Neuroprotection of Dopaminergic Neurons and their Subcellular Structures from Parkinson’s Disease-like Treatment

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This dissertation titled
Neuroprotection of Dopaminergic Neurons and their Subcellular Structures from
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ABSTRACT

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In this dissertation, I explore the molecular mechanisms involved in the neuroprotection of dopaminergic (DA) neurons from Parkinson’s disease (PD)-like treatment. PD is movement disorder characterized by the slow, gradual death of DA neurons in the substantia nigra pars compacta. Neuroprotection is the idea that these cells could be rescued or protected from pathology. I establish a Drosophila primary culture system to study PD-like neurodegeneration and screen potential neuroprotective therapies. DA neurons, as well as their subcellular structures, are quantified and compared between treatment groups. I show that 1-methyl-4-phenylpyridinium (MPP⁺), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), selectively damages DA neurons and their mitochondrial morphology. I also show that the dopamine type 2 (D2) receptor agonist quinpirole can prevent, but not reverse, MPP⁺-induced degeneration. Furthermore, I show that D2 autoreceptors (presynaptic receptors which respond to autologous DA release) are required for the rescue by quinpirole. However, even in DA neurons lacking D2 autoreceptors, MPP⁺-induced degeneration was prevented by reducing cellular excitability: either with the Na⁺-channel blocker tetrodotoxin (TTX) or by Ca²⁺ chelation. Moreover, DA neurons with mutated Ca²⁺ channels were inherently protected from MPP⁺ treatment. Lastly, I describe the time-
course of MPP⁺-induced degeneration within this model system. DA neurons are near-completely post-mitotic by 3 days in vitro (DIV), which is the time point when cultures are treated with drugs. I find that damage to mitochondria precedes the death of DA neurons, and that rescue therapy must be given almost concomitantly with PD-inducing treatment to be effective.
DEDICATION

This work is dedicated to the many flies who gave their lives in the name of science.
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LIST OF ABBREVIATIONS

BrdU = bromodeoxyuridine (5-bromo-2'-deoxyuridine)

cac = cacophony

D2R = dopamine type-2 receptor

DA = dopamine/dopaminergic

DAPI = 4',6-diamidino-2-phenylindole

DAT = dopamine transporter

DD2R = Drosophila dopamine type 2 receptor

ddH₂O = double distilled water

DIV = day(s) *in vitro*

DMSO = dimethylsulfoxide

drp1 = dynamin-related-protein-1

EGTA = ethylene glycol tetraacetic acid

GECI = genetically encoded Ca²⁺ indicator

GFP = green fluorescent protein

MPP⁺ = 1-methyl-4-phenyl-pyridinium ion

NGF = nerve growth factor

opa1 = optic-atrophy-1

PD = Parkinson’s disease

QUIN = quinpirole
ROS = reactive oxygen species

TH = tyrosine hydroxylase

TH-GFP = fly line UAS-GFP;TH-GAL4

THmtGFP = fly line UAS-mtGFP/CyO;TH-GAL4

TTX = tetrodotoxin
CHAPTER 1: INTRODUCTION

Parkinson’s disease (PD) is an age-related movement disorder associated with progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (Dauer and Przedborski, 2003). However, no current medication protects against DA neurodegeneration, perhaps because the etiology and pathogenesis of PD are still not adequately understood. There is no clear event or property in early PD that can be targeted by an intervening therapy to prevent degeneration (Loehle and Reichmann, 2010; Bezard et al., 2012). Nevertheless, prevention of DA neurodegeneration is an obvious therapeutic goal. Therefore, a popular area of PD research that this manuscript will emphasize is neuroprotection – an attempt to slow the progression of the disease or stop it from developing (Herrero et al., 2011; Smith et al., 2012).

In my work I develop a model of PD to study neuroprotection. PD is modeled in vitro using Drosophila cell cultures treated with a toxin that promotes cellular damage specifically in DA neurons. In this model, I quantify DA degeneration after toxin treatment by measuring the number of DA neurons and the morphological properties of mitochondria. I explore therapeutic targets that may prevent neurodegeneration in PD, and I test their mechanisms of action. These subcellular structures in the vulnerable DA neurons may serve as bellwethers of the progression of PD pathology that could be used as molecular biomarkers in developing neuroprotective treatments. Thus, I study the early events in degeneration and the subsequent changes in mitochondria that predicate cell death. Understanding these elements of PD-like pathology should help in the development of future therapies that accomplish neuroprotection in PD.
To explain the salient premises of my research, the following introduction provides a general background on the topics of PD, cell death in PD, the effect of PD pathology on subcellular structures (e.g. mitochondria), neuroprotection, and the use of *Drosophila* for studying PD.

**PD and Models of PD**

PD can arise from genetic or environmental causes; the majority (>90%) of cases are idiopathic. In idiopathic PD, the current hypothesis is that a lifetime of environmental insults selectively degenerate DA neurons. At the molecular level, these vulnerable neurons are characterized by mitochondrial dysfunction, an increase in reactive oxygen species (ROS), and the presence of Lewy bodies – protein aggregates made of misfolded α-synuclein (αSyn) protein and ubiquitin (Dawson et al., 2010; Jellinger, 2012). All these elements (mitochondria, ROS, and protein misfolding) are hypothesized to play a role in the pathogenesis of PD. Briefly, a primary pathogenic/oxidative event, or many small, chronic ones, begins the degenerative process specifically in DA neurons (Manning-Boğ et al., 2007). These DA neurons begin to degenerate at the terminals and gradually “die back” (Levy et al., 2009; Court and Coleman, 2012). A patient is noticeably symptomatic and a PD diagnosis can be made only after about 70% of the DA cell bodies are gone (Jellinger, 2012; Smith et al. 2012). The degeneration gradually spreads to other brain regions, which is associated with late-stage symptoms of the disease, e.g., dementia (Hansen and Li, 2012; Jellinger, 2012). At this point, neuroprotection is almost moot. Most of the DA neurons that will degenerate in PD have
already done so. Thus, models of neurodegeneration must mimic the above process and study neuroprotection at the correct time point.

Models of PD cause selective degeneration of DA neurons, and behavioral models also induce PD-like movement disorders. Reliable models can be developed using mutations or toxins. Genetic PD models have been produced from mutating genes first identified in familial forms of PD. These models display both DA cell degeneration and behavioral defects. The function of the genes is not completely understood, but the most well studied genes (PINK1, Parkin, and αSyn) are associated with mitochondrial dysfunction and altered mitochondrial morphology (Lu and Vogel, 2009; Imai and Lu, 2011).

Two major toxin models are 1-methyl-4-phenylpyridinium (MPP⁺, the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP) and rotenone, both of which inhibit complex I in the mitochondria and increase ROS (Lu and Vogel, 2009, Cicchetti et al., 2009). Rotenone treatment highlights the selective vulnerability of DA neurons because it affects all cells (a global insult), yet primarily degenerates DA neurons. MPP⁺, however, is imported into cells via the DA transporter protein, and thus damages DA neurons specifically. MPP⁺ is also the most well studied toxin, and it mimics PD both in terms of the neuronal circuitry that is affected and in PD-like behaviors produced (Manning-Boğ et al., 2007; Ghosh et al., 2012). Furthermore, MPP⁺ has produced PD-like symptoms and degeneration in humans (Langston et al., 1983).

Our main concern is that the model we use mimics the early events of actual PD. Preventing a degenerative mechanism in our model will not be useful if the same
mechanism does not exist in actual PD. Unfortunately, PD is difficult to study in the pre-symptomatic stages (before patients are diagnosed). Thus, which model best represents early PD processes is unknown (Tieu, 2011). Our neuronal culture model uses MPP\(^+\) because: 1) it is specific to DA neurons and thus, we can compare neurodegeneration between DA neurons and non-DA neurons within treatment groups, 2) the mechanism is consistent with a leading putative cause of PD as theorized in recent literature – degeneration via ROS stemming from mitochondrial dysfunction (Cicchetti et al., 2009), and 3) we can easily control when PD-like degeneration happens and subsequently track time-dependent changes following treatment.

Cell Death in PD and Selective Vulnerability of DA Neurons

Although the exact mechanisms of cell death in PD are unknown, DA neurons are thought to degenerate through mitochondrial-dependent apoptosis (Perier and Vila, 2012). Indeed, ROS and mitochondrial dysfunction are implicated in PD degeneration (Dawson et al., 2010; Nakamura et al., 2011; Saxena and Caroni, 2011; Botella et al., 2011). Genetic models and familial forms of PD also implicate mitochondrial dysfunction (Schapira, 2009; Nakamura et al., 2011). Furthermore, mutant αSyn also leads to mitochondrial fragmentation and apoptosis (Xie and Chung, 2012). Even wild-type αSyn, when overexpressed, interacts with mitochondria by inducing their fragmentation (Nakamura et al., 2011) and promoting apoptosis (Berman et al., 2008).

Yet, it is unknown why DA neurons are more prone to ROS-induced apoptosis compared to other neurons. Also unknown is the initial cause of the ROS increase,
although mitochondria are suspected as the major source (Surmeier et al., 2011). Many specific properties of DA neurons have been proposed as vulnerabilities to ROS-induced degeneration and indeed there are several factors involved in increased ROS levels (Double, 2012). One noteworthy idea is that certain types of calcium channels present on vulnerable DA neurons may add an increased Ca\(^{2+}\) load on the cell. The increase in Ca\(^{2+}\) can depolarize the membrane of mitochondria, inhibit their function, and limit their ability to sustain the metabolic needs of the cell (Surmeier et al., 2011). Even DA itself is a reactive molecule that can increase ROS levels if not carefully packaged in synaptic vesicles (Park et al., 2007; Ulusoy et al., 2012).

In contrast to specific insults, some familial forms of PD have mutations in genes that are expressed ubiquitously (Herrero et al., 2011), yet DA neurons still die selectively compared to other cell types (Saxena & Caroni, 2011). It is likely that all of the factors listed above (and many others not mentioned) each add to the cell’s ROS load. Eventually, the insults summate until ROS levels pass a survivable limit, i.e., beyond the ability of the cell’s defenses (Levy et al., 2009; Westerman, 2010; Herrero et al., 2011; Double, 2012). ROS potently damages DNA, RNA, lipids, and proteins (Miwa et al., 2008). The subsequent damage is detected by cell-death regulators such as the Bcl-2 family of proteins, which signal to mitochondria to release cytochrome c, activate caspases, and promote apoptosis (Taylor et al., 2009). Thus, in the case of PD, DA neurons may simply be more sensitive to ROS than other neurons.
Mitochondrial Dynamics and Relationship with PD pathology

Mitochondria are a dynamic collective of organelles that, in addition to supplying to the cell with energy, respond to cellular stress. They vary their morphology via fission and fusion (Berman et al., 2008). Fusion and the proteins that govern it are generally neuroprotective, while excessive fission (fragmentation) is associated with apoptosis and mitophagy (Berman et al., 2008; Perier & Vila, 2012). However, fission is also linked to synapse development and mitochondrial turnover and biogenesis (Berman et al., 2008; Perier and Vila, 2012).

Although the range of possible morphologies is wide and dependent on cell-type, damaged mitochondria are in general fragmented and round/swollen (Berman et al., 2008; Chang and Reynolds, 2006; Perier and Vila, 2012). Fragmentation contributes to mitochondrial membrane permeabilization and the release of cytochrome c (Kreisner and White, 2009), both of which lead to cell death. These fragmented/damaged mitochondria, which also show functional deficits in ATP production, appear both in mutant PD models and in cells treated with inhibitors of complex I of the electron-transport-chain (Imai and Lu, 2011; Wang et al., 2011; Perier and Vila, 2012). Apoptosis-related fragmentation occurs in both vertebrates and invertebrates (Kreisner and White, 2009). Furthermore, a study using Drosophila cells induced towards apoptosis by ecdysone treatment showed that damaged mitochondria precede the appearance of phosphatidylserine, caspase activation, and the subsequent cell death (Goyal et al., 2007). Lastly, the mitochondrial fragmentation and subsequent cell death can be prevented by inhibiting proteins that promote fission or by overexpressing genes
that promote fusion. This effect has also been shown in vitro using both genetic and neurotoxin models of PD (Perier and Vila, 2012). Thus, changes in mitochondrial morphology are likely an early event in PD pathology, and PD models could be used to screen for neuroprotective treatments by examining mitochondria. Indeed, some researchers suggest using mitochondria as a therapeutic target (Ghosh et al., 2010; Perier and Vila, 2012).

Quantitative comparison of mitochondrial morphology is still imperfect. Most studies report the morphology qualitative description, while others report only one parameter, e.g., the length (Wang et al., 2011) or “mass” (Liang et al., 2007). The most comprehensive method to date measures four parameters: number, size, interconnectivity, and elongation (Dagda et al., 2009), but has not been further developed or utilized by other groups. A comprehensive analysis is preferred because one variable will not accurately describe the nature of the changes in morphology. The shape, size, and number of mitochondria may change drastically in normal functioning. Thus, if researchers report a change in length of the mitochondria (as in Wang et al., 2011), but not report a decrease in the number of mitochondria, it may be unknown if the cell was in a state of degeneration or normal restructuring.

The use of mitochondria to study neuroprotection in PD is still somewhat controversial (Perier & Vila, 2012). Mitochondria could be rearranging their morphology as a protective function (Change & Reynolds, 2006) or independently of the PD pathology (Krieser & White, 2009). Moreover, some types of cell death occur regardless of mitochondrial signaling (Leist & Jaattela, 2001; Tait & Green, 2010). To date,
However, strong evidence exists that changes in mitochondrial morphology are predictive of cell death (Wang et al., 2011, Martinou & Youle, 2011).

**Neuroprotective Mechanisms**

A true neuroprotective therapy for PD would decrease DA neurons’ vulnerabilities, stop Lewy body (LB) pathology from spreading, keep ROS at basal levels, maintain the cell’s normal functioning, and ultimately stop DA neurons from degenerating. Because ROS is a key factor in PD, an effective neuroprotective therapy might simply reduce ROS by addressing a vulnerability specific to DA neurons. Alternatively, a neuroprotective therapy could increase the capacity for handling ROS, i.e., raising the threshold that determines if a cell succumbs to ROS. Treatments for PD could modulate ROS either by antioxidant effect or an event downstream of DA signaling (Herrero et al., 2011). However, current treatments for PD merely manage symptoms. Some medications for PD have been touted as neuroprotective, but recent studies provide insufficient evidence that they halt, slow down, or modify the progression of PD (Loehle and Reichmann, 2010; Singer, 2013). PD progresses in patients regardless of treatment.

Besides antioxidants, another possible route to neuroprotection is preventing excitotoxicity. Dopamine D2 receptors are coupled to G-proteins and include ion channels as effectors. D2 receptors generally have an inhibitory effect on the cell’s membrane potential (Neve et al., 2004). The D2 agonist quinpirole hyperpolarized the cell membrane and decreased the firing rate of neurons *in vitro* (Fasano et al., 2010). Thus, D2 agonists might accomplish neuroprotection by reducing cellular excitability.
Decreased activity may help vulnerable DA neurons because they putatively have an inordinate intracellular Ca\(^{2+}\) burden (Surmeier et al., 2012). Ca\(^{2+}\) is tightly regulated by cells, but the DA neurons that are vulnerable in PD have Ca\(^{2+}\)-channels which allow a large Ca\(^{2+}\) current into the cell. Thus, vulnerable DA cells suffer Ca\(^{2+}\) influxes beyond what is typical for a neuron because instead of using monovalent ion channels for their autonomous pacemaking activity, they rely on L-type Ca\(^{2+}\)-channels (Costa et al., 2008; Surmeier et al., 2011). L-type Ca\(^{2+}\)-channels are normally expressed in the human brain (Striessnig et al., 2006), but do not normally allow large Ca\(^{2+}\) currents as they do in DA neurons vulnerable to PD pathology (Surmeier & Schumacker, 2013). Intracellular Ca\(^{2+}\) must be safely stored or exported against a large concentration gradient and this process consumes ATP. Mitochondria normally buffer intracellular Ca\(^{2+}\) by storing it in the mitochondrial matrix (normally more electrically negative relative to the intracellular environment), which depolarizes the mitochondrial membrane potential (Surmeier et al., 2011). But if Ca\(^{2+}\) levels are excessive, the excess Ca\(^{2+}\) in the matrix will collapse the mitochondrial membrane potential, increase ROS, reduce ATP synthesis, and will eventually open the permeability transition pore and promote cell death (Surmeier et al., 2011; Hurley and Dexter, 2012). Thus, hyperpolarizing the cell membrane or reducing activity may be neuroprotective by preventing damaging Ca\(^{2+}\) influxes and the subsequent apoptosis signaling. If simply hyperpolarizing the cell membrane is neuroprotective, ion channels make obvious targets. Indeed, L-type Ca\(^{2+}\)-channel blockers prevented degeneration in a toxin-based, mouse model of PD (Ilijic et al., 2011). Activation of D2R also indirectly decreases activity of L, N, and P/Q Ca\(^{2+}\)-channels in
cultured mouse neurons (Neve et al., 2004). D2 agonists also block the opening of the mitochondrial permeability transition pore – preventing the collapse of the mitochondria membrane and the release of pro-death signaling molecules. Moreover, this result does not appear to be from antioxidant effects (Sayeed et al., 2006; Parvez et al., 2010).

Just as there are several ways to promote cell death signaling through mitochondria, there are likely many ways to prevent it. Antioxidants are an obvious choice, but even if they were adequately neuroprotective, preventing the cell death cascade may be best achieved by reducing excitotoxicity, which several molecules can accomplish with more selectivity and efficacy than antioxidants.

Using *Drosophila* to Test Neuroprotection in PD

*Drosophila* provides both behavioral and cellular models for PD-like degeneration, and has provided important advances to the understanding of PD (Muqit & Feany, 2002; Bilen & Bonini, 2005). Models of the disease can arise from either genetic mutations or toxin treatment (Lu and Vogel, 2009). Similar to actual PD, the behavioral defects from mutant or toxin models are rescued by feeding flies the DA precursor levodopa – the major drug therapy used to relieve symptoms in PD. However, just as in actual PD, levodopa did not rescue DA cell degeneration (Lu and Vogel, 2009; Smith et al., 2012). Thus, *Drosophila* closely models PD as it is observed in humans.

Most human genes implicated in PD are conserved in the *Drosophila* genome (Adams et al., 2000; Whitworth, 2011; Estaquier et al., 2012). One important gene missing from the fly genome is alpha-synuclein (*αSyn*). Although flies lack an *αSyn*
ortholog, the panneural expression of either mutant or wild-type human αSyn induced DA degeneration and behavioral defects mirroring PD (Feany and Bender, 2000; Pendleton et al., 2002). Thus, Drosophila appears to reliably model PD both in terms of pathogenesis and treatment effects.

Experiments with mutant Drosophila PD models have provided much evidence linking PD pathology to mitochondrial dysfunction. Two examples are the genes parkin and pink1. Both genes were identified in familial forms of human PD, and mutations in their Drosophila orthologs cause degenerative effects involving mitochondria (Lu and Vogel, 2009, Dawson et al., 2010). Furthermore, experiments in Drosophila show that the products of these two genes function in a linear pathway. Several other Drosophila mutant models of PD (such as flies with mutations for DJ-1 and LRRK) also show a convergence of mitochondrial dysfunction and oxidative stress in the degenerative process (Botella et al., 2009). Thus, Drosophila research can provide useful insight into the degenerative process of PD via several useful tools unique to the fly.

Drosophila remains a popular research animal because of its rapid life-span, ease of husbandry, and its suite of genetic tools. A well-known genetic tool used in the fly is the GAL4/UAS system, which conditionally expresses genetic constructs (e.g. RNAi or mutant genes) in specific cell populations. An example is the fly line UAS-GFP;TH-GAL4. The yeast transcription factor GAL4 is located downstream of the promoter for tyrosine hydroxylase (TH) – a DA specific precursor, and therefore GAL4 is expressed concomitantly with TH. GAL4 binds to its respective upstream activating sequence (UAS, also from yeast) and activates the expression of green fluorescent protein (GFP).
Therefore, DA neurons can be specifically identified by a GFP signal using a fluorescent microscope.

The mechanisms for mitochondrial morphology and apoptosis also appear to be well conserved between vertebrates and invertebrates (Krieser and White, 2009; Estaquier et al., 2012). This makes *Drosophila* an excellent animal model to study the steps involved in neurodegeneration. And although many studies show that some treatments (such as D2 agonists) prevent DA cell degeneration, few have examined the mechanisms of the protective effect. The few studies which do, claim the treatments possess an intrinsic antioxidant effect (Uberti et al., 2007). A simple cell-culture model using *Drosophila* could rapidly test DA neurons for specific vulnerabilities to PD-like treatments as well as the mechanisms that protect against them.

**Preview of Results**

Mechanisms of neuroprotection are poorly understood. Neuroprotective treatments should be tested not only whether they keep cells alive, but also if the cells are functional. The experiments outlined in the following chapters explore neuroprotective mechanisms and study early effects of PD-like treatment in our model.

Chapter 3 explores the neuroprotective mechanisms of D2 agonists, focusing on their inhibitory effect through the activation of D2 receptors. Na$^+$ and Ca$^{2+}$ channels are also manipulated to test the hypothesis that electrical inhibition is neuroprotective against PD-like treatment.
Chapter 4 expands upon those findings, testing neuroprotection with mitochondrial morphology as an experimental endpoint. First, we validate a quantifiable model for analyzing mitochondrial morphology in Drosophila neuronal cultures. We then test neuroprotective drugs against PD-like treatment using mitochondrial morphology as an endpoint. Next, we track the changes in mitochondrial morphology over time. We study mitochondrial morphology early in the degenerative process and test if neuroprotective therapies prevent changes in mitochondrial morphology following PD-like treatment.
CHAPTER 2: METHODS

Fly Lines

All lines were obtained from the Bloomington Drosophila Stock Center unless otherwise noted. Flies were kept at 25°C and raised on standard cornmeal agar diet. Fly food was made by adding 21 g yeast (deactivated, dry Saccharomyces cerevisiae), 9 g agar, 48.75 g dextrose, and 21 g sucrose into 1 L of double distilled water (ddH2O). The mixture is then boiled using a microwave. Then 60 g cornmeal (yellow, enriched and degeminated) is added while stirring. The mixture is boiled again, cooled to a comfortable handling temperature (≈70°C), 4 mL propionic acid added as a preservative, stirred, and then ≈25 mL of food is poured into 6-ounce, individual fly bottles. This recipe yields ≈5 bottles. Bottles were changed every 7 to 10 days.

A ‘Cantonized’ white eye stock w118 served as wild-type. UAS-mitoGFP/CyO;TH-GAL4 flies were gifts from Dr. Leo Pallanck at the University of Washington. Morphometric control experiments used DRP1ICT26/CyO-GFP and OPA1CS3475/CyO-GFP (both also gifts from Leo Pallanck). The DD2R RNAi experiments used 1407-GAL4 and UAS-DD2R-RNAi lines as well as permanent line of the two (stock #’s 8751 and 26001, respectively; exact genetic background of permanent line was w;1407-GAL4;UAS-DD2R-RNAi). A deficiency line dfDD2R/FM-GFP was also used for experiments with DD2R (stock # 9351). Experiments with Ca2+ were performed with elav-GAL4,cacHCl129/FM7i,UAS-ActGFP (stock # 8579). The tyrosine hydroxylase (TH)-GAL4 line was a gift from Dr. S Birman at the Dev. Biol. Institute of Marseille, France (Friggi-Grelin et al., 2003).
Drosophila Primary Neuronal Cultures

Cultures were prepared from fly embryos. Embryos were collected by placing adult flies in empty, 6-ounce, individual fly bottles capped with egg-laying plates lined with rows of yeast paste. The bottles were taped shut and placed upside down (egg-laying plate was floor) and left for ≈4 hours in darkness (covered) at room temperature. Flies usually lay few eggs the first time this process is done, but will lay more eggs when repeated 1-2 days later.

Egg-laying plates were prepared using lids of 35 mm plastic culture dishes filled with agar gel: 8 g agar was boiled in 200 mL of ddH2O, cooled to 65° C, 2 mL 100% ethanol added, 2 mL glacial acetic acid added, stirred, poured, and then stored at 4° C until use. This recipe yields 50 plates. Yeast paste was made by mixing 20 mL yeast (active, dry, baking Saccharomyces cerevisiae) with 20 mL water and then heated in microwave at least 20 s on high setting (to kill yeast). The yeast paste must have the consistency of decorative cake icing. If too watery, 1 mL yeast was added and put in microwave again for 20 s. This was repeated until consistency was as desired. Then yeast paste was put into a 10 cc syringe (without needle) and stored at 4° C. For collecting embryos, egg-laying plates were brought to room temperature, and the agar lined with yeast paste (leaving space for the flies to land and not get stuck in the yeast paste).

Once collected, embryos were rinsed off the egg-laying plates and dechorionated in 50% bleach. Mid-gastrula embryos at developmental stage 6.5-7 were harvested in a
laminar-flow hood (Forma Scientific, model 1849). Cells were dissociated and plated onto round, glass coverslips (Bellco Glass, Inc., Vineland NJ, USA) as previously described (Park and Lee, 2006; Park et al., 2007). Two embryos were used per coverslip except for balanced lines, in which single-embryo cultures were prepared for each coverslip. Cultures were incubated in 4-5% CO$_2$ at 24-25° C for up to 9 days *in vitro* (DIV). Culture medium (DDM1) was a mixture of high glucose Hams’s F-12/Debecco’s medium (Irvine Scientific, Santa Ana, CA, USA), L-glutamine (2.5 mM; Irvine Scientific), HEPES (20mM), and 100 µL each 4 supplements: putrescine (100 µM), progesterone (20 ng/mL), transferrin (100 µg/mL), and insulin (50 µg/mL). At 3 DIV (prior to drug treatment), all cultures had 50% of the culture medium replaced with new medium.

**Pharmacological Treatments**

All drugs were added to cultures at 3 DIV after baseline images were acquired, except for experiments with delayed use of rescue therapy. Drug remained in the dish once treated (i.e. was never washed out). Cultures were handled and treated in a laminar flow hood (Forma Scientific, model 1849). Drugs dissolved in ddH$_2$O were sterilized by filtration through a 0.2 µm cellulose acetate filter before use/storage. Drugs dissolved in DMSO were not sterilized by filtration; cultures treated with these drugs did not suffer contamination more often than usual. MPP$^+$ iodide (Sigma) was prepared as a 40 mM stock solution dissolved in ddH$_2$O and stored in darkness at -70° C. MPP$^+$ was handled according to guidelines reviewed in Przedborski et al. (2001). Stock solutions of 10 mM
quinpirole (Sigma) and 1 mM tetrodotoxin (TTX; Sigma) were dissolved in ddH₂O, sterilized, and kept at -20° C until use. Control groups were treated with equal amounts of DMSO (e.g. 2 µL of DMSO into 2 mL of culture medium) when other drug-treated groups required DMSO as a solvent.

**Live Imaging of DA Neurons**

For the UAS-GFP;TH-GAL4 fly line, in which the live-GFP signal could be tracked over time, cultures were prepared on photo-etched coverslips. These coverslips contain a labeled grid of unique numbers and letters so that a specific field of view and the cells within it can be repeatedly found and measured at different time points. Baseline images (prior to drug treatment) were collected from several areas with between 2-7 GFP+ cells. Images were then collected at a later DIV from the same area. The number of DA neurons at the later DIV was compared to the number of DA neurons at baseline for each measured area. A GFP signal was considered a DA neuron if its intensity was 3x the background intensity and if it overlapped a cell as shown by the bright-field image. A DA neuron was considered degenerated if its signal intensity dropped below 3x the background intensity when measured at a later time point.

Live imaging was also used for experiments in propidium iodide (PI, Invitrogen, Carlsbad, CA, USA) staining. Cultures were stained with PI at a concentration of 1 µL/mL for 5 minutes followed by a 5 minute wash in external solution (see recipe below in Action Potential Recording). Images were collected of “dim” GFP signals. The brightness of the images was digitally increased and sub-threshold GFP+ neurons were
examined for overlapping with PI signals. The average fluorescent intensity of sub-threshold GFP+ neurons was roughly 50% of the threshold.

Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde for 40 minutes on ice, and then washed 3 times, 10 minutes for each wash. Wash solution was 10 mM phosphate buffered saline containing 0.5% bovine serum albumin. All washes were at room temperature (≈25° C). Blocking and permeabilization was performed using 0.1% Triton X-100 and 5% normal goat serum (Sigma) for 30 minutes on ice. After 1 more wash for 10 minutes, permeabilized cultures were incubated overnight (≈16 hours) at 4° C with a 1:1000 ratio of primary antibody (mouse anti-tyrosine hydroxylase) diluted in wash. The next day, primary antibody was removed and cultures were washed 3 times, 10 minutes for each wash. Primary antibody was recycled and reused no more than 2 times, and treated with 0.1% NaN₃ after each use. Cultures were next treated with a 1:2000 ratio of secondary antibody (FITC or TRITC labeled goat anti-mouse) diluted in wash and placed on ice for 1 hour. Secondary antibody was then removed and cultures were again washed 3 times, 10 minutes for each wash. Coverslips were next mounted onto glass slides upon rows of fluorogel with Tris buffer (Electron Microscopy Sciences, 17985-10), covered with an extra drop of fluorogel, then topped with coverglass (Electron Microscopy Sciences, 72200-40), and edges sealed with clear fingernail polish.
Action Potential Recording

Cultures were transferred into a recording chamber containing the following external solution (mM): 140 NaCl, 1 CaCl₂, 4 MgCl₂, 3 KCl, and 5 HEPES, pH = 7.2. Action potentials (APs) were extracellularly recorded with cell-attached patch pipettes (tip resistance 4-6 MΩ, previously described in Yuan & Lee, 2007). The pipette was filled with internal solution containing the following ingredients (mM): 120 CsOH, 120 D-gluconic acid, 0.1 CaCl₂, 2 MgCl₂, 20 NaCl, 1.1 EGTA, and 10 HEPES, pH = 7.2. An axopatch 200B amplifier (Axon Instruments, Inc., Union City, CA, USA) was used to measure action potentials (APs).

DA neurons were identified via the live-GFP signal in UAS-GFP;TH-GAL4 flies. The D2 agonist quinpirole was focally applied using a Picospritzer III (Parker Hannifin Corp, Fairfield, NJ, USA) for 30 seconds. AP frequency was analyzed using the Minianalysis detection software (Synaptosoft, Decatur, GA, USA).

Microscopic Detection of Dopaminergic Mitochondria

Live images of dopaminergic (DA) mitochondria could be taken directly from the GFP signal of UAS-mitoGFP/CyO;TH-GAL4 cultures. To observe DA mitochondria in other lines, cultures were stained with both MitoTracker Orange (Invitrogen, 919826) and anti-TH antibody. At 7 DIV but before staining, cultures were treated with 50 nM (final concentration) MitoTracker in their original culture medium and placed back in the incubator for 1 hour. Culture medium was then removed and cultures fixed and stained as usual (described above) with anti-TH primary antibody and FITC-labeled secondary
antibody. The colocalization of signals between MitoTracker and anti-TH staining
specifically identified DA mitochondria for analysis.

Image Acquisition and Analysis

Prepared/stained cultures were viewed under a fluorescent microscope (Olympus
IX71). Images were taken using a Spot CCD digital camera (Diagnostic Instruments,
Sterline Heights, MI, USA). After images were acquired, they were analyzed using
ImageJ software. ImageJ allows for users to write programs (typically macros) in a
“Java-like” language. For experiments in mitochondria morphology, the morphometrics
were measured using a macro adapted from the plugin created by Dagda et al., 2009
(Appendices A and B). Major differences in the macro from Dagda’s original, were
changing the threshold to 20% of the maximum intensity and the measured particle range
to 5-500 pixels. This macro quantifies mitochondrial morphometrics using four
parameters: 1) number of mitochondria, 2) size, 3) interconnectivity, and 4) elongation.
These four parameters taken together provide a gestalt “snapshot” of the mitochondrial
phenotype. Basing the cell’s health only on the number of mitochondria is incomplete:
healthy cells have higher number of mitochondria, are larger, more interconnected, and
are less round compared to unhealthy cells (Dagda et al., 2009; Perier & Vila, 2012). But
an unhealthy cell may still have a large number of mitochondria, but those mitochondria
may have an unhealthy morphology (i.e. are non-functional). Thus, measuring only one
parameter may incorrectly assess a cell as having healthy mitochondria. To analyze the
images, the signal from mitochondria is painted by the threshold function in ImageJ
based on the signal intensity. For a 12-bit image, the intensity ranges from 0 (no signal) to 4095 (maximum saturation). To determine the best threshold for our analysis, the raw signal of several images of DA mitochondria was compared to its “ideal” threshold level. The ideal threshold level was considered the one where a manual count of the mitochondria from the raw image gave the same results as the ImageJ macro. The mean ideal threshold level was then set as the threshold to be applied for all future analysis. This threshold was determined to be 20% of the maximum intensity, which is about 4x the typical background intensity. The macro only considers signals within a size range. With our microscope settings and image size, the mean size for mitochondria is around 40 pixels$^2$ (1.4 µm$^2$). Thus, the lower and upper limits of the size range had to be decreased from Dagda’s original macro. If the lower limit was too low, it would include tiny artifacts as signals, which do not provide meaningful parameter values. The upper limit is less of a concern. In healthy neurons, many of the mitochondria are interconnected, combining to a pixel area much greater than the average mitochondria size, which is the phenomenon that is measured by the interconnectivity score. Because the soma is always completely saturated in our images, it is not included in analysis. This is easily accomplished by simply drawing around the soma with the selection tool in ImageJ (Figure 1). An upper limit of 500 pixels$^2$ was used to exclude what are probable artifacts (e.g. the occasional piece of broken glass). Thus, a particle size range of 5-500 pixels$^2$ was chosen for non-somatic mitochondria.
Figure 1. Example images of mitochondrial morphology quantification. Cultures were prepared from UAS-mtGFP/CyO;TH-GAL4 flies, which express GFP specifically in the mitochondria of DA cells. (A) A brightfield image of neuronal culture at 7 DIV (scale bar = 20 µm). (B) Live-GFP signal of mitochondria in a DA neuron. The soma is indicated by a white arrow. (C) Threshold painting of GFP signal. The grey line indicates the selected area to be analyzed (soma excluded). (D) Zoom-in of area in blue box from C. Outlines of mitochondrial signals are drawn in black and numbered in red. Some mitochondrial are long, interconnected and abstract shapes, while others are small and round.

Determining the 4 parameters of mitochondrial morphology requires computation. Counting the number of mitochondria and determining the mean size is a straightforward task; the ImageJ software simply counts each signal and simultaneously determines the pixel area, perimeter, and circularity for each signal. From these measurements, the ImageJ macro calculates a score for interconnectivity and elongation as the following:
Equation (1):

\[
\text{interconnectivity} = \frac{\text{mean area}}{\text{mean perimeter}}
\]

Equation (2):

\[
\text{elongation} = \frac{1}{\text{circularity}}
\]

Where circularity is:

Equation (3):

\[
\text{circularity} = 4 \times \pi \times \left(\frac{\text{mean area}}{\text{mean perimeter}}\right)^2
\]

Interconnectivity describes the network of the mitochondria, and is calculated by dividing the mean area by the mean perimeter of all the particles analyzed. Higher scores for interconnectivity signify that mitochondria have more physical connections, while lower scores signify that the mitochondria are more fragmented. Elongation is best thought of as the shape of mitochondria. Higher values are more abstract shapes, while a value of 1 would be considered a perfect circle.

For the experiments requiring the colocalization of signals to identify DA mitochondria, a new macro was written combining the macro above with the colocalization highlighter plugin from the WCIF version of ImageJ (Appendix B). The macro essentially works the same as in Appendix A, but analyzes the colocalized signal from two images: one image showing signals for DA neurons and their neuritic projections, and another image showing signals for mitochondria. To visualize
mitochondria, cultures were treated with 50 nM MitoTracker Orange (Invitrogen M-7510) in the original culture medium for 1 hour at 25°C prior to immunocytochemistry staining with an antibody to tyrosine hydroxylase (anti-TH). Images of the MitoTracker and anti-TH signal were then collected and analyzed using the macro in Appendix B. The macro applies a threshold to both images (the MitoTracker and anti-TH) that is 20% of the maximum intensity. The images are then combined into a single new image showing only the colocalized signals above threshold in each former image. In Figures below using this technique, the colocalized signals above threshold are shown in white.

Quantification of Dopamine Neurons

To determine the number of DA neurons between groups, we counted the number of anti-TH+ neurons per 1000 cells in each treatment group. Cells are counted using a nuclear dye 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR, USA) as previously described (Park & Lee, 2006). Briefly, images of the anti-TH and DAPI signals are collected from 5-8 random areas on a coverslip. Coverslips can contain a maximum of ≈10,000 cells (from two embryos), and each image captures ≈800 cells. Anti-TH+ cells are counted during image collection, but DAPI signals are counted using ImageJ. A cell is considered TH+ if the signal intensity is > 4x the background intensity. The DAPI image is painted using the Threshold tool with automatic settings. The total area painted (i.e. the sum of the DAPI signals above threshold) is measured in pixels. Next, a sample area is manually counted (typically ≈45 cells), and the corresponding area of the DAPI signals in the sample is measured. The total DAPI area is divided by the
sample DAPI area to estimate the number of DAPI+ cells in the image. Finally, TH+ neurons are normalized per 1,000 DAPI+ cells.

Statistics

All statistics are reported as mean ± 1 standard error of the mean. Analysis is performed using either ANOVA with Tukey’s Honest Significant Difference correction or Student’s t-test. Significance scores are: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. All distributions are tested for normality and homogeneity of variance before testing.

Notes

Note on Grammar

For Drosophila, the names of recessive mutants are always lower case even when appearing at the beginning of a sentence.

Note on Figures

All scale bars indicate a length of 20 µm unless otherwise stated.

Note on Materials

All materials (excluding fly lines) were obtained from Sigma (St. Louis, MO, USA), unless stated otherwise.
Note on Computer Hardware and Software

All analysis was performed on a Hewlett-Packard Pavilion dv6 model laptop running Windows 7 64-bit version with intel i3 core processor. ImageJ version 1.46r (64-bit) was used for cell quantification and for measurements of mitochondrial morphology from live-GFP signals. The older WCIF ImageJ version 1.37c was used for mitochondrial quantification for the immunocytochemistry method, and was run in compatibility mode on the same machine.
CHAPTER 3: SELECTIVE DEGENERATION OF DA NEURONS AND RESCUE BY D2 AUTORECEPTORS

The past success of genetic PD models in the fly provides encouragement for testing models using toxins. Only a few studies in *Drosophila* have examined PD processes in non-genetic models, e.g. rotenone or paraquat (Coulom & Birman, 2004; Chaudhuri et al., 2007; Lawal et al., 2010). In this chapter, I describe the methods and results from a cellular PD-like model that our lab has developed (Park & Lee, 2006; Park et al., 2007). Using primary *Drosophila* neuronal cultures, I show that MPP⁺ induces neurodegeneration specifically in DA neurons, and that D2 agonists can prevent this degeneration through the activation of *Drosophila* D2 receptors (DD2R). Moreover, I explore the mechanism of this protective effect. Because DD2R activation inhibits neuronal activity, I also test if electrical inhibition is neuroprotective. Lastly, because of the importance of Ca²⁺ in the health of the cell and its relationship to apoptosis, I test if modulation of Ca²⁺ is also protective in our model.

Effect of MPP⁺ on *Drosophila* Neuronal Culture: Live-Tracking.

MPP⁺ is used to model PD-like degeneration in our cell cultures. MPP⁺ enters DA neurons specifically through the DA transporter (DAT) and inhibits complex I in the mitochondria, leading to an increase in ROS and eventual cell death (Levy et al., 2009). Our lab has developed a variety of methods to quantify the degeneration of DA neurons, most involve live-tracking of the DA neurons or immunocytochemistry using antibodies.
to tyrosine hydroxylase (anti-TH). Both methods succeed in quantifying DA cell death differences between treatment groups.

The live-tracking method uses the fly line UAS-GFP;TH-GAL4 (TH-GFP). This line employs the UAS-GAL4 system (Brand & Perrimon, 1993) to conditionally express green fluorescent protein (GFP) only in cells also expressing TH, i.e., DA neurons. The GFP signal is tracked over time using photo-etched coverslips. These coverslips contain a labeled grid of unique numbers and letters so that a specific field of view and the cells within it can be repeatedly found and measured at different time points (Figure 2A, next page). For our experiments, we collected baseline images at 3 days in vitro (DIV). Next, dishes were randomly split into one of four different treatment groups: control, 10 µM MPP⁺, 40 µM MPP⁺, or 100 µM MPP⁺. Subsequent images were collected at 5 and 9 DIV (i.e. after 2 and 6 days of treatment, respectively). The number of DA neurons at the later DIV was compared to the number of DA neurons at baseline. For control groups, the number of DA neurons at 9 DIV increased to 142 ± 6% of baseline levels. 40 µM MPP⁺ decreased the number of DA neurons by 37% when measured at 9 DIV, while the 10 µM MPP⁺ group showed only a 13% reduction (Figure 2B). Upon visual examination of the neuronal projections in the cultures, the high dose of MPP⁺ also damaged non-DA neurons, and thus was not included in the analysis.
**Figure 2.** MPP⁺ live-tracking experiment
MPP⁺ reduces the number of DA neurons. (A) Overlapped image of bright-field and live-GFP signal for a specific area on a photo-etched coverslip (area 8K in this case). DA neurons are indicated by white arrows. Cultures were prepared from UAS-GFP;TH-GAL4 flies, which express GFP specifically in DA neurons. *Inset* An enlarged view of the dotted area in (A) (scale bars = 20 µm). (B) Plot showing the change in the number of DA neurons over time for each treatment group. The number of DA neurons at 5 or 9 DIV is normalized to baseline at 3 DIV. There was no significant difference between groups at 5 DIV, but there was a difference for the 40 µM MPP⁺ group at 9 DIV (Student’s t-test; n = number of images, from 6 separate experiments: control = 110, 10 µM MPP⁺ = 55, 40 µM MPP⁺ = 61; ** p < 0.01). *Inset* Bar plot showing no difference in the number of DA neurons between groups at baseline. From Wiemerslage et al., 2013.
Differentiation of DA Neurons

In experiments similar to ours, Park et al., (2007) hypothesized that the increase of DA neurons seen in the control group is due to a maturation of the GFP signal (i.e. DA cells became brighter over time, so that more were above threshold for detection). To determine if the percent increase from baseline seen in the control group was due to an increase in the proliferation of DA neurons, we tested if cells in our cultures are still dividing at 3 DIV. The mitotic marker bromodeoxyuridine (BrdU; Boone & Doe, 2008) was added to cultures at either 0 DIV (immediately after preparation) or 3 DIV. Cultures were stained with anti-BrdU antibody after 3 days of incubation with BrdU. When BrdU was added at 0 DIV, most of the cells – including DA neurons – were BrdU+. However, when BrdU was added at 3 DIV, less than 2% of cells – again including DA neurons – were BrdU+ (Figure 3C). This shows that our cultures have mostly finished dividing by 3 DIV, and that the decrease of DA neurons seen previously (Figure 2B) is due to a reduction of the number of DA neurons, and not merely by a prevention of proliferation.
Cultured neurons are post-mitotic by 3 days in vitro. Cultures from TH-GFP flies expressing green fluorescent protein (GFP) specifically in DA neurons were treated with BrdU at either 0 or 3 DIV and then stained 3 days later with anti-BrdU antibody at either 3 or 6 DIV respectively. (A) Overlapped image of bright-field and anti-BrdU (red) signals at 3 DIV, where BrdU was added at 0 DIV. Most neurons are BrdU+ except those indicated by arrows. Cells were rarely BrdU+ when BrdU was added at 3 DIV (not shown) (scale bar = 20 µm). (B) Overlapped image of anti-BrdU (red) and GFP signals (green) when BrdU added at 0 DIV. Overlapped (yellow) signals indicated by arrows are BrdU+ DA neurons. (C) Bar plot showing the levels of BrdU+ cells when BrdU was added at either 0 or 3 DIV. The group treated at 0 DIV shows that DA neurons do not differentiate at different rates compared to other cell types. The group treated at 3 DIV shows that both DA neurons as well as other cells only rarely incorporate BrdU past 3 DIV. From Wiemerslage et al., 2013

Effect of MPP⁺ on Drosophila Neuronal Culture: Immunocytochemistry

To further support the idea that MPP⁺ treatment is decreasing the number of DA neurons in our cultures, we used immunocytochemistry to quantify the number of DA neurons (Figure 4A). We tested cultures from both wild-type and UAS-GFP;TH-GAL4 flies. Cultures were stained with anti-TH antibody and the nuclear dye 4',6-diamidino-2-phenylindole (DAPI) and the number of DA neurons was quantified by comparing the number of TH+ cells to the number of DAPI+ cells. First, we tested if the number of DA neurons changed over time. Cultures from both the wild-type and UAS-GFP;TH-GAL4 strains were stained at either 3 or 9 DIV and the number of DA neurons were quantified
at each time point. Contrary to the live-tracking results, the immunocytochemistry experiments found a roughly 25% decrease over time for the number of DA neurons in the control group. There was no difference in the number of DA neurons between the wild-type and TH-GFP strains – both are considered control strains (Figure 4B).

Next, we tested if MPP\textsuperscript{+} decreases the number of DA neurons as evidenced by immunocytochemistry. At 3 DIV, cultures were treated with either 40 µM MPP\textsuperscript{+} or as control. At 9 DIV, cultures were stained with anti-TH antibody. Next, images were collected and the number of TH\textsuperscript{+} cells was quantified between groups. MPP\textsuperscript{+} treatment decreased the number of DA neurons roughly 50% (Figure 4C).

**Figure 4.** MPP\textsuperscript{+} treatment reduces the number of DA neurons. Immunocytochemistry experiments quantifying the number of DA neurons. Cultures were prepared from either wild-type or UAS-GFP;TH-GAL4 strains and treated with 40 µM MPP\textsuperscript{+} at 3 DIV. Then they were stained 6 days later at 9 DIV with anti-TH antibody. (A) Fluorescent image from neurons stained with an antibody to tyrosine hydroxylase (anti-TH). Five DA neurons (indicated with arrows) were observed in this field of view. *Inset* Shows TH\textsuperscript{+} neuritic processes in addition to the soma (scale bars = 20 µm). (B) Bar plot shows that TH\textsuperscript{+} neurons quantified at 3 and 9 DIV in wild type (WT) and TH-GFP lines differed significantly between 3 and 9 DIV (Student’s t-test, data from 6 experiments; * p < 0.05 and ** p < 0.01). Within each DIV, however, there was no difference between the two fly strains. (C) Bar plot shows that 40 µM MPP\textsuperscript{+} treatment reduced the number of TH\textsuperscript{+} neurons compared to control. Cells were treated at 3 DIV, and then stained and quantified at 9 DIV (Student’s t-test, data from 4 experiments). From Wiemerslage et al., 2013.
Comparison of GFP and Anti-TH Signals

Because we use two methods to identify DA neurons, we tested the overlap between the signals from each method. Generally the live-GFP signal identifies more DA neurons and the number of neurons increases over time, while the anti-TH antibody signal detects fewer neurons and the number of DA neurons detected over time decreases. Thus, with the number of GFP signals increasing over time and the number of anti-TH signals decreasing, the overlap between the two might be expected to worsen. Cultures from UAS-GFP;TH-GAL4 (THGFP) flies were stained at either 3 or 9 DIV, and random images were taken to check the overlap between the two signals. The number of cells that were both anti-TH+ and GFP+ were divided by the total number of anti-TH+ cells. The percent overlap decreased 24% from 81% at 3 DIV to 57% at 9 DIV. Neurons that are GFP+ yet anti-TH- are likely neurons which initially expressed TH and GFP at an early DIV, but have now suppressed TH expression. The GFP signal remains because it is stable in the cell (Tsien 1998; Day & Davidson, 2009). Neurons that are anti-TH+ but GFP- still show some GFP fluorescence, but are below the threshold (4x the background intensity) to be considered GFP+. Moreover, others have found similar results as ours concerning overlap between genetically controlled signals and antibody signals. In the adult fly brain, there was a 69% overlap between GFP+ and anti-TH+ cells (Pesah et al., 2005). A similar mismatch percentage also exists in rodents and other vertebrates (Matshushita et al., 2002), and the overlap is only 30% in zebrafish (Meng et al., 2008). Overall, both methods of detection are useful, adequate, and consistent for detecting differences in the number of DA neurons between treatment groups. And although the
overlap between the signals warrants further investigation, such research is beyond the scope of this work.

Table 1

Overlap of GFP and TH signals.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Cell Number:</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>TH+/GFP+</td>
<td>TH+ only</td>
<td>GFP+ only</td>
<td>% overlap</td>
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<td>3 DIV</td>
<td>89</td>
<td>106</td>
<td>332</td>
<td>81</td>
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<td>9 DIV</td>
<td>82</td>
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<td>57</td>
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Totals come from control groups of experiments employing immunocytochemistry.

DA Transporter Modulates Sensitivity to MPP⁺

Our experiments use a 40 µM dose of MPP⁺, but experiments using MPP⁺ in rodents achieved neurodegeneration with as low as 1-10 µM (Marini et al., 1989; Sanchez-Ramos et al., 1997; Bains et al., 2007). Thus, we tested if overexpressing the DA transporter (DAT) would increase the sensitivity of our cultured neurons to MPP⁺ treatment. DAT was over-expressed specifically in DA neurons by crossing two transgenic lines: TH-GAL4 and UAS-DAT. In cultures from the F₁ progeny of this cross, the lower 10 µM dose of MPP⁺ significantly decreased the number of TH⁺ cells to an equal magnitude (roughly 50%) of the decrease seen in 40 µM MPP⁺ (Table 2). Whereas previously, 10 µM MPP⁺ did not significantly reduce the number of DA neurons (Figure 2). Thus, Drosophila could have less DAT activity than rodents in terms of transporting MPP⁺.
Table 2

Over-expression of dopamine transporter in DA neurons increases sensitivity to MPP⁺

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Num. of DA Neurons per 1000 cells</th>
<th>n (number of images)</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.8 ± 0.2</td>
<td>68</td>
</tr>
<tr>
<td>10 µM MPP⁺</td>
<td>1.9 ± 0.2*</td>
<td>73</td>
</tr>
<tr>
<td>40 µM MPP⁺</td>
<td>1.6 ± 0.3*</td>
<td>59</td>
</tr>
</tbody>
</table>

* p < 0.05, from Wiemerslage et al., 2013.

MPP⁺-Induced Degeneration is Specific to DA Neurons

We next tested if MPP⁺ is toxic to non-DA neurons. We tested 40 µM MPP⁺ in both cholinergic and GABAergic neurons, both of which are major cell-types in the adult fly brain as well as in primary neuronal culture (Lee & O’Dowd, 1999; Lee et al., 2003). We prepared a fly strain expressing Cha-GAL4 x UAS-GFP and Gad1-red fluorescent protein (RFP). Thus, GFP will be expressed in cholinergic neurons while RFP is expressed in GABAergic neurons. Cells were quantified by the live-tracking method. The number of cells were measured at 5 and 9 DIV, and compared to the number at the 3 DIV baseline. MPP⁺ had no effect on either cell-type (Figure 5). Thus, 40 µM MPP⁺ is the appropriate dose for our experiments because its effect is robust yet specific to DA neurons.
Figure 5. Degeneration is specific to DA neurons. Cultures were prepared from flies expressing GFP in cholinergic neurons and RFP in GABAergic neurons. Cultures were treated with 40 μM MPP+ at 3 DIV. Then cells were quantified at 5 and 9 DIV by live-tracking and compared to baseline at 3 DIV. (A) Image showing GFP and RFP signals. No signals overlap, indicating specificity of markers (scale bar = 20 µm). (B) Plots showing no change between control and MPP+ groups over time for either cell-type (n = number of images: cholinergic = 20, GABAergic = 20). From Wiemerslage et al., 2013.

MPP+ Treatment Induces Cell Death

We next tested if MPP+ treatment induces DA cell death in our model. Cell death can be detected by the dye propidium iodide (PI) or by antibodies to caspases (Figures 6A and 7B, respectively). PI enters and stains cells in which the membrane is compromised, i.e. unable to prevent the passage of small molecules from the extracellular fluid to the cytosol (as happens in cell death). Caspases are proteins that activate during cell death processes, and cleave cell structures into pieces (Taylor et al., 2008).

Dead/dying DA neurons were identified by the colocalized signals from genetically expressed GFP and PI staining. Cultures were prepared from UAS-GFP;TH-GAL4 flies, which conditionally express GFP in DA neurons. These cultures were
treated with or without 40 µM MPP⁺ at 3 DIV, and then stained with PI at 9 DIV. There were no “bright” GFP signals that colocalized with PI signals. In the live-tracking experiments described above, we considered a DA neuron to be dead if the GFP signal intensity dropped below a threshold of 3x the background intensity. Thus, for this experiment we considered a dead DA neuron to be one with a “dim” GFP signal that colocalized with a PI signal. The percent of dead DA neurons per group was found by dividing the number of GFP signals colocalized with PI signals by the total number of GFP signals. MPP⁺ increased the percentage of dead DA neurons compared to control about 6-fold from 0.6% ± 0.3% to 4.2% ± 1% (Figure 6B).

Figure 6. MPP⁺ causes DA cell death in our model. Cultures were prepared from UAS-GFP;TH-GAL4 flies (TH-GFP), which express GFP specifically in DA neurons. Cultures were treated with 40 µM MPP⁺ at 3 DIV and were stained at 9 DIV with PI. A dead DA neuron was considered as a cell with colocalized GFP and PI signals. (A) Shows a dead DA neuron overlapped with PI (white arrow, scale bar = 20 µm). A healthy DA neuron is on the right. (B) Bar plot shows that MPP⁺ treated cultures contain higher amounts of dead DA neurons (Student’s t-test, data from 4 experiments; ** p < 0.01). From Wiemerslage et al., 2013.

Dead/dying DA neurons were also identified by anti-caspase antibody. Again, a dying DA neuron was considered to be one with a “dim” GFP signal colocalized with the
anti-caspase antibody signal. UAS-GFP;TH-GAL4 cultures were treated with MPP\(^+\) at 3 DIV and then stained with anti-caspase antibody at 9 DIV. MPP\(^+\) treatment increased the percentage of dead DA neurons compared to control 106% from 13.3\% ± 1.1\% to 27.4\% ± 3\% (Figure 7). These results demonstrate that MPP\(^+\) causes DA cell death in a caspase-dependent manner in our model. Thus, our model mimics a current theorized mechanism of cell death in PD.

*Figure 7. MPP\(^+\) promotes apoptosis in DA neurons in our model. Cultures from UAS-GFP;TH-GAL4 flies were treated with 40 \(\mu\)M MPP\(^+\) at 3 DIV and were stained at 9 DIV with an antibody to caspase. (A) Brightfield image of neuronal culture at 9 DIV (scale bar = 30 \(\mu\)m). (B) overlapped image shows signals from genetically driven GFP and anti-caspase staining. Green signals are DA neurons, and orange signals indicate cells in a state of apoptosis. (B-1) Shows a healthy GFP+ cell with a strong GFP signal intensity and no overlap with the anti-caspase signal. (B-2) Degenerated neuron displaying a dim GFP signal overlapped with anti-caspase signal. (C) Bar plot demonstrates that MPP\(^+\) treated cultures have more degenerated DA neurons (Student’s t-test, data from 3 experiments; * \(p < 0.05\)).
Rescue of MPP\(^+\) Treatment by the D2 Agonist Quinpirole.

D2 agonists are used in the clinic to treat PD symptoms (Schapira and Olanow, 2004; Ferrari-Toninelli et al., 2008). Thus, we tested if D2 agonists protect DA neurons from PD-like degeneration in *Drosophila* primary cell cultures. Two different D2 agonists were used: quinpirole because it has an effect on *Drosophila* behavior (Andretic & Hirsh, 2000; Draper et al., 2007), and bromocriptine because of its use in PD patients and association with neuroprotection (Bozzi & Borrelli, 2006). Concomitant treatment with 10 \(\mu\)M quinpirole partially rescued the MPP\(^+\)-induced degeneration of DA neurons at 9 DIV (Figure 8A). 10 \(\mu\)M bromocriptine had a similar level of rescue, i.e. only partial. Furthermore, treatment with either D2 agonist did not change the number of DA neurons compared to control (Figure 8C).
Figure 8. D2 agonists rescue MPP⁺ induced neurodegeneration. Cultures from UAS-GFP;TH-GAL4 flies were tracked over time via a live-GFP signal expressed in DA neurons. The number of GFP+ cells was counted at 5 and 9 DIV, and then compared to a pre-treatment baseline (3 DIV). This experiment utilized photo-etched coverslips so that the same GFP+ cells could be repeatedly analyzed. (A) The number of GFP+ cells increased over time in control, but did not increase in the MPP⁺ treated group. Quinpirole (10 µM) added concomitantly with 40 µM MPP⁺ partially prevented DA neurodegeneration. (B) Bar plot showing the neuroprotective effect of the D2 agonists quinpirole and bromocriptine (10 µM) against MPP⁺-induced neurodegeneration. The percent difference of total GFP+ neurons in a square was counted at 9 DIV and compared to the baseline at 3 DIV (Student t-test, data from 4 experiments; ** p < 0.01, and *** p < 0.001). (C) Neither quinpirole nor bromocriptine alone (treated without MPP⁺) showed any difference in the number of GFP+ neurons compared to control. From Wiemerslage et al., 2013.

Lastly, to show that the effect of the D2 agonists were through D2 receptor activation, we test whether the rescue effect of quinpirole is blocked by the D2 antagonist haloperidol. Indeed, 10 µM haloperidol abolished quinpirole’s rescue effect (Table 3). From all the above, the rescue effect of quinpirole and bromocriptine appears to be through D2 receptors activation.
Table 3

Rescue of MPP\(^+\)-induced neurotoxicity by quinpirole is suppressed by the D2 antagonist haloperidol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Num. of DA Neurons per 1000 cells</th>
<th>n (number of images)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.9 ± 0.1</td>
<td>55</td>
</tr>
<tr>
<td>MPP(^+)</td>
<td>2.5 ± 0.1***</td>
<td>28</td>
</tr>
<tr>
<td>MPP(^+)+Quin</td>
<td>3.5 ± 0.3</td>
<td>105</td>
</tr>
<tr>
<td>MPP(^+)+Quin+Hal</td>
<td>2.3 ± 0.1***</td>
<td>90</td>
</tr>
</tbody>
</table>

*** p < 0.001, from Wiemerslage et al., 2013.

D2R is Necessary for Rescue by Quinpirole.

To further support our claim that D2R activation is required for rescue by D2 agonists, we use *Drosophila*’s suite of genetic tools to remove or knock down D2 receptors, rather than merely block them pharmacologically. Four DA receptors have been cloned and characterized in *Drosophila*: dDA1, DAMB, DopEcR, and DD2R (Gotzes et al., 1994; Han et al., 1996; Srivastava et al., 2005; Mustard et al., 2005). *Drosophila* D2-like receptor (DD2R) is the only D2 receptor identified to date (Hearn et al., 2002). Thus, we tested if DD2R is necessary for quinpirole to rescue DA neurons from MPP\(^+\) treatment. We used two fly lines: 1) df(DD2R)/FM-GFP, which lacks the gene for DD2R, and 2) UAS-DD2R-RNAi;1407-GAL4, which expresses panneural RNAi to DD2R – knocking down DD2R in all neurons. Both lines were used because the DD2R deficiency line possibly lacks other important genes, so the results may not be
due to the loss of DD2R functionality alone. Also, the DD2R-RNAi line may not sufficiently knock down DD2R expression. However, if quinpirole is unable to rescue DA neurons from MPP\(^+\) treatment in either line, then DD2R is likely necessary.

Normally, 10 µM quinpirole rescues the degenerative effect of MPP\(^+\). But without DD2R, quinpirole did not rescue DA neurons from concomitant 40 µM MPP\(^+\) treatment in either fly strain (Figure 9). Thus, DD2R is required for quinpirole’s rescue of MPP\(^+\) induced degeneration.

**Figure 9.** Cells lacking DD2R are not rescued by quinpirole.
(A) Bar plot for results from the DD2R deficiency line. Single-embryo cell cultures were prepared from the balanced strain df(DD2R)/FM-GFP. At 3 DIV, coverslips were selected by the presence/intensity of a live-GFP signal (refer to Darya et al., 2009). Coverslips with no GFP signal were df(DD2R) homozygous and thus lacked DD2R. Coverslips with an intense live-GFP signal were used as controls to confirm that quinpirole did protect against MPP\(^+\) treatment (data not shown). At 3 DIV, cultures were randomly split into 3 treatment groups: control, 40 µM MPP\(^+\), or 40 µM MPP\(^+\) plus 10 µM quinpirole. Cultures were then stained with anti-TH at 9 DIV. The number of TH\(^+\) neurons was reduced by MPP\(^+\) with no rescue observed in cultures treated simultaneously with quinpirole (Student t-test, \(n = \) number of images: control = 81, MPP\(^+\) = 50, and MPP\(^+\)+quinpirole = 59; ** \(p < 0.01\)).

(B) Bar plot of the results from cultures expressing DD2R-RNAi transgene driven by a pan-neuronal driver 1407-Gal4. As in df(DD2R), no rescue was observed at 9 DIV in groups treated with quinpirole (Student t-test, \(n = \) number of images: control = 49, MPP\(^+\) = 59, and MPP\(^+\)+quinpirole = 35; ** \(p < 0.01\)). Again, at 3 DIV cultures were treated with either 40 µM MPP\(^+\) with or without quinpirole, and then stained with anti-TH at 9 DIV. From Wiemerslage et al., 2013
D2 Autoreceptors are Necessary for Rescue by Quinpirole

D2 receptors are generally autoreceptors (Neve et al., 2004), and electrophysiological experiments confirm that DD2R can function as an autoreceptor (Vickrey and Venton, 2011). However, DD2R is also expressed in non-DA neurons (Hearn et al., 2002). Thus, it is possible that the protective effect of quinpirole is due to non-DA D2Rs, D2 autoreceptors, or both. To remove DD2R specifically in DA neurons (i.e. knock down D2 autoreceptors) we crossed UAS-GFP;TH-GAL4 flies with UAS-DD2R-RNAi flies. The F1 progeny of this cross expresses DA neurons tagged with GFP and lacking DD2R. We cultured cells from this cross onto photo-etched coverslips, treated with drug at 3 DIV, and tracked the cells over time. Quinpirole did not rescue DA cells lacking DD2R autoreceptors (Figure 10). Thus, DD2R autoreceptors are required for the quinpirole-mediated rescue of MPP+-induced degeneration.

Another interesting finding was that the control group for this experiment did not have an increase in the number of DA cells over time as in previous experiments. This is likely due to a gene-dosage effect. This cross only contains the UAS-GFP and TH-GAL4 constructs on one chromosome (i.e. heterozygous for these transgenes), whereas the previous experiments were performed in a homozygous, permanent line.
Figure 10. Live-tracking: DD2R autoreceptors are required for rescue by quinpirole. Knock down of DD2R in DA neurons (DD2R autoreceptors) prevents quinpirole from protecting against MPP+ treatment. DD2R autoreceptors were removed by expressing RNAi to DD2R specifically in DA neurons by crossing UAS-GFP-TH-GAL4 flies with UAS-DD2R-RNAi flies. Cultures were plated onto photo-etched coverslips and treated at 3 DIV (baseline). DA cells were tracked over time via the live-GFP signal and compared to pre-treatment, baseline measurements taken at 3 DIV. Quinpirole failed to rescue DA neurons from degeneration (Student’s t-test, n = number of images: control = 48, MPP+ = 35, and MPP+ + Quin = 45; ** p < 0.01). From Wiemerslage et al., 2013.

We also confirmed the above results via immunocytochemistry. Cultures were prepared from the same cross as above, treated with drug at 3 DIV, and then stained with anti-TH antibody at 9 DIV. As above, there were three treatment groups: control, MPP+, and MPP+ + quinpirole. Compared to control, MPP+ decreased the number of DA neurons about 50% either with or without quinpirole (Figure 11). Again, DD2R autoreceptors were required for the quinpirole-mediated rescue of MPP+-induced degeneration.
Immunocytochemistry: DD2R autoreceptors are required for rescue by quinpirole.

Cell cultures were prepared from F1 progeny of a cross between UAS-DD2R-RNAi and TH-GAL4 flies. At 3 DIV, cultures were treated with 40 µM MPP\(^+\) alone or concomitantly with 10 µM quinpirole. At 9 DIV, cells were stained with anti-TH antibody and quantified. The number of TH\(^+\) neurons was reduced by MPP\(^+\) while no rescue was observed in cultures treated simultaneously with quinpirole (ANOVA, n = number of images: CONT = 51, MPP = 27, MPP\(^+\)+Q = 54; ** p < 0.01).

Reduced Excitability Protects DA Neurons from MPP\(^+\) Treatment.

Next, we explored the mechanisms of the quinpirole-mediated rescue through DD2R. D2R regulates the excitability of DA neurons, and helps prevent excitotoxic cell death (Piallat et al., 1996; Schapira & Olanow, 2003). The D2 agonist quinpirole has an inhibitory effect on DA neurons (Fasano et al., 2010). Electrophysiological experiments...
from our lab also confirm that 10 μM quinpirole focally applied to DA neurons decreases the firing rate of DA neurons to 45 ± 10% of control (Figure 12). Thus, quinpirole reduces the excitability of DA neurons in our system.

**Figure 12.** Quinpirole decreases DA cell excitability.
Quinpirole reduces the number of action potentials (APs) in DA neurons. DA neurons were identified using live-GFP signal in UAS-GFP;TH-GAL4 cultures. APs were measured via patch clamp (as described in Yuan & Lee, 2007). 10 μM quinpirole was focally applied to DA neurons following baseline recordings. (A) Example traces of APs recorded in a GFP+ DA neuron aged 3 DIV. The APs in DA neurons were inhibited by the D2 agonist quinpirole. (B) Bar plot shows a significant decrease in the number of APs for DA neurons treated with quinpirole (Student’s t-test, n = 5; * p < 0.05). Experiment performed by Archan Ganguly. From Wiemerslage et al., 2013.

Because quinpirole has an inhibitory effect on the cell and because quinpirole is capable of protecting against MPP⁺ treatment, we tested if reducing cellular excitability in cells lacking DD2R was neuroprotective. Therefore, we added 1 μM of the classic
Na\(^+\)-channel blocker tetrodotoxin (TTX) to cultures treated with 40 \(\mu\)M MPP\(^+\). TTX was tested in cultures with pan-neural DD2R-RNAi. This was to confirm whether the rescue effect of the reduced excitability is enough. The DD2R-RNAi strain was used rather than the DD2R-deficiency mutant because the deficiency mutant may lack other genes besides DD2R which could affect the results of the experiment and because the same results were found in both strains when quinpirole as tested as a protective treatment (Figure 9).

Thus, RNAi is likely adequately disabling the expression of DD2R.

TTX rescued DA neurons from MPP\(^+\) treatment in cells expressing RNAi to DD2R. Cultures treated with 40 \(\mu\)M MPP\(^+\) and concomitant 1 \(\mu\)M TTX treatment were no different than control, and compared to MPP\(^+\) alone and MPP\(^+\) plus quinpirole, TTX increased the number of DA neurons by 119\% from 1.4 ± 0.2 to 3.1 ± 0.2 (Figure 13). Thus, TTX is protective, presumably from its blocking of sodium channels and subsequent inhibition of cellular excitability.
Figure 13. Reduced cell excitability protects DA neurons. Cell cultures were prepared from UAS-DD2R-RNAi;1407-GAL4 flies, and treated at 3 DIV with one of four treatments: control, 40 µM MPP⁺ alone, or 40 µM MPP⁺ plus 1 µM TTX. At 9 DIV cells were stained with anti-TH and quantified. TTX returned the number of TH⁺ cells to control levels (Student’s t-test; n = number of images: control = 49, MPP = 59, MPP⁺+TTX = 35; ** p < 0.01). From Wiemerslage et al., 2013.

Lastly, TTX is also protective against MPP⁺ in cells lacking DD2R autoreceptors. Cultures were prepared from TH-GAL4 and UAS-DD2R-RNAi flies, treated at 3 DIV and then stained with anti-TH antibody at 9 DIV. Unlike quinpirole, TTX increased the number of neurons 60% from 1.5 ± 0.2 to 2.4 ± 0.3 (Figure 14).
Figure 14: TTX rescues DA neurons lacking DD2R autoreceptors. Cell cultures were prepared from F1 progeny of a cross between UAS-DD2R-RNAi and TH-GAL4 flies. At 3 DIV, cultures were treated with 40 µM MPP⁺ alone or concomitantly with 10 µM quinpirole. At 9 DIV, cells were stained with anti-TH antibody and quantified. The number of TH⁺ neurons was reduced by MPP⁺ while no rescue was observed in cultures treated simultaneously with quinpirole (ANOVA, n = number of images: CONT = 51, MPP⁺ = 27, MPP⁺+TTX = 48; ** p < 0.01).

Ca²⁺ Chelation is Neuroprotective to DA neurons

The electrical inhibition caused by either quinpirole or TTX appears be preventing cell death processes by reducing excitotoxicity. Ca²⁺ is an important factor in excitotoxicity and apoptosis (Orrenius et al., 2003; Szydlowska & Tymianski, 2010), and some suggest that Ca²⁺-channels may make DA neurons selectively vulnerable (Surmeier et al., 2012). Thus, we next tested if chelation of extracellular Ca²⁺ is neuroprotective.
We tested the effects of 1 mM ethylene glycol tetraacetic acid (EGTA). This dose of EGTA was calculated (based on a formula by Tsien, 1980; Grynkiewicz et al., 1985; Haugland, 2001) to reduce the amount of free Ca$^{2+}$ in the culture medium 90% from 1 mM to 0.1 mM. Cell morphology was not affected at this dose of EGTA.

EGTA rescued the number of TH+ cells in our model. Compared to control, 40 µM MPP$^+$ decreased the number of DA neurons 54% from 3.9 ± 0.4 to 1.8 ± 0.2. The concomitant addition of EGTA and MPP$^+$, however, increased the number of DA neurons 83% compared to MPP$^+$ alone, from 1.8 ± 0.2 to 3.3 ± 0.4. The EGTA rescue group was not significantly different from control (Figure 15). This result supports the idea that inhibitory mechanisms may be neuroprotective and also that calcium may be a potential source of vulnerability in DA neurons.
Figure 15: EGTA rescues the number of DA neurons from MPP treatment. Cultures were prepared from UAS-GFP;TH-GAL4 flies. At 3 DIV, cultures were treated with MPP\textsuperscript{+}, MPP\textsuperscript{+} + EGTA, or as control. At 9 DIV, cultures were stained with anti-TH and the number of DA neurons was quantified in each group. 1 mM EGTA maintained the number of DA neurons within the level of control (Tukey's HSD test; n = number of images: CONT = 59, MPP\textsuperscript{+} = 85, MPP\textsuperscript{+} + EGTA = 72; *** p < 0.001).

Ca\textsuperscript{2+}-Channel Mutants are Inherently Protected from MPP\textsuperscript{+} Treatment

The last experiment performed in this section shows that the genetic removal of Ca\textsuperscript{2+}-channels protects DA neurons from MPP\textsuperscript{+} induced degeneration. This experiment used homozygous embryos from the fly strain cac\textsuperscript{HCl29}/Act-GFP, in which the chromosome containing the cacophony (cac) mutant gene is balanced by a chromosome containing GFP-tagged actin. cac\textsuperscript{HCl29} is a non-functional version of the cac Ca\textsuperscript{2+}-
channel. Homozygous coverslips were selected by lack of GFP signal (coverslips with no GFP signals were homozygous Ca\(^{2+}\)-channel mutants, while coverslips with bright GFP signals were used as control). Ca\(^{2+}\)-channel mutant cultures were inherently protected against 40 µM MPP\(^+\) treatment. MPP\(^+\) had no effect on the number of DA neurons in \(cac\) mutants; there was no difference between control and MPP\(^+\) treatment (1.1 ± 0.3 compared to 1.1 ± 0.4 respectively). In the Act-GFP controls, however, MPP\(^+\) maintained its degenerative effect – decreasing the number of DA neurons 39% from 1.4 ± 0.02 to 0.8 ± 0.06 (Figure 16). Thus, calcium channels may be an enabling factor in the degeneration of DA neurons.

![Figure 16: Ca\(^{2+}\)-channel mutants are inherently resistant to MPP\(^+\) treatment](image)

For this experiment, coverslips with an intense live-GFP signal were used as controls (labeled as ActGFP). At 9 DIV, cells were stained with anti-TH and quantified. In the cac mutant cultures, there was no difference in the number of TH\(^+\) neurons between control and MPP\(^+\) groups. However, MPP\(^+\) did have a degenerative effect in the GFP-CONT group (Student’s t-test, *** = p< 0.01; n = number of images: cacCONT = 54, cacMPP\(^+\) = 63, ActGFP-CONT = 65, ActGFP-MPP = 64; ** p < 0.01).
Three Ca-channel mutants have been developed in Drosophila: *cacophony*, *DmcaD*, *Dma1G* (Worrell and Levine, 2008). *cac* was chosen for this initial experiment because it is the most well described and studied Ca\(^{2+}\)-channel in Drosophila, though it does not code for a putative L-type channels that would create vulnerability in DA neurons. *cac* codes for N,P, and Q type. Although interestingly, at the time of this writing, www.FlyBase.org, the authoritative database of fly mutations, lists *cac* as an L-type Ca\(^{2+}\)-channel. An L-type Ca\(^{2+}\)-channel mutant does exist in Drosophila (*DmcaD*), and could be easily tested. The exact Ca\(^{2+}\)-channel, however, may not be so important. A recent study convincingly found that inhibition of a T-type Ca\(^{2+}\)-channel was neuroprotective and that these T-type Ca\(^{2+}\)-channels could also be a source of vulnerability in DA neurons (Xiang et al., 2011).

A puzzling finding from the above results is the difference in the number of DA neurons between control groups of different experiments. The typical number of DA neurons in cell cultures is 4 DA neurons per 1000 cells. But the *cacophony* mutants gave a much lower ratio of 1 per 1000. We attribute this difference to inherent properties within the mutant lines that affect the rates of differentiation and survival of the DA neurons. This idea could be examined further, but is beyond the scope of this research.

**Summary of Chapter 3**

Our *Drosophila* model establishes a culture system to study PD-like neurodegeneration using the DA-specific toxin MPP\(^+\). D2 agonists protect against the MPP\(^+\) induced degeneration through the activity of D2 autoreceptors, which acts to
decrease cell excitability. Other blockades of cellular excitability, such as TTX or chelation of Ca$^{2+}$ also protect against MPP$^+$ treatment. Furthermore, Ca$^{2+}$ is indeed an important factor in the selective vulnerability of DA neurons (Figure 17).

![Figure 17](image.png)

*Figure 17*. Summary figure of Chapter 3.

MPP$^+$ enters DA neurons through the dopamine transporter (DAT) inhibits complex I of mitochondria. D2 agonists act on D2 autoreceptors to decrease the excitability of DA neurons. Reducing cellular excitability via blockade of sodium channels (NaCh) by tetrodotoxin (TTX) also prevents MPP$^+$ induced degeneration. Lastly, Ca$^{2+}$ is an important factor in determining whether excitotoxic events ultimately lead to neurodegeneration. Cells lacking Ca$^{2+}$-channels were inherently protected from MPP$^+$ induced degeneration.
CHAPTER 4: ASSESSMENT OF ALTERED MITOCHONDRIAL MORPHOLOGY
AND ITS RESCUE

Changes in mitochondria are implicated in PD (Dawson et al., 2010; Nakamura et al., 2011; Saxena and Caroni, 2011; Botella et al., 2011). Mitochondrial morphology is generally thought to indicate the status/health of the cell in which they reside. Some researchers are using mitochondria as a quantifiable endpoint in experiments studying neurodegeneration (Dagda et al., 2009, Wang et al., 2011). Testing the rates of cell death may not be the best way to measure neuroprotection because a living cell is not necessarily functionally rescued. Models should expand their analysis to include other endpoints. Mitochondria are an obvious choice because they are coupled to both apoptosis and the functionality of the cell (Rugarli and Langer, 2012).

In this chapter I modify our previous cell culture model of PD-like degeneration to quantify the mitochondrial morphology in DA neurons and explore how mitochondria are related to neurodegeneration and neuroprotection. First, I quantify different parameters of morphology using mutants with known morphological defects in their mitochondria. Next, I describe MPP\(^+\) treated DA neurons as having fragmented mitochondria – indicating these cells are in the early stages of cell death. In Chapter 3, I showed that D2R agonists prevent DA degeneration. Here again, I show that concomitant treatment with the D2 agonist quinpirole prevents the fragmented mitochondrial phenotype induced by MPP\(^+\). Moreover, I show that this rescue requires D2 autoreceptors by examining the morphology of DA neurons expressing RNAi to DD2R. Blocking cellular excitability prevented MPP\(^+\)-induced damage to mitochondrial
morphology even in DD2R mutants. Lastly, the mitochondria in DA neurons with mutated Ca\textsuperscript{2+} channels were inherently protected from MPP\textsuperscript{+} treatment.

Quantifying Mitochondria in Dopamine Neurons

As mentioned in the introduction, quantifying mitochondrial morphology is still imperfect. Our method builds on previous work that analyzes mitochondrial morphology using four parameters: number, size, interconnectivity, and elongation (Dagda et al., 2009). This analysis gives a comprehensive assessment of the mitochondrial morphology, whereas other studies may measure only one parameter and ignore other potential factors in the mitochondrial network. Moreover, the locale of the mitochondria is important. Our method quantifies non-somatic mitochondria (i.e. in the dendrites and axons) because these are likely the most important for DA neuronal signaling.

Our lab developed an immunocytochemistry method for quantifying mitochondrial morphology specifically in DA neurons: staining cultures with both MitoTracker and an antibody to tyrosine hydroxylase (TH) and then using an overlapped image of the two to single out DA mitochondria from the co-localization of the two signals (Figure 18).
Figure 18. Example images from immunocytochemistry method of quantifying mitochondria in DA neurons.

(Green/top image) DA neurons are identified by antibody to tyrosine hydroxylase (anti-TH). (Red/middle image) mitochondria are identified by MitoTracker staining. (Overlapped/bottom image) Colocalized signals from anti-TH and MitoTracker show as white and represent mitochondria specifically from DA neurons. Image taken from wild-type at 7 days in vitro (DIV) (scale bar = 20 µm).
The immunocytochemistry method for quantifying mitochondrial morphology was validated using mutant lines with known defects/phenotypes in mitochondrial morphology: dynamin-related protein 1 (drp1) mutant, which prevents mitochondrial fission, and optic atrophy 1 (opa1) mutant, which prevents mitochondrial fusion. Four morphological parameters of mitochondria in DA neurons were measured in three groups: mutant drp1, mutant opa1, and control. Using the fly lines drp1/CyO-GFP and opa1/CyO-GFP, single-embryo cultures were prepared. Individual coverslips were selected by the presence/intensity of a live-GFP signal. Coverslips with no GFP signal were homozygous for the drp1 or opa1 mutation. Coverslips with an intense live-GFP signal (10x more luminous than background) were used as controls. All three groups were stained with MitoTracker Orange prior to immunocytochemistry staining with anti-TH. The colocalization signal from the MitoTracker and antibody identifies DA mitochondria specifically for analysis.

drp1 mutants have elongated and widely connected mitochondria, while opa1 mutants have isolated and round mitochondria (Figure 19). Both drp1 and opa1 reduced the number of mitochondria (per DA neuron) from 96 ± 7 to 41 ± 11 (57% decrease) and 58±5 (43% decrease) respectively (Figure 20). A decreased number of mitochondria in drp1 mutants was expected because mitochondria are highly interconnected in the drp1 mutants. Thus, the method successfully detected a difference in the number of mitochondria.
Figure 19. Quantification of mitochondrial morphology in control and mutant mitochondria.

(Top row) DA cells for control, \textit{drp1} mutants, and \textit{opa1} mutants. Signal is from antibody to tyrosine hydroxylase (TH), selectively marking DA neurons. (Middle row) images of MitoTracker staining for control, \textit{drp1} and \textit{opa1}. Mitochondria from all cell types are stained. (Bottom row) overlapped image of anti-TH and MitoTracker signals. Colocalized signals are shown in white, i.e. are mitochondria from DA neurons. Single-embryo cell cultures on individual glass coverslips were prepared from \textit{drp1}/CyO-GFP and \textit{opa1}/CyO-GFP fly lines. At 3 DIV, coverslips were selected by the presence/intensity of the live-GFP signal. Coverslips without a live-GFP signal were homozygous for \textit{drp1} or \textit{opa1}. Coverslips with the brightest live-GFP signals were used as controls. At 7 DIV, coverslips were stained with anti-TH and MitoTracker and images were taken for analysis (scale bar = 20 µm).
For size, interconnectivity, and elongation, the trend for the three treatment groups was the same: \textit{drp1} had the highest value, \textit{opa1} the lowest, and the GFP control fell in the middle. \textit{drp1} mitochondria had mean area of $2.8 \pm 0.2 \ \mu m^2$, 131\% larger than control at $1.2 \pm 0.1 \ \mu m^2$. \textit{opa1} mitochondria were 37\% smaller than control at $0.8 \pm 0.1 \ \mu m^2$. \textit{drp1} mitochondria were 40\% more interconnected than control, with an interconnectivity score of $1.85 \pm 0.07$ compared to $1.33 \pm 0.03$. \textit{opa1} mitochondria were 15\% less interconnected compared to control with an interconnectivity score of $1.12 \pm 0.07$. Lastly, \textit{drp1} mitochondria were 40\% more elongated than control, with an elongation score of $1.85 \pm 0.07$ compared to $1.33 \pm 0.03$. \textit{opa1} mitochondria were 8\% less elongated compared to control with an elongation score of $1.12 \pm 0.07$ (Figure 20). These data validate our technique as able to detect differences in each parameter. Moreover, our results for mitochondrial size match lengths commonly reported across species (0.5 to 1 \mu m) (Rafelski & Wallace, 2008; Bereiter-Hahn 1990).
`drp1` mutants had larger, more interconnected, and elongated mitochondria compared to control and `opal`, while `opal` had smaller, less interconnected, and less elongated mitochondria compared to `drp1` and control. Both `drp1` and `opal` had fewer mitochondria than control – likely because in `drp1` cells, the mitochondria are all connected and thus not counted as separate, and in `opal` cells because the fragmented mitochondria are translocated to the soma where they are not included in analysis. At 7 DIV, images were taken for analysis (Tukey’s HSD test; n = number of experiments, DRP n = 3, GFPCONT n = 7, OPA n = 3; *** p < 0.001).
Genetic Tools to Quantify DA Mitochondria

We also used a genetic construct to quantify DA mitochondria. The fly line UAS-mtGFP/CyO;TH-GAL4 tags mitochondria with green fluorescent protein (GFP) specifically in DA cells using the GAL4-UAS genetic tool. We verified the expression of GFP in mitochondria by staining with MitoTracker. MitoTracker stained the same signals identified by the live-GFP signal (Figure 21).

![Live GFP](image1)
![MitoTracker](image2)

Figure 21. Overlap of live GFP signals with MitoTracker staining. (Green/left image) DA mitochondria are identified by live-GFP signal in UAS-mtGFP/CyO;TH-GAL4 cultures. (Red/middle image) mitochondria from all cells are identified by MitoTracker staining. (Overlapped/right image) MitoTracker successfully stains the same mitochondria identified by live-GFP signal. Colocalized signals are shown in white. Images taken at 7 DIV (scale bar = 20 µm).

Degeneration of Dopaminergic Mitochondria by PD-like Treatment

After validation of the method, mitochondrial morphology was tested in our PD model. We tested if 40 µM of MPP⁺ would alter mitochondrial morphology using both visualization methods: the UAS-mtGFP/CyO;TH-GAL4 strain and the immunocytochemistry method (using a wild-type strain). For both methods, cultures were treated with MPP⁺ at 3 DIV. At 7 DIV, images were taken from the live-GFP signal from the UAS-mtGFP/CyO;TH-GAL4 cultures and quantified. Staining with the
immunocytochemistry method in wild-type cultures was also performed at 7 DIV. For both methods, control mitochondria appeared elongated and more interconnected, while MPP⁺ treated mitochondria displayed a fission-like phenotype: fewer, smaller, and rounder (Figure 22).

**Figure 22.** MPP⁺ gives mitochondrial morphology a fragmented phenotype. A) Example images of control and MPP⁺-treated DA neurons at 7 DIV. Images show the live-GFP signal for mitochondria in DA neurons (scale bar = 20 µm). Single-embryo cell cultures on individual glass coverslips were prepared from UAS-mtGFP/CyO;TH-GAL4 flies (THmtGFP), which possess GFP-tagged mitochondrial in DA neurons. At 3 DIV, coverslips were selected by the presence/intensity of the live-GFP signal. Coverslips with the brightest live-GFP signals were likely homozygous for mtGFP. Also at 3 DIV, cultures were treated with either 40 µM MPP⁺ or as control. At 7 DIV, images were taken for analysis. B) Example images of control and MPP⁺-treated DA neurons from immunocytochemistry colocalization method (anti-TH and MitoTracker). Cultures were treated at 3 DIV and stained for analysis at 7 DIV. DA mitochondria are shown in white (scale bar = 20 µm).
In the UAS-mtGFP/CyO;TH-GAL4 strain, MPP\textsuperscript{+} significantly decreased all the measured parameters compared to control: the number of mitochondria decreased 50% from 74 ± 4 to 37 ± 2, the size of mitochondria decreased 22% from 1.8 ± 0.1 to 1.4 ± 0.1 µm\textsuperscript{2}, interconnectivity decreased 9% from 1.97 ± 0.03 to 1.80 ± 0.03, and elongation decreased 6% from 1.18 ± 0.01 to 1.11 ± 0.01. The immunocytochemistry method mirrored those results. MPP\textsuperscript{+} also significantly decreased all the measured parameters compared to control: the number of mitochondria decreased 53% from 88 ± 8 to 41 ± 3, the size of mitochondria decreased 47% from 1.5 ± 0.1 to 0.8 ± 0.1 µm\textsuperscript{2}, interconnectivity decreased 27% from 1.56 ± 0.04 to 1.14 ± 0.03, and elongation decreased 7% from 1.50 ± 0.02 to 1.40 ± 0.02 (Figure 23). Thus, MPP\textsuperscript{+} fragments mitochondria: decreasing the number, shrinking them, breaking down the network, and making them rounder.
Figure 23. Mitochondrial damage shown in both methods. In both methods, all four parameters showed a significant decrease from control when treated with MPP⁺. At 7 DIV, images were taken for analysis (Student’s t-test; n = number of cells: THmtGFP-CONT n = 133; THmtGFP-MPP n = 165, WtCONT = 29, WtMPP =51; *** p < 0.001).
Time Course of MPP⁺-Induced Mitochondrial Damage

Drugs that are neuroprotective in the laboratory fail to prevent degeneration in actual PD (Loehle and Reichmann, 2010). One possible explanation is that these therapies are prescribed too late in the disease, i.e., too much degeneration has already occurred and the cells remaining are beyond saving. This is consistent with laboratory results, as most animal models are given the rescue drug concomitantly with the toxic one (Herrero et al., 2011). One way to improve PD models might be to change the treatment pattern: treating with toxic drugs first, waiting until the disease manifests, and then adding the rescue therapy. This would test if a therapy could reverse, halt, or slow degeneration after it has begun rather than preventing degeneration while the cells are normal. This would also match how the medications are currently used in the clinic. Surprisingly, few model studies have used this simple modification. Perhaps current therapies for PD are neuroprotective, but not utilized early enough in the disease.

Previously, we showed that 40 µM MPP⁺ degenerates DA neurons in our cell cultures at 6 DIV (treated with MPP⁺ at 3 DIV). The results in the previous section, however, show damaged mitochondria at 4 DIV (treated with MPP⁺ at 3 DIV). This supports the general hypothesis that mitochondrial damage precedes cell death, but we wished to fully test the idea. Specifically, we determined the timing of the two events in our model. To do this we measured both the amount of cell death and mitochondrial damage at 1, 2, 3, and 4 DIV after treatment with MPP⁺. Mitochondrial morphology was measured over time via the live GFP signal from UAS-mtGFP/CyO;TH-GAL4 cultures. Cell death was quantified by comparing the number of DA neurons between control and
MPP⁺ groups via immunocytochemistry (previously described in Wiemerslage et al., 2013).

As early as 1 DIV after MPP⁺ treatment, mitochondria showed significant changes in mitochondrial morphology in all but one parameter – number of mitochondria. At 4 DIV compared to the baseline at 3 DIV, MPP⁺ decreased the size 31 ± 5%, decreased the interconnectivity score 10 ± 3%, and decreased the elongation score 10 ± 2%, while the control group saw an increase of 9 ± 8% in size, a 2 ± 4% increase in interconnectivity, and a 2 ± 2% decrease in elongation. The number of mitochondria was not significantly decreased until 7 DIV. From 7 DIV compared to the baseline at 3 DIV, the MPP⁺ group had 34 ± 7% fewer mitochondria, while the control group had 8 ± 13% fewer (Figure 24).
Figure 24. PD-like treatment damages mitochondrial morphology early in disease process.
The above plots showing changes in the morphological parameters of mitochondria in DA neurons over time. Single-embryo cell cultures were prepared from UAS-mtGFP/CyO;TH-GAL4 flies. At 3 DIV (baseline), coverslips were selected by the presence/intensity of the live-GFP signal and treated with either 40 µM MPP⁺ or as control. Images were collected for analysis at 3, 4, 5, and 7 DIV. MPP⁺ significantly decreased size, interconnectivity, and elongation as early as 4 DIV and remained decreased. The number of mitochondria in the MPP⁺ group was not different from control until 7 DIV (Student’s t-test; n = 6, number of experiments; * p < 0.05, ** p < 0.01, *** p < 0.001)
The number of dopamine neurons, however, did not significantly decrease until 3 DIV after treatment at 3 DIV. At 6 DIV, MPP\(^+\) significantly decreased the number of DA neurons 28% from 4.7 ± 0.1 to 3.4 ± 0.1 (Figure 25). Thus in our model, MPP\(^+\) treatment first alters mitochondrial morphology, and then subsequently leads to cell death days later.

*Figure 25.* DA cell death happens after changes in mitochondria. Plot showing number of DA neurons at different time points between control and MPP+-treated cultures. Cultures were prepared from UAS-GFP;TH-GAL4 flies and later treated with MPP+ at 3 DIV. Subsets of cultures were then immunostained at the following timepoints: 4, 5, 6, 7, and 8 DIV. For each timepoint, the number of DA neurons was quantified and compared between control and MPP+. MPP+ did not significantly reduce the number of DA neurons until 6 DIV (Student’s t-test; n = 8, number of experiments; *p < 0.05*).
Prevention of MPP\(^+\)-Induced Changes in Mitochondrial Morphology by D2 Agonist

D2R agonists prevent the degeneration of DA neurons in our model (Wiemerslage et al., 2013). Because mitochondrial dysfunction is implicated in PD and because our model shows changes in mitochondrial morphology when treated with a PD toxin, we next tested if 10 µM of the D2R agonist quinpirole would rescue mitochondrial morphology from MPP\(^+\) treatment. At 3 DIV, cultures from UAS-mtGFP/CyO;TH-GAL4 flies were treated with either 40 µM MPP\(^+\) alone, MPP\(^+\) plus 10 µM quinpirole, or as control. Mitochondrial morphology was analyzed from the live GFP signal at 7 DIV.

Concomitant treatment with both 40 µM and 10 µM quinpirole prevented changes in mitochondrial morphology (Figure 26). Quinpirole prevented MPP\(^+\)-induced changes in all 4 parameters. Compared to MPP\(^+\) quinpirole increased the number of mitochondria 54% from 37 ± 2 to 67 ± 4, increased the size 18% from 1.4 ± 0.1 to 1.7 ± 0.1 µm\(^2\), increased the interconnectivity score 9% from 1.80 ± 0.03 to 1.96 ± 0.05, and increased the elongation score 5% from 1.11 ± 0.01 to 1.16 ± 0.01 (Figure 27). None of the parameters in the group treated with both MPP\(^+\) plus quinpirole were significantly different from control. Thus, D2R activation prevents changes induced by MPP\(^+\).
Figure 26. Mitochondria are protected from PD-like treatment by D2 agonist (images). Example images of control, MPP+, and MPP+quinpirole treatment groups at 7 DIV (scale bar = 20). Images show the live-GFP signal for mitochondria in DA neurons. Single-embryo cell cultures on individual glass coverslips were prepared from UAS- mtGFP/CyO;TH-GAL4 flies, which possess GFP-tagged mitochondrial in DA neurons. At 3 DIV, coverslips were selected by the presence/intensity of the live-GFP signal. Coverslips with the brightest live-GFP signals were likely homozygous for mtGFP. Also at 3 DIV, cultures were treated with either 40 µM MPP+ alone, MPP+ plus 10 µM quinpirole, or as control. At 7 DIV, images were taken for analysis.
Figure 27. Quinpirole rescues mitochondrial morphology from PD-like treatment. All four parameters are protected from MPP+ treatment by concomitant exposure with the D2 agonist quinpirole. At 7 DIV, images were taken for analysis (Tukey’s HSD test, n = number of cells: CONT n = 133, MPP n = 165, MPP+Q n = 62; ** p < 0.01, *** p < 0.001).
Following the above experiments, we investigate how soon neuroprotective treatment must be added to be effective, i.e., we determined if quinpirole could reverse, halt, or slow degeneration after it has begun rather than merely preventing degeneration while the cells/mitochondria are normal. This would also possibly match how the medications are currently used in the clinic. Cultures from UAS-mtGFP/CyO;TH-GAL4 flies were treated with 40 µM MPP⁺ at 3 DIV, with 10 µM quinpirole added the next day (4 DIV, roughly 24 hours later), 2 days later (5 DIV), 3 days later (6 DIV), with MPP⁺ only, or as control. Mitochondrial morphology was analyzed from the live GFP signal at 7 DIV.

Delayed treatment with quinpirole did not rescue mitochondrial morphology as robustly as concomitant treatment. When quinpirole was added 2 or 3 DIV following MPP⁺ treatment, the mitochondrial morphology was no different than the group treated with MPP⁺ alone. When quinpirole was added 1 day after treatment with MPP⁺, however, the elongation score increased 2% from 1.11 ± 0.01 to 1.13 ± 0.01 compared to the MPP⁺ alone group (Figure 28). Thus, delayed treatment with quinpirole only slightly rescued mitochondrial morphology from MPP⁺ treatment. For a robust effect, rescue therapy must be added early in the disease process.
Figure 28. Quinpirole treatment must be added early for rescue of mitochondrial morphology from MPP+ treatment.

Single-embryo cell cultures on individual glass coverslips were prepared from UAS-mtGFP/CyO;TH-GAL4 flies, which possess GFP-tagged mitochondrial in DA neurons. At 3 DIV, coverslips were selected by the presence/intensity of the live-GFP signal. Coverslips with the brightest live-GFP signals were likely homozygous for mtGFP. Also at 3 DIV, cultures were treated with either 40 µM MPP+ alone, or as control. At 4, 5, and 6 DIV, a subset of MPP+ treated cultures were also treated with 10 µM quinpirole. At 7 DIV, images were taken for analysis. Only elongation was partially rescued when quinpirole was added at 4 DIV. No parameters were rescued with quinpirole was added at 5 or 6 DIV (Tukey’s HSD test; n = number of cells: CONT = 133, MPP = 165, MQ4 = 147, MQ5 = 157, MQ6 = 87; * p < 0.05, *** p < 0.001).
D2 Autoreceptor is Necessary for Rescue of Dopaminergic Mitochondria

The relationship between D2R and mitochondrial morphology is currently unknown. But as seen above, D2R activation prevents mitochondrial damage from MPP\(^+\) treatment. Some D2R agonists are thought to have antioxidant effects that may act on mitochondria (Ferrari-Toninelli et al., 2010; Cassarino et al., 1998; Herrero et al., 2011). This antioxidant effect may be quinpirole’s mechanism of action regarding the effects in mitochondrial morphology discussed above. Thus, we next tested if D2R activation was required for quinpirole to rescue mitochondrial morphology from MPP\(^+\) treatment. We used two different fly strains containing RNAi to *Drosophila* dopamine type 2 receptor (DD2R): UAS-DD2R-RNAi;1407-GAL4 and UAS-DD2R-RNAi crossed with TH-GAL4. The former contains pan-neural RNAi to DD2R, while the latter expresses RNAi only in DA neurons. The latter crossed line allows us to test whether D2 autoreceptors are necessary for the effect of D2 agonists. At 3 DIV, cultures were treated with either MPP\(^+\), MPP\(^+\) plus rescue treatment, or as control. Cultures were stained at 7 DIV and the dopaminergic mitochondria were analyzed by overlapping the signal from MitoTracker and anti-TH antibody.

Quinpirole did not prevent the changes in mitochondrial morphology induced by MPP\(^+\) in either of the two DD2R-RNAi lines. Furthermore, there was no significant difference between the MPP\(^+\) alone and MPP\(^+\)-plus-quinpirole groups in any of the measured parameters. In cultures with pan-neural DD2R-RNAi, compared to control, MPP\(^+\) decreased the number of mitochondria 68% from 95 ± 7 to 30 ± 3, decreased the size of mitochondria 43% from 1.4 ± 0.1 to 0.8 ± 0.1 \(\mu\text{m}^2\), decreased interconnectivity
27% from 1.43 ± 0.05 to 1.05 ± 0.03, and decreased elongation 5% from 1.52 ± 0.02 to 1.43 ± 0.02. Similar decreases were found between control and MPP⁺+quinpirole treatment (Figure 29).

*Figure 29. Mitochondrial morphology in cultures lacking D2 receptors. The D2 agonist quinpirole fails to rescue any parameter from MPP⁺ treatment. TTX, however, restores all parameters to control levels except for the number of mitochondria, where TTX did increase levels above MPP⁺ and MPP⁺+quinpirole (Tukey’s HSD test; n = number of cells: CONT n = 36, MPP⁺ n = 51, MPP⁺+Q n = 25, MPP⁺+TTX n = 33; * p < 0.05, ** p < 0.01, *** p < 0.001).
In the strain with DD2R-RNAi only in DA cells, compared to control, MPP\(^+\) decreased the number of mitochondria 66% from 86 ± 6 to 29 ± 2, decreased the size of mitochondria 47% from 1.5 ± 0.1 to 0.8 ± 0.1 \(\mu\text{m}^2\), decreased interconnectivity 27% from 1.48 ± 0.04 to 1.08 ± 0.02, and decreased elongation 5% from 1.48 ± 0.01 to 1.40 ± 0.02. Again, similar decreases were found between control and MPP\(^+\)+quinpirole treatment (Figure 30). Thus, DD2R autoreceptors are necessary for quinpirole to prevent MPP\(^+\)-induced changes in mitochondrial morphology, just as they are to prevent MPP\(^+\)-induced cell death.
Figure 30. Mitochondrial morphology in DA neurons lacking D2 autoreceptors. Results generally mirrored those in cells lacking D2 autoreceptors in all cell types (Tukey’s HSD test; n = number of cells: CONT n = 75, MPP+ n = 38, MPP+Q n = 73, MPP+TTX n = 44; * p < 0.05, ** p < 0.01, *** p < 0.001).
Electrical Inhibition Prevents MPP⁺-Induced Changes in DA Neurons’ Mitochondrial Morphology

In the previous chapter, we showed that the classic Na⁺-channel blocker, tetrodotoxin (TTX), prevents the MPP⁺-induced loss of DA neurons in cells lacking DD2R. Here we extend this work to test if electrical inhibition prevents MPP⁺-induced changes in mitochondrial morphology. For this analysis we again tested the two lines expressing DD2R-RNAi used previously (UAS-DD2R-RNAi;1407-GAL4 and UAS-DD2R-RNAi). At 3 DIV, all cultures were treated with 40 µM MPP⁺ added concomitantly with 1 µM TTX. Then at 7 DIV, mitochondria morphology was analyzed.

TTX treatment decreased MPP⁺-induced changes in mitochondrial morphology. Compared to the MPP⁺-alone group, the MPP⁺-plus-TTX group showed a significant increase in all the parameters for both strains. In the strain with pan-neural DD2R-RNAi, the number of mitochondria increased 93% from 30 ± 3 to 58 ± 5, the size of mitochondria increased 33% from 0.8 ± 0.1 to 1.2 ± 0.1 µm², interconnectivity increased 19% from 1.11 ± 0.05 to 1.32 ± 0.06, and elongation increased 6% from 1.43 ± 0.02 to 1.51 ± 0.02 (Figure 30). In the strain with DD2R-RNAi only in DA neurons, the number of mitochondria increased 66% from 29 ± 2 to 48 ± 4, the size of mitochondria increased 63% from 0.8 ± 0.1 to 1.3 ± 0.1 µm², interconnectivity increased 29% from 1.08 ± 0.02 to 1.39 ± 0.04, and elongation increased 6% from 1.40 ± 0.02 to 1.48 ± 0.02 (Figure 31).

Thus, electrical inhibition has a neuroprotective effect against MPP⁺-induced changes in mitochondrial morphology. The absence of DD2R had no effect on the rescue by TTX (Figures 29 & 30).
Calcium Modulation Prevents MPP⁺-Induced changes in DA Neurons’ Mitochondrial Morphology

As mentioned earlier, ROS is implicated in the degeneration of DA neurons, yet it is unknown why DA neurons are more prone to ROS-induced apoptosis compared to other neurons. Also unknown is the initial cause of the ROS increase, although mitochondria are suspected as the major source (Surmeier et al., 2011). Many specific properties of DA neurons have been proposed as vulnerabilities to ROS-induced degeneration and indeed there are several factors involved in increased ROS levels (Double, 2012). One noteworthy idea is that certain types of calcium channels present on vulnerable DA neurons may add an increased Ca²⁺ load on the cell. The increase in Ca²⁺ can depolarize the membrane of mitochondria, inhibit their function, and limit their ability to sustain the metabolic needs of the cell (Surmeier et al., 2011).

Therefore, we determined if Ca²⁺ modulation prevented MPP⁺-induced changes in mitochondrial morphology. This was tested using cultures from a fly strain with mutated Ca²⁺ channels. This experiment used homozygous embryos from the fly strain elav-GAL4,cac^{HC129}/FM7i,UAS-ActGFP, in which the chromosome containing the cacophony (cac) mutant gene is balanced by a chromosome containing GFP-tagged actin. cac^{HC129} has a non-functional version of the Ca²⁺-channel. Homozygous coverslips were selected by lack of GFP signal (i.e. coverslips with no GFP signals were homozygous Ca²⁺-channel mutants). Cultures were treated at 3 DIV with 40 µM MPP⁺, and then stained with MitoTracker and anti-TH antibody at 7 DIV to analyze mitochondria specifically from DA neurons. 3 out of the 4 parameters tested were protected from MPP⁺ treatment.
in the cultures with mutated \textit{cac}. The number of mitochondria, however, decreased 27% from 73 ± 5 to 53 ± 4 when treated with MPP⁺ (Figure 31). These results support the idea that inhibitory mechanisms may be neuroprotective and also that calcium may be a source of vulnerability in DA neurons.
Figure 31. *cac* mutant cells are resistant to MPP$^+$ treatment. Single-embryo cell cultures were prepared from the balanced strain *cac*-HC129/ACT-GFP. At 3 DIV, coverslips were selected by the presence/intensity of a live-GFP signal. Coverslips with no GFP signal were homozygous for the *cac* mutant and thus lacked functional Ca$^{2+}$ channels. Also at 3 DIV, cells from each strain were treated with or without 40 µM MPP$^+$. DA mitochondria in *cac* mutants are only partially protected from MPP$^+$ treatment. For this experiment, *cac* mutants were compared to wild-type cultures. Mitochondria were quantified by the costaining method at 7 DIV. MPP$^+$ decreased the number of mitochondria in *cac* mutants, but all other parameters were spared (Tukey’s HSD test; n = number of cells: *cac*CONT = 53, *cac*MPP = 36, WtCONT = 29, WtMPP$^+$ = 51; * p < 0.05, ** p < 0.01, *** p < 0.001).
Treatment Effects of Quinpirole and TTX

As a final experiment, we tested the effect on mitochondrial morphology from treatment with quinpirole or TTX alone. Neither drug significantly altered any of the parameters of mitochondrial morphology (Figure 32).

*Figure 32:* Neither Quinpirole nor TTX alone alters mitochondrial morphology. Cultures from UAS-mtGFP/CyO;TH-GAL4 flies were treated at 3 DIV. Images were taken for analysis from the live-GFP signal at 7 DIV. (Tukey’s HSD test, n = number of images: CONT = 133, Quin = 36, TTX = 48).
Summary of Chapter 4

In this chapter we expanded the use of our *Drosophila* primary cell culture model of PD-like degeneration to quantify mitochondrial morphology specifically in DA neurons. We showed that mitochondrial morphology is useful for determining the effectiveness of neuroprotective treatments. We quantified mitochondrial morphology using 4 parameters: number, size, interconnectivity, and elongation. Moreover, we validated our method using mutants with known morphological defects: *drp1* and *opa1*. Next, we found that MPP\(^+\)-treated DA neurons have fragmented mitochondria – indicating these neurons are in the early stages of cell death. Fragmentation was detected as early as one day after treatment with MPP\(^+\). However, DA neurons did not show significant cell death until 3 days after treatment. We also explored the neuroprotective mechanisms of D2 receptor agonists on mitochondrial morphology. Concomitant treatment with the D2 agonist quinpirole kept mitochondrial morphology at control levels. However, adding quinpirole one day after MPP\(^+\) treatment did not, except for the elongation parameter. It is unknown how important the elongation of mitochondria are for overall function, so this result cannot be adequately interpreted as a significant rescue effect. Quinpirole also did not prevent damage to mitochondrial morphology in DA neurons lacking D2 autoreceptors. Lastly, we explored the relationship between calcium and mitochondrial morphology in our model and found that either electrical inhibition or mutation of Ca\(^{2+}\) channels protected against MPP\(^+\) treatment. Our work establishes a PD-model for measuring mitochondrial morphology in Drosophila cell cultures. We show that PD-like treatment damages mitochondrial morphology and this degeneration
precedes neuronal death. Our results strongly suggest that neuroprotective treatment must be added early in the disease process to be effective. Furthermore, the neuroprotective effect of quinpirole is dependent on D2 autoreceptor signaling. Lastly, we support the theory that excess Ca\(^{2+}\) is a source of vulnerability for DA neurons. The summary figure below (Figure 33) updates the previous summary figure from Chapter 3. Here we suggest that mitochondrial damage from MPP\(^+\) treatment is a redundant pathway leading the cell towards degeneration in parallel to excitotoxicity.
Figure 33. Summary figure of Chapter 4. MPP\(^+\) enters the DA neuron through the DAT and inhibits complex I of the mitochondria: promoting the formation of superoxide, damaging the mitochondria, and subsequently leading to the fragmented phenotype typical for apoptosis. Excitotoxicity also leads to this fragmented phenotype by collapsing the mitochondrial membrane potential. Mitochondria will buffer excess Ca\(^{2+}\), but too much will stress their ability to produce ATP, cause them to release pro-death signaling molecules, and again produce the fragmented phenotype seen in apoptosis. As mitochondria are damaged, they divide into smaller, rounder versions (thus increasing in number) and then are trafficked back to the soma. Our results initially show no change in the number of mitochondria upon treatment with MPP\(^+\) until 3 DIV after treatment. At this point, the damaged mitochondria have been trafficked to the soma where they are no longer counted by our method.
CHAPTER 5: DISCUSSION

This work establishes a Drosophila primary culture system to study PD-like degeneration and screen potential neuroprotective therapies. DA neurons, as well as their subcellular structures, can be quantified and compared between treatment groups. We show that MPP⁺, the active metabolite of MPTP, selectively damages DA neurons and their mitochondrial morphology. We also show that the D2 agonist quinpirole can prevent, but not reverse MPP⁺-induced degeneration. D2 autoreceptors are required for the rescue by quinpirole. However, in DA neurons lacking D2 autoreceptors, MPP⁺-induced degeneration was prevented by reducing cellular excitability with the Na⁺-channel blocker TTX. Furthermore, DA neurons with mutated Ca²⁺ channels were inherently protected from MPP⁺ treatment. Lastly, we describe the time-course of MPP⁺-induced degeneration in our model. DA neurons are near-completely post-mitotic by 3 DIV, which is when cultures are treated with drugs in our model. We find that damage to mitochondria precedes the death of DA neurons, and that rescue therapy must be given almost concomitantly with PD-like treatment to be effective.

Drosophila for Research in PD

The model developed in this work continues to reinforce Drosophila’s usefulness as a model system for PD. Our model parallels PD to the best of the current understanding of the pathogenesis of PD. Idiopathic PD is an age-related disorder, and symptoms do not appear until after several decades of aging. Thus, our model exposes the DA neurons to toxins after they are post-mitotic. Variations of this treatment
schedule were considered. For example, in the experiments where quinpirole was added days later after MPP\(^+\) treatment, we considered washing out the MPP\(^+\) from the cultures. However, we decided against it because in actual PD, the disease process is still present when therapy is added. A true neuroprotective drug should prevent further decline by overcoming the disease process. Thus, our experiments try to reflect clinical conditions as much as possible and not remove the underlying PD-like pathology after its introduction.

Our *in vitro* system has two main strengths compared to others. First, cultures are easily treated with pharmacological regimens compared to *in vivo* experiments. Drugs may be added or removed at any time point, and one could rapidly screen combinations of drug therapies to screen for neuroprotective ones – an experiment can be performed in about a week. The time course experiment such as ours where the rescue therapy quinpirole was added days later following MPP\(^+\) treatment is one example where the timing of pharmacological treatment is manipulated. Furthermore, using the suite of genetic tools available in *Drosophila*, such as mutant models of PD, one could explore the relationship between genetic factors in PD versus environmental conditions (i.e. toxins like MPP\(^+\) or rotenone). In PD mutants, the pathological mechanism is constantly present throughout development, but in toxin models of PD, the pathological treatment can be added or removed at any time. It would be interesting to look for combinatorial effects of the PD-mutant pathology and the toxin pathology in our model. Exploring this combination of pathological forces is important because it could help explain the idiopathic nature of PD. Over 90% of PD cases are from unknown origin (Bossy-Wetzel
et al., 2004). More specifically, it is unknown whether people who develop PD have a vulnerability based on their genetic background, or if everyone would get PD merely by out-surviving their DA neurons.

The second major advantage of our *in vitro* system over an *in vivo* one is that cells in culture are easily accessible for functional assays such as electrophysiology. A particular cell type can be identified via live-GFP signal (as in our electrophysiology experiments) and used for single cell recordings. Moreover, the tracking of individual cells or sub-cellular structures (e.g. mitochondria) over time is largely impractical *in vivo*, but is easily accomplished in our system.

Overall, *Drosophila* will remain an important model for PD research because of its suite of genetic tools, ease of husbandry, rapid experimental turnaround, and high conservation of orthologs with mammals (Whitworth, 2011; Adams et al., 2000).

*Drosophila* has a library of transgenic GAL4 drivers that can be matched with an equally large library of transgenic UAS lines expressing RNAi or GFP-tagged molecules. Optogenetics (the genetic expression of light-activated ion-channels: blue light for excitatory cations, and yellow light for inhibitory Cl\(^{-}\) ions; Matsunaga et al., 2013) is also possible in the fly, and the light-activated channels can be conditionally expressed using the UAS-GAL4 system (Brand & Perrimon, 1993). Thus, neurons could be excited or inhibited without the use of globally-acting drugs. Moreover, their expression can be limited to a specific cell type. Another somewhat recent development is the UAS-GCaMP fly line, which is a transgenic fly expressing a genetically-coded Ca\(^{2+}\) indicator. The gene product is a fusion of calmodulin and GFP, but the GFP is not fluorescent
unless $\text{Ca}^{2+}$ is bound to the calmodulin part of the indicator (Akerboom et al., 2013). Several different fluorescent proteins are available (Akerboom et al., 2013), so one could propose some elegant experiments combing signaling molecules. For example, DA cells could express GFP in mitochondria and a red fluorescent $\text{Ca}^{2+}$ indicator. Then one could correlate mitochondrial morphology with $\text{Ca}^{2+}$ signaling. Moreover, a red-shifted signaling molecule can be combined with the blue-light activated optogenetic system (Fiala et al., 2010). Thus, a specific population of neurons could be activated/inhibited via optogenetics, and the subsequent $\text{Ca}^{2+}$ signaling measured.

Quantifying DA Neurodegeneration

We used two methods to quantify DA neuron degeneration: live-tracking with genetically expressed GFP specific to DA neurons, and immunocytochemistry. We used the live-GFP signal in UAS-GFP;TH-GAL4 flies to track DA neurons over time and found that the number of GFP+ cells increased from 3 DIV to 9 DIV, but did not increase in cultures treated with MPP+. We also find that this effect of MPP+ is specific to DA neurons, as both GABAergic and cholinergic neurons were unaffected by the same dose of MPP+ that damaged DA neurons.

Immunocytochemistry also detected a decrease in the number of DA neurons following treatment with MPP+. However, the total number of DA neurons detected by anti-TH antibody (TH+) decreased over time, as opposed to the GFP+ cells in the live-tracking experiments, which saw an increase in DA neurons over time. This prompted us to examine the overlap between the signals from the two methods. We found that overlap
is better at 3 DIV (81%) rather than at 9 DIV (57%). However, these percentages are similar to those found in other studies and in various models – including mammals (Pesah et al., 2005; Friggi-Grelin et al., 2003; Matsushita et al., 2002; Meng et al., 2008). More work should be done in correlating the GFP signal with TH expression, as the understanding of this could influence the interpretation of experiments such as ours. For example, the change in TH expression could be an early event in the degenerative process. Or, cells may change their TH expression pattern throughout the experiments regardless of treatment. For example, in a DA neuron from UAS-GFP;TH-GAL4 culture, at 3 DIV the DA neuron may express TH and GFP. In this line, GFP is driven by the same promoter as TH, so the cell should be positive for both GFP and anti-TH signals. However, at 9 DIV the cell may not be expressing TH at the same level as in 3 DIV, and thus is anti-TH negative. The TH negative cell remains GFP+, although, because GFP is very stable in the cell and is still present even though the cell is not actively expressing the TH driver for GFP. Thus, at 3 DIV more DA neurons are expressing TH or expressing TH at a higher level, than at 9 DIV. And this change in expression is responsible for the decrease in overlap between signals. Regardless, both methods detected a loss of DA neurons. And GFP+ DA neurons do disappear (i.e. die – confirmed by bright-field image) from one time point to the next. And this happens more often PD-like treatment groups than in control groups (Park & Lee, 2006).

Lastly staining with anti-caspase antibody or propidium iodide (PI) further supported that our measurements were detecting cell death. Dim GFP+ signals were more likely to be overlapped with markers of cell death in MPP+-treated cultures. This
makes sense, because the GFP signal is likely being degraded by caspases during the death process. Thus, GFP+ cells that lose their fluorescence are showing signs of degeneration. And this further supports our criteria for quantifying cell death in the live-tracking experiments as a decrease in fluorescent intensity.

Quantifying Mitochondrial Damage in DA Neurons

In addition to measuring cell death by counting the remaining number of DA neurons, we examined early events in the degenerative process by testing mitochondrial morphology between treatment groups. To examine the structural changes of mitochondria in the disease process, we employed a modified version of an ImageJ macro by Dagda et al., 2009 to analyze the mitochondria using 4 parameters. Using both genetic tools and immunocytochemistry, we measured mitochondrial morphology specifically in DA neurons.

Our method tested non-somatic mitochondria (i.e. mitochondria in the neuritic projections), which are likely more important for the synaptic signaling and functionality than somatic mitochondria (Cheng et al., 2010; Court & Coleman, 2012). Indeed, axons are thought to possess a unique, genetically-controlled self-destruction program which functions somewhat in parallel to apoptosis (Barrientos et al., 2011), and mitochondria are a major part of the mechanisms controlling axonal degeneration (Court & Coleman, 2012). We validated our quantification of non-somatic mitochondria by successfully finding differences in the DA mitochondria of cells with mutated DRP1 or OPA1, both of which show a distinct mitochondrial morphology. 

\textit{drp1} mutants have mitochondria
which are unable to divide and we found them to have fewer, but larger, more interconnected, and more elongated mitochondria compared to control. *opa1* mutants, which have decreased mitochondrial fusion, also had fewer mitochondria but were smaller, less interconnected, and less elongated as control. Thus, we successfully limited our analysis both spatially (measuring mitochondria only in dendritic/axonal projections) and specifically to a cell type (DA neurons).

This method measures 4 parameters of mitochondrial morphology: number, size, interconnectivity, and elongation. An example of the importance of measuring multiple parameters for mitochondrial morphology is illustrated by our results for *drp1* mutants. Cells with mutated DRP1 had an increase in size and interconnectivity compared to control, but the number of mitochondria was lower than in control. This decrease in the number of mitochondria is explained by the high interconnectivity score – there are fewer mitochondria because most of them are connected to each other and counted as one, large mitochondria. Although the increase in interconnectivity and size is normally associated with healthy mitochondria, homozygous *drp1* mutations are lethal. Thus, the decreased number of mitochondria are important feature in determining the health of the cell. Functional neurons are likely to have many large, well-connected, and elongated mitochondria. Any deviation, even in one parameter, could indicate dysfunction. For example, an increase in size may indicate a pathological swelling rather than normal increase in size from a rescue treatment. In summary, we recommend a comprehensive assessment of mitochondrial morphology using multiple parameters.
Both methods of detecting DA mitochondria (genetic expression of GFP and immunocytochemistry/MitoTracker-based) found that MPP\(^+\) damaged mitochondrial morphology (i.e. mitochondria were fragmented). Thus, our data support the link between PD and mitochondrial dysfunction. However, the observed values in some parameters were different between the two methods. Compared to the live-GFP method of detecting DA mitochondria, the immunocytochemistry method found a slightly higher number of mitochondria (91 versus 83), a slightly smaller size (1.4 versus 1.6), a much lower interconnectivity score (1.5 versus 2.0), and a much higher elongation score (1.5 versus 1.2). We attribute these changes to incomplete overlap between the anti-TH and MitoTracker signals or incomplete staining by MitoTracker. In immunocytochemistry colocalization method, both the anti-TH and MitoTracker signals are each painted by an independent threshold, which paints all the signals above a certain intensity. Optimizing the threshold for colocalization between each signal was challenging. Setting either too high would produce many overlap artifacts, while too low of a threshold missed measurements. The threshold for the MitoTracker signal was the same as that used for the live-GFP signal. In an experiment that stained UAS-mtGFP/CyO;TH-GAL4 cultures with MitoTracker (in which DA neurons have GFP tagged mitochondria and are also stained by MitoTracker), manual counts as well as morphometric analysis confirmed that the same intensity threshold for both signals gave less than a 10% difference in magnitude.

For the immunocytochemistry method, the anti-TH signal generally saturated the cell body and all the cellular projections, and usually separated from the background
intensity quite well. Thus, the threshold for the anti-TH signal had a sigmoidal, ON/OFF, pattern of painting, i.e., increases or decreases in the threshold did not alter the painted image. However, incomplete staining by MitoTracker or the anti-TH antibody could have produced “hard” edges that would make the colocalized signals appear less round and thus have a higher elongation score. Likewise, incomplete staining by MitoTracker could reduce the size and number of mitochondria measured during analysis.

Furthermore, a non-ideal painting of the signal by the threshold function could have over or undercounted the signals. For example, a large mitochondrion may not have been painted completely by the threshold and counted as two instead of one mitochondria. Thus, differences in the values of the parameters between both methods of detecting mitochondria are to be expected.

Another possibility for the difference in values between the two methods is that the GFP trafficked to the mitochondria may affect their structure/function. GFP is generally considered nontoxic to cells (Goldman & Roy, 2000). Some reports, however, do show that GFP negatively affects cellular functions (Liu et al., 1999; Baens et al., 2006). Thus, it is possible that the difference in mitochondrial morphology in the UAS-mtGFP/CyO;TH-GAL4 flies is due to GFP toxicity. Regardless, both methods (immunocytochemistry and genetic labeling with GFP) found the same trend between control and MPP⁺-treated groups – a decrease in all parameters when treated with MPP⁺.
Neuroprotection by D2 Agonists

A true neuroprotective therapy for PD would decrease DA neurons vulnerabilities, keep ROS at basal levels, maintain the cell’s normal functioning, and ultimately stop the DA neurons from degenerating. We address the last point by showing that quinpirole indeed prevents the loss of DA neurons in our cell culture model (Wiemerslage et al., 2013), and we explore the preceding points by testing mitochondrial morphology. Normally shaped mitochondria should be a sign of normal cell functionality and thus, neuroprotection. We found that at 7 DIV, quinpirole fully prevented changes in mitochondrial morphology from PD-like insults. However, at 9 DIV, quinpirole only partially rescued the number of DA neurons. The timing of when the measurements were taken can explain this mismatch in rescue effect. Measurements for mitochondrial morphology were taken at 7 DIV, while measurements for actual cell death were taken at 9 DIV. It is possible that when measurements were taken at 7 DIV, MPP⁺+quinpirole treated cells had not yet succumbed to their eventual degeneration, i.e., quinpirole’s rescue effect may not be true protection, but merely a slowing of the degenerative process – the cells are going to degenerate, but simply are not yet showing the effects at 7 DIV. Thus, our results show a correlation between rescue by quinpirole treatment and mitochondrial morphology. However, this “rescue” by quinpirole is only a temporary or incomplete one. The underlying disease process (i.e. the MPP⁺ treatment) is still present, and will eventually cause cell death. However, this would suggest that quinpirole is altering the progression of the disease, yet clinical studies find that D2 agonists do not prevent further progression of PD (Loehle and Reichmann, 2010). It
would be interesting to determine if quinpirole extends the lifespan of DA neurons, and if so, whether mitochondria are an important factor.

Alternatively, the rescue in mitochondrial morphology by quinpirole may not be indicative of complete neuroprotection. Quinpirole treatment may be keeping the cell’s mitochondria healthy and functioning, but the neurons are still under the influence of MPP\(^+\) and are going to degenerate regardless of the status of their mitochondria. Thus, future experiments should confirm whether a rescue in mitochondrial morphology indeed maintains cellular functionality.

D2 Autoreceptors are Required for Rescue by D2 Agonists

Quinpirole did not rescue DA neurons or their mitochondrial morphology from PD-like treatment in cells lacking D2 receptors or D2 autoreceptors. Neither the number of DA neurons, nor any of the parameters for mitochondrial morphology were rescued in either pan-neural DD2R-RNAi or with DD2R-RNAi limited only to DA neurons. Thus, D2 autoreceptors are required for the rescue of DA neurons and their mitochondrial morphology by quinpirole.

D2 receptors have an inhibitory effect on the cell’s membrane potential (Neve et al., 2004). Furthermore, quinpirole hyperpolarizes the cell membrane and decreases the firing rate of DA neurons in vitro (Fasano et al., 2010; Wiemerslage et al., 2013). We suggest that this inhibitory signaling from D2R activation could be a neuroprotective mechanism. Indeed, the classic Na\(^+\)-channel blocker TTX rescued the number of DA neurons even in cells lacking DD2R. We recapitulate those findings with experiments
examining mitochondrial morphology: TTX also rescued mitochondrial morphology from MPP\(^+\) treatment, even in cells lacking DD2R. However, the observed rescue was only partial – TTX did not keep the number of mitochondria at control levels, but all other parameters of mitochondrial morphology were maintained. Thus, the inhibitory effect of quinpirole is not the only neuroprotective mechanism in our model.

Other likely neuroprotective mechanisms of quinpirole are antioxidant effects or changes in G-protein signaling. Our results do not support the idea that D2 agonists have antioxidant effects. If quinpirole did function as an antioxidant, then the DD2R mutants should show at least some protection from MPP\(^+\) treatment. 10 \(\mu\)M Quinpirole, however, had no rescue effect against MPP\(^+\)-induced mitochondrial morphology or cell death in cells lacking DD2R. Nonetheless, other D2 agonists do have antioxidant effects (Herrero et al., 2011). The D2 agonist pramipexole has antioxidant properties at doses \(\geq 5 \ \mu\)M in cultured mouse cells (Le et al., 2000). Other D2 agonists, such as ropinirole, require higher concentrations (in the mM range) for ROS scavenging activity (Iida et al., 1999). Nonetheless, antioxidants fail to show neuroprotective benefits in the clinic (Loehle & Reichmann, 2010), and our results also offer no support for antioxidant effects of D2 agonists. Thus, it seems that the cell’s best defense against ROS is to simply limit the reactions that produce them by regulating the level of \(O_2\) and \(Fe^{2+}\) in the mitochondrial matrix (Miwa et al., 2008). Mitochondria can reduce the conversion of \(O_2\) to superoxide radicals in the matrix by allowing \(H^+\) ions into the matrix to form water with \(O_2\) (Islam et al., 2012). \(Fe^{2+}\) forms hydroxyl radicals via Fenton chemistry, so limiting the amount of Fenton chemistry-capable metal ions will minimize hydroxyl formation (Miwa et al., ...)
Thus, antioxidants are probably not going to be the most fruitful direction of study in neuroprotective research because changing the properties/levels of $O_2$ or $Fe^{2+}$ will have major consequences for the cell. An efficacious neuroprotective therapy will likely reduce ROS indirectly by other signaling pathways, such as reducing cellular excitability.

TTX may not fully rescue the number of mitochondria because it only blocks $Na^+$ to reduce excitability, i.e., the G-protein signaling properties (and subsequent inhibitory signaling) from quinpirole are not activated. D2 receptors are coupled to G-proteins and include ion channels as effectors (Neve et al., 2004). In addition to an inhibitory effect, quinpirole may also activate downstream signaling molecules such as PKA. Indeed, in a collaboration, we show that forskolin (an activator of PKA) rescues mitochondrial morphology and cell death (Huang et al., in review). Thus, because quinpirole led to a full rescue of mitochondrial morphology but TTX did not, the inhibitory effect is not sufficient for total rescue. Quinpirole could have additional mechanisms including downstream signaling effects. One possible effector is the dopamine transporter (DAT). An in vivo study in Drosophila showed that TTX reduced the activity of DAT (Makos et al., 2009). Thus, the neuroprotection we see in our model could be due to less MPP$^+$ entering the cell through the DAT.

High Levels of $Ca^{2+}$ is a vulnerability in DA neurons

The results from our experiments with the $Ca^{2+}$-chelator EGTA and the $Ca^{2+}$-channel mutants support the theory that $Ca^{2+}$ signaling is a source of vulnerability in DA
neurons. EGTA rescued the number of DA neurons from PD-like treatment. Furthermore, cultures with mutated Ca\textsuperscript{2+}-channels were inherently protected from PD-like treatment: both in mitochondrial morphology and in the number of DA neurons.

DA neurons are hypothesized to have an inordinate intracellular Ca\textsuperscript{2+} burden (Surmeier et al., 2012). Ca\textsuperscript{2+} is tightly regulated by cells, but the DA neurons that are vulnerable in PD have Ca\textsuperscript{2+}-channels which are open more than usual. Vulnerable DA cells suffer Ca\textsuperscript{2+} influxes beyond what is typical for a neuron because instead of using monovalent ion channels for their autonomous pacemaking activity, they rely on L-type Ca\textsuperscript{2+}-channels (Costa et al., 2008; Surmeier et al., 2011). Intracellular Ca\textsuperscript{2+} must be safely stored or exported against a large concentration gradient and this process consumes ATP. Moreover, mitochondria store excessive Ca\textsuperscript{2+} in the matrix, which consequently depolarizes the mitochondrial membrane potential, increases ROS, reduces ATP synthesis, and will eventually open the permeability transition pore and promote cell death if levels are high enough (Surmeier et al., 2011, Hurley and Dexter, 2012). Thus, hyperpolarizing the cell membrane or reducing activity may be neuroprotective by preventing damaging Ca\textsuperscript{2+} influxes and the subsequent apoptosis signaling. If simply hyperpolarizing the cell membrane is neuroprotective, ion channels make obvious targets. Indeed, L-type Ca\textsuperscript{2+}-channel blockers prevented degeneration in a toxin-based, mouse model of PD (Ilijic et al., 2011). And interestingly, activation of D2R also decreases activity of L, N, and P/Q Ca\textsuperscript{2+}-channels (Neve et al., 2004).

Ca\textsuperscript{2+} levels were manipulated in two ways in our experiments: chelation with EGTA and exclusion via mutations of Ca\textsuperscript{2+} channels. Ultimately, both are preventing an
increase in cytosolic Ca\(^{2+}\) concentration, but both accomplish this via different mechanisms. EGTA is a highly specific Ca\(^{2+}\) chelator. Thus, intracellular Ca\(^{2+}\) levels should decrease from EGTA treatment by disrupting the concentration gradient of Ca\(^{2+}\). And in the Ca\(^{2+}\)-channel mutants, less Ca\(^{2+}\) should be entering the cell. Thus, cells in both conditions should be less susceptible to excitotoxic Ca\(^{2+}\) influxes and the subsequent cell death signaling. When excessive Ca\(^{2+}\) enters the cell and is buffered by the mitochondria, ROS increases (Goldberg et al., 2012; Dryanovski et al., 2013). And this increase in ROS is thought to lead to cell death (Herrero et al., 2011). Ca\(^{2+}\)-channel mutant cultures were protected from MPP\(^{+}\)-induced degeneration in terms of mitochondrial morphology, as well as cell death. EGTA treated cultures were also resistant to MPP\(^{+}\)-induced cell death. Thus, any treatment that decreases the intracellular Ca\(^{2+}\) concentration may be protective to DA neurons. Indeed, several studies have found a variety of Ca\(^{2+}\) signaling modulations that are neuroprotective: blocking Ca\(^{2+}\) channels with fendiline restored climbing behavior in a Drosophila model of PD (Hillman et al., 2012), an inhibitor of store-operated Ca\(^{2+}\) entry (SOCE) protected PC12 cells from MPP\(^{+}\)-induced degeneration (Chen et al., 2013), and in rats, modulation of a T-type Ca\(^{2+}\)-channel protected DA neurons from PD-like treatment (Xiang et al., 2011).

However, not all intracellular Ca\(^{2+}\) leads to pro-death signaling. Ca\(^{2+}\) signaling between the endoplasmic reticulum (ER) and mitochondria regulates cellular energetics and can promote cell survival. The relationship between intracellular Ca\(^{2+}\) levels and cell survival is parabolic, i.e., too much or too little intracellular Ca\(^{2+}\) leads to cell death (Michel et al., 2013). Too little Ca\(^{2+}\) storage in the ER activates SOCE and causes a
prolonged increase in cytosolic Ca\(^{2+}\) levels (Parekh & Putney, 2005). Thus, therapeutic manipulations of Ca\(^{2+}\) need to be well planned to keep the cell functioning normally. In our experiments, the Ca\(^{2+}\)-channel mutation did not appear to be an ideal neuroprotective therapy because there was only a partial rescue in mitochondrial morphology.

Time-Course of Neurodegeneration

We also explored the time-course of the disease process in our model. We showed that MPP\(^+\) decreases the number of DA neurons after 3 days of chronic exposure (from 3 DIV to 6 DIV). However, changes in mitochondrial morphology happen as early as 24 hours after treatment – supporting the theory that mitochondrial dysfunction is an early event in PD. Others found similar results in other models. Goyal et al., 2007 observed in Drosophila that damaged mitochondria preceded caspase activation, apoptosis, and the appearance of phosphatidylserine. Overall, strong evidence exists that changes in mitochondrial morphology are predictive of cell death in PD (Wang et al., 2011, Martinou & Youle, 2011), and our results further support this idea.

When to take measurements was determined based on experience with the live-tracking experiments and experiments with BrdU. Few (roughly 1 per 1000) cells incorporated BrdU when it was administered at 3 DIV. Cells at 3 DIV also have defined projections, and GFP signals from UAS-mtGFP/CyO;TH-GAL4 flies were much brighter than at 1 or 2 DIV. Thus, 3 DIV was the time-point in which we added drugs and took baseline measurements, as these cells were considered post-mitotic. We quantified the number of DA neurons at 9 DIV because MPP\(^+\) did not have an effect at early DIV (e.g. 5
DIV). Our cultured neurons are viable up to roughly 14 DIV. Past 14 DIV, most neurons are showing blebbing, shorter projections with altered morphology. Measurements for mitochondrial morphology were made at 7 DIV because we anticipated mitochondrial degeneration to be an early event, and thus wanted to take measurements before the cells were degenerated.

Another aspect of timing in our experiments was when to add rescue therapy. As mentioned earlier, D2 agonists fail to show neuroprotective benefits in PD patients (Loehle and Reichmann, 2010), likely because they are not used before a majority of DA cell death has occurred. We find that quinpirole added concomitantly with MPP\(^+\) prevented changes in mitochondrial morphology (was neuroprotective), but does not repair cells or augment the disease when added 24 hours later following MPP\(^+\) treatment (was not neurorestorative). Overall, healthy mitochondria were greater in number, larger, more interconnected, and more elongated. Thus, our results suggest that maintaining this healthy phenotype will rescue mitochondria. Indeed, when mitochondria are driven towards fusion by mutation of \(drp1\), DA cells are protected from PD-like treatment (Berman et al., 2008). However, it remains unknown if any therapy can reverse mitochondrial damage after it has been induced by MPP\(^+\) treatment. Such a therapy would likely be neuroprotective in PD. If mitochondrial damage is an early, causative event, then reversing the damage should slow the progression of actual PD. It is unknown, however, if it is possible for a cell to recover in this way. Continued survival of the cell is considered implausible once the membrane permeability pore has formed and released cytochrome c into the cytosol (Tait & Green, 2010). But some experiments
have shown that mitochondrial recovery is possible. Cells can survive laser ablation of individual mitochondria and their release of cytochrome c, so long as less than 15% of mitochondria are damaged (Khodjakov et al., 2004). Moreover, neuronal mitochondria can reabsorb previously released cytochrome c and restore their morphology when treated with nerve growth factor (NGF; Martinou et al., 1999). This rescue, however, has not yet been shown in cells afflicted with PD-like pathology, but our model could rapidly test therapies for this effect.

Final Conclusions and Future Directions

In summary, this work develops a unique method for screening neuroprotective agents for PD. Our results support the idea that mitochondrial damage/dysfunction is an early event in PD. We found that rescue agents must be added almost concomitantly with the disease onset to be neuroprotective. We found that the neuroprotective mechanism of D2 agonists is largely (but not entirely) from an inhibitory effect on the cell and requires the expression of D2 autoreceptors. Lastly, we support the theory that Ca$^{2+}$ is a source of vulnerability for DA cells in PD.

Future experiments could explore the relationship between inhibition and neuroprotection in PD. Using optogenetics, one could easily limit excitation and inhibition both spatially (to specific cell populations like DA neurons) and temporally (testing many different treatment patterns of light excitation: 1 minute on/off, strobe, constant, etc.). An experiment could also test the effect of excitation and inhibition on mitochondrial morphology. Excessive excitation would likely damage mitochondrial
morphology by increasing intracellular Ca\(^{2+}\) levels. Excessive inhibition, however, may also damage mitochondrial morphology. Some studies show that DA neurons need a certain level of stimulation to survive (Michel et al., 2013). The excessive inhibition may prevent adequate levels of Ca\(^{2+}\) and signal for an overall decrease in mitochondria.

Questions remain about the signaling pathway between D2Rs and mitochondrial morphology. D2R agonists activate Gai subunits in *Drosophila* (Hearn et al., 2002), which inhibit the production of cAMP (Birnbaumer, 2007). Several transgenic lines are available in *Drosophila* that manipulate G-protein subunits and cAMP signaling/production. Thus, one could easily test intracellular events that modulate the rescue effect of D2 agonists.

Lastly, genetically encoded Ca\(^{2+}\) indicators (GECIs) such as GCaMP could be used to test how mitochondria, Ca\(^{2+}\), and PD are interrelated. GCaMP could be used to confirm whether DA cells treated with EGTA or DA cells in the Ca\(^{2+}\)-channel mutants had a lower level of intracellular Ca\(^{2+}\). And a specific GECI tagged to the mitochondrial matrix (Akerboom et al., 2013) could be used to examine Ca\(^{2+}\) levels specifically in mitochondria. We would expect Ca\(^{2+}\) levels in mitochondrial to increase following PD-like treatment and decrease following treatment with quinpirole.
REFERENCES


Keeney, PM, Xie, J, Capaldi, RA, and Bennett, JP, Jr (2006). Parkinson’s disease brain mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and misassembled. *Journal of Neuroscience* 26, 5256–64.


mitochondrial fission by the Parkinson disease-associated protein alpha-synuclein.


Neve KA, Seamans JK, Trantham-Davidson H (2004). Dopamine receptor signaling.

*Journal of Receptors and Signal Transduction* 24(3):165-205.


APPENDIX A: CODE FOR ANALYSIS OF MITOCHONDRIAL MORPHOLOGY  
(LIVE-GFP METHOD)

// Measures the mitochondrial morphology of mitochondrial particles in a cell

macro "Measure mitochondrial Morphology [F8]" //opens image for analysis when F8 is
//pushed
{
    open();
    run("Set Measurements...", "area integrated limit display redirect=None decimal=3");
    setTool(3); //activates freehand selection tool
}

macro "Process RGB Image [F9]" //applies threshold when F9 is pushed
{
    run("Clear Results");
    setThreshold(850, 4095); //adjust threshold here if necessary
}

macro "Measure Mitochondria [F10]" //performs calculations and prints results when
//F10 is pushed
{
    run("Clear Results");
    run("Set Measurements...", "area perimeter circularity redirect=None decimal=2");
    run("Analyze Particles...", "minimum=5 maximum=500 bins=1000 show=Outlines
display summarize");
    for (i=0; i<nResults; i++) {MP+= getResult('Perim.', i);
        MA +=getResult('Area', i);
        MC +=getResult('Circ.', i);
    }
    AMP= (MP/i); //calculates mean perimeter
    AMA= (MA/i); //calculates mean area
    AMC= (MC/i); //calculates mean circularity
    Rmorph= (AMA/AMP); // Measures the area perimeter ratio (interconnectivity) of
//mitochondria
    print(getTitle());
    print("Count:" +i);
    print("Total Area Mitos:" +MA);
    print("Perimeter Mitos:"+MP);
    print("Circularity mitos:"+MC);
    print("Avg. Perimeter:" +AMP);
    print ("Avg. Area:" + AMA);
    print ("Avg. Circularity:"+ AMC);
print("Area/Perim:"+ Rmorph);
selectWindow("Results");
selectWindow("Log");
}

class macro "Close All Windows [F4]" //closes all active windows with F4 is pushed
{
    while (nImages>0) {
        selectImage(nImages);
        close();
    }
}
APPENDIX B: CODE FOR ANALYSIS OF MITOCHONDRIAL MORPHOLOGY

(IMMUNOCYTOCHEMISTRY METHOD)

// Displays the colocalization of two images and then measures the morphometrics of the
// overlapped signal

macro "Close All Windows [F11]" // F11 used as a shortcut to close all active windows
{
    while (nImages>0) {
        selectImage(nImages);
        close();
    }
}

macro "Open Images for colocalization [F8]" // F8 used as shortcut
{
    open(); // select red image
    run("8-bit"); // makes image 8-bit
    open(); // select green image
    run("8-bit"); // makes images 8-bit
    run("Colocalization Highlighter", "ratio=50 threshold_channel_1=52 threshold_channel_2=52 display=255 colocalized"); // adjust threshold here if necessary
    setTool(3); // activates freehand selection tool for drawing around region of interest
    selectWindow("Colocalized points (8-bit) "); // selects image containing only colocalized // signals
    setThreshold(15, 255); // threshold setting not important here
}

macro "Measure Morphometrics [F10]" // F10 used as shortcut
{
    run("Clear Results");
    run("Set Measurements...", "area perimeter circularity redirect=None decimal=2");
    run("Analyze Particles...", "minimum=5 maximum=500 bins=100 show=Outlines display summarize"); // set size range of particles here
    for (i=0; i<nResults; i++)
    {
        MP += getResult('Perim.',i);
        MA += getResult('Area', i);
        MC += abs(getResult('Circ.', i)); // old version of ImageJ reports circularities of -1, so
        \absolute value needed here
    } // loop to assign variables from results
    AMP= (MP/i); // calculates mean perimeter
    AMA= (MA/i); // calculates mean area
AMC = (MC/i); // calculates mean circularity
Rmorph = (AMA/AMP); // measures the area perimeter ratio, a.k.a. interconnectivity
print(getTitle());
print("Count:" + i);
print("Total Area:" + MA);
print("Avg. Perimeter:" + AMP);
print("Avg. Area:" + AMA);
print("Avg. Circularity:" + AMC);
print("Area/Perim:" + Rmorph);
selectWindow("Results");
selectWindow("Log");
}