 Behavioural assessment on adult zebrafish post-cerebroventricular microinjection

To assess locomotion secondary to CVMI of rotenone, zebrafish that were injected with rotenone or DMSO were placed into a 30x20x20cm standalone glass tank individually. Zebrafish

were allowed to acclimatize to their new environment for 30 minutes before being recorded for 10 minutes with a Nikon D3100 camera. For each individual fish, a random 3-minute clip from each video was used to be processed and analyzed:

For the midline crossing behavioral assay, the midline depth of the tanks was marked with a tape, the number of times each fish crossed the midline were counted manually.

For distance travelled assessing experiments, the position of the fish in the tank were traced manually every 10 milliseconds using the Tracker 4.11.0 program (Open Source Physics). Each position was given corresponding x- and y- coordinates. Distance traveled every 10 milliseconds was calculated by the Pythagorean formulation: $D = \sqrt{dx^2 + dy^2}$. Where dx is the difference between the x-coordinates of the points and dy is the difference between the y-coordinates of the points. Total distance was assembled and calculated in Microsoft Excel. All behavior experiments were performed in biological triplicates.

2.7 RNA isolation, cDNA synthesis and quantitative RT-PCR

Zebrafish were raised to various time points of development and euthanized with an overdose of MS-222. Zebrafish RNA was isolated from whole embryo lysate of 20 to 50 embryos from the same treatment using RNeasy Mini kit (Qiagen). RNA concentration and quality were confirmed by Agarose gel electrophoresis and quantified using a NanoDrop 2000 spectrophotometer (ThermoScientific). Only RNA samples with OD_{260}/OD_{280} nm absorption ratio >1.95 were used. The RNA samples were then reverse transcribed into cDNA using the iScript RT Supermix (BioRad), with a total amount of RNA of 500µg, in accordance to the manufacturer's instructions. The primers used for PCR reactions are listed in table 2.1. All primer sets were

Table 2.1: List of primers used in qRT-PCR

Forward and reverse PCR primer pairs against mitochondria fission 1 (*fis1*), dynamin-1-like (*dnm1l*), optic atrophy 1 (*opa1*), mitofusin 2 (*mfn2*), RNA polymerase (*rnap*), elongation factor 1 alpha (*ef1a*), and Ribosomal protein L13a (*rpl13a*) are listed. Primers were diluted in ddH_2O to a 10µM stock concentration and kept at -20ºC until selected for use.

Gene of interest	Forward primer	Reverse primer
fis1	GGCCCTGGAGCTAGAAAAAC	CACGGCCAAACCAATAAGAC
dnm1l	TCCTACTTCTGTGCCTCGTG	GGCCTTCATCTCCCTTCTTC
opa1	CCTGCTTGTGATGCCCTTGT	GGTGGTCTCTGTGGGTTGTT
mfn2	GCGCCTACATCAAAGAAAGC	ATGTGTCTTCGGGACAGGAC
dat	AGACATCTGGGAAGGTGGTG	ACCTGAGCATCATACAGGCG
rnap	CCAGATTCAGCCGCTTCAAG	CAAACTGGGAATGAGGGCTT
ef1a	GTACTTCTCAGGCTGACTGTG	ACGATCAGCTGTTTCACTCC
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG

designed to span an exon-intron boundary and were optimized to an equal annealing temperature of 60°C in accordance to the MIQE guideline (Taylor et al. 2010).

The components of each reaction were prepared to a total volume of 10 µl: 0.5 µl forward primer (0.5 µM), 0.5 µl reverse primer (0.5 µM), 5.0 µl PowerUp SYBR Green Master Mix (ThermoScientific) and 4 µl cDNA (6.25ng/µL) was added as PCR template. The reactions were performed using the ECO Real-Time PCR system (illumina) under the following cycles: UDG activation (50ºC for 2 minutes), denaturation program (95°C for 2 minutes), PCR cycling and quantification program repeated 40 times (95°C for 15 seconds, 60°C for 1 minute), melting curve program (95°C for 15 seconds, 60ºC for 1 minutes and 95ºC for 15 seconds with a ramp rate of 5.5°C per second and a continuous fluorescence measurement) and finally a cooling step to 50°C. The Cq value was defined as the point at which the fluorescence rises appreciably above the background fluorescence. Relative expression was assessed by normalization with a combination of two or three reference genes: *β-actin*, *rpl13a* and *ef1a*. Batches of zebrafish treated on different days were considered biological replicates in this experiment.

2.8 Zebrafish whole larval cell dissociation and flow cytometry

To prepare a single cell suspension of 3dpf *Tg(dat:eGFP),* about 500 zebrafish embryos were dechorionated with a 0.5mg/ml Pronase E protease (Sigma-Aldrich) solution. Dechorionated embryos were sacrificed with an overdose of MS-222, washed 3 times with Ringer's solution (116mM NaCl, 2.9mM KCl, 1.8mM $CaCl_2$, pH 7.2) and were de-yolked by vigorously pipetting in a 50mL tube of deyolking buffer (55mM NaCl, 1.8mM KCl, 1.25mM $NaHCO_3$). Using the rubber end of a plunger from a 0.5mL syringe, the embryos were dissociated by pushing them

through multiple cell strainers (VWR, Canada): 100µm once and 40µm twice. The single cell suspension was then resuspended in FACSmax solution (Genlantis) after every straining. As a control, a cell suspension of stage-matched, wild-type (WT) embryos was prepared to set the sorting gate. Cell suspension was then stained with trypan blue to ensure cell survival. The cell suspension was first analyzed with a flow cytometer to find out desired GFP-positive population and exclude background.

For 5dpf zebrafish cell disruption, in addition to the previously described procedure, before straining and after deyolking, the fish were incubated in 0.25% Trypsin, 1mM EDTA (Invitrogen) for either 5 minutes, 15 minutes, 30 minutes or 1 hour at 28ºC (enzymatic disruption).

After that, the *Tg(dat:eGFP)* and the WT cell suspensions were then read on a Gallios flow cytometer (Beckman-Coulter) using a laser with a wavelength of 520nm (GFP). All flow cytometry reads were normalized to 50,000 events.

2.9 Adult zebrafish brain dissociation, Poly-D-Lysine coating and plating

All the experiments described in this section were performed in a biological safety cabinet.

For plate coating, Poly-D-Lysine powder (Sigma) was prepared as a 10x stock solution by adding 50mL of autoclaved dH_2O to 5mg of powder in a glass bottle. The 10x stock solution was then distributed into 5mL aliquots in 50mL tubes. The aliquots were then put at -20ºC for storage and were thawed only once in in a 37ºC incubator when needed. Upon thawing, 45mL of autoclaved dH_2O was added to the 5ml of poly-D-lysine to make a working 1x solution. In a 6-well plate (Corning), 2mL of 1x poly-D-lysine was added to each well. The plate was incubated

at 37ºC in an incubator for 30 minutes to 1 hour. Poly-D-lysine was removed by aspiring with a glass Pasteur pipette. To wash the wells, 2mL of autoclaved dH_2O was added, let stand for 4 minutes and was gently aspirated. The plates were then left in the cell culture hood with UV light on with the cover off for 30 minutes to dry. Extra unused coated plates are stored in the biosafety hood for later uses.

Adult *Tg(dat:eGFP)* zebrafish, 10 months to 1 year of age, were euthanized with 2-4ºC ice-cold water for a minimum of 5 minutes. The brain was then dissected in a 1x PBS solution. The olfactory bulb and the telencephalon were isolated and transferred into a 1.5mL tube for dissociation. Those regions were then enzymatically digested with 500µL of 0.25% Trypsin, 1mM EDTA (Invitrogen) at 37ºC for 10 minutes. After incubation, the brain tissue was gently dissociated by pipetting up and down 10 times using a 1000µL pipet tip cut at about 5 to 6 millimeters from the bottom. The tissue was then incubated again at 37ºC for 10 minutes followed by pipetting up and down 10 times using an uncut 1000µL pipet tip. The enzymatic reaction was terminated by adding 12.5µL of Hi-FBS (Life Technology). The cell suspension was then centrifuged at 800 x g for 10 minutes at room temperature. The supernatant was removed followed by the addition of fresh L-15 medium. The cell suspension was then strained through a 40µm cell strainer (VWR Canada) and confirmed under a microscope (Olympus CK 2).

The suspension was seeded in plates coated with poly-D-lysine in L-15 medium at 37ºC. The plates were monitored daily using the JULI fluorescent cell analyzer (Digital Bio).

2.10 Statistical analysis

All data quantification and statistical analysis were performed with GraphPAD PRISM 6.0. The difference between the control and treatment groups of the following experiments were analyzed with two-tailed unpaired Student's t-test: mitochondria fragmentation, and adult dopaminergic cell count. qRT-PCR data were assessed for significance by one-factor analysis of variance (ANOVA). Assessments of adult zebrafish behaviour post-CVMI were performed with Kruskal–Wallis one-way analysis of variance. Data were presented as mean ± standard deviation (SD) and all experiments were independently repeated at least three times. All cell counts were performed in a blinded fashion which each data point consisted of 25-50 cells. For each sample in a qRT-PCR experiment, about 50 embryos were used to generate a single mRNA extract. The number of biological replicate used in each experiment was indicated in the corresponding section of text or figure legend. P-values < 0.05 were considered as statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

CHAPTER 3: RESULTS

3.1 Zebrafish dopaminergic mitochondria morphology analysis

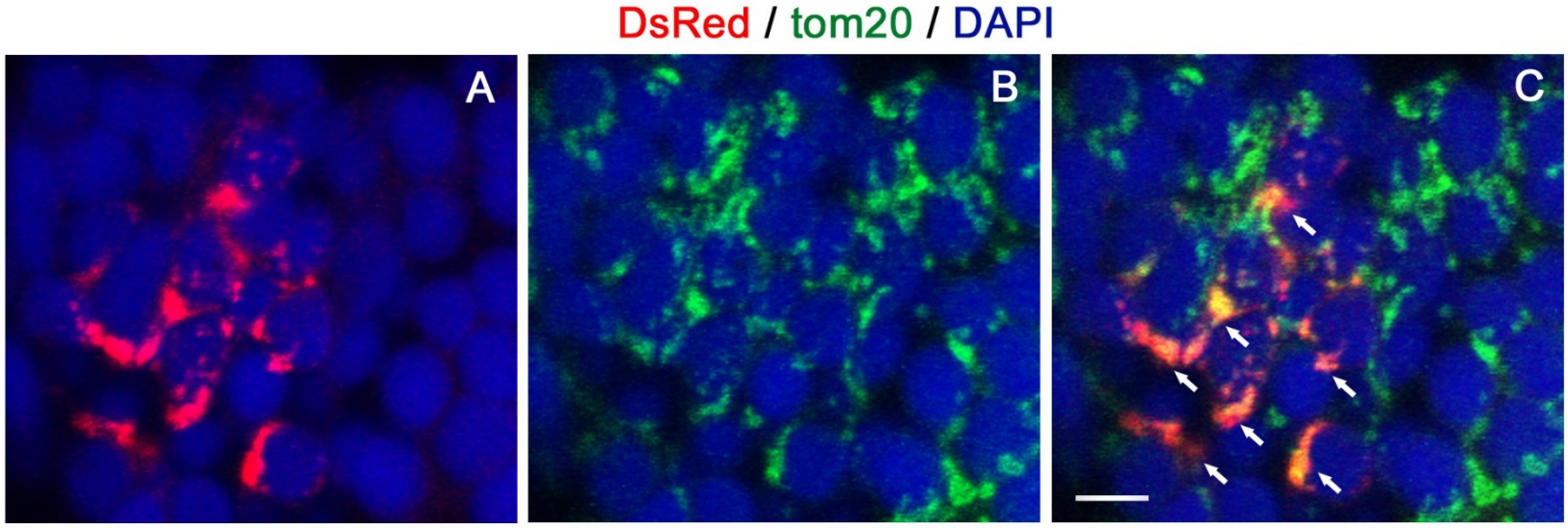
The initial objective of this project was to follow the mitochondria in the dopaminergic neurons of the zebrafish. Specifically, we analyzed the *in vivo* changes to the mitochondria morphology secondary to rotenone neurotoxicity. To visualize the mitochondria, immunohistochemistry coupled with fluorescence microscopy has been a solid method to achieve our goal. We therefore sought to optimize the conditions that would allow us to image the mitochondria with the highest resolution.

Traditionally, antibodies against mitochondrial markers such as tom20, VDAC or Hsp60 have been used to visualize the mitochondria in cell cultures. However, in animal tissues, the use of those antibodies could be unspecific as they stain mitochondria in every cell type. The transgenic zebrafish line *Tg(dat:tom20 MLS-mCherry)* expresses the red fluorescent protein mCherry exclusively in the mitochondria of DA neurons (Noble et al. 2015). Therefore, we have been doing immunohistochemistry on frozen sections using an anti-DsRed antibody on the *Tg(dat:tom20 MLS-mCherry)* larvae to study dopaminergic mitochondria. Since mCherry is a derivative of DsRed, the anti-DsRed antibody also recognizes mCherry.

To confirm that mCherry correctly labels the mitochondria, we performed immunolabelling with tom20 antibody on the *Tg(dat:tom20 MLS-mCherry)* 7dpf larvae. Comparing the signal of mCherry fluorophores that survived fixation and tom20 staining, we observed good colocalization (arrows, Figure 3.1C). Thus, immunostaining with anti-DsRed on the *Tg(dat:tom20 MLS-mCherry)* line was a valid method to follow changes in morphology of the DA mitochondria.

Figure 3.1: Imaging of zebrafish dopaminergic mitochondria.

Frozen sections (12µm thickness) of 7dpf *Tg(dat:tom20 MLS-mCherry)* embryos were immunostained with anti-tom20 (green) and DAPI (blue). mCherry survived fixation and was exclusive to dopaminergic mitochondria (A), anti-tom20 stained all mitochondria in the tissue (B). Merged image showed colocalization of mCherry and tom20 indicated with arrows (C). Scale bar = 5µm.


DsRed / tom20 / DAPI
A
B
C

3.2 Rotenone exposure resulted in developmental defects in larval zebrafish

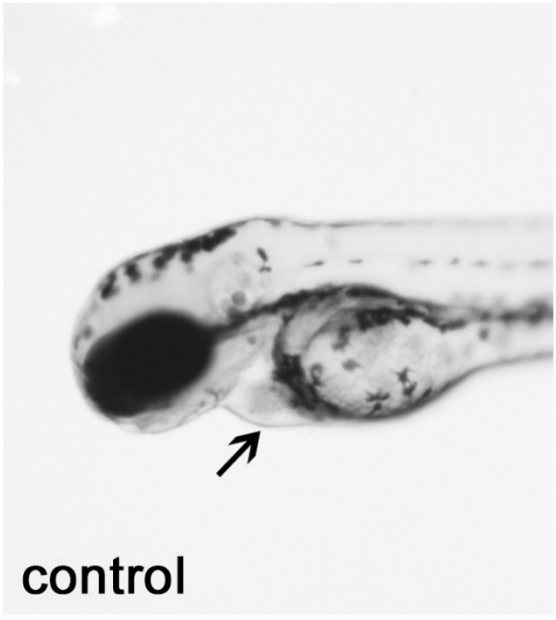
Rotenone, as a piscicide, has been demonstrated to be highly toxic to aquatic life. To increase survival of zebrafish to higher doses of rotenone, embryos were raised at 22ºC (RT) instead of at 28.5ºC. Lower temperatures decrease the zebrafish metabolic rate. Thus, we hypothesized that treatment at the standard 28.5ºC could be either too low to produce any response or too high resulting in rotenone killing the fish before it exhibits any neurotoxic effect. We have done multiple dose testing and 100nM was the highest concentration that ensured DA degeneration and at least 80% survival at RT. By 3dpf, about 90% of embryos treated with rotenone showed massive cardiac edema (arrow, Figure 3.2). The zebrafish also failed to hatch normally and required manual dechorionation. The phenotype persisted until 5dpf. However, at 7dpf, the defects seemed to be attenuated (data not shown). This is consistent with previous studies that involve rotenone treatment with zebrafish (Keow et al. 2016 , unpublished, Bretaud et al. 2004).

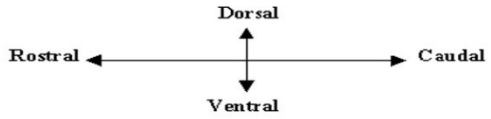
3.3 Rotenone caused an increase in dopaminergic neurons with fragmented mitochondria

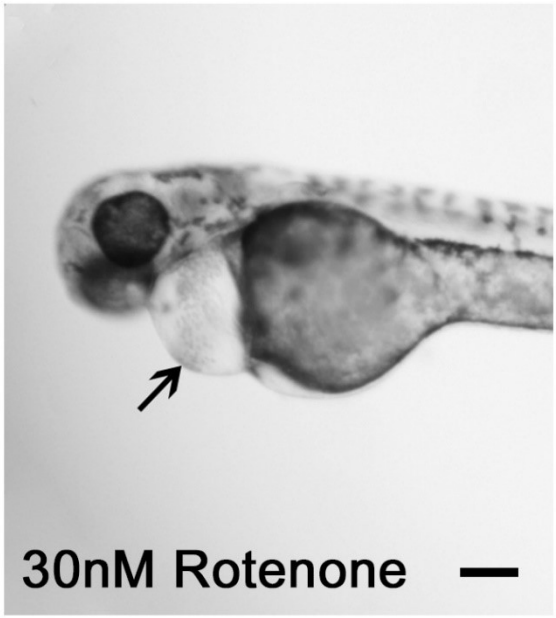
We adapted the rotenone administration procedure to reproduce the neuron degeneration reported by Martel and colleagues (Martel et al. 2015). *Tg(dat:tom20 MLS-mCherry)* embryos were exposed to either 100nM rotenone or DMSO and then cryosectioned and immunostained with DsRed antibody. We focused on imaging the ventral diencephalon region for two reasons. First, the DA loss following rotenone treatment was reported to be the most pronounced in this region (more than 50% number of cells decrease) (Martel et al. 2015). Second, as mentioned earlier, DA neurons in this region have been suggested to link with the human nigrostriatal pathway.

Figure 3.2: Zebrafish embryos treated with rotenone showed massive cardiac edema.

Zebrafish embryos exposed at 24 hours post-fertilization to 30nM rotenone or higher exhibited non-specific developmental deficits such as cardiac edema by 4dpf (bottom panel). These abnormalities persisted until 7dpf. Control groups treated with DMSO exhibited no abnormalities (top panel). Arrows showed the difference between the two groups. Scale bar = 100μm.


control


Dorsal
Rostral
Caudal
Ventral


30nM Rotenone

The z-stack planes were processed using maximum intensity projection, which made the 3D conformation of the mitochondria into a 2D presentation. The mitochondrial morphology of each cell was scored according to the criteria in Figure 3.3A, in a blinded fashion:

Normal: mitochondria are uniformly distributed and linked to each other around the nucleus.

Fragmented: mitochondria appear in less volume and are apart from each other.

Partially fragmented: Fragmentations were only observed in less than 50% portion of the network.

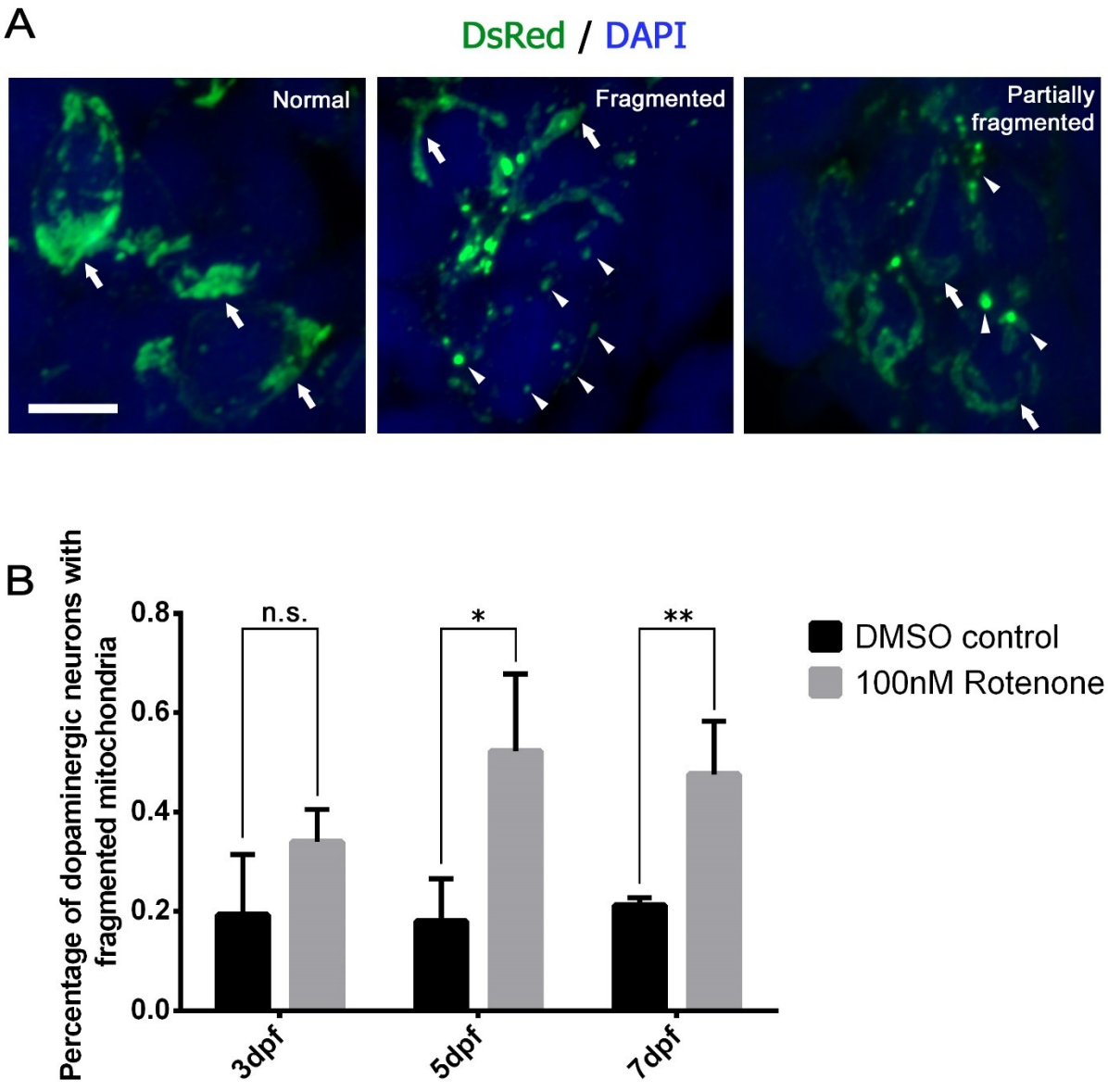
Embryos exposed to 100nM rotenone demonstrated no significant differences in neurons with fragmented mitochondria phenotype at 3dpf compared to controls (n=3, at least 20 cells each fish). However, at 5dpf and 7dpf, increases of 34% and 26%, respectively, were observed (n=4-5, at least 30 cells each fish) (Figure 3.3B).

3.4 Quantitative RT-PCR of genes involved in mitochondrial fission and fusion

In order to confirm our observations of rotenone induced mitochondrial fragmentation, we sought to investigate the molecular changes that accompany the zebrafish mitochondria network response to the oxidative damages caused by rotenone. We performed qRT-PCR to quantify mRNA expression levels of several genes involving the fission/fusion machinery including *dynamin-1-like (dnm1l), fis1, mitofusin 2 (mfn2)* and *optic atrophy 1 (opa1).* We compared the changes in the expression of these genes at 3dpf, 5dpf and 7dpf in a 25-50 pooled whole zebrafish lysate. We also investigated the expression of *dopamine transporter (dat)* at 3dpf to confirm the neurodegeneration at this developmental stage. For this experiment, zebrafish treated

Figure 3.3: Rotenone caused an increase in dopaminergic neurons with fragmented mitochondria.

Frozen sections (12µm thickness) of embryos exposed to 100nM rotenone or 0.1% DMSO were immunostained with anti-DsRed (green) and DAPI (blue) in the ventral diencephalic cluster (vDC). Cells were ranked as normal (left), fragmented (middle) or partially fragmented (right). Arrows mark normal elongated mitochondria. Arrowheads mark fragmented mitochondria (A). Zebrafish exposed to 100nM rotenone at 3dpf showed no significant different (n=3). However, at 5dpf, rotenone-exposed zebrafish have increased percentage (34%) of neurons with fragmented mitochondria (n=4, * $p < 0.05$). At 7dpf, the difference between treatment of 100nM and DMSO were 26% (n=4-5, ** $p < 0.01$) (B). Scale bar = 5µm.


A
DsRed / DAPI
Normal
Fragmented
Partially fragmented
B
Percentage of dopaminergic neurons with fragmented mitochondria
0.8
0.6
0.4
0.2
0.0
n.s.
*
**
3dpf
5dpf
7dpf
DMSO control
100nM Rotenone

from different batches are considered different biological replicates (n=3). Expression at all genes were normalized to two reference genes: *ef1a*, *rnap* and *rpl13a.*

We saw no significant change in *dnm1l* and *fis1* expression at either 3dpf, 5dpf or 7dpf, in zebrafish exposed to 100nM rotenone. Interestingly, there was a decrease in the expression of *opa1* (22%) in 3dpf embryos compared to controls. This change was magnified at 5dpf with *opa1* transcript levels decreased by 40%. However, by 7dpf, expression of *opa1* in larvae exposed to rotenone increased by 54% relative to controls. With *mfn2*, we also saw a decrease in expression levels at 3dpf (21% decrease). By 5dpf and 7dpf, expression of *mfn2* appeared to return to control levels.

The expression of *dat* in 3dpf zebrafish exposed to rotenone also decreased by 45% compare to DMSO controls (Figure 3.4).

3.5 Sensitivity of adult zebrafish to Cerebroventricular Microinjection of rotenone and MPTP

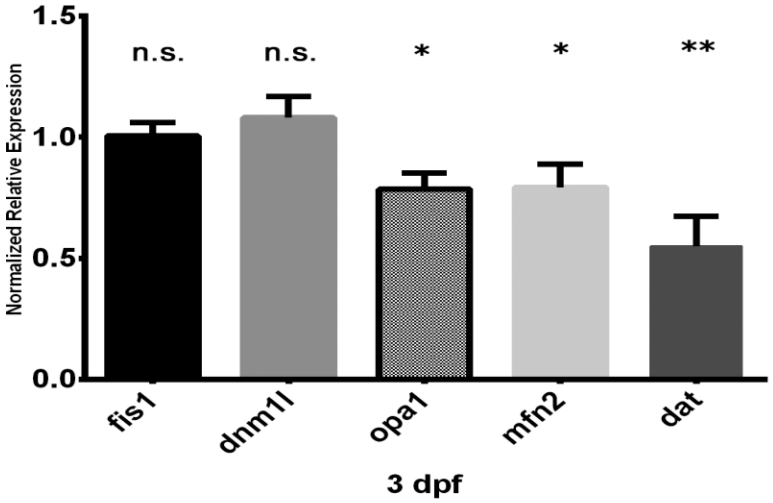
Our next objective was to induce dopaminergic neuron loss in adult zebrafish by delivery of rotenone specifically to the brain. To reach this goal, we used the cerebroventricular microinjection (CVMI) method described by Kizil and colleagues (Kizil and Brand 2011). The CVMI technique is briefly described in Figure 3.5A. The first goal was to find out the highest dose for each neurotoxin that would result in a 100% survival throughout the whole treatment process. Using this approach, a much higher dose can be used than treatment done by dissolving the compound into the fish water. The concentration of rotenone can be up to 1.5mM and the concentration of MPTP can be up to 50mM compared to nM and μM level in several previous

Figure 3.4: Effects of rotenone on mRNA levels of some mitochondria regulator genes

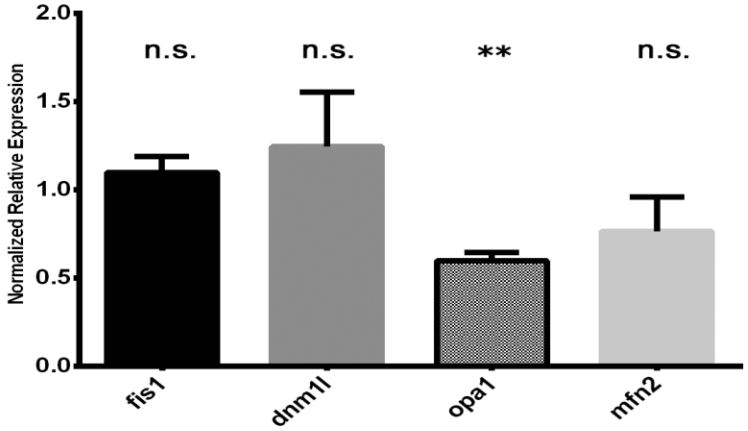
Zebrafish embryos exposed to DMSO or 100nM rotenone were raised to either 3dpf (top), 5dpf (middle) or 7dpf (bottom) for qRT-PCR analysis. All quantifications were performed in biological triplicates and normalized to two reference genes (n=3).

Levels of *dynamin-1-like dnm1l* and *fis1* in rotenone-exposed zebrafish larvae were not significantly different from controls at any developmental stage. mRNA levels of optic atrophy 1 *opa1* decreased by 22% and 40% in rotenone-exposed zebrafish larvae at 3dpf and 5dpf, respectively, compared to DMSO controls but increased by 54% at 7dpf. Transcripts of *mitofusin 2 mfn2* only significantly decreased in rotenone-exposed zebrafish at 3dpf.

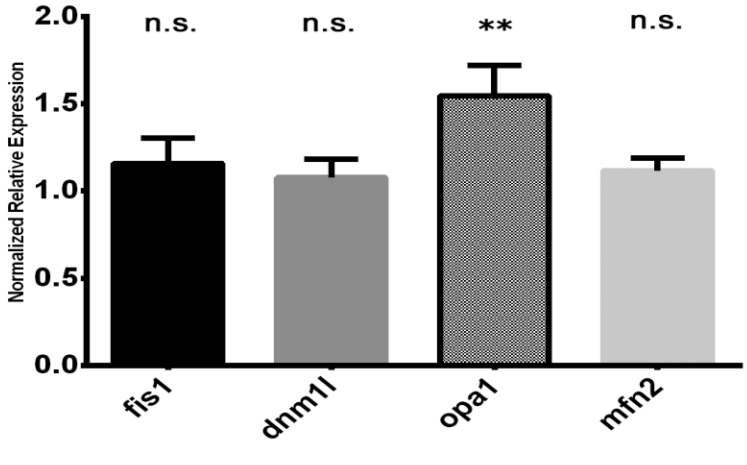
Relative expression of dopamine transporter *dat* of 3dpf zebrafish exposed to 100nM rotenone was also analyzed with a 45% decrease in transcript levels (* $p<0.05$, ** $p<0.01$).



3 dpf



5 dpf

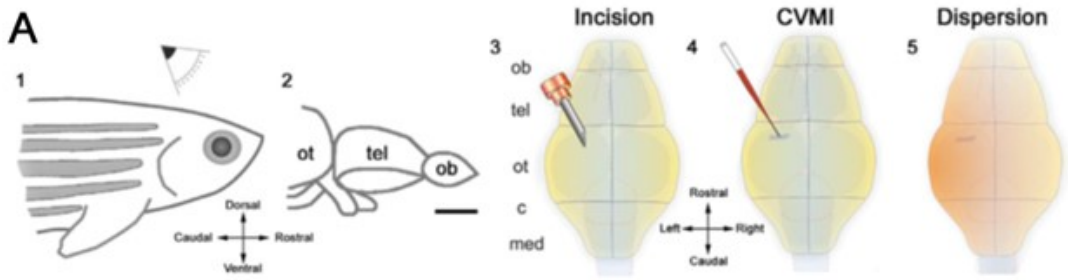


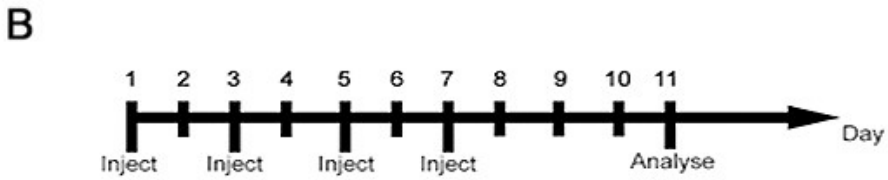
7 dpf

Figure 3.5: Procedure for cerebroventricular microinjection of rotenone and MPTP in adult zebrafish.

Zebrafish was anaesthetized with the dorsal surface of the head faced upwards (1). CVMI was developed to target mostly the forebrain including the optic tectum (ot), telencephalon (tel) and the olfactory bulb (ob). First, an incision was made into the skull over the optic tectum (ot) using a sharp needle (3). Using thin glass capillary microinjection needles, the solution was injected through the incision (4). The injected liquid dispersed throughout the brain (5). Image adapted from Kizil et al. 2012. (A).

The injections were performed every two days, for a total of 4 times. Four days after the last injection, the fish were recorded for behavioural assays; and then sacrificed and brain-dissected for immunohistochemistry (B).


A
1
2
ot
tel
ob
Dorsal
Caudal
Rostral
Ventral
Incision
CVMI
Dispersion
3
4
5
ob
tel
ot
c
med
Rostral
Left
Right
Caudal


B
1
2
3
4
5
6
7
8
9
10
11
Day
Inject
Inject
Inject
Inject
Analyse

studies (reviewed in table 1.2 and 1.3). For each concentration of rotenone, five wild type (WT) fish were used. Control fish were injected with DMSO instead of rotenone.

All adult zebrafish injected with 2mM of rotenone died within the first day after injection. With 1.5mM rotenone, two zebrafish died after the first injection and one died after the second injection. Zebrafish injected with either 1mM, 500µM, 300µM rotenone or DMSO survived all four injections (Table 3.1).

Zebrafish seemed to have variable response to MPTP. Therefore, we increased the number of fish for each concentration to eight. The control group was injected with saline. No fish survived after the first injection of MPTP at a concentration of 100mM. For fish injected with 50mM and 35mM of MPTP, at least one fish died every injection. The sublethal dose for MPTP CVMI was determined to be 25mM as all zebrafish survived at the end of the treatment. A similar result was obtained with a MPTP concentration of 10mM (Table 3.1).

We also noticed a big deficit in locomotion in treated fish and the fish had respiratory arrest right after injection of rotenone. The phenotypes included rapid flapping of the operculum, resting at the bottom of the tank after a few bursts of movement, abnormal spinning movement while swimming. However, the effect is temporary and disappears after several minutes (Figure 3.6A). For the rotenone injections that led to mortality, these phenotypes persisted until death. However, we did not observe this behaviour in fish injected with MPTP (Figure 3.6B). In contrary, death observed in zebrafish following CVMI of MPTP was rapid. The fish died in less than 5 minutes post-injection.

Moreover, the fish injected with rotenone showed a decreased pigmentation as well as increased redness in the gills (data not shown). This could be signs of stress during the treatment process.

Table 3.1: Summary of the effect of rotenone and MPTP Cerebroventricular Microinjection on adult zebrafish.

Drug	*Dose*	*Initial number of fish*	*Number of survivals after the last injection*
rotenone	DMSO	5	**5**
	2mM	5	**0**
	1.5mM	5	2
	1mM	5	**5**
MPTP	Saline	8	**8**
	100mM	8	**0**
	35mM	8	4
	25mM	8	**8**
	10mM	8	**8**

3.6 Cerebroventricular microinjection of rotenone caused a decrease in the telencephalic dopaminergic neurons

The *Tg(dat:eGFP)* transgenic zebrafish line express the green fluorescent protein in most of their dopaminergic neurons (Xi, Yu, et al. 2011). We have been using the *Tg*(*dat:eGFP)* fish to analyze changes in DA levels following rotenone microinjection. Four days after the last injection of rotenone, the fish were euthanized, and their brains were dissected. The brains were fixed and cryosectioned. We performed immunolabeling on 20μm frozen sections from *Tg(dat:eGFP)* adult brains using anti-GFP antibody. Specifically, we were interested in the DA neurons in the telencephalon for 2 reasons:

- As reviewed in a previous section, one of the hypotheses pointed out the DA clusters in this region might be responsible for voluntary movement in zebrafish.

- The DA distribution in this region is close to the ventricular zone where the drug disperses. Therefore, theoretically, this DA neuron clusters would be the most vulnerable.

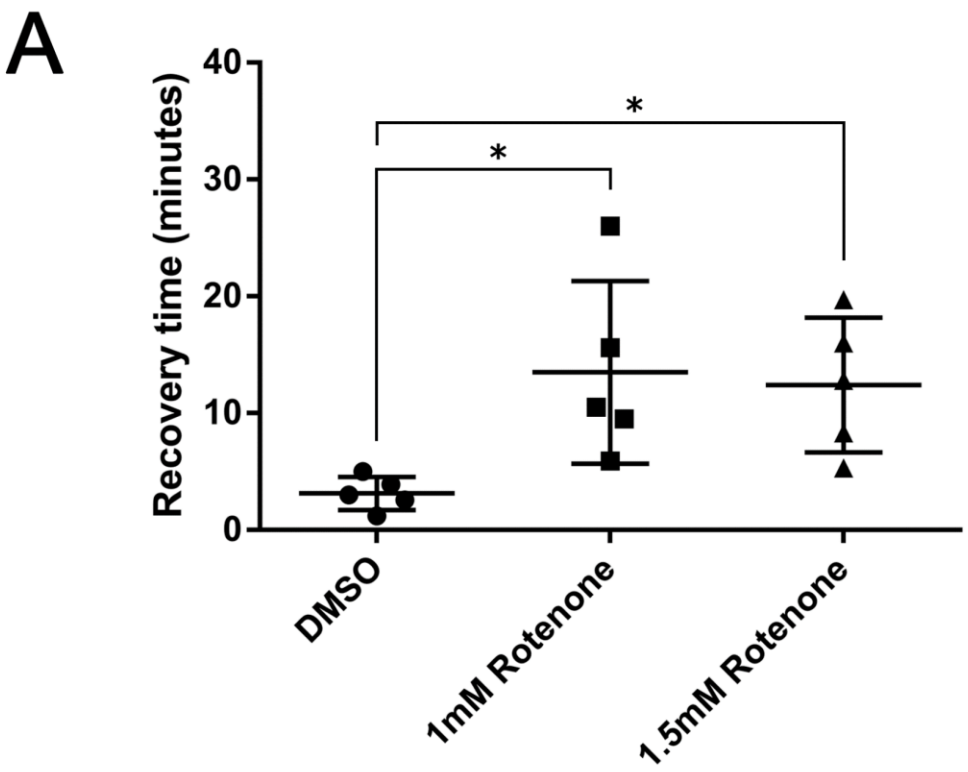
The number of DA neurons were counted in four sections in the telencephalon, in a blinded fashion. We found out that in the fish injected with 1mM rotenone, there was a mild decrease (20%) in the number of GFP-positive cells compared to controls (Figure 3.7B).

3.7 Behavioural assays of adult zebrafish

To test if the decrease in DA level observed above could manifest into locomotion deficits, we performed two tests: midline cross and total distance traveled. Four days after the last injection, the fish were put out of the dark in a static tank and were recorded using a Nikon camera. However, there were no deficit in movement of those fish compared to the control as

Figure 3.6: Zebrafish showed abnormal behaviour after cerebroventricular microinjection and took much longer time to recover.

The fish were anaesthetized with MS-222 before the injection procedure. Right after CVMI of the neurotoxin, the fish were put into a recovery tank right after the injection completed. The amount of time it took for the fish to wake up and regain normal swimming pattern were recorded. All zebrafish injected with DMSO recovered to their usual swimming behaviour in less than 5 minutes while fish injected with rotenone took up to 28 minutes to recover ($n=5$, * $p<0.05$) (A). No significant change in the recovery time of surviving zebrafish injected with MPTP at any concentration ($n=4$-8) (B).


A
Recovery time (minutes)
40
30
20
10
0
*
*
DMSO
1mM Rotenone
1.5mM Rotenone

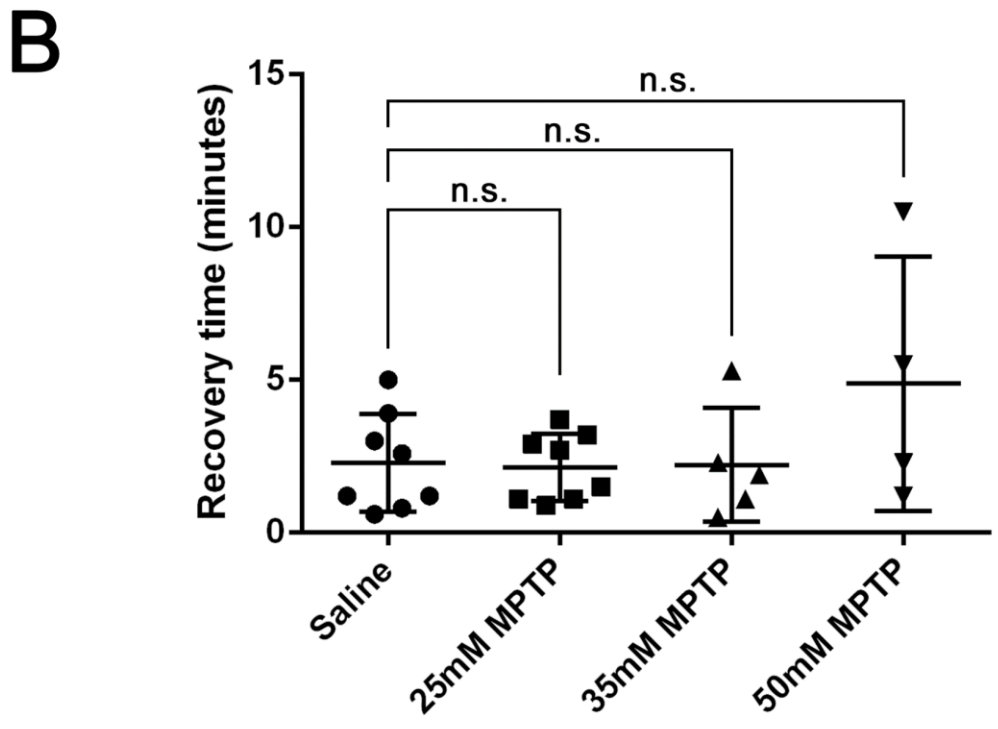
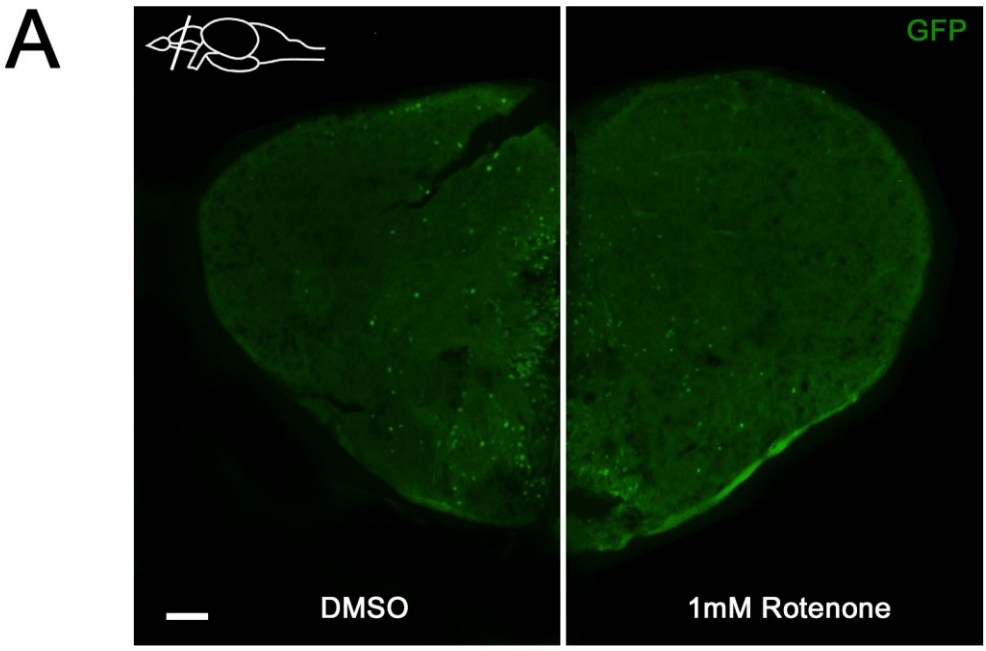
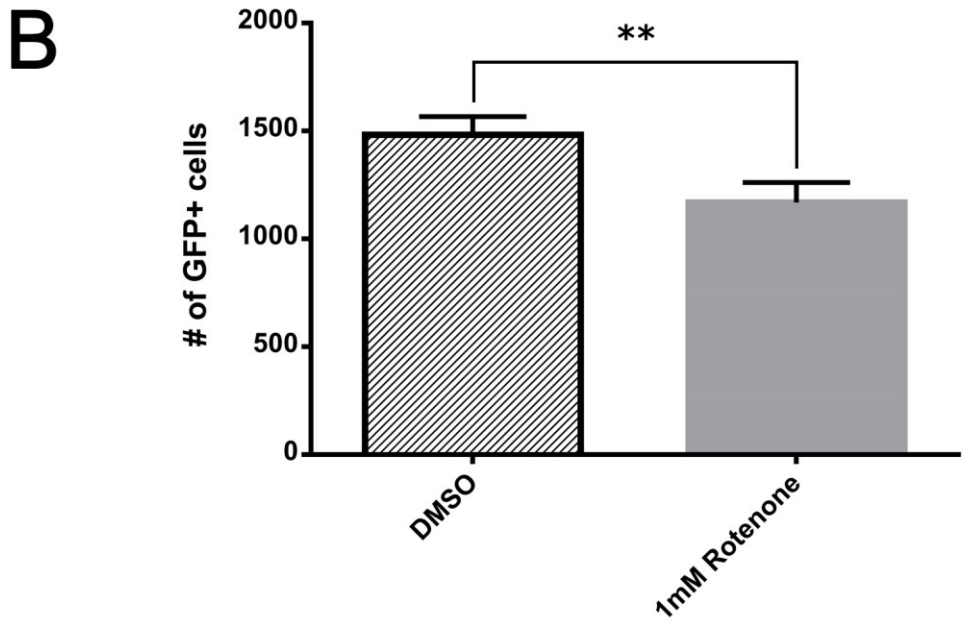

B
Recovery time (minutes)
15
10
5
0
n.s.
n.s.
n.s.
Saline
25mM MPTP
35mM MPTP
50mM MPTP

Figure 3.7: CVMI of rotenone into adult zebrafish induced a decrease in the number of dopaminergic neurons in the telencephalon.

Immunostaining of frozen sections (20µm thickness) with anti-GFP (green) in the telencephalic region of 10-month-old *Tg(dat:eGFP)* zebrafish injected with 0.1% DMSO (left) or 1mM rotenone (right) (A). The number of DA neurons in zebrafish injected with 1mM rotenone decreased 20% compared to controls (n=3, ** $p < 0.01$). Scale bar = 50µm


A
GFP
DMSO
1mM Rotenone


B
2000
1500
1000
500
0
# of GFP+ cells
**
DMSO
1mM Rotenone

suggested by comparable distance traveled (Figure 3.8A). The fish also showed no sign of anxiety-like behaviour as oppose to a previous study when zebrafish exposed to rotenone during embryogenesis showed a reduced midline crossing occurrences (Figure 3.8B).

These findings suggested that the observed loss in DA neurons in the telencephalon did not translate into any bradykinetic phenotype.

3.8 Cell dissociation and flow cytometry of dopaminergic neurons

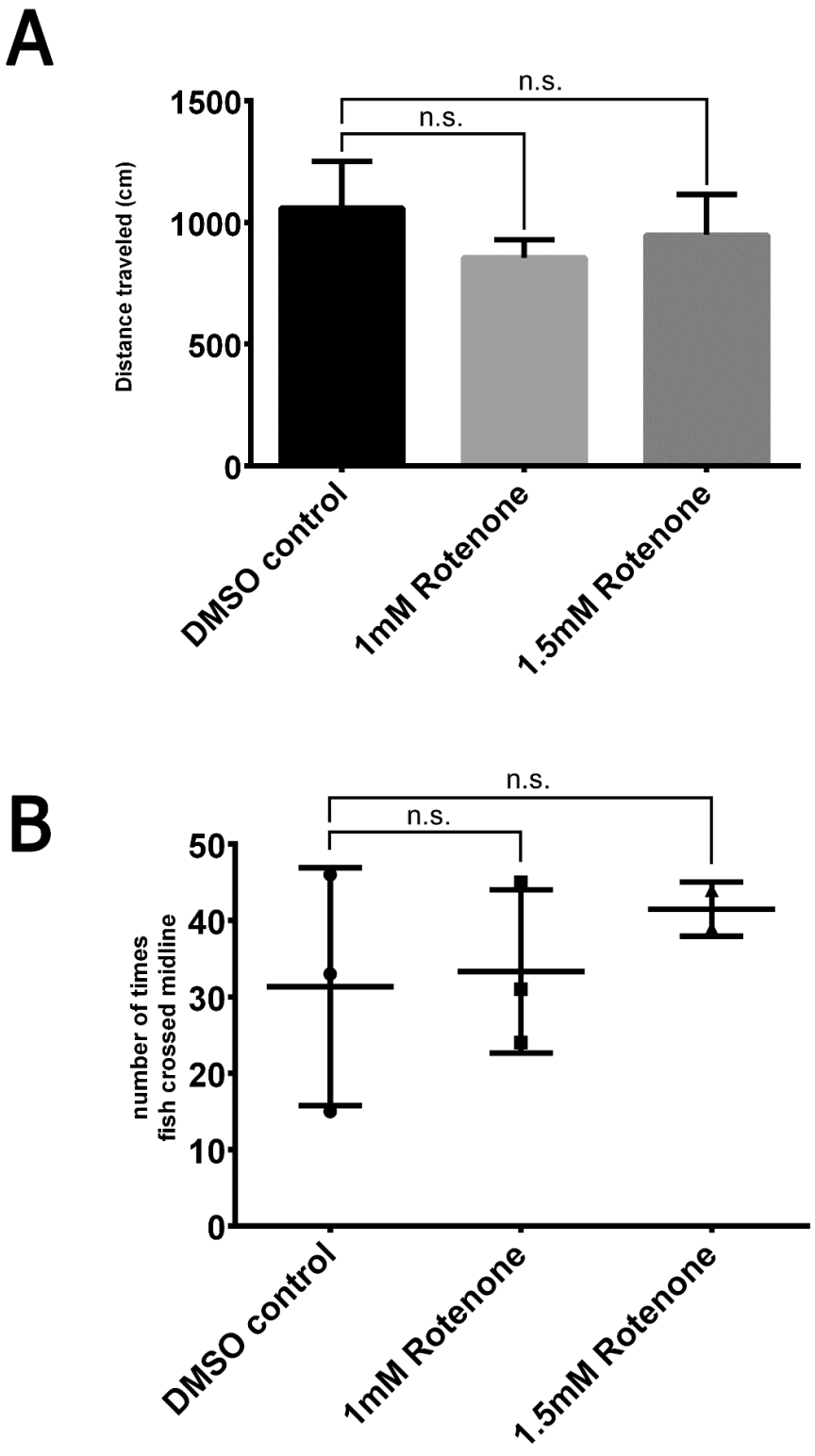
Due to the complexity of the brain, a powerful approach is to generate a primary culture of a type of neurons of interest for simpler manipulations and observations. Usually, the method involves dissecting the tissue, dissociating it into single cells, and growing the cells *in vitro*. Since DA neurons have been the research interest of several scientists, being able to keep these neurons in culture would allow a number of experimental tools and techniques to be accessible in a highly controllable environment. Time-lapse observations of multiple complexed phenomena can be made simple with primary culture: molecular trafficking, morphological changes, neurotoxicology or cell-cell interactions.

First, we wanted to develop a mean to isolate the DA neurons in zebrafish to a suitable number for culture. Secondly, we wanted to maintain the DA neurons in appropriate conditions. Therefore, using the GFP signal in the DA neurons of the *Tg(dat:eGFP)* line, we developed a method to enrich DA neurons with fluorescent-activated cell sorting (FACS).

Zebrafish embryos at 3dpf were mechanically dissociated by pushing them through multiple cell strainers: 100µm once and 40µm twice. As a control, a cell suspension of stage-matched, wild type embryos was prepared to set the sorting gate. The cell suspension was firstly

Figure 3.8: Behavioural tests showed that adult zebrafish injected with rotenone have no distinct behavioral phenotype nor locomotion deficits.

Adult zebrafish injected with either 1mM rotenone, 1.5mM rotenone or 0.1% DMSO were placed in a standalone tank and were tracked for position every 10 milliseconds in a total time of 3 minutes to calculate the distance traveled (A) or monitored for 4 minutes for the number of times they crossed the midline of the tank (B). All test in any concentration showed no significant change compared to controls (n=3).


A
n.s.
n.s.
1500
1000
500
0
Distance traveled (cm)
DMSO control
1mM Rotenone
1.5mM Rotenone
B
n.s.
n.s.
50
40
30
20
10
0
number of times
fish crossed midline
DMSO control
1mM Rotenone
1.5mM Rotenone

analyzed with a flow cytometer to find out the desired GFP+ population and exclude background. We found out from a cell suspension of 500 embryos at 3dpf, compared to control, 0.11% of the events were gated to be our target DA cell population based on particle size and relative fluorescent intensity (Figure 3.9A). However, this percentage was too low to sort out a viable number of cells to culture. Therefore, we sought to use later developmental stages hoping to solve this problem.

Zebrafish larvae at 5dpf were subjected to the same protocol. However, we did not find the GFP-positive population observed in the 3dpf cell suspension (Figure 3.9B). We also tried a combined method of mechanical and enzymatic dissociation with trypsin. However, none of these protocols succeeded to provide a sufficient population of GFP-positive DA neurons.

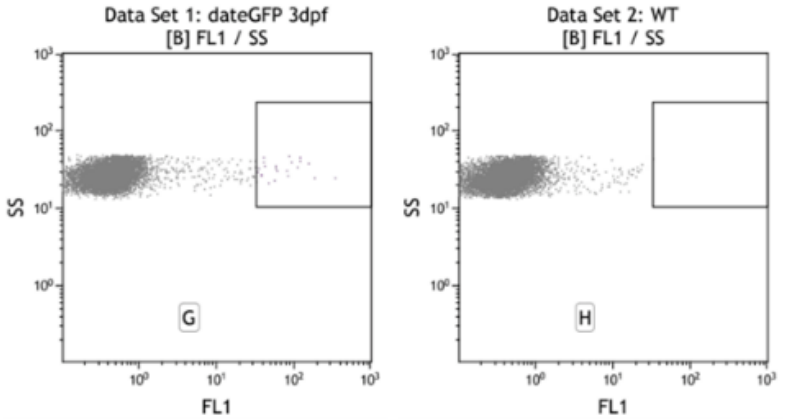
3.9 Primary culture of adult zebrafish brain cell suspension

Another attempt to culture zebrafish primary DA neurons was conducted using adult *Tg(dat:eGFP)* brains. Even though adult tissues are generally harder to dissociate, DA neurons are much more abundant in the adult brain. We adapted a protocol developed for culturing adult zebrafish neural stem cells (Lopez-Ramirez et al. 2016). The brains of adult *Tg(dat:eGFP)* were dissected and regions with the highest number of dopaminergic clusters, notably the olfactory bulb and the telencephalon, were isolated. Those regions were then enzymatically digested with trypsin to generate single cell suspension. The suspension was seeded in plates coated with poly-D-lysine in L-15 medium at 37ºC. The plates were monitored daily using a fluorescent cell analyzer. As a control, the same procedure was performed on WT zebrafish brain of similar age.

Figure 3.9: Flow cytometry analysis of zebrafish cell suspension.

500-1000 embryos of *Tg(dat:eGFP)* (left) and wild type (right) were dissociated either mechanically or enzymatically at 3dpf (A) or 5dpf (B) and were analyzed by flow cytometry. Each dot represents a detected event. Data were plotted against side scatter (SS) and GFP (FL1). Rectangle marks a distinct GFP-positive population of *dat:eGFP* sample compared to WT control.

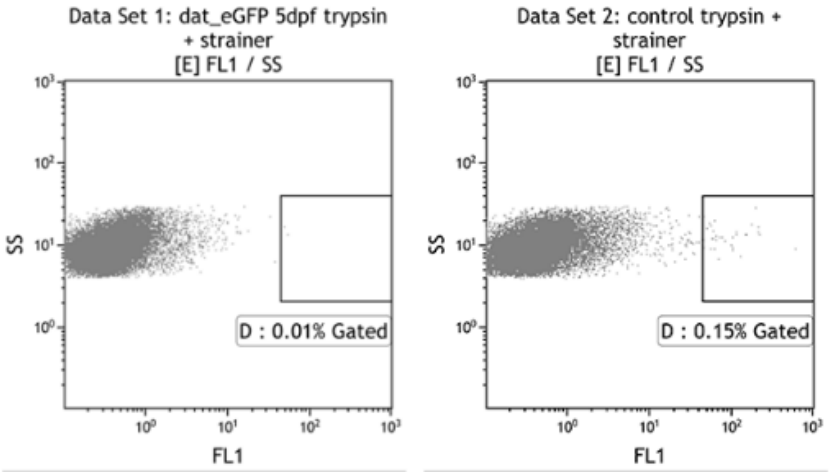
A



Gate	%Total
All	53.45
G	0.11

Gate	%Total
All	43.82
H	0.00

B



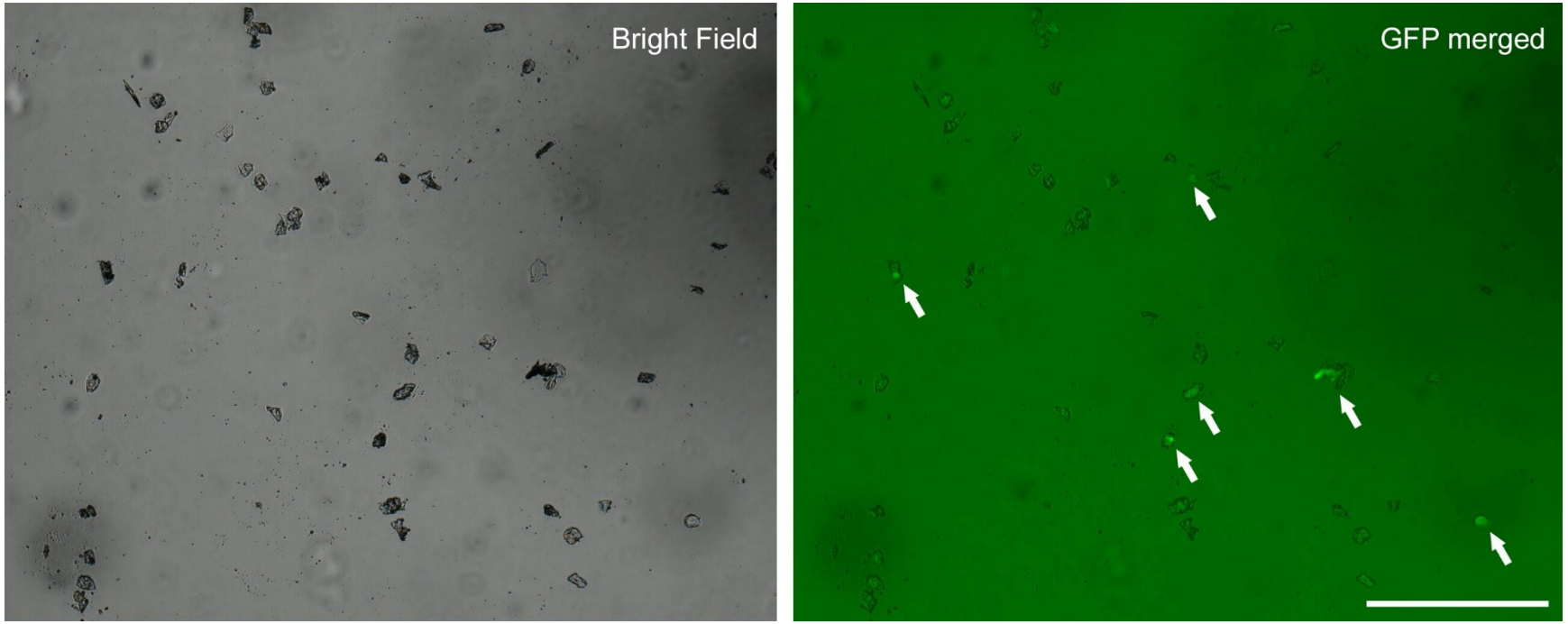
Gate	%Total
All	44.70
D	0.00

Gate	%Total
All	48.06
D	0.07

After two days of incubation, a population of various cells were observed growing in both plates. In *Tg(dat:eGFP)* plates, multiple cells attached to the coating were identified to be GFP-positive. WT culture plates did not have this population (data not shown), suggesting the cells observed in the *Tg(dat:eGFP)* plates were the transgenic GFP-expressing DA neurons (arrows, Figure 3.10).

Figure 3.10: Culture of adult zebrafish brain cell suspension.

Telencephalon and olfactory bulb of 10-month-old *Tg(dat:eGFP)* zebrafish were digested with trypsin to generate a cell suspension. The suspension was seeded into a 6-well plate coated with poly-D-lysine in L15 medium mixed with N2 supplemental and was incubated at 37ºC. The plate was imaged with a fluorescent cell analyzer after two days under bright field (left) and fluorescence for GFP (right). Arrows mark GFP-positive cells. Scale bar = 50µm.


Bright Field
GFP merged

CHAPTER 4: GENERAL DISCUSSION

Through experiments conducted in this thesis, we were able to assess the use of zebrafish as a model for Parkinson's Disease. Particularly, we were able to demonstrate that environmental hazards may contribute to the deterioration of vulnerable dopaminergic neurons by oxidative stress and mitochondrial dysfunction.

Part of this project was to follow the mechanism of action of rotenone, previously described to elicit bradykinetic dopaminergic neurodegeneration in zebrafish. We were able to reproduce the general toxic effects of rotenone by exposure methods documented by Martel and Keow (2016) such as decreased locomotor function, cardiac deformities and lowered dopamine transporter *dat* transcripts. We were able to examine zebrafish mitochondria by various microscopy approaches. We observed that rotenone caused changes in the morphology of the zebrafish dopaminergic mitochondria network. We also investigated the expression levels of various genes involved in mitochondrial fusion and fission in response to rotenone exposure. The transcript levels of these genes were observed to be variable. Neither *dnm1l* nor *fis1,* two well studied pro-fission genes, showed no significant differences. For fusion process, mRNA levels of *mfn2* slightly decreased only at 3dpf while *opa1* decreased from 3dpf to 5dpf but increased at 7dpf.

As Parkinson's Disease is clearly prevalent in older patients, we investigated the possibility of using adult zebrafish as a toxin-based model for the disease. We used a cerebroventricular microinjection approach to target two neurotoxins, rotenone and MPTP, to the telencephalon. We were able to optimize the dosing and the injection procedure to ensure maximum bioavailability of the drug. Adult zebrafish showed a 20% decrement in the number of dopaminergic neu-

rons after microinjection of rotenone. However, we did not see any change in locomotion phenotype in these fish.

Unfortunately, due to lack of an established protocol, we were not able to develop a primary culture of zebrafish dopaminergic neurons. Using flow cytometry and fluorescence-activated cell sorting, we identified a population of GFP-positive cells from 500 of *Tg(dat:eGFP)* embryos. However, this population was too small to provide sufficient neurons to establish a culture.

4.1 Challenges in mitochondria imaging of zebrafish dopaminergic neurons

The mitochondria have been challenging objects for microscopy. The diameter of mitochondria is nearly at the resolution limit of light microscopes, making numerous cellular processes involving these organelles elusive to the eyes of researchers. Most frequently, to resolve mitochondrial structure, fluorescent tagging combined with laser scanning confocal microscopy is used. Since mitochondria are highly dynamic organelles, live imaging may be a more informative strategy to observe the various series of intrinsic movements. However, compared to other widely used models such as cell lines and mice, the size of a zebrafish cell is much smaller. The diameter of a zebrafish DA neuron is about 5μm (about a fourth of a mouse cortical neuron or a HEK293 cell), making the process of resolving zebrafish mitochondria challenging. In addition, due to the risk of phototoxicity and the need to follow rapid events, live imaging usually uses a lower power laser with fast scanning time. This makes high resolution live imaging difficult. No detailed analysis of the morphology of zebrafish DA mitochondria was found. The only report in

in vivo live imaging of the zebrafish DA mitochondria was limited to observation of axonal transport that are close to the surface of the fish (Dukes et al. 2016).

In order to visualize DA mitochondria with better resolution than with fluorescence microscopy, electron microscopy was also previously conducted. However, the method became more and more problematic as it lacked specificity to DA mitochondria and immunogold produced too many artefactual signals. For this reason, in this body of work, using cryosections on a confocal microscope with an objective with numerical aperture (NA) of 1.4, we were only able to clearly distinguish DA neuron with fragmented mitochondria from DA neurons with normal mitochondria. We were not able to perform several quantifications that are commonly conducted in mitochondrial analysis such as mitochondrial length, mitochondrial motility, cristae structure analysis or protein localization.

Furthermore, DA neurons usually stay in clusters, meaning they stay in very close proximity to each other. This is also a nuisance in the process of analysis as one may mistakenly account the mitochondria of one cell to another. Therefore, we chose to analyze the mitochondria of the DA neurons in the diencephalon not only because neurodegeneration in this region was most pronounced but also because DA neurons in the diencephalon were more sparingly spaced.

4.2 Rotenone-induced neurodegeneration precedes mitochondrial fragmentation

It was reported that rotenone caused changes to zebrafish DA neuron patterning as early as 2dpf through 15dpf (Martel et al. 2015). Rotenone's mechanism of action strongly suggests that it causes neurodegeneration by inhibition of mitochondrial complex I resulting in the formation of ROS. Subsequently, accumulation of ROS impairs mitochondrial function and causes

mitochondria to become fragmented. This mechanism was supported in the current study by the fact that the mitochondria of DA neurons became highly fragmented at 5dpf and 7dpf. Moreover, same phenomena were witnessed in mouse primary astrocytes (Deheshi et al. 2015). However, our analysis in rotenone-exposed zebrafish at 3dpf showed no significant disproportion of DA neurons with fragmented mitochondria. To check if DA neurodegeneration happened at 3dpf, we performed qRT-PCR of *dat* on a cDNA sample obtained from 3dpf embryos treated with 100nM rotenone. There was a 45% decrease in the transcript levels of *dat* in rotenone-exposed zebrafish. Although the decrease was milder than the previous report by Keow and colleagues (73.1%) (Keow et al., unpublished), this suggested that rotenone appeared to disrupt the zebrafish DA system at earlier developmental stages in a different complex I inhibition-independent mechanism.

It is not surprising that the toxic effects of rotenone are not specific to DA neurons. There have been reports which claimed that rotenone may exhibit its toxicity by microtubule depolymerization (Ren et al. 2005; Choi et al. 2011). In zebrafish, we also observed several abnormalities during embryogenesis of rotenone-exposed larvae such as cardiac edema and gut malformation (Figure 3.2). Moreover, quantification before 5dpf is usually variable because at this stage, zebrafish develop at variable speed. Also, the DA system of the zebrafish is not fully developed until 5dpf.

Therefore, we propose that during the first two days of exposure, the complex I inhibitory effect of rotenone does not yet lead to sufficient ROS accumulation to induce a change in mitochondrial structure. Thus, the observed decreased levels of DA neurons at 3dpf are due to non-specific growth delay. By 5dpf (4 days of exposure), the ROS build-up is big enough to over-

whelm endogenous free radical scavengers in DA neurons, ultimately leading to oxidative damage-mediated cell death.

Overall, considering how energy-demanding DA neurons are, as well as the basal oxidative stress from the process of dopamine production, these data confirmed the theory that rotenone induces its neurotoxic effects through mitochondrial damage. We were able to create a link to the complex I deficiency observed in the brain of PD patients (Schapira et al. 1990), as well as how mitochondrial structure can directly affect the cell's bioenergetic demand and mortality.

4.3 Rotenone altered the gene regulation of genes involving in the mitochondrial fusion machinery

The fusion/fission dynamics, as previously reviewed, is very important in maintaining the functional integrity of mitochondria. Noteworthily, mitochondrial fusion and fission have been heavily associated with cell death/survival mechanisms (Chen et al. 2007; Suen et al. 2008). Fission may help to isolate damaged mitochondria portions to be later eliminated by autophagy (Twig et al. 2008). Fusion have been implicated in mitochondrial quality control processes such as mtDNA damage prevention or dilution of damaged mitochondrial segments (Parone et al. 2008).

Therefore, we sought to follow changes of the gene expression of the proteins directly responsible for mitochondrial fusion and fission machinery: *Dynamin-1-like dnm1l*, *mitochondrial fission 1 fis1, Optic Atrophy 1 opa1* and *Mitofusin 2 mfn2*. Both *Mitofusin 1 mfn1* and *Mitofusin 2 mfn2* were initiallychosen as genes of interest. However, both zebrafish functional paralogs *mfn1a* and *mfn1b* have poorly annotated sequences in most databases. Moreover, *mfn1* and mfn2

seem to have redundant function (Chen et al. 2003). Thus, analysis of *mfn1* gene expression was omitted in this study.

Overall, there was no change in the expression of genes involved in mitochondrial fission, namely *dnm1l* and *fis1*, in larvae exposed to 100nM rotenone. Expression of the pro-fusion gene *mfn2* was decreased at 3dpf. Expression of optic atrophy 1 *opa1*, which is also involved in fusion, decreased at 3dpf and 5dpf. Looking at the general trend in expression, one may conclude that the previously described mitochondrial fragmentation resulting from rotenone exposure was likely to link to the downregulation of fusion and unlikely to be the result of fission upregulation. Interestingly, mRNA levels of *opa1* appeared to significantly increase in 7dpf zebrafish exposed to 100nM rotenone even though fragmentation persisted. The OPA1 protein helps to regulate the shape of mitochondria by playing a key role in the fusion of the IMM. It is also involved in a respiratory process called oxidative phosphorylation to derive energy. Additionally, OPA1 is also believed to play a role in mitochondrial DNA maintenance, and in apoptosis (Ishihara et al. 2006; Song et al. 2007; Song et al. 2009). We hypothesized that, at earlier stages, the gene was downregulated to reduce fusions events and to enforce mitochondrial morphology control. However, at later stages, the toxic effects accumulated. The *opa1* gene was upregulated to take a role in other functions such as the attempts to increase respiration or apoptosis.

Most of fission mechanisms were proposed from studies in yeast and *Drosophila*. While the precise mechanism of fission in higher animals is not as well studied, it is assumed that the process is the same as in yeast. Dnm1l localizes in the cytosol, is recruited to the mitochondria and facilitates the constriction of the OMM by direct or indirect interaction with Fis1 (Yoon et al. 2003; Yu et al. 2005). Most probably, the determining factor for Dnm1l translocation and activity is strongly dependent on the post-translational modification of the protein. Therefore, the

lack of significant change in *dnm1l* transcripts observed in rotenone-exposed zebrafish may not fully reflect the activity of the dynamin-like protein. On the other hand, knockdown of Fis1 in mammalian cell lines blocks mitochondrial fission without affecting Dnm1l localization to mitochondria (Lee 2004). This also suggests that Dnm1l may be able to translocate to the mitochondria by different pathways.

Additionally, the transcripts quantified in this experiment were not exclusive to DA neurons but were from all tissues from a pooled sample of at least 20 individuals. Since different types of cells require different levels of energy to function, it is possible that the mitochondria network in DA neurons are regulated differently. Besides, different individuals may have different reactions in response to a drug. Together, this could explain most of the non-significant observations in the transcript of studied genes.

4.4 A 20% loss in the telencephalic dopaminergic neurons was not enough to induce locomotor deficits

Realizing that no neurotoxin model of PD has been established in adult zebrafish and the need for generating one, we created a protocol to minimize any off-target effects that could possibly cause by rotenone by specifically delivering the drug to the brain. We adapted the Cerebroventricular Microinjection (CVMI) technique that was firstly developed by Kizil and colleagues to target the adult zebrafish ventricular zone in the telencephalon with morpholino oligonucleotides to knock down adult neurogenesis expression in progenitor stem cells (Kizil and Brand 2011). Given that rotenone is highly lipophilic and can easily cross the blood-brain barrier, we assumed that upon being delivered into the ventricular fluid, the drug would disperse throughout

the brain. We also expected that the most vulnerable DA neuron cluster following rotenone CVMI would be the "A16" cluster at the subpallium as it is localized in very close proximity to the ventricular zone (Figure 3.7A). We injected an array of concentrations of rotenone CVMI: 100μM, 300μM, 1mM, 1.5mM and 2mM. We determined that the sublethal dose of rotenone for the procedure was 1mM.

Initially, as a pilot study, the injection was performed only once. We analyzed the brain of *dat:eGFP* fish injected with 1.5mM rotenone one day post-injection. No changes in the number of DA neurons were observed in the telencephalon of these fish (data not shown). To ensure maximum bioavailability of rotenone to the fish, we increased the injection process to a total of four times. One day of rest was spared between each injection to avoid handling stress that may occur to the fish. With this procedure, we were able to ablate 20% of the telencephalic DA neurons. This finding also correlates with a previous study confirming that long term exposure of rotenone reproduces PD phenotypes including decreased DA levels in rats (Cannon et al. 2009) as well as in the previously described larval zebrafish.

Zebrafish are hyperactive and curious fish. They require constant movement unless they are sleeping or being locomotor impaired. They are likely to explore the whole tank that contains them and prefer to be in a school of at least 5 to 10 individuals. Translation of zebrafish behaviour to human symptoms in the context of PD has been debatable. Currently, the most accepted method in assessing a bradykinetic phenotype in zebrafish is measuring the distance traveled. In this study, distance traveled in each zebrafish was derived manually from tracing the position of the fish every 10 milliseconds, ensuring high accuracy. Unfortunately, our behavioural tests failed to demonstrate that the loss of DA neurons by CVMI of rotenone causes locomotor defi-

cits. However, this result might due to the low number of biological replicates (n=3). Increasing the number of fish per treatment may be worthwhile to increase the reliability of this result.

We then concluded that a 20% loss of dopaminergic neuron was not enough to induce a noticeable movement change as the brain still had thousands more cells to attenuate the loss. Most evidently, in the case of PD patients, degeneration in SNc DA neurons can be up to 60%. In MPTP treatment of multiple animal models, locomotor impairments were detected following at least a 40% reduction of DA levels (Langston et al. 1984; McKinley et al. 2005; Cannon et al. 2009; Xi, Yu, et al. 2011). Yet, as reviewed before, there has been a debate about the functional homolog of midbrain dopaminergic system in zebrafish. Some believe that the zebrafish's equivalent nigrostriatal dopaminergic pathway lies in the projection of clusters from the ventral diencephalon to the telencephalon based on the structures of other catecholaminergic signaling systems. Others believe the pathway is the projection of DA clusters within the subpallium itself. The fact that our reported ablation of DA neurons in the telencephalon did not cause any disruption in movement supports the former hypothesis.

4.5 The toxic effects of rotenone on aquatic life

Rotenone is slightly toxic to mammals but is extremely toxic to fish and has been commercially used to kill invasive fish in rivers and lakes (Ling 2003). We found out that by changing conditions (temperature), larval zebrafish can survive up to a concentration of 100nM.

It is likely that the most prominent toxic effect of rotenone to aquatic life is through respiratory inhibition. During the process of CVMI, rotenone, zebrafish clearly had trouble breathing and the amount of time it took to recover was dependent on the dose. This result seemed to

be due to the leakage of rotenone into the gills from the skull which causes respiratory arrest. The increased redness observed in the gills of rotenone-injected fish also supports this conclusion. This could also explain the failure of inducing neurodegeneration in adult zebrafish by dissolving the drug in water in previous studies (reviewed in table 1.3) as rotenone appears to have higher affinity to the gills and kills the fish by hypoxia.

Interestingly, exposure to rotenone in fish might increase their movement instead of impairing it. It is reported that rotenone exposure to trout and medaka increased their movement. Rotenone has also been used in small-scale sampling by ecologists to study the biodiversity of marine lifeforms to collect camouflaged or hidden fishes in the shorelines (Robertson and Smith-Vaniz 2008). The reason for this may due to the fact that fish get oxygen-contained water into their gills by moving forward. Treatment of rotenone impairs the fish's respiration and in response their movement increases in attempt to increase oxygen uptake. This is a noteworthy point for researchers who utilize rotenone to reproduce PD phenotypes on aquatic models.

Rotenone's neurotoxic effect seems to have slow kinetics. Because the drug only stayed in the skull for a few hours after CVMI, it is possible that the 20% decrease in DA neurons after 4 consecutive injections is because the drug did not have time to ablate enough cells in the area before being flushed out of the system. In the Lewis rat model, a daily dose of rotenone for four weeks was required to induce 45% degeneration of DA neurons in the SNc (Cannon et al. 2009).

We then sought to utilize a drug with fast action to magnify the loss in a shorter time span and MPTP was chosen. Sharing the same mechanism of action with rotenone, MPTP has been the first and the best studied toxin-based model of PD. Self-injection of MPTP in human was reported to elicit symptoms of PD within three days (Langston 2017). We were able to optimize the dose for CVMI of MPTP with a sublethal dose of 25mM. Even though MPTP and rotenone

have the same mechanism of action, it is understandable that CVMI of MPTP did not result in the same transient acute respiratory arrest post-injection observed with CVMI of rotenone (Figure 3.6B). MPTP was not specifically produced to be a piscicide. Moreover, MPTP is acutely specific to DA neurons as it is uptaken into the cell by dopamine transporter (Gainetdinov et al. 2002). No reports that describe non-Parkinsonism effects of MPTP has been found. However, due to time constrain, we were not able to investigate DA neuron levels in MPTP-injected fish.

4.6 The dopaminergic neuron population is not sufficient to overcome background fluorescent signals in flow cytometry

The use of flow cytometry and fluorescent-activated cell sorting (FACS) with transgenic zebrafish has been developed over the years to separate specific populations of cells expressing fluorescent reporter protein for RNA isolation and characterize gene expression in single cells. For instance, Gallardo and colleagues employed FACS combined with microarrays to enrich the transcription profile of zebrafish lateral line cells (Gallardo and Behra 2013). Single cardiac progenitor cell isolation and characterization was made possible by Samsa and colleagues with FACS (Samsa et al. 2016). To push the application further, Ma and colleagues were able to characterize the structure of zebrafish hematopoietic stem cells and were able to transplant these cell into irradiated adult zebrafish (Ma et al. 2011). Interests in culture of DA neurons were first suggested thanks to the *Tg(dat:eGFP)* line.

We adapted the protocol described by Manoli and colleagues (Manoli and Driever 2012) to create a single-cell suspension of primary cells from whole zebrafish embryos, generated by the mechanical dissociation of 500 *Tg(dat:eGFP)* zebrafish embryos at 3dpf based on size and

relative fluorescence intensity compared to a WT (non-transgenic) suspension. It is noteworthy that the dopaminergic neuron population is estimated to be less than 1% of the total cell in the body. By 3dpf, the DA system in zebrafish is not fully developed. In addition, not 100% of the obtained embryos were transgenic as they were bred from the natural spawning of about 15 heterozygote *Tg(dat:eGFP)* parents and manual screening of over 500 embryos was nearly impossible. Therefore, the number of sorted cells was determined to be insufficient for subsequent experiments.

To further complicate the procedure, the *Tg(dat:eGFP)* line has unspecific expression in the jaws (Xi, Yu, et al. 2011) that could interfere with the purity of the flow cytometry results. To resolve this, we proposed to double label DA neurons with two fluorescent proteins. The transgenic line *Tg(HuC:loxP-DsRed-loxP-GFP)* expresses DsRed in neuronal precursor cells during embryogenesis, and then high expression levels persist in most regions of post-hatching and larval brain under the control of the *HuC* promoter. Breeding *Tg(dat:eGFP)* with this line allow DA neurons to possess both GFP and DsRed. The procedure is illustrated in figure 4.1A. We also confirmed the simultaneous expression of two fluorophores in DA neurons by double immunostaining of anti-DsRed and anti-GFP (arrowheads, figure 4.1B). Nevertheless, this did not solve the problem of low DA neuron yield.

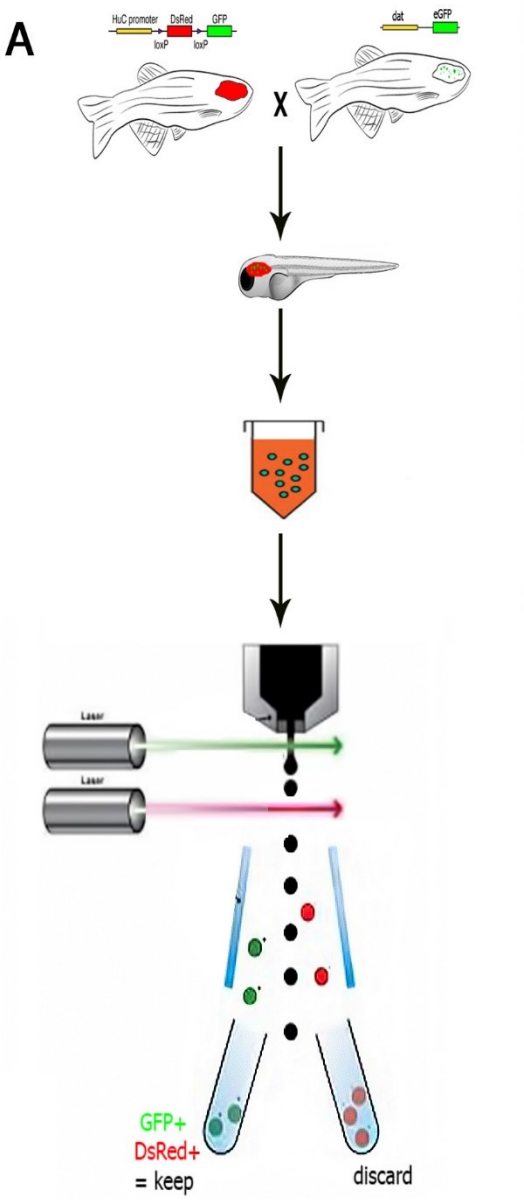
Therefore, we sought to use later developmental stages as a starting material. We decided to use 5dpf embryos as, at this stage, the DA system of the zebrafish is more fully developed. We avoided using embryos older than 5dpf because the cells become cross-linked and more difficult to dissociate as the tissue becomes older. Due to this, we employed a combination of mechanical and enzymatic dissociation to generate single-cell suspension. Surprisingly, flow cytometry of

Figure 4.1: Strategy of fluorescent-activated cell sorting on double fluorescent DA neurons and autofluorescence in the yolk.

Embryos obtained from a cross between *Tg(HuC:loxP-DsRed-loxP-GFP)* and *Tg(dat:eGFP)* parents possess DA neurons expressing both GFP and DsRed. Cell dissociation followed by flow cytometry are then conducted. Cells that are excited by both GFP and RFP lasers are DA neurons (A).

Frozen sections (14µm thickness) of 7dpf *Tg(HuC:loxP-DsRed-loxP-GFP / dat:eGFP)* embryos were double-immunostained with anti-DsRed (red), anti-GFP (green). Arrowheads mark colocalization of GFP and DsRed-positive cells (B).

Live imaging of wild type zebrafish at 5dpf under green fluorescent filter. Dotted lines indicate the shape of the fish. Arrow marks the autofluorescence in the yolk (C).


A
HuC promoter
DsRed
GFP
loxP
loxP
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eGFP
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DsRed+
= keep
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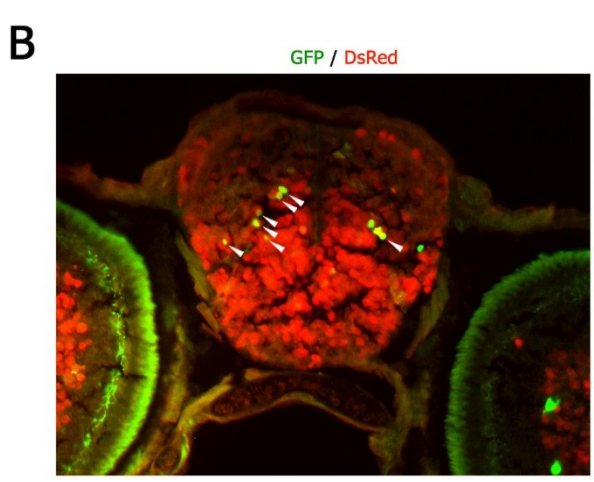
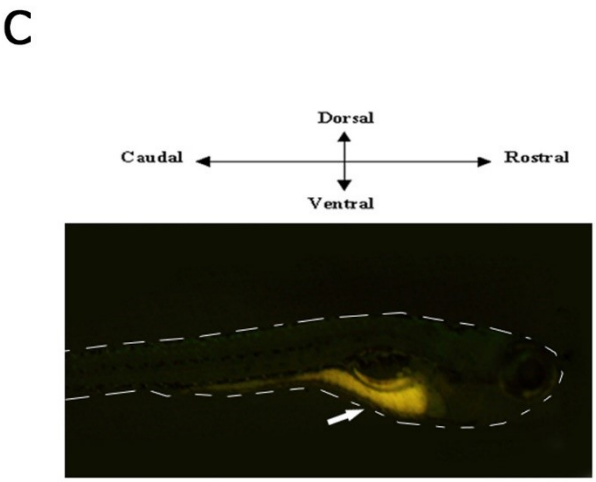

B
GFP / DsRed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Dorsal
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this cell suspension did not yield any GFP-positive population in the transgenic data set and the GFP signal from the WT population was even higher (Figure 3.9B). By carefully analyzing the 5dpf cell suspension under a fluorescence cell analyzer, we identified the main source of background fluorescence signal was from the yolk. At 5dpf, the gut system has developed over the surface of the yolk, making the deyolking step inefficient. The yolk was the biggest source of autofluorescence and, when homogenized, it broke down into various sized particles, making sorting much more difficult (Figure 4.1C).

We also sought to use the adult zebrafish brain to culture DA neurons. DA neurons are much more abundant in the adult zebrafish brain as they constantly generate new neurons throughout their life by the adult neurogenesis process. Dissection of one adult brain theoretically yields at least 50,000 DA neurons. Dissociation of the adult brain also minimizes the autofluorescence from non-neuronal tissues. We adapted a protocol developed for culturing a mixed population of adult zebrafish brain-derived neurospheres (Lopez-Ramirez et al. 2016). By two days, we observed a GFP-positive population in the culture (Figure 3.10). This population might be the GFP-expressing DA neurons from the *dat:eGFP* transgenic brain.

Another challenging step is to optimize the conditions to gently dissociate the neurons and to grow them in good health. In this experiment, we used trypsin-EDTA digestion to dissociate the neurons. This enzyme has been long used for dissociating and de-anchoring multiple cell lines from culture plates. Experts in primary neuron culture communities argue that trypsinization might be too harsh and might damage the neurons during the process. Growing conditions such as temperature and CO_2 supplement might also alter growth and neuronal morphology. Together, it is implied that the neuronal populations we acquired were not in their healthy form. As

cell culturing in zebrafish is currently not developed, further identification will be needed to characterization these cells.

4.7 Future directions

Recently, imaging methods that break the resolution limit of light microscopy, super-resolution microscopy, are becoming more and more developed. Various methods of super-resolution microscopy have been reported to be able to resolve mitochondrial membrane interactions (Brown et al. 2011; Ishigaki et al. 2016) and distinguish the inner and outer membrane (Jans et al. 2013). Employing super-resolution microscopy could potentially be useful to study the morphology of zebrafish mitochondria. To uncover the mechanisms of fission/fusion in response to rotenone, mitochondrial fractionation followed by Western blot may be more representative than qRT-PCR to show how dnm1l translocates to the mitochondrial membrane.

Since PD is a multifactorial disease, combination of both genetics and environmental models may provide more mechanistic insights. Recently our laboratory has been working on the knockout of zebrafish PARL's homologs *parla* and *parlb*. Due to PARL's role in mitophagy, treatment of rotenone in the *parla* and *parlb* mutants might increase the susceptibility to the drug.

To model PD in adult zebrafish, further optimization of CVMI and injection of various other chemicals implicated with PD can also be conducted.

Identification of the cultured cells can be conducted with neuronal markers. After that, multiple trials with less damaging dissociation methods such as the use of papain combined to the optimization of culture condition should help provide a viable primary DA population.

CHAPTER 5: CONCLUSION

The primary objective of this research is to explore the mechanism of action of rotenone in zebrafish to develop it as a model for the environmental aspect of Parkinson's Disease. Throughout this body of work, we were able to uncover the toxic effects of rotenone in zebrafish. We identified that rotenone caused an increase in oxidative stress which leads to mitochondrial fragmentation and impairs the mitochondrial fusion/fission machinery. We proposed that rotenone-induced neurodegeneration in larval zebrafish might occur in two pathways: During early stages, rotenone impairs neurodevelopment and, at the same time, gradually builds up ROS in existing DA neurons. Later on, ROS accumulate to the point that mitochondria become fragmented and subsequently induce unrestrained neuronal cell death. We were also able to demonstrate that exposure to rotenone, specifically in the brain of the adult zebrafish, decreased DA levels. This may be a potential approach for utilizing adult zebrafish as a model for Parkinson's Disease.

Overall these results gave us more information about one of the potential the physiological mechanisms leading to dopaminergic degeneration and allow for the development of therapeutic strategies for Parkinson's Disease.

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