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Parkinson's disease and a dopamine-derived neurotoxin, 3,4-Dihydroxyphenylacetaldehyde : implications for proteins, microglia, and neurons

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PARKINSON'S DISEASE AND A DOPAMINE-DERIVED NEUROTOXIN,
3,4-DIHYDROXYPHENYLACETALDEHYDE: IMPLICATIONS FOR PROTEINS,
MICROGLIA, AND NEURONS

by

Laurie Leigh Eckert

<u>An Abstract</u>

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of
Philosophy degree in Pharmacy (Medicinal and Natural Products Chemistry) in the
Graduate College of The University of Iowa

December 2012

Thesis Supervisor: Associate Professor Jonathan A. Doorn

ABSTRACT

Parkinson's disease (PD) is a prevalent neurodegenerative disorder for which the greatest risk factor is age. Four to five percent of 85-year-olds suffer from this debilitating disease, which is characterized by the selective loss of dopaminergic neurons within the substantia nigra and the presence of protein aggregates known as Lewy bodies. While the etiology of this disease is still unknown, recent research implicates oxidative stress, activated microglia, and reactive dopamine (DA) metabolites to play a role in the initiation or progression of the disease. Activated microglia cause injury to dopaminergic neurons via a host of mechanisms, including reactive oxygen species production, release of cytokines, and phagocytic activity. Microglial activation has been detected in the brains of PD patients, but the source of this activation has not been elucidated. Previous research has shown electrophiles and endogenous neurotoxins to play a role in this microglial activation. The interaction between the neurotoxic metabolite of DA, 3,4-dihydroxyphenylacetaldehyde (DOPAL), and microglia has not been explored.

DOPAL is a highly reactive, bifunctional electrophile produced by oxidative deamination of DA by monoamine oxidase (MAO). DOPAL is oxidized in the major metabolism pathway to 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase (ALDH). DOPAL has previously been shown to be 100-fold more toxic than DA *in vitro* and *in vivo*. Potent inhibition of the rate-limiting enzyme in DA biosynthesis, tyrosine hydroxylase, by DOPAL has been well-established. DOPAL-mediated aggregation of α-synuclein, the primary component of PD-hallmark Lewy bodies, has been suggested but was further explored in this work.

Results presented in this body of work include further determination of the aggregation of α-synuclein by DOPAL, including evidence of covalent modification. The interaction of DOPAL with BV-2 microglia, an immortalized cell line, was addressed in depth through exploration of DOPAL catabolism, toxicity, and generation of an activational response. Metabolism of DOPAL to DOPAC was altered in activated microglia, with the production of DOPAC reduced by ~40%. Metabolism of DOPAL to DOPAC was also inhibited by both 4-hydroxynonenal and malondialdehyde, gold standards of lipid peroxidation. Both of these compounds were found to be significantly toxic to BV-2 cells at concentrations well below those considered toxic to dopaminergic cells. Alternatively, DOPAL and DA were found to be non-toxic to this cell line, while DOPAL was shown to be significantly toxic to dopaminergic cells at concentrations as low as 10 μM.

Significant activation of BV-2 microglia by DOPAL was observed at 10 μM and above by release of TNF-α. Morphological changes, release of IL-6, and changes in expression of COX-2 also indicated activation by DOPAL but not DA or DOPAC. BV-2-conditioned media, generated by incubation with DA, DOPAL, or DOPAC, was added to MN9D cells, and toxicity was measured by the MTT assay. BV-2 conditioned media generated by DOPAL incubation produced the greatest toxicity for MN9D cells. These results implicate DOPAL in dopaminergic cell death through microglial activation.

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Thesis Supervisor

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Thesis Supervisor: Associate Professor Jonathan A. Doorn

Graduate College
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Iowa City, Iowa

CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Pharmacy (Medicinal and Natural Products Chemistry) at the December 2012 graduation.

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Classrooms and labs!
Loud boiling test tubes!
Sing to the Lord a new song!

He has done marvelous things.
I too will praise Him with a new song!

Herbert F. Brokering,
"Earth and All Stars"

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide .... MTT

3,4-Dihydroxyphenylacetaldehyde .... DOPAL

3,4-Dihydroxyphenylacetic acid .... DOPAC

3,4-Dihydroxyphenylalanine ....DOPA

3,4-Dihydroxyphenylethanol ....DOPET

3-Methoxy-4-hydroxyphenylacetaldehyde ....MOPAL

3-Methoxytyramine.. 3-MT

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid) .... HEPES

4-Hydroxy-2-nonenal.. 4HNE

8-Anilino-1-naphthalenesufonic acid..ANS

α-Synuclein ....α-syn

Aldehyde dehydrogenase .... ALDH

Aldose-Ketose Reductase .... AKR

Bicinchoninic acid .... BCA

Bovine Serum Albumin ....BSA

Catechol-*O*-methyltransferase ....COMT

Circular dichroism ....CD

Cyclooxygenase 2 ....COX-2

Deep Brain Stimulation..DBS

Dimethyl sulfoxide...DMSO

Dopamine...DA

Dulbecco's Modified Eagle Medium...DMEM

CHAPTER ONE

INTRODUCTION: PARKINSON'S DISEASE

History, Symptoms, and Treatment

James Parkinson published "An Essay on the Shaking Palsy" in 1817, in which he described six cases to illustrate the disease he had observed.[1] Each of the patients (or people on the neighborhood street) had a resting tremor and difficulty walking. This publication was the first on the disease that would later be named for its author. Jean-Martin Charcot spoke of Parkinson's work 50 years later and suggested naming the disease after Parkinson himself. To this day, the disease is known for the person to first publish a thorough description of the disease's signs and symptoms.[2]

Toward the end of the 19th century and into the beginning of the 20th century, a number of scholars began to describe some of the pathological characteristics of the disease, including loss of pigment, gliosis, and degradation of the substantia nigra in the striatal region of the brain, (Blocq and Marinesco, Brissaud, Tretiakoff) and the presence of Lewy bodies (Lewy, Tretiakoff).[2, 3] Further studies of the disease pathology continued into the 20th century, and the publication of the work of Greenfield and Bosanquet in 1953 led to a widespread acceptance of the disease pathology: loss of cells within the substantia nigra and presence of Lewy bodies (cytoplasmic protein aggregates).[2-4]

In 1957, two key findings were published that ultimately led to a much greater understanding of Parkinson's disease (PD). Montagu first reported presence of dopamine (hydroxytyramine, DA) in brain tissue from various mammals,[5] and Carlsson indicated that administration of a racemic mixture of 3,4-dihydroxyphenylalanine (DOPA, the metabolic precursor to DA, Scheme 1.1) to mice treated with reserpine led to an

antagonistic action toward the tranquilization produced by the drug.[2, 6] A further understanding of DA quickly followed with work demonstrating DA was localized to the striatal region of the brain.[7] It was with this publication Carlsson first suggested the symptoms of PD could be duplicated with treatment of reserpine, which depletes striatal DA levels. Carlsson went on to win a Nobel Prize for this and related discoveries.[2] Confirmation of the depletion of DA in the striatum in PD was performed in 1960,[8] and two years later, two groups (Birkmayer and Hornykiewicz and Barbeau) explored administration of racemic and L- DOPA in PD patients.[2, 9] In 1997, Spillantini et al. established α-synuclein as a primary component of Lewy bodies in idiopathic PD.[10]
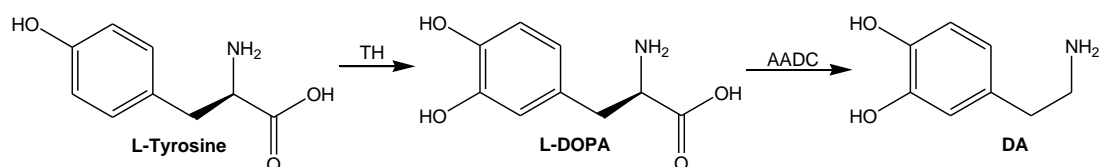
Scheme 1.1   Biosynthesis of DA from L-Tyrosine by tyrosine hydroxylase (TH) and amino acid decarboxylase (AADC).

It is estimated over 1 million Americans suffer from PD today. Risk of incidence increases with age, with 4-5% of people 85 years old affected.[11] PD patients diagnosed today would be cited as having a number of motor and non-motor symptoms Parkinson described himself. Motor symptoms include bradykinesia, or difficulty in making voluntary movements; resting tremor, most often in the limbs; rigidity; and changes in gait, typically observed in a shuffle with little lifting of the feet and a leaning forward of the torso.[12] Non-motor symptoms may include, but are not limited to, loss of olfaction, depression, dementia, sleep disorders, pain, nausea, and hallucinations.[13] It is thought

that patients have usually lost at least 75% of their dopaminergic neurons in the substantia nigra by the time symptoms appear.[3] Symptoms typically continue to worsen as patients age and more neurons die or lose the capacity to store supplemental DA received through L-DOPA therapy.

While researchers are currently trying to gain greater understanding of the disease, the etiology of PD still remains unknown. For this reason, in part, little has changed in the treatment of PD patients from treatments established in the 1960s. Frequently those with PD are administered L-DOPA to reduce symptoms. Unfortunately L-DOPA has a 'wearing-off' effect, which leaves patients without alleviation of symptoms at times. Additionally, long-term treatment with L-DOPA leads to complications, including dyskinesias during peak drug efficacy. In a study by Shaw et al. of 178 patients with PD undergoing L-DOPA therapy, only 37 patients still saw the same benefit of therapy after 6 years of treatment.[14] In part these effects are thought to be due to a continual loss of dopaminergic neurons as the disease continues to progress, and a loss of capability of these neurons to effectively metabolize or store DA.[15, 16]

Recent work has led to exploration of additional pharmacological intervention, including use of monoamine oxidase-B (MAO-B) inhibitors to prevent metabolism of DA to its aldehyde metabolite, 3,4-dihydroxyphenylacetaldehyde (DOPAL) and use of DA agonists to provide a more continuous delivery of DA to the brain and reduce and/or delay the administration of L-DOPA.[17] Halting the metabolism of DA with an MAO-B inhibitor would, in theory, allow for increased 'on' time for patients as metabolism of DA is slowed and reduce the dose of L-DOPA required.[18, 19] DA agonists were well-established for improvement of continuous DA delivery in PD patients by the end of the

1980s. Their use has allowed a delayed administration of L-DOPA and formation of dyskinesias in patients. Additionally, use of DA agonists has been shown to have some neuroprotective effects, including inhibition of apoptotic processes.[20] DA agonist administration has more recently been linked to adverse drug reactions including impulse control disorders and leg edema.[17]

Surgical intervention in the form of deep brain stimulation (DBS) has become more widely used in extreme cases of bradykinesias and dyskinesias. During DBS, specific regions of the brain, e.g. the subthalamic nucleus and globus pallidus internus, are stimulated which improve motor problems. This procedure does alleviate a number of symptoms, but unfortunately has its own risks, including the general risk of surgery for older patients; the population frequently affected with PD.[21]

<div align="center">Proposed Etiologies</div>

<div align="center">Genetics</div>

The majority of cases of diagnosed PD (70-90%) are considered sporadic; patients with the disease appear to have no genetic explanations for developing the disease.[22] However, recent work has begun to establish a number of genes that are altered in patients with genetic forms of PD may also play a role in the sporadic forms of the disease. While a number of genes have been implicated, including *SNCA, PRKN, PINK1, DJ-1* and *LRRK2*, for the purpose of this discussion, two genes and their corresponding proteins will be addressed; *SNCA* which expresses the synaptic protein α-synuclein and *DJ-1* which expresses the protein DJ-1.[23]

α-Synuclein is expressed throughout the brain as a pre-synaptic protein, where it associates with synaptic vesicles.[22] The small, 140-amino acid protein is known to play a

role in regulating levels of DA in the pre-synaptic terminal, likely through its interaction with synaptic vesicles.[23] Its wild-type sequence contains a region that assembles into an α-helix and a region that is highly prone to forming amyloid-type aggregates, which are observed even in sporadic cases of PD. These aggregates assemble into protofibrils and then insoluble fibril polymers, which contain β-pleated sheet conformations.[24] Protofibrils have been implicated as toxic in PD and aggregates of α-synuclein are the main component of the hallmark Lewy bodies seen in PD pathology.[22, 25] Two prevalent mutations, A53T and A30P have been observed. The more common A53T mutation leads to a more rapid formation of protofibrils and clinical manifestations of a more rapid deterioration. Aggregation of the A30P α-synuclein protein seems to parallel that of the wild-type seen in sporadic PD, as does the progression of the disease.[26]

DJ-1 is expressed throughout the brain and other tissues. It is most frequently observed as a cytosolic protein, but has been seen localized to the nucleus and mitochondria.[26] Function of the protein is not entirely well-characterized, but is thought to play a role in regulation of transcription, apoptosis, and act as a chaperone that inhibits aggregation of α-synuclein.[22] The most well-known and explored function of this protein is as a sensor of oxidative stress.[24, 27] The protein has been indicated to play some role in the control of the oxidative environment in the cell. Wild-type protein has been shown to be sensitive to covalent modification by DA and DA-quinone.[27] Mutations in the *DJ-1* gene have been indicated to change localization of the protein, likely preventing its ability to sense and regulate the oxidative environment in the cytosol.[26]

Transition Metals

Transition metals with redox capabilities are thought to be involved in the etiology and progression of PD, due to a loss of homeostasis. Increased levels of iron have been observed in the substantia nigra of patients with PD as compared to controls.[28] Increased Fe(II) can promote oxidative stress, a well-established component of PD. Increased levels of iron are able to participate in the well-established Fenton reaction to produce free hydroxyl radicals, Scheme 1.2.[29] Also, iron catalyzes lipid peroxidation through formation of peroxyl radicals. The final products of these radicals can rearrange to form the gold standard products of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxynonenal (4HNE).[30]

Copper (II), known to be elevated in the CSF of PD patients, can also participate in the Fenton reaction and alter the redox environment of the cell.[31] In addition to participation in the Fenton reaction, the ability of Cu(II) to catalyze oligomerization of α-synuclein has been well-established.[30, 31] Fe(II) is also indicated to play a role in α-synuclein aggregation.[29] Both of these metal-induced aggregates are thought to be even more toxic than the protofibrils of α-synuclein alone.

$$H_2O_2 \; + \; Fe^{2+} \quad \longrightarrow \quad OH\bullet \; + \; OH^- \; + \; Fe^{3+}$$

Scheme 1.2   The Fenton reaction leading to production of hydroxyl radical, mediated by Fe(II). This reaction can also take place in the presence of Cu(II).

Pesticide Exposure

Due to the high percentage of cases of PD that are sporadic, scientists have turned

to the environment to look for potential causes of the disease. A significant correlation

has been repeatedly observed between pesticide exposure and diagnosis of PD.[32] These

pesticides are of a number of classes, including insecticides, herbicides and fungicides,

some of which are shown in Figure 1.1.
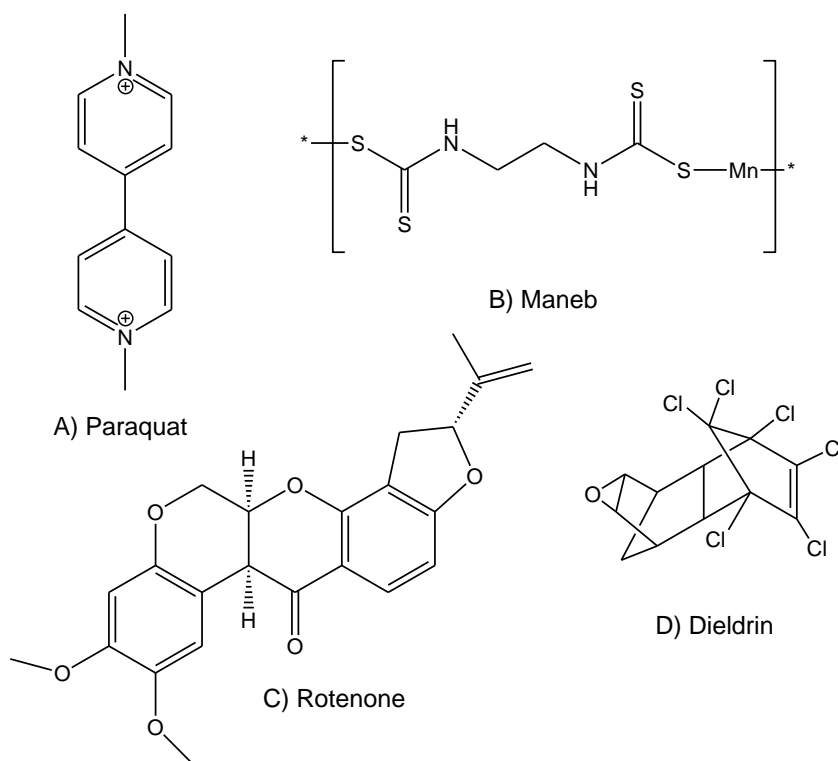
Figure 1.1   Structures of PD-relevant pesticides, including paraquat (A),
            maneb (B), rotenone (C), and dieldrin (D).

Exposure to the herbicide paraquat has been purported to play a role in disease

progression through oxidative stress. Paraquat (Figure 1.1, A) has frequently been used

as an animal model since its correlation to PD was discovered.[33] As a charged species, it

is thought to enter the brain through an amino acid transporter. Within a cell, the molecule generates high levels of oxidative stress through redox cycling and formation of superoxide anion.[34] Levels of reduced glutathione are dramatically depleted as the molecule also prevents reduction of oxidized glutathione. While paraquat is selective for striatal dopaminergic cells, only 20-30% cell death is observed after repeated administration.[33, 34]

Frequently administered with paraquat, both in pesticide application and PD animal models, the fungicide maneb is able to inhibit mitochondrial complex III.[35] Maneb (Figure 1.1,B) is a polymer of manganese ethylenebisdithiocarbamate. Co-administration of maneb and paraquat in animal models of PD leads to a synergistic toxic effect to the dopaminergic system.[34] The use of this 'multi-hit' model likely mimics a more similar situation to that for humans, however administration is frequently intra-venous or intra-peritoneal, which diminishes the relevance of the model to human exposure.[35]

The insecticide rotenone is a naturally-occurring molecule that has been well-characterized. It is known to act through inhibition of mitochondrial complex I.[33] Its lipophilicity lends easy crossing of the blood-brain barrier. Additionally, rotenone has been shown to inhibit proteasome activity.[35] It has a relatively short environmental half-life of 1-3 days, therefore, it is unlikely to produce PD via environmental exposure. In animal PD models, it is used as a mitochondrial inhibitor, producing 30% loss of substantia nigra dopaminergic neurons as compared to control, following intravenous infusion for 3 weeks.[33] However, the mitochondrial inhibition has been observed to be

non-specific. While this model may produce some PD-like symptoms, again, the

likelihood of PD in humans being caused by exposure to rotenone is very minimal.[34, 35]

Dieldrin is a persistent, bioaccumulative, organochlorine insecticide that has been

detected in the brains of patients with PD at levels greater than control subjects.[36] Early

symptoms of exposure include headache, nausea, and vomiting. The half-life of dieldrin

in humans was determined to be 396 days in the bloodstream of humans ingesting

dieldrin for 18 months. Cellular toxicity is thought to be due to a number of mechanisms,

including oxidative stress, mitochondrial dysfunction, and apoptotic mechanisms, to

name a few.[37] Recent research has shown elevations in the neurotoxic metabolite of DA,

DOPAL, in dopaminergic cells following exposure to dieldrin.[38]

Oxidative Stress

While a number of the proposed etiologies of PD discussed above produce

increased levels of oxidative stress, much research has been dedicated to understanding

the observed increases in oxidative stress in PD in and of themselves. Reduced levels of

GSH (reduced glutathione) are observed in the brains of PD patients, but the cause for

this decrease is unknown.[39] Another indicator of oxidative stress frequently observed in

PD is that of protein oxidation, specifically formation of products of lipid peroxidation.[40]

The gold standards used as evidence of this process include 4HNE and MDA, depicted in

Figure 1.2. Both of these molecules have been measured at increased levels in the brains

of PD patients, further indicating the presence of oxidative stress.[41, 42] Both electrophiles,

4HNE and MDA, are capable of forming stable, covalent adducts with nucleophilic

groups on proteins. Their reactivity with both amines and thiols can be detrimental for

various cellular processes. 4HNE and MDA have both been demonstrated capable of

inhibiting aldehyde dehydrogenase (ALDH).[43-46] Inhibition of this enzyme prevents

metabolism of the neurotoxic DA metabolite, DOPAL. This will be further discussed in

a later section.

In addition to increased lipid peroxidation products, research has indicated the

formation of reactive oxygen species (ROS) by DA and DOPAL.[47, 48] Both catechols are

capable of being oxidized to quinones through a one- or two-electron mechanism, which

produces reactive oxygen species as byproducts. Both the oxidized DA species and the

ROS are toxic to dopaminergic cells. The mechanism for this oxidation can be either

spontaneous or via metal or enzyme catalysis.

A) 4-hydroxy-2-nonenal   B) Malondialdehyde
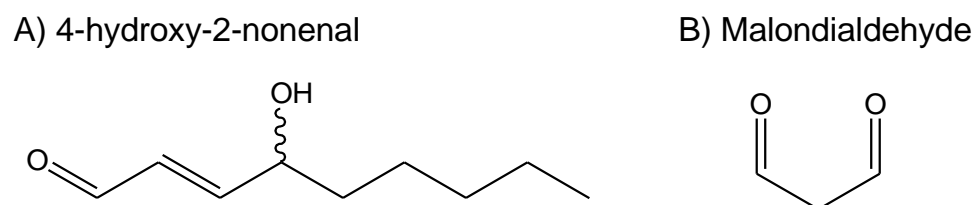
Figure 1.2   Structures of the gold standard products of lipid peroxidation,
4-hydroxy-2-nonenal (A) and malondialdehyde (B).

Reactive Dopamine Metabolites

Due to the selective loss of dopaminergic cells observed in PD, research has long

pointed to DA as the neurotoxic factor in the progression of the disease. DA itself is

toxic both *in vitro* and *in vivo*. The products of DA oxidation, including superoxide

anion, hydrogen peroxide, and hydroxyl radical have been shown to be neurotoxic.

Oxidized DA has also been implicated in depurination of DNA adducts and microglial

activation.[49-52] However, the MAO metabolite of DA, DOPAL, has been shown to be at least 100 times more toxic *in vitro* and even more toxic *in vivo*.[53-57]

DOPAL is produced upon oxidative deamination of DA by MAO on the outer mitochondrial membrane.[57] DOPAL can be further oxidized to 3,4-dihydroxyphenylacetic acid (DOPAC) by ALDH or reduced by aldose-ketose reductases (AKRs) to 3,4-dihydroxyphenylethanol (DOPET) (Scheme 1.3).[57] DOPAL catabolism is thought to be altered in PD for a number of reasons. One, MAO is upregulated with age.[11] As PD affects primarily those advanced in age, this increase in MAO expression leads to a greater turnover of DA to DOPAL. Two, as previously mentioned, lipid peroxidation products 4HNE and MDA inhibit ALDH and prevent metabolism of DOPAL to DOPAC. This also leads to elevated levels of DOPAL, as observed by Goldstein et al. Their work with post-mortem brain tissue of PD patients illustrated a 4-fold increase in the ratio of DOPAL to DA and DOPAL to DOPAC as compared to control.[58] Three, *in vitro* inhibition of mitochondrial complex I, led to a decrease in oxidation of DOPAL to DOPAC and an increase in metabolism of DA to DOPAL. This would lead to further aberrant levels of DOPAL.[59] Four, decreased expression of the cytosolic ALDH1 was observed in the substantia nigra of patients with PD.[60]

Aberrant levels of DOPAL are of great concern for those suffering from PD. Stereotactic injections of DOPAL into the substantia nigra of rats led to dose-dependent toxicity to dopaminergic neurons and increased glial proliferation at sub-cytotoxic concentrations.[55] DOPAL is a bifunctional electrophile that has also been demonstrated to be capable of covalent protein modification and causing protein crosslinking, including

aggregation of α-synuclein.[61-63] In addition to generating protein aggregates, DOPAL is able to cause inhibition of the rate-limiting enzyme in DA biosynthesis, TH (Scheme 1.1).[53, 64] Further evidence of damage in the presence of increased DOPAL includes work by Mallajosyula et al. Upregulation of astrocytic MAO-B in their animal model produced a PD-like pathology, which included the presence of activated microglia.[65]
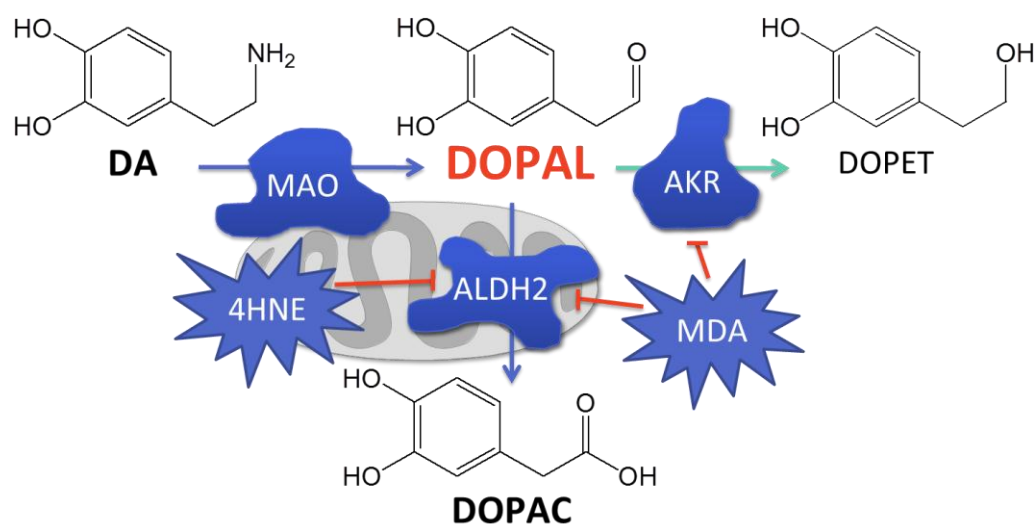
Scheme 1.3   DA metabolism involves MAO to convert DA to DOPAL, followed by ALDH activity to DOPAC or AKR activity to convert DOPAL to DOPET. Products of lipid peroxidation, MDA and 4HNE, inhibit ALDH and AKR as shown.

Activated Glial Cells

Microglia are specialized macrophages native to the central nervous system. They were first described in the 1920s by Pio del Rio-Hortega, having been previously discovered by Nissl and Robertson independently.[66-68] These cells are responsible for protecting the central nervous system from infections and injury, frequently clearing cellular debris through phagocytic activity. When these cells are presented with minute

alterations in their environment, they elicit a response known as activation. Activation occurs rapidly and allows the cells to change morphology from a ramified (resting) state to an active ameboid state.[69] Proliferation of these cells increases upon activation, and upregulation of major histocompatibility complex (MHC) antigens and complement receptors occurs. Release of proinflammatory and neurotoxic factors (e.g. tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β)) and reactive oxygen and nitrogen species also accompanies activation, which can lead to neuroinflammation.[66, 70]

Neuroinflammation has been suggested to be a significant part of the etiology or progression of PD for a number of reasons. Evidence of activated mircroglia exists in the substantia nigra of PD patients. Activated microglia observed were human leukocyte antigen-DR positive and inducible nitric oxide synthase (iNOS) positive.[71, 72] Molecules known to be a part of the inflammatory process have been measured at increased levels in the substantia nigra pars compacta and cerebrospinal fluid of PD patients as compared to controls, including MHC molecules, both class I and II, and various cytokines.[71, 73] Dopaminergic cell death can occur easily within an inflammatory environment containing high levels of cytokines like TNF-α and IL-1β, nitric oxide, and increased expression of cyclooxygenase-2 (COX-2).[71, 74] Microglia are thought to generate environments such as these, but the mechanism of glial activation is unknown. There is *in vitro* evidence for activation by α-synuclein. Primary murine microglia were treated with α-synuclein and activation was observed through a number of endpoints, including increased extracellular superoxide, TNF-α, and nitric oxide. Additionally, the presence of microglia in co-culture with dopaminergic neurons isolated from embryonic mice enhanced α-synuclein neurotoxicity. The observed neurotoxicity increased in direct correlation with an increase

in the number of microglia present in co-culture.[75] DA-quinone and hydrogen peroxide-modified dopaminergic cell membranes have been established as capable of producing microglial activation,[52] but beyond this work there has been little attention given to the interaction of DA itself and its metabolites with microglia.

Changes in the DA metabolic environment described above make a strong case for the presence of elevated levels of DOPAL and its increased opportunity to interact with proteins, neuronal, and non-neuronal cells. Further understanding of these interactions is needed to work toward discovery of biomarkers, treatment, and prevention of PD.

CHAPTER TWO

STATEMENT OF HYPOTHESIS

<u>Introduction</u>

Parkinson's disease (PD) affects 4-5% of 85-year-olds and over 1 million

Americans.[11] It is a neurodegenerative disease that selectively affects the dopaminergic

cells within the substantia nigra. Symptoms of the disease do not appear until roughly

75% of the dopaminergic cells have died, making early diagnosis a challenge.[3] Along

with the loss of dopaminergic neurons controlling movement, PD causes a characteristic

pathology of protein aggregates in the substantia nigra, known as Lewy bodies. The main

component of these bodies is the pre-synaptic protein α-synuclein.[10]

The etiology of the disease remains unknown, but recent research points to

oxidative stress, activated microglia, and reactive dopamine (DA) metabolites as

significant contributors in the progression of the disease.[11, 40, 52, 53, 56, 71] High levels of

oxidative stress have been observed in the substantia nigra of PD patients, including

increased products of lipid peroxidation, i.e. 4-hydroxynonenal (4HNE) and

malondialdehyde (MDA).[41, 42]

Activated microglia have been observed in the PD-affected substantia nigra, along

with the presence of various cytokines indicating activation, including tumor necrosis

factor α (TNF-α) and interleukin-1β. Microglia that have undergone activation have been

shown to have deleterious, toxic effects on dopaminergic neurons.[72, 73, 76, 77]

The selective degeneration of dopaminergic neurons has led to the hypothesis that

DA itself is the neurotoxin responsible for the disease. However, the aldehyde metabolite

of DA, 3,4-dihydroxyphenylacetaldehyde (DOPAL), is 100-fold more toxic *in vitro* and

in vivo.[54, 55, 57] DOPAL is a bifunctional electrophile, capable of covalently modifying and crosslinking proteins, causing loss of enzyme function, and generating reactive oxygen species.[47, 53, 61-64] DOPAL was shown to cause aggregation of the PD-relevant α-synuclein by Burke et al., but this work ignored the reactivity of DOPAL with amines and was performed in tris buffer, leaving a gap in the understanding of the role of DOPAL in this key protein aggregation affiliated with Lewy bodies.[63]

While research has shown activated microglia are capable of creating oxidative stress, and this can cause dopaminergic cell death, little work has been done to investigate the direct interaction between microglia and DOPAL, both with respect to metabolism and activation. The goal of this work was to explore the interaction of DOPAL with three PD-relevant systems; proteins, microglia, and neurons.

Hypothesis

DOPAL, a reactive metabolite of dopamine, causes sub-cellular and cellular changes relevant to Parkinson's disease, including protein aggregation and microglial activation.

Specific Aims

Three specific aims were proposed to test the above hypothesis:

Specific Aim 1: Ascertain the reactivity of DOPAL toward amino acids, peptides, and proteins relevant to PD. Work completed under this aim will demonstrate the ability of DOPAL to covalently modify proteins and cause aggregation of α-synuclein, the main protein component of Lewy bodies.

Specific Aim 2: Determine the metabolism and toxicity of dopamine metabolites, specifically DOPAL, in microglia. Completion of this aim will establish the capability of

microglia to metabolize DOPAL and the toxicity DOPAL elicits in a non-neuronal cell line.

Specific Aim 3: Define the microglial response to aberrant levels of DOPAL and elucidate the functional consequence of microglial activation generated by DOPAL treatment for dopaminergic neurons. Accomplishment of this aim will illustrate the effect DOPAL has on microglial activation, which has not been explored in depth prior to this work. Also, the capability of microglia activated by DOPAL to cause toxicity to a dopaminergic cell line through incubation with glial-conditioned media, will be determined through this aim.

CHAPTER THREE

PARKINSON'S DISEASE AND DOPAL: IMPLICATIONS FOR PROTEINS

<u>Introduction</u>

Parkinson's disease (PD) is a selective neurodegenerative disorder that affects the substantia nigra. This selective dopaminergic cell death has led scientists to a prevailing hypothesis that dopamine (DA) itself may play a role in the initiation or progression of the disease, due to its neurotoxicity.[49, 78] Toxicity of DA has been reported *in vitro* and *in vivo* in neuronal and non-neuronal cells. Additionally, products of DA oxidation, including DA-quinone, have been indicated as neurotoxic, via a number of mechanisms, including free radical production (e.g. superoxide anion, hydrogen peroxide, and hydroxyl radical), formation of depurination DNA adducts, and activation of microglia.[49-52] DA and DA-quinone are capable of protein aggregation and modification which can lead to loss of protein function and ultimately cellular death.[79] These molecules have been implicated specifically in associating with the PD-relevant protein, α-synuclein.[25, 27, 80-82]

While reactivity of DA is clearly relevant in work related to a disease that affects dopaminergic neurons, a metabolite of DA has been shown to be far more toxic *in vitro* and *in vivo*.[53-57, 83] This metabolite, 3,4-dihydroxyphenylacetaldehyde (DOPAL), contains both a catechol and an aldehyde and is the product of DA oxidative deamination by monoamine oxidase (MAO). DOPAL is oxidized by aldehyde dehydrogenase (ALDH) to 3,4-dihydroxyphenylacetic acid (DOPAC) in the major catabolism pathway. Following this oxidation, the catechol is methylated by catechol-*O*-methyltransferase (COMT) to form homovanillic acid (HVA, 3-methoxy-4-hydroxyphenylacetic acid) as

shown in Scheme 3.1. COMT has also been known to act on many of the intermediate

metabolites, generating other metabolic species, also shown in Scheme 3.1.[84, 85]
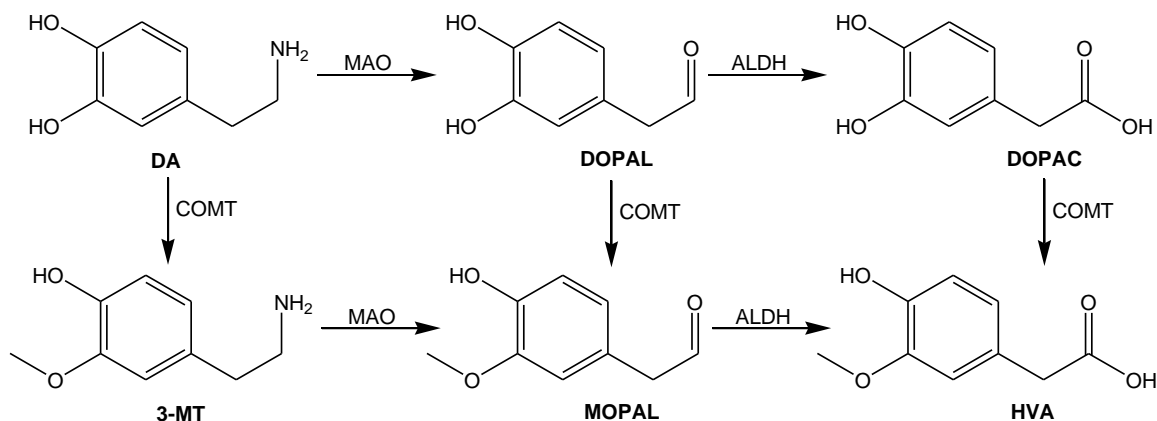
Scheme 3.1   DA is catabolized by MAO to DOPAL, which is oxidized by
ALDH to DOPAC. DOPAC is methylated by COMT to the
excreted metabolite, HVA, in the major DA metabolism pathway.
COMT can alternatively methylate DA or DOPAL to form 3-MT
and MOPAL, respectively.

COMT methylation of DA generates 3-methoxytyramine (3-MT), which can be oxidized

by MAO to form 3-methoxy-4-hydroxyphenylacetaldehyde (MOPAL). MOPAL can be

produced via COMT activity on DOPAL, alternatively.[84, 86] Prior to the research shown

in this chapter, reactivity of MOPAL had not been addressed. Reactivity of MOPAL was

an interesting avenue to pursue, as it is a DOPAL analog without the ability to form an

ortho-quinone. The lack of oxidative capacity of the molecule allows for a greater

understanding of DOPAL's reactivity and the necessity of the catechol moiety. DOPAL

has been shown to be reactive toward amines, including lysine, with a determinable rate

constant.[62] Comparison of that rate to the one determined for MOPAL will be discussed

in this chapter, along with examination of peptide and protein modification by DOPAL and comparisons to the non-catechol MOPAL.

Oxidation of a catechol is a mechanism through which the reactivity of the catechol is enhanced. An additional method of examining the necessity of the catechol present in DOPAL's structure is the addition of antioxidants, e.g. ascorbate and N-acetyl cysteine, to determine reactivity under reduced conditions.[87] Hastings et al. explored the toxicity of DA quinone via injection of high concentrations of DA directly into rat striatum. The animals injected with DA alone showed demonstrated significant increases in protein-bound cysteinyl-DA and cysteinyl-DOPAC after 24 hours. These levels were significantly attenuated following co-injection of DA and ascorbate or glutathione (GSH). Additionally, the high levels of dopaminergic cell-specific toxicity observed following DA injection was attenuated in animals co-injected with GSH or ascorbate.[88] These results warrant the use of ascorbate and other antioxidants to prevent oxidation of DOPAL to a quinone *in vitro* to further probe the necessity of the catechol for DOPAL's reactivity. Proposed DOPAL reactivity with proteins, including one area of examination found in this chapter is depicted in Scheme 3.2.

As previously mentioned, DA has been implicated in aggregation of α-synuclein, the main protein component of Lewy bodies.[25, 82] α-Synuclein is a 14 kDa, pre-synaptic protein associated with synaptic vesicles, and also a primary part of the hallmark protein aggregates found in neurons of PD patients. α-Synuclein is known to self-aggregate, even in its wild-type form (in addition to the A53T mutant known to be a part of the familial form of PD).[89] These self-aggregates can assemble into various structures, including protofibrils (thought to be toxic) and fibrils (thought to be inert).[25, 82]
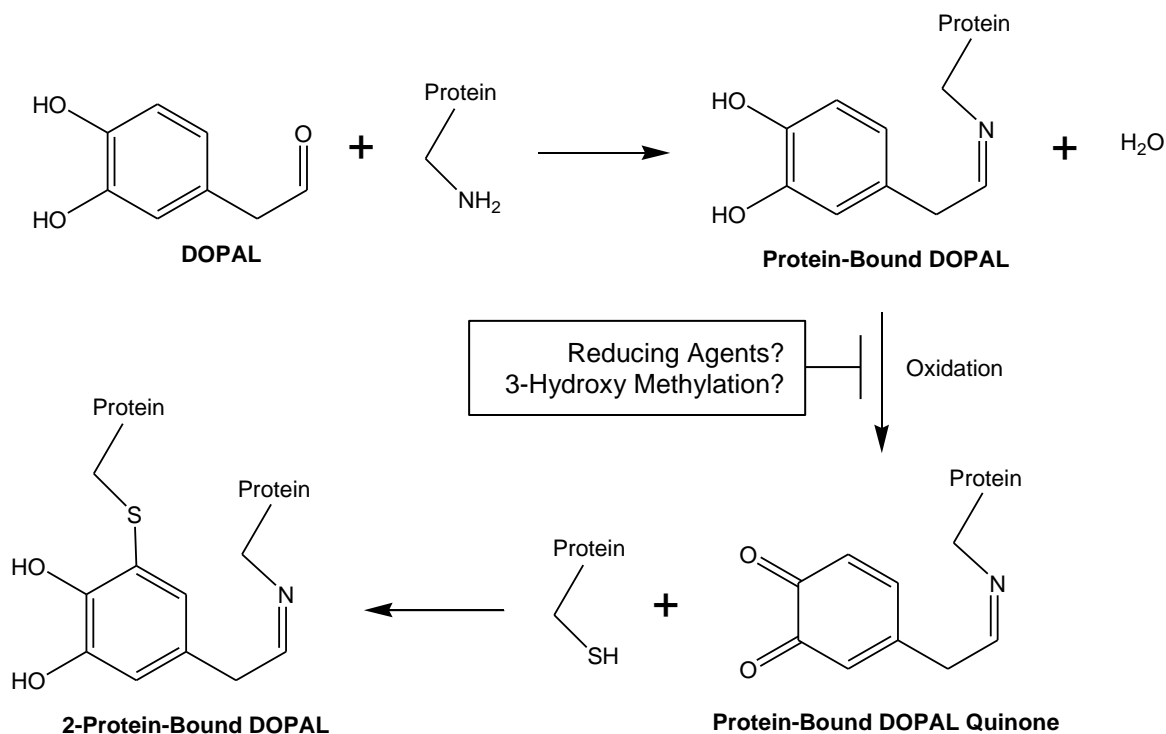
Scheme 3.2   Proposed mechanism of protein modification by DOPAL. DOPAL reacts through a Schiff base with protein amines (i.e. lysine or arginine) to form a protein-bound DOPAL imine. Upon oxidation, DOPAL rearranges to a protein-bound quinone which reacts with protein thiols (i.e. cysteine) to form a covalent bond via Michael-type addition with an additional protein. This could also take place with a protein thiol on the same protein already bound. The proposed impact of 3-hydroxy methylation and reducing agents are also shown.

Conway et al. studied the role of DA in α-synuclein aggregation and found DA stabilized the protofibrillar structure of the protein via a covalent adduct. The aggregation required an oxidative environment, suggesting catechol oxidation with subsequent modification by DA quinone.[25] Others have demonstrated changes in α-synuclein structure in the presence of DA through a variety of methods, including a novel microscopy method and circular dichroism (CD),[82] along with radioactive labeling with gel electrophoresis and NMR.[81] It is important to note that work with α-synuclein and DA in some of this

research required test-tube incubations from 3 days to one week,[25, 82] and a 2 hour incubation of α-synuclein with DA exhibited relatively minor changes in protein aggregation.[81] Burke et al. delved into the interaction of DOPAL with α-synuclein and observed significantly more aggregation with the more reactive DOPAL than DA or other metabolites.[63] However, the incubation of DOPAL with recombinant protein was performed in tris buffer, which contains a free primary amine, predicted to react with DOPAL. Additionally, concentrations of DOPAL that were required to observe significant aggregation of α-synuclein were considerably greater than the physiologically relevant 2-3 μM observed *in vivo*.[63, 90] Further exploration of the interaction between α-synuclein and DOPAL will be presented in this chapter.

<u>Experimental Procedures</u>

Materials

DOPAL was obtained via either a previously described biosynthetic method, involving isolated rat or mouse liver MAO, with DA as the starting material, or a previously described synthetic method with epinephrine as the starting material.[47, 91, 92] MOPAL was synthesized using the biosynthetic method described above with 3-MT as the starting material. Concentration of both aldehydes was determined by an ALDH assay with nicotinamide adenine dinucleotide (NAD) and high-performance liquid chromatography (HPLC) analysis, which also were both previously established.[62, 93] DA, epinephrine, NAD, and all other chemicals, unless otherwise noted, were purchased from Sigma Aldrich. Human recombinant α-synuclein was provided as a generous gift from Dr. Jean-Christophe Rochet at Purdue University.

Evaluation of the Reactivity of MOPAL and DOPAL

N-acetyl-L-lysine was added to a solution of 100 μM MOPAL at varying

concentrations (1, 2.5, 5, 7 and 10 mM). The reaction was carried out in 50 mM sodium

phosphate buffer at a pH of 7.4, with 5 mM sodium cyanoborohydride for adduct

stability, and was incubated at 37° C. Aliquots were taken at 0, 15, 30, 45, 60, 90, 120,

150, 180 and 210 minutes and quenched at a ratio of 1:5 with 1.0 % trifluoroacetic acid.

Samples were analyzed by HPLC as described below. Data for N-acetyl-arginine were

determined in a similar fashion as listed above. Briefly, N-acetyl arginine was added to a

solution of 100 μM DOPAL in 50 mM sodium phosphate buffer (pH 7.4) at the

concentrations listed above for N-acetyl lysine and incubated at 37°C. Aliquots were

collected at the same time points and quenched at a ratio of 1:6 with 1.0% trifluoroacetic

acid. Values from each concentration's time course were converted to % control, with

control being the area under the curve at time 0. (This work was performed in

collaboration with Dr. Jennifer Rees, who also previously obtained data for N-acetyl

lysine modification by DOPAL in a similar fashion.)

Determination of MOPAL and DOPAL Levels by HPLC

An Agilent 1200 Series Capillary HPLC system was used for area under the curve

determinations of MOPAL. Briefly, separation was accomplished with a Phenomenex

C18 Luna microbore column (1x150 mm, 100Å) using isocratic conditions consisting of

0.1% trifluoroacetic acid in water with 10% acetonitrile (v/v) at a flow rate of 50 μL/min.

MOPAL was detected with a photo-diode array with absorbance set at 202 and 280 nm.

The retention time of MOPAL was 10.3 min. Analysis of DOPAL levels took place in

the same fashion, with isocratic conditions of 0.1% trifluoroacetic acid in water with 6%

acetonitrile (v/v), at a retention time of 8.0 minutes.

Identification of MOPAL and DOPAL Adducts

via MALDI-TOF

A model peptide (10 µM RKRSRAE) was incubated with 100 µM of DOPAL or

MOPAL (10 mM tricine buffer, pH 7.4, 4 hours at 37°C) and analyzed via MALDI-TOF

mass spectrometry. Briefly, 1 µL of sample was diluted 1:1 with 0.1% TFA and mixed

with 1 µL of water saturated with α-cyano-4-hydroxycinnaminic acid on a plate. The

mixture was allowed to air dry and analyzed using a Bruker Bioflex MALDI-TOF mass

spectrometer in reflectron mode. Calibration was performed using the peptides

angiotensin I and bradykinin. Samples for the ascorbate-containing experiments were

treated with 200 µM DOPAL and 5 mM ascorbate in conditions as described above, for 6

hours. Spectra shown have been normalized to relative intensity of the molecular ion

peak.

Identification of DOPAL Adducts via IT-TOF

A model peptide, neurotensin (AA8-13, N-acetylated, 10 µM, Ac-RRPYIL), was

incubated with 500 µM DOPAL (due to low rate constant for reactivity), in 10 mM

tricine, and was incubated at 37°C for 4 hours prior to analysis by IT-TOF. Five µL of

sample was directly injected into a Shimadzu IT-TOF mass spectrometer. The mobile

phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). An

isocratic flow of 50% B was used with a flow rate of 0.1 mL/min. Positive ion scanning

was performed from *m/z* 200 to 2000 in 3.3 msec.

Determination of Competition for Protein Binding

Between DOPAL and MOPAL

Bovine Serum Albumin (BSA) (0.5 mg/mL) was incubated with 50 μM DOPAL, 50μM MOPAL, 50 μM DOPAL : 50 μM MOPAL, 50 μM DOPAL : 100 μM MOPAL, or 50 μM DOPAL : 500 μM MOPAL to determine the competition for protein binding between DOPAL and MOPAL. The reaction was run in 50 mM sodium phosphate buffer, pH 7.4, with 5 mM sodium cyanoborohydride for adduct stability, at 37° C for 4 hours. Five μg of protein were run through SDS-PAGE (7.5% acrylamide gel). Upon completion of SDS-PAGE, proteins were transferred from the gel to nitrocellulose membrane with a semi-dry transfer apparatus (70 min, 20 V). The membrane was placed in 0.24 mM NBT in 2 M potassium glycine buffer (pH 10.0), in which it was incubated at 4° C overnight. The membrane was rinsed with distilled H_2O. Competition for protein binding between MOPAL and DOPAL was determined by the intensity of the staining in those lanes, quantified by the program NIH Image J, version 1.37.

Determination of Protein Crosslinking

with DOPAL vs. MOPAL

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was incubated with 100 μM DOPAL, 100 μM MOPAL or alone at a concentration of 0.3 mg/mL in 50 mM sodium phosphate buffer, pH 7.4, at 37° C for 4 hours. These samples were produced in duplicate and 9 μg of protein of each sample was run through SDS-PAGE (10% acrylamide gel). Upon completion of SDS-PAGE the gel was stained with Coomassie blue.

Incubation of DOPAL and Metabolites

with α-Synuclein for Western Blot Analysis

Samples were produced containing 0.15 μg/mL α-synuclein (~10 μM) and varying concentrations of DOPAL or DA or DOPAC as indicated for each experiment. Experiments containing ascorbate or N-acetyl cysteine included increasing concentrations of the antioxidant as shown. All incubations were performed in 50 mM sodium phosphate buffer, pH 7.4. Samples were incubated for 4 hours at 37°C and frozen or added to a gel immediately following.

Western Blot of α-Synuclein Samples

Protein samples, produced as described above, were run through SDS-PAGE (100 ng/lane, 15% acrylamide gel) and subjected to Western blotting. Membranes were treated for 2 hours in 5% milk in TBS-T (w/v) at room temperature. Primary antibody (rabbit anti-α-synuclein, Sigma Aldrich, 1:5000) was added and membranes were incubated at 4°C overnight. Membranes were rinsed in TBS-T and incubated for 1.5 hours at room temperature with secondary antibody (goat anti-rabbit, Santa Cruz Biotechnology, 1:20,000). Following TBS-T rinses, membranes were incubated with 3 mL ECL Prime (GE/Amersham) for 5 minutes. Film was exposed to the membranes and developed.

Measurement of α-Synuclein Conformational Changes

by Circular Dichroism

Samples were prepared for CD analysis in 50 mM sodium phosphate buffer, pH 7.4, containing 2 mg/mL α-synuclein and 0, 10, 100, or 500 μM DOPAL. The samples were incubated for 4 hours at 37°C. Samples were analyzed on a Jasco

Spectropolarimeter, model J815. Analysis was performed using the online resource, Dichroweb (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml).[94, 95] Results were deconvoluted using the CDSSTR algorithim.[96]

<div align="center">Preparation of α-Synuclein + DOPAL Samples</div>

<div align="center">for Mass Spectrometry Analysis</div>

DOPAL (50 μM) was incubated with 0.15 mg/mL α-synuclein for 4 hours at 37°C in ammonium bicarbonate (pH 7.4). The sample was then treated with $NaCNBH_3$ for 30 minutes to reduce any imines formed via DOPAL and protein amines to secondary amines. The sample was finally subjected to trypsin for 8 hours at 37°C and analyzed via IT-TOF MS, as described below. (This work was performed in collaboration with Dr. Lydia Vermeer.)

<div align="center">Determination of Covalent Modification</div>

<div align="center">of α-Synuclein by DOPAL</div>

A Shimadzu IT-TOF mass spectrometer, coupled with a Shimadzu HPLC was used for analysis of DOPAL-modified α-Synuclein. Fifteen μL of sample was injected, and separation of peptides was achieved with a Phenomenex Aeris Widepore XB-C18 column (100 x 2.1 mm), with a particle size of 3.6 μm and a pore size of 200 Å. Gradient conditions were used to accomplish the desired separation, with a mobile phase of 0.1% formic acid in water (v/v, A) and 0.1% formic acid in acetonitrile (v/v, B), (0 min: 5% B, 5-15 min: 5-50% B, 15-30 min: 50-90% B, 31 min: 95% B, 35 min: 50% B, 36-40 min: 5% B. Positive ion scanning was performed from m/z 200 to 2000 in 3.3 msec. Results were compared to those found with the University of California, San Francisco Protein Prospector MS-Digest program. A tryptic digest of α-synuclein was determined by the

program, allowing 2 missed cleavages, variable modifications of pyroglutamate and

oxidation of methionine, and peptide mass of *m/z* of 200 – 5000. (This work was

performed in collaboration with Dr. Lydia Vermeer.)

<div align="center">Results</div>

<div align="center">DOPAL and MOPAL React with N-Acetyl Lysine</div>

<div align="center">with a Determinable Rate Constant</div>

Determination of the second order rate constants for DOPAL and MOPAL with

N-acetyl lysine were determined following DOPAL and MOPAL (100 μM) incubation

with N-acetyl lysine from 1 to 10 μM for 4 hours. Aliquots were collected at 0, 15, 30,

45, 60, 90, 120, 150, 180 and 210 minutes, and samples were quenched as described

above to prevent further reaction and analyzed by HPLC. The rate constant determined

for MOPAL reactivity required the addition of sodium cyanoborohydride. HPLC

analysis determined amount of DOPAL or MOPAL loss over time, and second order rate

constants were determined by use of pseudo-first order rate constant graphs. Second

order rate constants for MOPAL and DOPAL are shown below in Figure 3.1. DOPAL

was found to have a significantly greater rate of reaction with N-acetyl lysine than that

for MOPAL, which required addition of $NaCNBH_3$. (This work was performed in

collaboration with Dr. Jennifer Rees and previously published; the line shown for

DOPAL is provided for comparison.[62])
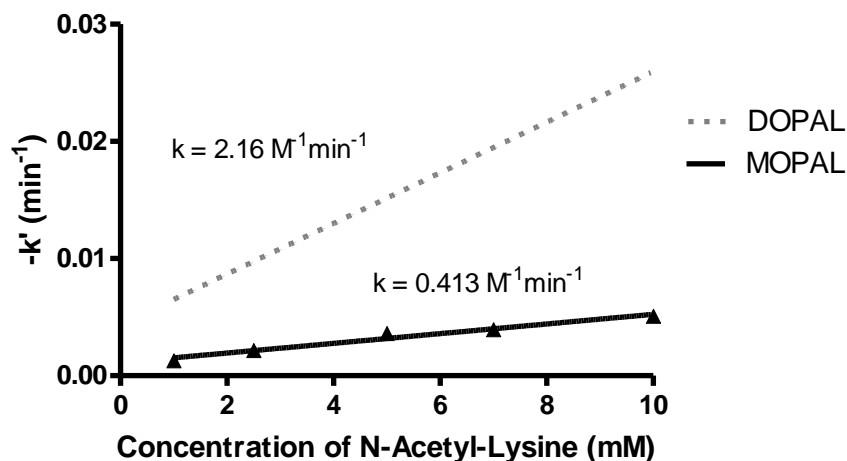
Figure 3.1   DOPAL and MOPAL react with N-acetyl lysine with determinable
              second order rate constants. DOPAL reacts at a significantly
              greater rate than its 3-methoxy metabolite (MOPAL) at a rate of
              2.16 $M^{-1}min^{-1}$, compared to the rate for MOPAL of 0.413 M^{-1}
              min^{-1}. Rate constant determination for MOPAL required addition
              of $NaCNBH_3$ to the incubation.

<div align="center">

DOPAL Reacts with N-Acetyl Arginine

with a Determinable Rate Constant

</div>

Determination of the second order rate constant (k) for DOPAL with N-acetyl

arginine was performed in the same fashion as that described above for DOPAL + N-

acetyl lysine. Briefly, DOPAL was incubated with a range of concentrations of N-acetyl

arginine for 3.5 hours, with time points collected and DOPAL concentration determined

via HPLC. The second order rate constant was determined by averaging pseudo-first

order rate constant graphs from three experiments. A representative graph of the

normalized data from one experiment is shown in panel A, and the average rate constant

graph is shown in panel B. The average rate constant graph (B) takes into consideration

the spontaneous loss of DOPAL over time. Results for the reaction of DOPAL with N-

acetyl arginine were determined to be considerably slower than that for DOPAL with N-acetyl lysine.
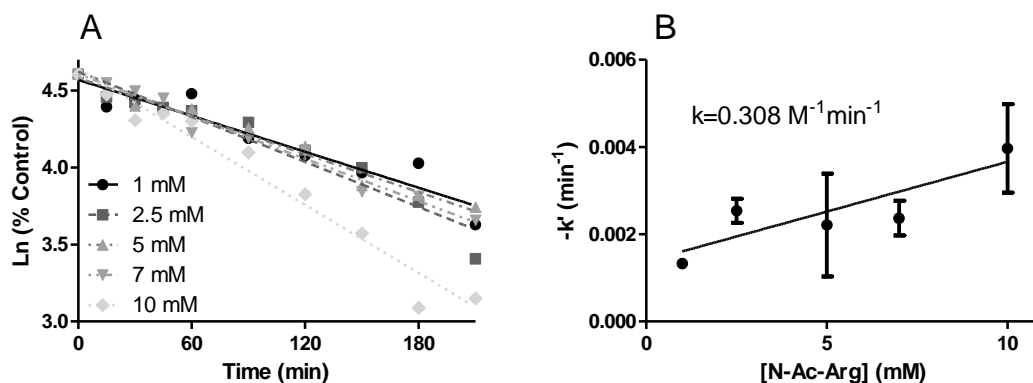
Figure 3.2   DOPAL reacts with N-acetyl arginine with a determinable rate constant. A representative graph of the normalized data is shown in panel A, and the average rate second order rate constant is depicted in panel B. The second order rate constant for the reaction of DOPAL with N-acetyl arginine was found to be 0.308 $M^{-1}min^{-1}$. ($n=3 \pm$ SD)

DOPAL and MOPAL Covalently Modify an

Arginine- and Lysine-Containing Peptide

The above experiments observed loss of DOPAL and MOPAL over time by HPLC. To further probe the reaction taking place between the aldehydes and the amino acids, DOPAL and MOPAL were incubated with an arginine- and lysine-containing peptide (RKRSRAE) for 4 hours. Samples were prepared and subjected to MALDI-TOF-MS analysis to explore covalent modification of the peptide by DOPAL and MOPAL. Changes in *m/z* for the peptide corresponding to single and multiple adducts of

DOPAL or MOPAL were detected, indicating covalent modification of the peptide, likely

on both lysine and arginine residues. Results are shown in Table 3.1

Table 3.1   Observed and calculated *m/z* for peptide
RKRSRAE + DOPAL or MOPAL, measured by
MALDI-TOF analysis.

Compound	Theoretical *m/z*	Experimental *m/z*	Adduct (Da)
Peptide (RKRSRAE)	902.57	901.974	
Peptide + DOPAL	1036.62	1037.870	135.9
Peptide + 2 DOPAL	1170.67	1169.830	133.9 (x2)
Peptide + MOPAL	1050.63	1051.943	149.7
Peptide + 2 MOPAL	1198.69	1201.946	150.0 (x2)

DOPAL Covalently Modifies

Arginine-Containing Peptides

The results from the previous experiment with the RKRSRAE peptide established

the formation of a covalent bond between DOPAL and a peptide containing both lysine

and arginine. Due to arginine's considerably lower rate constant for the reaction with

DOPAL, it was necessary to examine the interaction of DOPAL with a peptide

containing no lysine residues. To explore the ability of DOPAL to modify arginine

covalently, DOPAL and the N-acetylated Neurotensin (aa 8-13, Ac-RRPYIL-OH)

peptide were incubated for 4 hours prior to analysis by IT-TOF mass spectrometry. The

observed addition of a molecular mass of 135.188 Da is strongly indicative of an adduct

being formed between an arginine on the peptide and DOPAL. Results are shown below

in Table 3.2.

Table 3.2   IT-TOF mass spectrometry of an arginine-containing peptide
incubated with DOPAL, indicating a Schiff base adduct.

Peptide	Modified Peptide (m/z)	Unmodified Peptide (m/z)	Adduct (m/z)
Ac-RRPYIL-OH	993.680	859.492	134.188

Ascorbate Reduces the Number of Products

of DOPAL-Mediated Peptide Modification

Thus far in this chapter, DOPAL and MOPAL have been seen to be capable of

modifying arginine and lysine residues, and lysine residues, respectively. MOPAL is not

capable of undergoing oxidation to a quinone species, due to its 3-methoxy group, and

was shown to have a significantly lower second-order rate constant with N-acetyl lysine

than DOPAL. An additional approach was used to explore the necessity of DOPAL

oxidation for peptide modification. The RKRSRAE peptide was incubated with DOPAL

in the presence and absence of 5 mM ascorbate. Results are shown in Figure 3.3. The

peptide alone showed one major peak at the expected m/z (902.57 Da, panel A). Peptide

treated with DOPAL (panel B) exhibited a number of major peaks, including those for

peptide + 2 DOPAL (m/z 1169.48) and values close to peptide + 3 and 4 DOPAL. The

higher m/z observed for 3 and 4 DOPAL adducts were a few Da different from the

calculated values, which could be due to unique DOPAL rearrangements not yet

characterized. No significant peak was observed for the peptide + DOPAL calculated

value. When ascorbate was added to the incubation (panel C), the peaks observed for

multiple DOPAL modifications were no longer present and the major product was the

peptide + 1 DOPAL adduct at *m/z* 1036.54 (theoretical, *m/z* 1036.62). This adduct of 134

Da is indicative of a Schiff base product.

Compound	Theoretical *m/z*	Experimental *m/z*	Adduct (Da)
Peptide (RKRSRAE)	902.57	902.57	
Peptide + 2 DOPAL	1170.67	1169.48	133.4 (x2)
Peptide + DOPAL	1036.62	1036.54	134
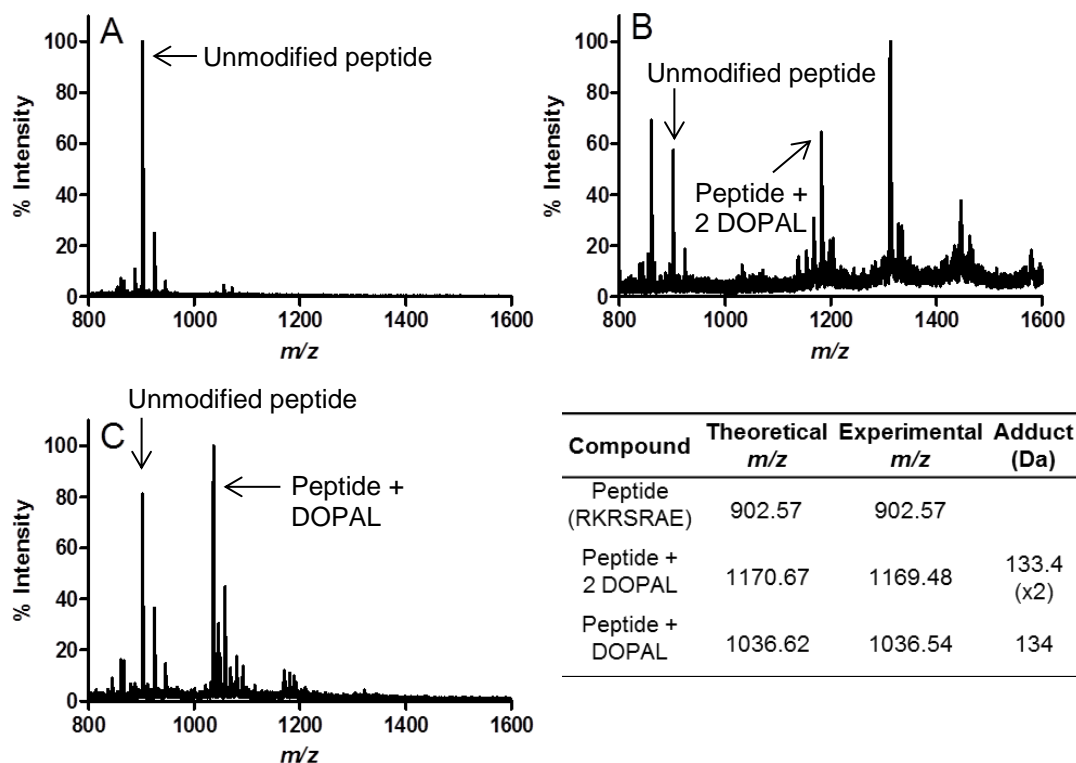
Figure 3.3   MALDI-TOF results following incubation of an arginine- and lysine-containing peptide (RKRSRAE, 902.57 Da) alone (A), with DOPAL (B) and with DOPAL + ascorbate (C). Peptide treated with DOPAL shows a predominant peak at *m/z* 1169.48 (Peptide + 2 DOPAL), and peptide treated with DOPAL and ascorbate shows an adduct at *m/z* 1036.54 (theoretical *m/z* 1036.62). Detection of an adduct with a molecular weight of 134 Da is indicative of a Schiff base product.

DOPAL Reactivity Toward a Model Protein

Determined by a Catechol-Sensitive Dye

DOPAL reactivity toward bovine serum albumin (BSA) as a model protein was

determined following incubation with NBT. NBT is a redox-cycling dye, known to stain

nitrocellulose membranes containing redox-sensitive functional groups like catechols.[97]

Staining in this experiment should only be seen in lanes containing DOPAL. In Figure

3.4, DOPAL has out-competed MOPAL for protein binding, as detected by nitroblue

tetrazolium (NBT) catechol staining. Protein-bound MOPAL does not stain, as it is not a

catechol. Even at 10-fold excess MOPAL, the DOPAL band remains at 88% intensity

compared to control (BSA + 50 μM DOPAL).

Lane	Sample	Densitometry (% Control)
1	BSA + 50 μM DOPAL	100 %
2	BSA + 50 μM MOPAL	0
3	BSA + 50 μM DOPAL + 50 μM MOPAL	100 %
4	BSA + 50 μM DOPAL + 100 μM MOPAL	77 %
5	BSA + 50 μM DOPAL + 500 μM MOPAL	88 %
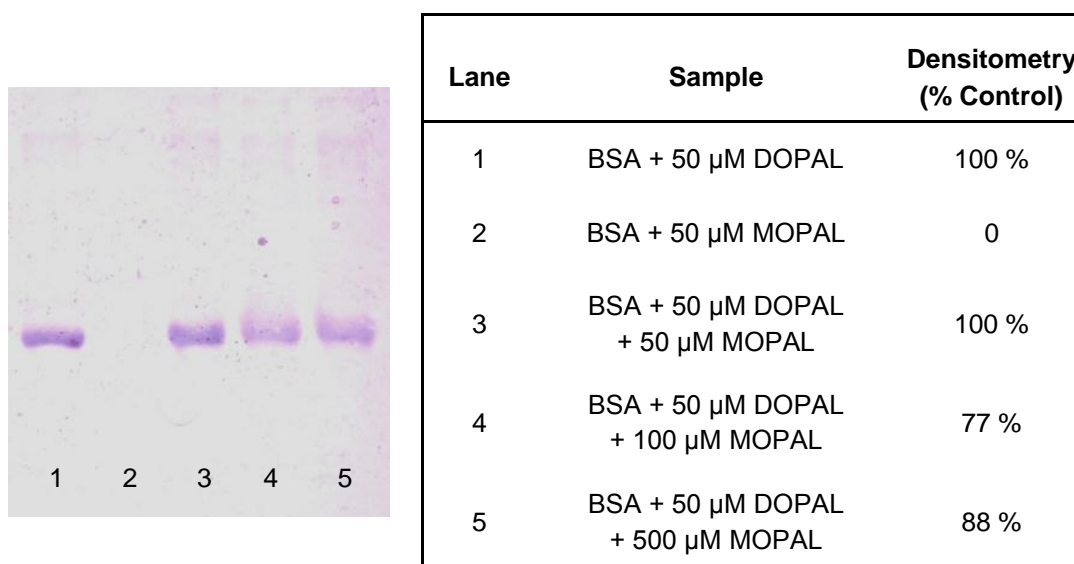
Figure 3.4   DOPAL out-competes MOPAL for protein binding with a model protein,
               BSA. DOPAL binding to protein is determined by NBT stain, a dye
               selective for catechols. Protein treated with MOPAL alone (Lane 2) does
               not show staining, as MOPAL is not a catechol. Additionally, MOPAL
               interferes minimally with DOPAL binding of BSA even up to 1:10
               DOPAL:MOPAL. Percent control was determined based on BSA + 50
               μM DOPAL alone, Lane 1.

DOPAL, but not MOPAL,

Causes Aggregation of a Model Protein

The ability of DOPAL to react with proteins and cause aggregation is thought to be due to its properties as a bifunctional electrophile (i.e. aldehyde and catechol/quinone). A proposed mechanism for DOPAL-mediated protein crosslinking can be seen in Scheme 3.2. Exploration of this proposed crosslinking took place with the model protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is a pro-survival protein important for neuronal health, thought to play a role in in PD.[98, 99] The protein was treated with DOPAL or MOPAL in duplicate, subjected to SDS-PAGE and stained with Coomassie Blue (Figure 3.5).

Lanes	Sample
1, 4	GAPDH (Control)
2, 5	GAPDH + 100 µM DOPAL
3, 6	GAPDH + 100 µM MOPAL
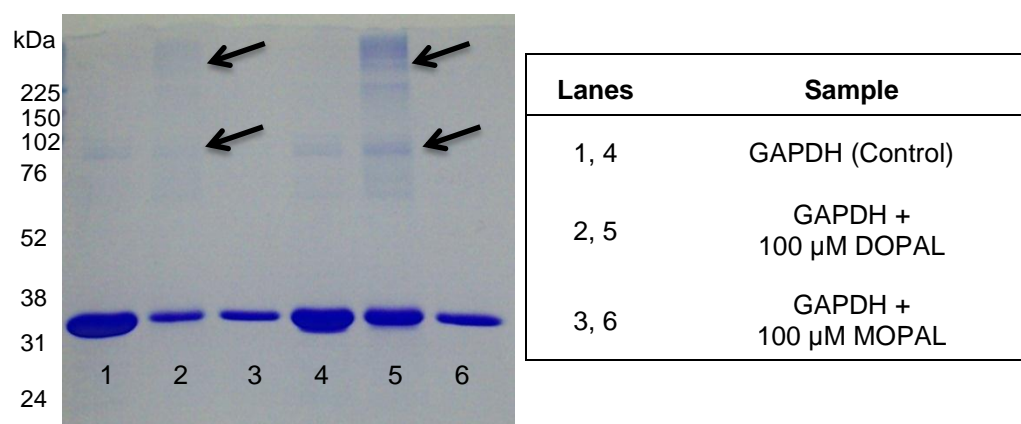
Figure 3.5   DOPAL, but not MOPAL, causes significant protein aggregation of a model protein (GAPDH), as observed by SDS-PAGE. Arrows indicate sites of protein aggregation in Lanes 2 & 5.

Lanes 2, 4, 5 and 7 show a lack of crosslinking, or an absence of higher molecular weight bands, as expected for non-treated and MOPAL-treated GAPDH, as MOPAL does not contain the catechol moiety to allow for redox cycling and thiol adduction. Lanes 3 and

6, containing DOPAL and GAPDH, show crosslinked protein at higher molecular weight, indicated by arrows. The observed results indicate the catechol is critical to the ability of DOPAL to crosslink proteins.

<div align="center">

DOPAL, but not DA or DOPAC,

Facilitates Aggregation of α-Synuclein

</div>

The main component of Lewy bodies, α-synuclein, has been shown to aggregate in the presence of DA and DOPAL.[25, 63, 81, 82] However, previous work utilized extended incubations of α-synuclein with DA (up to a week) and very high concentrations of DA or DOPAL (up to 2 mM and 1.5 mM for DA and DOPAL, respectively). Some of the incubations were performed in Tris buffer, which contains a free primary amine that could react with the aldehyde of DOPAL. For these reasons, α-synuclein was incubated with DA, DOPAL and DOPAC for only 4 hours in sodium phosphate buffer, pH 7.4, and the samples were analyzed by Western blot (Figure 3.6).

Lane	Sample
1	α-synuclein
2	α-synuclein + 50 µM DA
3	α-synuclein + 50 µM DOPAL
4	α-synuclein + 50 µM DOPAC
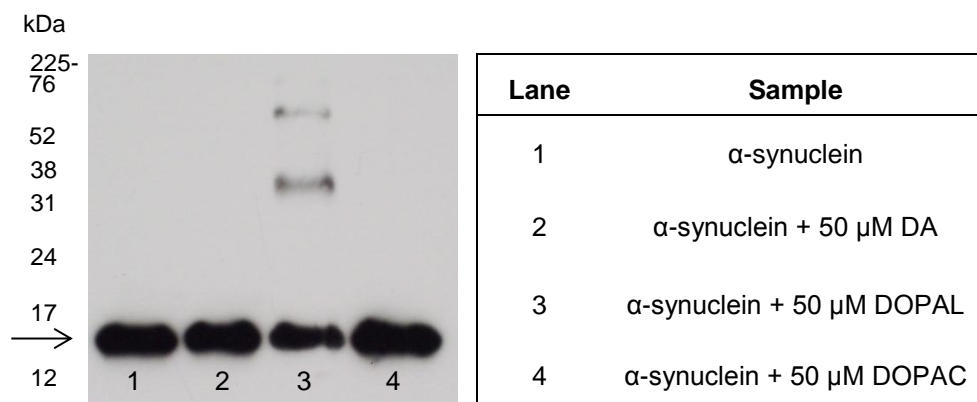
Figure 3.6   DOPAL causes aggregation of the PD-relevant α-synuclein after four hours, while DA and DOPAC do not. DOPAL-aggregated α-synuclein is observed in the dimeric and additional multimeric forms in Lane 3. The molecular weight of the monomer is marked with an arrow.

DOPAL elicited significant aggregation of α-synuclein, into its dimeric and other multimeric forms. A loss in staining of the monomeric band can be observed for the lane of protein treated with DOPAL, compared to the untreated, DA and DOPAC lanes.

DOPAL Induces α-Synuclein Aggregation

Dose-Dependently

DOPAL-mediated α-synuclein aggregation was further explored by examination of the dependence of dose of DOPAL. PD is a progressive disease, and while native concentrations of DOPAL are thought to be between 2-3 μM,[90] it is feasible these levels could be elevated rapidly under aberrant metabolism conditions, such as in the presence of lipid peroxidation products known to inhibit ALDH.[43] α-Synuclein (~10 μM) was treated with DOPAL for 4 hours in sodium phosphate buffer, pH 7.4. Samples were then analyzed by Western blot to observe changes in protein aggregation (Figure 3.7).

DOPAL Causes Aggregation of α-Synuclein

Time-Dependently

Bisaglia et al. demonstrated a time-dependent formation of α-synuclein oligomers induced by DA quinone treatment.[81] Aggregates were observed by ^{14}C label on DA rather than Western blot and were very faint. Examination of the changes in DOPAL-mediated α-synuclein aggregation over time was warranted for comparison to changes shown with DA quinone aggregation, which included the presence of sodium periodate. Additionally, no control for α-synuclein incubated alone was shown. α-Synuclein is known to self-aggregate in certain conditions,[100] so the absence of the control makes the results shown incomplete. The experiment shown in Figure 3.8 was continued out to 4 hours beyond the 2 hours demonstrated in the Bisaglia work. Panel A depicts samples of

α-synuclein incubated in 50 mM sodium phosphate buffer alone, with aliquots that were collected at 0, 1, 2, 4, 8, and 12 hours. In panels B and C, the α-synuclein time-courses with 10 and 50 μM DOPAL, respectively, are shown. α-Synuclein aggregation is again dose-dependent and also time-dependent.

Lane	Sample
1	α-synuclein (α-syn)
2	α-syn + 1 μM DOPAL
3	α-syn + 5 μM DOPAL
4	α-syn + 10 μM DOPAL
5	α-syn + 25 μM DOPAL
6	α-syn + 50 μM DOPAL
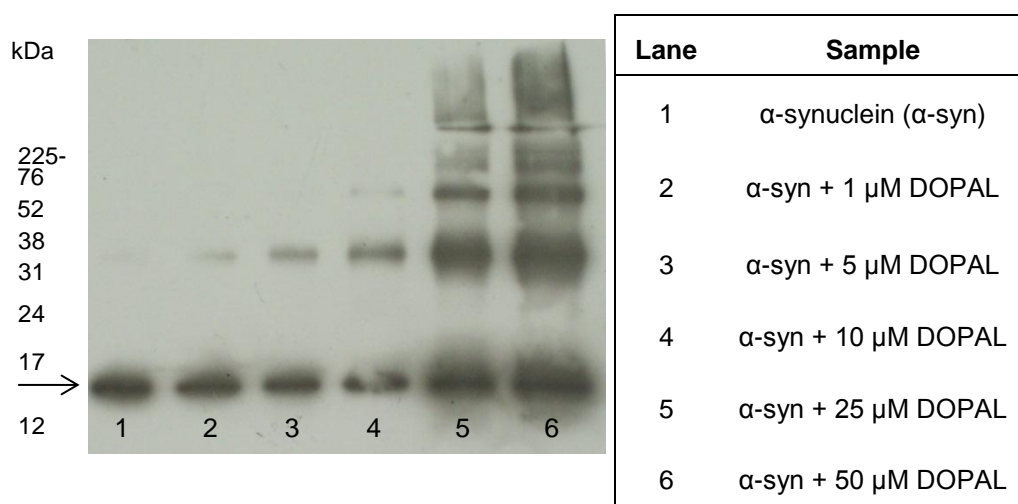
Figure 3.7   DOPAL causes α-synuclein aggregation dose-dependently after 4 hours. As α-synuclein is incubated with greater concentrations of DOPAL, an increase in protein aggregates at the dimer, trimer, and further multimeric molecular weights occurs. A decrease in the monomeric form of α-synuclein is also observed with increase in concentration of DOPAL. Lane 4 represents ~ 1:1 ratio of α-synuclein to DOPAL. The molecular weight of the monomer is marked with an arrow.

Ascorbate Inhibits

DOPAL-Mediated α-Synuclein Aggregation

Previous work in this chapter demonstrated changes in DOPAL modification of a model peptide in the presence of ascorbate (Figure 3.3). Ascorbate prevents oxidation of catechols, reducing their reactivity but does not react covalently with DOPAL itself.[87]
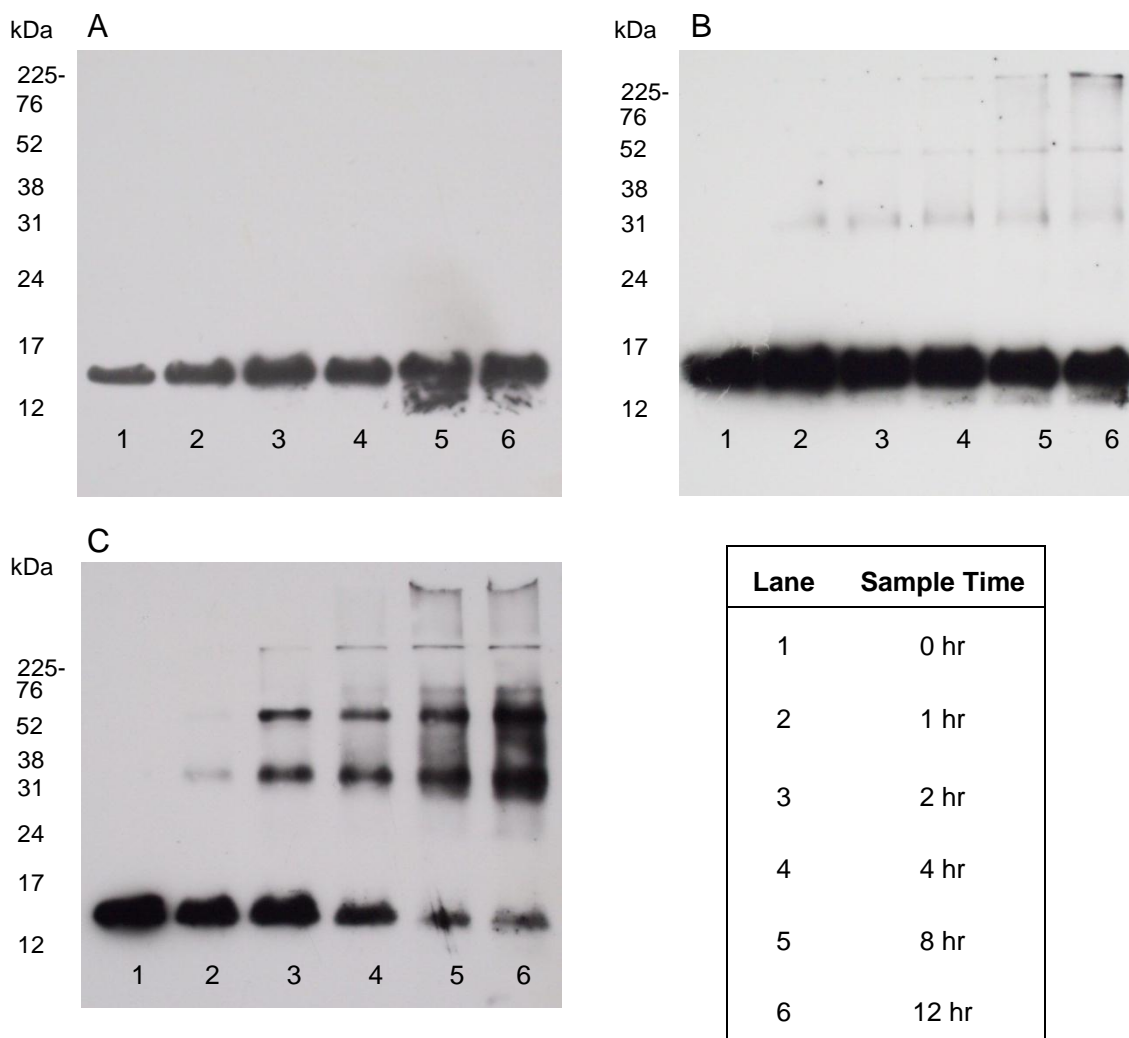
Figure 3.8   α-Synuclein aggregation caused by DOPAL is time-dependent. Aliquots of α-synuclein incubated alone (A) or with DOPAL (10 μM, B or 50 μM, C) over time were subjected to Western blot analysis to observe changes in aggregation over time. α-Synuclein demonstrated little aggregation on its own, but DOPAL caused aggregation in both a time- and dose-dependent fashion.

While ascorbate was used to quench the reaction for the gel radioanalysis of DA quinone

and α-synuclein in the work by Bisaglia et al., no further examination has been performed

to explore the role antioxidants may play in catechol-produced α-synuclein aggregation.

Figure 3.9 presents Western blots following α-synuclein (10 μM) aggregation in 0 (panel A) and 50 μM DOPAL (panel B), in the presence of increasing concentrations of ascorbate. Samples were incubated for 4 hours. No changes were observed in the control blot (A) where no DOPAL was present in the reaction. In panel B, α-synuclein oligomers can be seen following incubation of α-synuclein and 50 μM DOPAL alone, but addition of ascorbate, even at 5 μM (a 2:1 ratio of α-synuclein to ascorbate), prevented oligomer formation.

<div align="center">

N-Acetyl Cysteine Inhibits

α-Synuclein Aggregation Caused by DOPAL

</div>

As ascorbate potently inhibited DOPAL-generated α-synuclein aggregation (Figure 3.9), examination of inhibition by other antioxidants was important. N-acetyl cysteine is a known antioxidant that our lab has found to inhibit crosslinking of other proteins, such as GAPDH, by DOPAL.[62] Figure 3.10 shows Western blots that were produced with samples of α-synuclein (10 μM) that were incubated as above with increasing concentrations of N-acetyl cysteine (A) or N-acetyl cysteine and 50 μM DOPAL (B). No aggregation was observed in the absence of DOPAL (A) and no change was observed upon addition of increasing concentrations of N-acetyl cysteine. N-Acetyl cysteine was a less potent inhibitor of α-synuclein oligomerization, following α-synuclein treatment with 50 μM DOPAL, as the dimer band remained visible even up to treatment with 500 μM N-acetyl cysteine (NAC), while in Figure 3.9, panel B, ascorbate prevented dimerization completely at 100 μM.
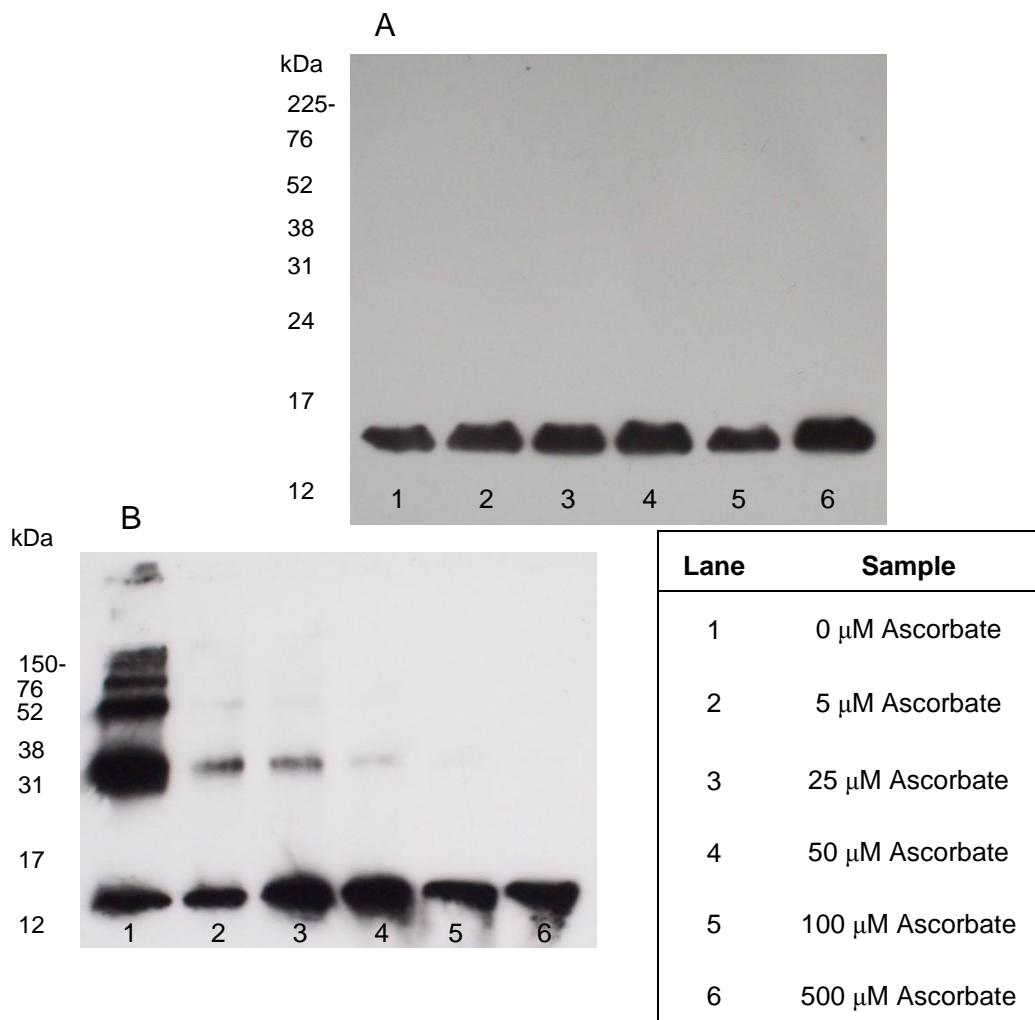
Figure 3.9   Ascorbate prevents aggregation of α-synuclein in the presence of DOPAL. Panel A contains α-synuclein treated with ascorbate alone as shown in the list of lane contents. Panel B depicts α-synuclein incubated with 50 μM DOPAL in addition to ascorbate as shown in the lane content list. Ascorbate prevented aggregation of α-synuclein potently at 5 μM, which was one-tenth molar equivalent of the concentration of DOPAL present in the sample and one-half the molar equivalent of the concentration of α-synuclein.
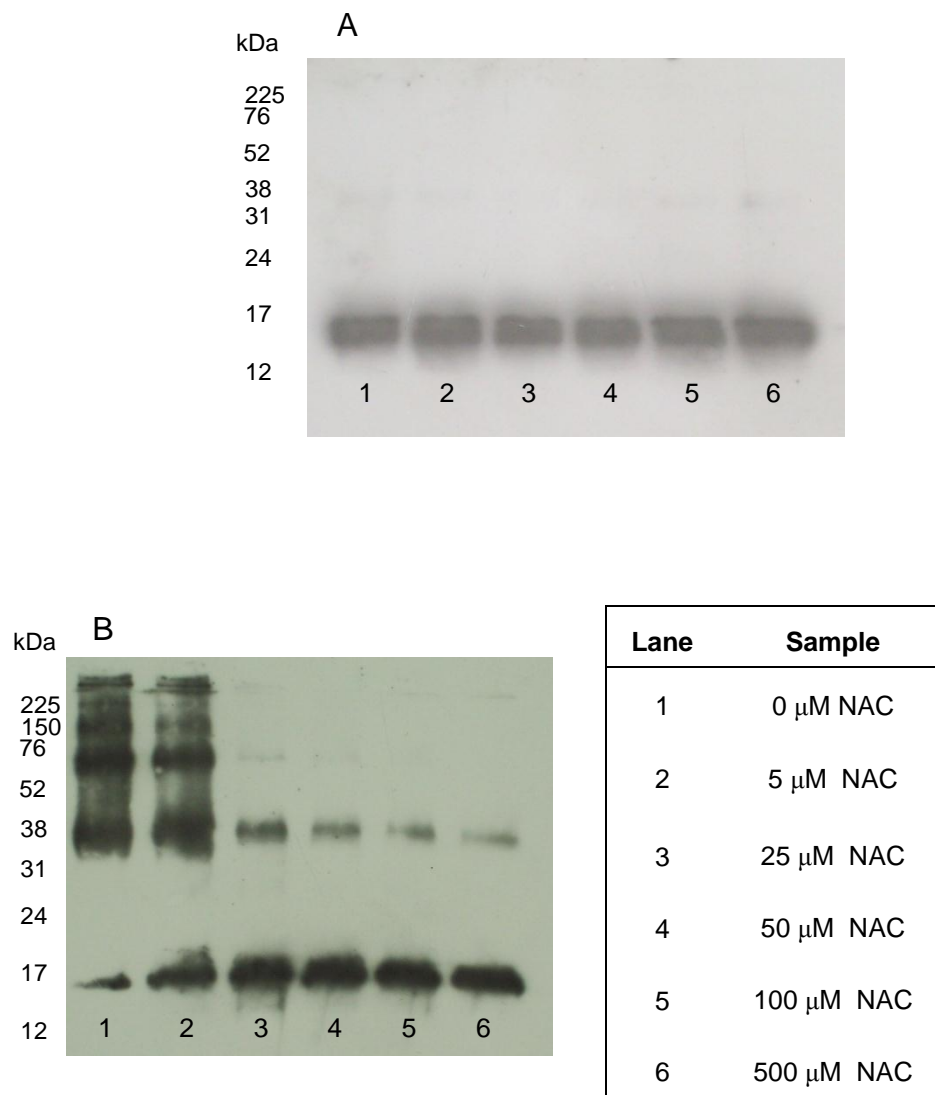
Figure 3.10   DOPAL-mediated α-synuclein oligomerization is inhibited by N-acetyl cysteine(NAC). α-Synuclein (10 μM) was treated with increasing concentrations of N-acetyl cysteine alone (A) or along with 50 μM DOPAL (B). N-acetyl cysteine exhibits less potent inhibition of α-synuclein oligomerization. The dimer can still be observed after treatment with 50 μM DOPAL and 500 μM N-acetyl cysteine (B), indicating a less potent inhibition than that generated by ascorbate.

Analysis of Structural Changes to

α-Synuclein by DOPAL by Circular Dichroism

A respected method for determining changes to protein structure, CD was employed by Outeiro et al. to examine DA-induced changes to the secondary structure of α-synuclein.[82] Changes in aggregation of the protein have clearly been observed through Western blotting, but DOPAL-mediated changes to the native structure of α-synuclein in solution needed further exploration. A similar method to the one used by Outeiro et al. was used for this experiment.[82] Samples were treated with 0, 10, 100 or 500 μM DOPAL for 4 hours and then subjected to analysis by a spectropolarimeter. Analysis was performed using an online tool, Dichroweb, as discussed in the Experimental Procedures section. Figure 3.11, panel A presents the conformational changes of α-synuclein generated by DOPAL, monitored by CD. Graphical representation of the structural changes is depicted in the graphs in panels B and C. Panel B shows the percent of total secondary structure determined to be α-helical. The decrease seen upon increasing concentration of DOPAL treatment matches the decrease in minimum ellipticity at 198 nm. The increase observed in the percent of β-strand per total secondary structure following DOPAL treatment (panel C) matches the increase in minimum ellipticity at 220 nm. These results are similar to those previously published in the granular formation of α-synuclein amyloid fibrils and in the nitration of α-synuclein.[101, 102]
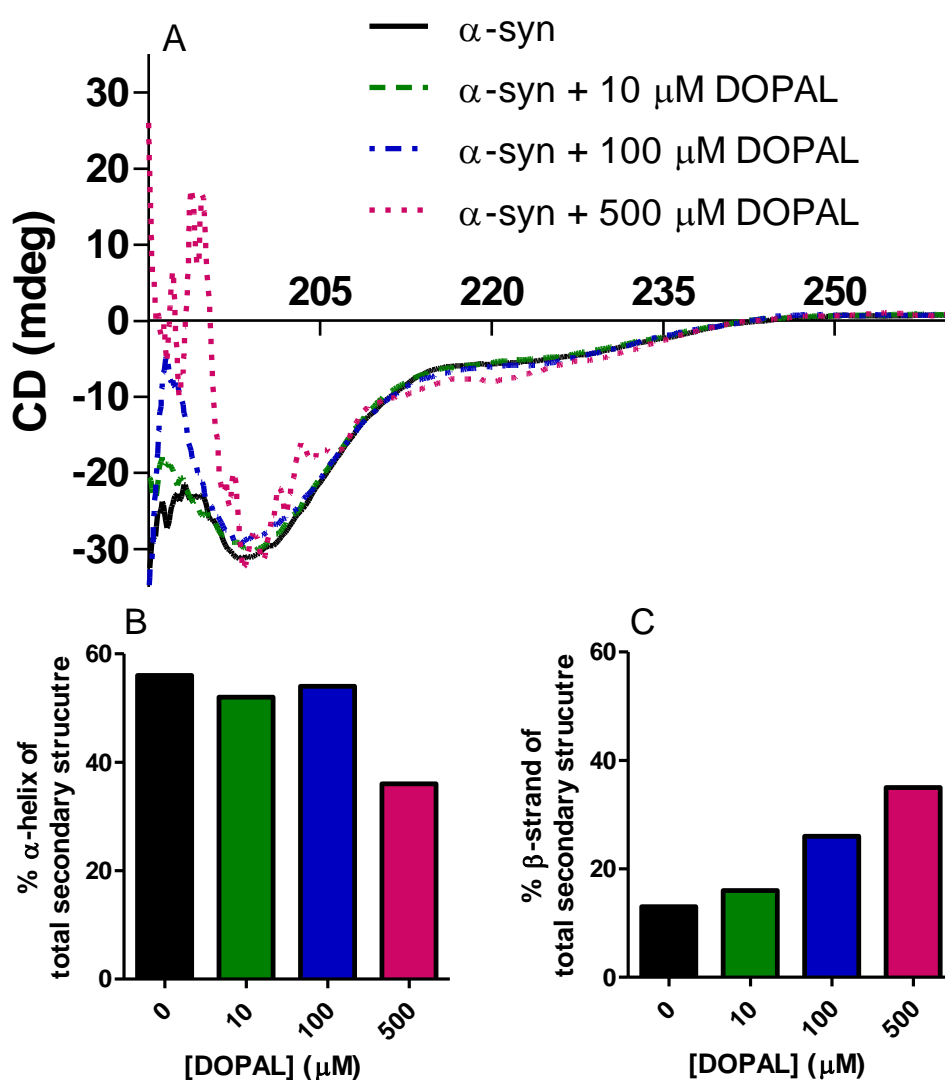
Figure 3.11   DOPAL induces changes to the structural conformation of α-synuclein, as determined by CD analysis (Panel A). Panel B indicates changes in the % of total secondary structure deconvoluted to represent α-helices, which decreases with increase of DOPAL. The % of total secondary structure deconvoluted to represent β-strands is shown in panel C and rises with increase in DOPAL concentration, indicating formation of fibril-like aggregates.

DOPAL Covalently Modifies α-Synuclein

DOPAL-generated changes in α-synuclein structure and oligomerization have

been observed throughout this chapter. The final experiment in this work to explore

DOPAL modification of α-synuclein was to look at covalent modification of the protein.

The protein was incubated with 0 or 50 μM DOPAL for 4 hours and then trypsinized for

8 hours. Following trypsinization, the peptides were subjected to HPLC-IT-TOF

analysis. Total ion chromatograms for both control and DOPAL-treated α-synuclein are

shown in Figure 3.12. DOPAL adducts were found on three peptides of the α-synuclein

protein treated with DOPAL, and no matching peptides were observed in the control

sample. Each of these peptides contains a lysine residue, which is proposed to be the site

of adduction. The reactivity for DOPAL with free amines, specifically that of lysine is

well characterized and proposed to be the primary method of protein modification.

Results are depicted below in Figure 3.13 with an image generated through the NCBI

Molecular Modeling Database,[103, 104] showing the modified lysine residues on the protein,

along with tabular results in the upper right corner of the image. The mass of the adduct

at K6 (133.49 Da) represents formation of a Schiff base imine between DOPAL and the

lysine residue. Mass of the DOPAL adducts at K32 and K88 (~136 Da) are indicative of

a Schiff base modification that has been reduced to an amine. Protein coverage observed

for the untreated sample was 84 % and 98% for the DOPAL-modified α-synuclein. (This

work was performed in collaboration with Dr. Lydia Vermeer.)
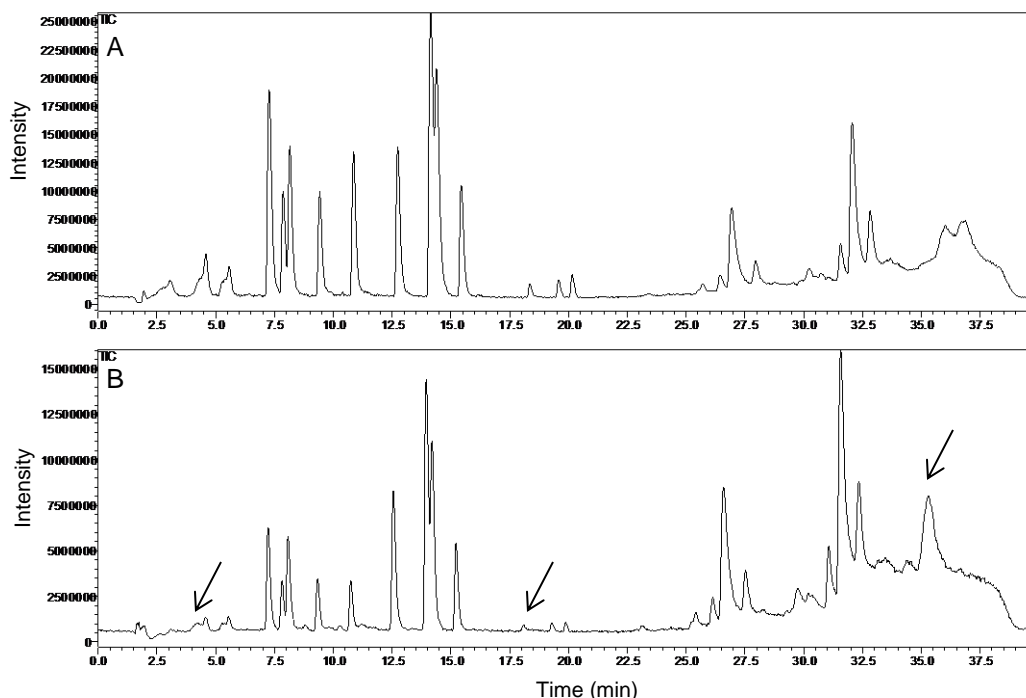
Figure 3.12   Total ion chromatograms of trypsinized α-synuclein (A)
and trypsinized α-synuclein + DOPAL (B) following 4
hours incubation with 0 or 50 μM DOPAL and 8 hours
incubation with trypsin, all at 37°C. Arrows indicate
retention times for DOPAL-modified peptides.

Discussion

DOPAL has been established as a PD-relevant neurotoxicant that is ten-fold more

toxic than its metabolic precursor, DA.[53-57, 83, 105] A number of mechanisms for cellular

damage and toxicity by DOPAL have been suggested, including inhibition of tyrosine

hydroxylase, generation of free radicals, induction of the mitochondrial transition pore

and protein modification and crosslinking.[47, 53, 62-64, 83, 106, 107] Probing these mechanisms

requires a deeper understanding of how DOPAL interacts with various cellular

components it may encounter, which for this study, begins at the amino acid level.

Previous work demonstrated a lack of reactivity for DOPAL toward N-actetyl cysteine.[62]

Peptide (AA)	RT (min)	Modified (Da)	Unmodified (Da)	Adduct (Da)
1-10	35.3	1321.079	1187.5799*	133.499
24-32	4.2	966.4367	830.3832	136.054
84-126	18.1	4996.73	4860.0095*	136.721
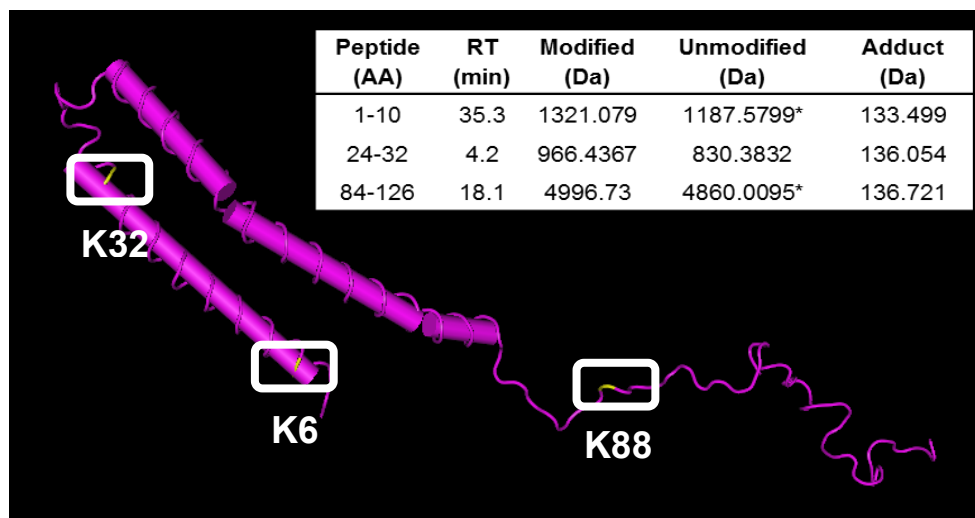
Figure 3.13   DOPAL covalently modifies α-synuclein on three lysine residues, as determined by IT-TOF. Tryptic peptides shown in the table inset are amino acids 1-10, 24-34 and 88-126. Modification at all 3 lysines is indicative of formation of a Schiff base, with the K6 modification a Schiff base imine and the K32 and K88 modifications reduced Schiff base primary amines. *, values for unmodified peptide were determined via Protein Prospector, due to absence of exact peptide in control mass spectrum.

Results shown in this chapter indicate DOPAL is reactive toward lysine and arginine

residues, including free N-acetylated amino acids, those within peptides, and also those

present in whole proteins. Additionally, both electrophilic portions of DOPAL are

necessary for its rate of reactivity toward lysine, as evidenced by a significantly

decreased rate constant observed for MOPAL with N-acetyl lysine that required the

presence of sodium cyanoborohydride. MOPAL was unable to out-compete DOPAL for

modification of a model protein, BSA, even at a ratio of 1:10, DOPAL: MOPAL.

MOPAL also was unable to cause protein crosslinking in another model protein,

GAPDH, unlike DOPAL, which caused significant aggregation.

Reactivity of DOPAL toward amino acids, peptides, and proteins also was also

indicated to require an oxidative environment, likely due to oxidation of the catechol to a

quinone.[47] In the presence of the antioxidant ascorbate, modification of a peptide by DOPAL was significantly diminished. Further study into the reactivity of DOPAL toward arginine in the presence of antioxidants would be valid to better understand the relationship between DOPAL and arginine.

The results observed regarding the reactivity of DOPAL with various model systems allowed further investigation with a PD-relevant protein, α-synuclein. As the main component in the protein-aggregate Lewy bodies, the fibrillar form of α-synuclein has been suggested to be pathogenic.[25] The etiology of these protein aggregates is currently unknown, and it has been suggested that DA or DOPAL could play a role in this aggregation.[25, 63, 81, 82] Previous work with α-synuclein and DA or DOPAL included week-long incubations and use of amine-containing buffers. This chapter has clearly shown DOPAL as the mediator of α-synuclein aggregation, as compared to both DA and DOPAC, with only a four-hour incubation. Additionally, α-synuclein oligomerization by DOPAL was both dose- and time-dependent. Evidence was once again presented that DOPAL oxidation is necessary for its crosslinking capabilities, as both antioxidants, N-acetyl cysteine and ascorbate, previously established to prevent crosslinking of GAPDH prevented α-synuclein aggregation.[62, 108] Additionally, DOPAL caused conformational changes to the native state of α-synuclein in solution, as determined by CD, similar to those changes observed in previous publications indicating formation of amyloid fibrils.[101, 102] Finally, covalent modification of α-synuclein by DOPAL was observed by mass spectrometry, indicating DOPAL modification via Schiff base at three lysine residues.

DOPAL levels are thought to be 2-3 µM in healthy human brain.[90] However, a variety of changes occur in the brains of PD patients that are anticipated to lead to changes in levels of DOPAL. First, MAO is upregulated with age, and risk of incidence of PD also increases with age, with 4-5% of 85-year-olds affected.[11] This upregulation of MAO would lead to greater turnover of DA to DOPAL. Second, cytosolic ALDH1 expression is decreased in the brains of PD patients.[60] Third, ALDH has been shown to be inhibited by products of lipid peroxidation, including 4-hydroxynonenal (4HNE) and malondialdehyde (MDA).[43, 45, 61] Both 4HNE and MDA have been shown to be elevated in the substantia nigra of PD patients.[41, 42] ALDH inhibition by these products of oxidative stress leads to increased levels of DOPAL and decreased levels of DOPAC. Fourth, research has indicated inhibition of mitochondrial complex I leads to decreased oxidation of DOPAL to DOPAC via loss of mitochondrial NAD production.[59] Complex I dysfunction has been observed consistently in the brains of PD patients, with unknown cause.[109, 110] In addition to these reasons for increased DOPAL, research indicates there is a significant decrease in reduced glutathione in the substantia nigra of PD patients.[110] A loss of antioxidant defenses would produce an environment in which DOPAL modification of protein could occur rapidly and indiscriminately. While not shown in this chapter, GSH prevents DOPAL-mediated protein crosslinking, similar to the inhibition observed here for α-synuclein by ascorbate and N-acetyl cysteine.[108] Each of these factors contributes to the case for DOPAL as a highly relevant participant in the etiology and progression of PD. Further understanding of the role DOPAL plays in various PD-relevant systems will eventually allow for development of pharmacological or physiological interventions to prevent progression of this debilitating disease.

CHAPTER FOUR

PARKINSON'S DISEASE AND DOPAL: METABOLISM AND TOXICITY OF

DOPAL IN BV-2 MICROGLIA

<u>Introduction</u>

Parkinson's disease (PD) is a neurodegenerative disorder affecting over 1 million

Americans. Risk of incidence increases with age, with 4-5% of 85-year-olds being

affected.[11] The pathogenesis of the PD-characteristic dopaminergic cell death within the

substantia nigra has not been elucidated, but recent research implicates oxidative stress,

activated glial cells, and reactive metabolites of dopamine (DA) as potential factors in the

progression of the disease.[11, 40, 52, 53, 56, 57, 71] While oxidative stress increases upon

microglial activation, and microglial activation has been shown to lead to dopaminergic

cell death, little research has been done to investigate the direct interaction between

microglia and DA and its metabolites, both with respect to metabolism and activation.[11,
52, 76, 111] Jinsmaa et al. demonstrated products of oxidative stress are able to inhibit

enzymes critical to DA catabolism within dopaminergic cells, and this inhibition

contributes to aberrant levels of a DA metabolite that is an endogenous neurotoxin.[43]

DA is metabolized to 3,4-dihydroxyphenylacetaldehyde (DOPAL) by monoamine

oxidase (MAO). DOPAL is then further oxidized to 3,4-dihydroxyphenylacetic acid

(DOPAC) by aldehyde dehydrogenase (ALDH). In a minor metabolism pathway,

DOPAL can also be reduced by aldehyde reductases to 3,4-dihydroxyphenylethanol

(DOPET) (Scheme 4.1). DOPAL has been shown to be significantly more toxic to

dopaminergic cells than DA and other DA metabolites (e.g. DOPAC and homovanillic

acid) *in vitro* and *in vivo*.[53-55, 59, 83] However, the toxicity of DOPAL for non-neuronal

cells, along with its metabolism and ability to activate microglia, remains unexplored.
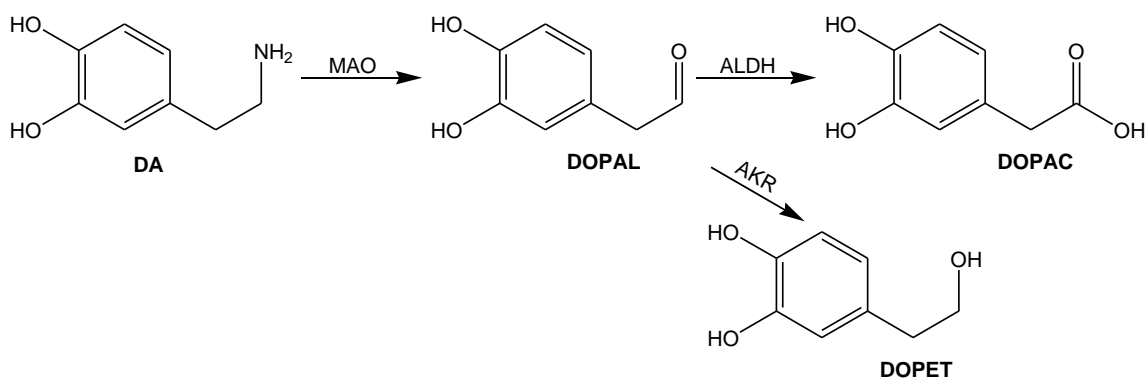
Scheme 4.1 Dopamine (DA) is oxidatively deaminated to DOPAL by
monoamine oxidase (MAO). DOPAL is further oxidized by
aldehyde dehydrogenase (ALDH) to DOPAC, the acid metabolite.
DOPAL can be reduced via an aldehyde-ketone reductase (AKR)
to DOPET, the alcohol, by a minor pathway.

   Neuroinflammation has been proposed as a mechanism in PD progression for a

number of reasons. Reactive microglia have been detected within the substantia nigra of

PD patients.[71, 72] Elevated levels of inflammatory-associated molecules, such as major

histocompatibility complex-related molecules and cytokines, have been detected in the

substantia nigra pars compacta (7- to 15-fold greater than control) and cerebrospinal fluid

of PD patients.[52, 71, 77] This inflammatory environment has been shown to cause

dopaminergic cell death through high levels of nitric oxide, TNF-α, IL-1β, and increased

expression of cyclooxygenase-2 (COX-2).[71, 74] It has been proposed that activated

microglia are the primary contributors of the increased levels of these inflammatory

molecules. Zhang et al. demonstrated that α-synuclein, the major component of PD-

characteristic Lewy bodies (abnormal aggregates of protein within neurons), activates microglia. Upon activation those cells were toxic toward dopaminergic neurons cultured from embryonic mice.[75]

Le et al. demonstrated microglial activation by DA-quinone and hydrogen peroxide-modified dopaminergic cell membranes[52] but no work has explored the direct interaction of DOPAL with microglia. Previously, it was suggested that DA itself was a toxin responsible for the neuronal degeneration that occurs in PD.[49, 78] However, DOPAL has been found to be several-fold more toxic than DA *in vitro* and *in vivo*, as mentioned above, and recent work by Goldstein et al. has demonstrated significantly elevated ratios of DOPAL to DA and DOPAL to DOPAC in the post-mortem brains of PD patients as compared to controls of similar age.[58] The majority of PD patients are advanced in age, and research has shown a significant upregulation of MAO occurs with age in various parts of the brain, especially in the substantia nigra.[112] This upregulation would be expected to increase DA metabolism, yielding elevated levels of DOPAL. Furthermore, research has demonstrated the ability of products of oxidative stress, known to be elevated in PD, to inhibit DOPAL oxidation to the less-reactive DOPAC via ALDH.[40, 43]

Gold standard products of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxy-nonenal (4HNE), have specifically been demonstrated to inhibit both ALDH and aldehyde reductases and ALDH, respectively, within dopaminergic cells at sub-cytotoxic levels,[43] however their ability to inhibit DA catabolism within microglia has not been investigated. Also shown in the Jinsmaa et al. paper was a several-fold increase in levels of extracellular DOPAL following MDA or 4HNE exposure, with no observed change in the intracellular DOPAL concentration.[43] This finding indicates potential for microglial

exposure to elevated levels of DOPAL. Additionally, it was important to compare changes in metabolism within activated microglia to that in resting microglia. The following study was performed to investigate the response of an established murine microglial cell line (BV-2 cells) to DOPAL exposure, with respect to DOPAL metabolism and toxicity, in the presence and absence of MDA or 4HNE.

Experimental Procedures

Materials

DOPAL was obtained via either a previously-described biosynthetic or synthetic method using DA or epinephrine, respectively, as the starting material.[47, 91, 92] DOPAL concentration was determined by an ALDH assay with nicotinamide adenine dinucleotide (NAD$^+$) and high-performance liquid chromatography (HPLC) analysis, as previously established.[62, 93] MDA was synthesized via a previously described method; briefly, 1,1,3,3,-tetramethoxypropane was acidified with hydrochloric acid (1:1) and diluted into 50 mM sodium phosphate buffer (pH 7.4), with concentration determined spectrophotometrically.[43] 4HNE was synthesized as previously described, with concentration determined spectrophotometrically.[113] DA, epinephrine, NAD, Lipopolysaccharide (LPS) and all other chemicals were purchased from Sigma-Aldrich unless otherwise noted.

Cell Culture

BV-2 microglia were obtained via a generous gift from Dr. John Hong, Head, Neuropharmacology Section, NIEHS, NIH and were cultured as previously described.[114] Briefly, the cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/mL penicillin

and 100 μg/mL streptomycin (Invitrogen) at 37°C, 5% CO_2 in a humidified atmosphere.

Cells were plated at 4×10^4 per well in 6-well plates and incubated for two days prior to

experiment with one media change. Activated cells for evaluating DOPAL metabolism

were generated by treatment with 1 μg/mL LPS overnight prior to experiment.

<div style="text-align:center">

Treatment of Cells with DOPAL

and MDA or 4HNE and DA Metabolites

</div>

Two- and four-hour metabolism and toxicity experiments were carried out in

Krebs-Ringer-HEPES buffer (131 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM

$MgSO_4$, 0.4 mM KH_2PO_4, 6 mM glucose and 20 mM HEPES, pH 7.4). Cells were rinsed

with the buffer and allowed to equilibrate for 15 minutes at 37°C, 5% CO_2. In

metabolism experiments containing MDA or 4HNE, 50 μM DOPAL was added to each

well prior to the 15-minute equilibration time. In all other experiments, DA or DOPAL

at indicated concentrations was added at time 0. Aliquots of the extracellular media were

collected at times 0, 30, 60, 120, 180, and 240 minutes into 6% perchloric acid to

precipitate proteins, spun at 10,000xg, and analyzed via HPLC. Toxicity experiments

involving MDA or 4HNE allowed incubation with varying concentrations of inhibitor for

4 hours following a 15-minute equilibration time with buffer only.

<div style="text-align:center">

Quantification of DOPAL and Metabolites by HPLC

</div>

Extracellular DA, DOPAL and metabolites were quantified via HPLC analysis as

previously described.[43, 62] Proteins were precipitated and spun out of the media as

described above, and then samples were injected on an Agilent 1200 Series Capillary

HPLC system. Separation was accomplished with a Phenomenex C18 Luna microbore

column (1x150 mm, 100Å) using isocratic conditions consisting of 0.1% trifluoroacetic

acid in water with 6% acetonitrile (v/v) at a flow rate of 50 μL/min. Metabolites were detected with a photodiode array with absorbance set at 202 and 280 nm. Area under the curve was determined for DA, DOPAL, DOPAC, and DOPET. Concentration of metabolites was generated from standard curve measurements.

<div align="center">Cell Viability Assays</div>

Cell viability was measured by both the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) assays. The MTT assay evaluates cell viability by the ability of mitochondrial reductases to reduce the yellow tetrazolium salt into a purple formazan dye. Following 4-hour exposure to DOPAL, MDA, or 4HNE (as described above), BV-2 cells were incubated with MTT (0.5 mg/mL) for 2 hours at 37°C. Following this incubation, the extracellular medium was removed and centrifuged at 10,000xg for 5 minutes to pellet any formazan dye in floating cells. The pellet of dye and dye within each well were dissolved in DMSO. Absorbance of each product was measured using a Molecular Devices Spectra-Max plate reader (Sunnyvale, CA) at 650 nm and 570 nm. The absorbance at 650 nm was subtracted from the absorbance at 570 nm and then compared to the untreated control values to determine percent control cytotoxicity. Cell viability was evaluated by the LDH assay with a kit from Roche (Indianapolis, IN), performed by manufacturer's instructions. The LDH assay is a determination of cell wall permeability, indicative of cell viability.

<u>Results</u>

DA Catabolism

BV-2 microglia were treated with DA to explore their capability for metabolism. As it has been suggested that DA is responsible for the neurodegeneration observed in PD,[49, 78] examination of the interaction of the microglia with DA was important. When treated with increasing concentrations of DA, BV-2 cells metabolized some DA to DOPAC in 2 hours, which was released into the extracellular media (Figure 4.1). Levels of DOPAC produced, even after treatment with 100 μM DA, were only around 2 μM. Similar results were observed after 4 hours.
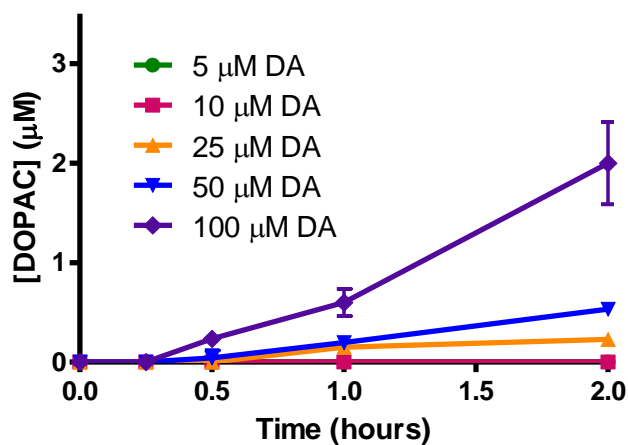
Figure 4.1   BV-2 microglia metabolize DA to low levels of DOPAC in a dose-dependent manner. Upon treatment with 100 μM DA, BV-2 cells only release 2 μM DOPAC after 2 hours.

DOPAL Catabolism

As previous work has demonstrated release of DOPAL against a concentration gradient in a dopaminergic cell line,[43] it was important to examine the capability of microglial cells to metabolize DOPAL to its less toxic, downstream metabolites. BV-2 cells were treated with varying concentrations of DOPAL (5, 10, 25, and 50 μM) for four hours (with aliquots collected at 0, 0.5, 1, 2, 3, and 4 hours). The microglia converted DOPAL (Figure 4.2, panel A) to DOPAC (4.2 panel B) and DOPET (4.2 panels B and C) nearly completely. DOPAL consumption over time paralleled DOPAC (solid line) and DOPET (dashed line) production, indicating DOPAL consumption was due to ALDH and aldehyde reductase activity. The predominant product was DOPAC, with only minor DOPET production observed.

DOPAL Catabolism in Activated Microglia

As activated microglia have been detected in the brains and CSF of patients with PD, and activation leads to changes in protein expression and levels of oxidative stress,[40] it was important to examine potential changes in DOPAL catabolism within activated microglia. DOPAL consumption (Figure 4.3, panel A) by activated cells (treated overnight with 1 μg/mL LPS prior to experiment, represented by open symbols and dotted lines) remained identical to that of resting cells. However, the production of DOPAC was reduced by roughly 40% for all concentrations of DOPAL treatment (4.3, panel B).
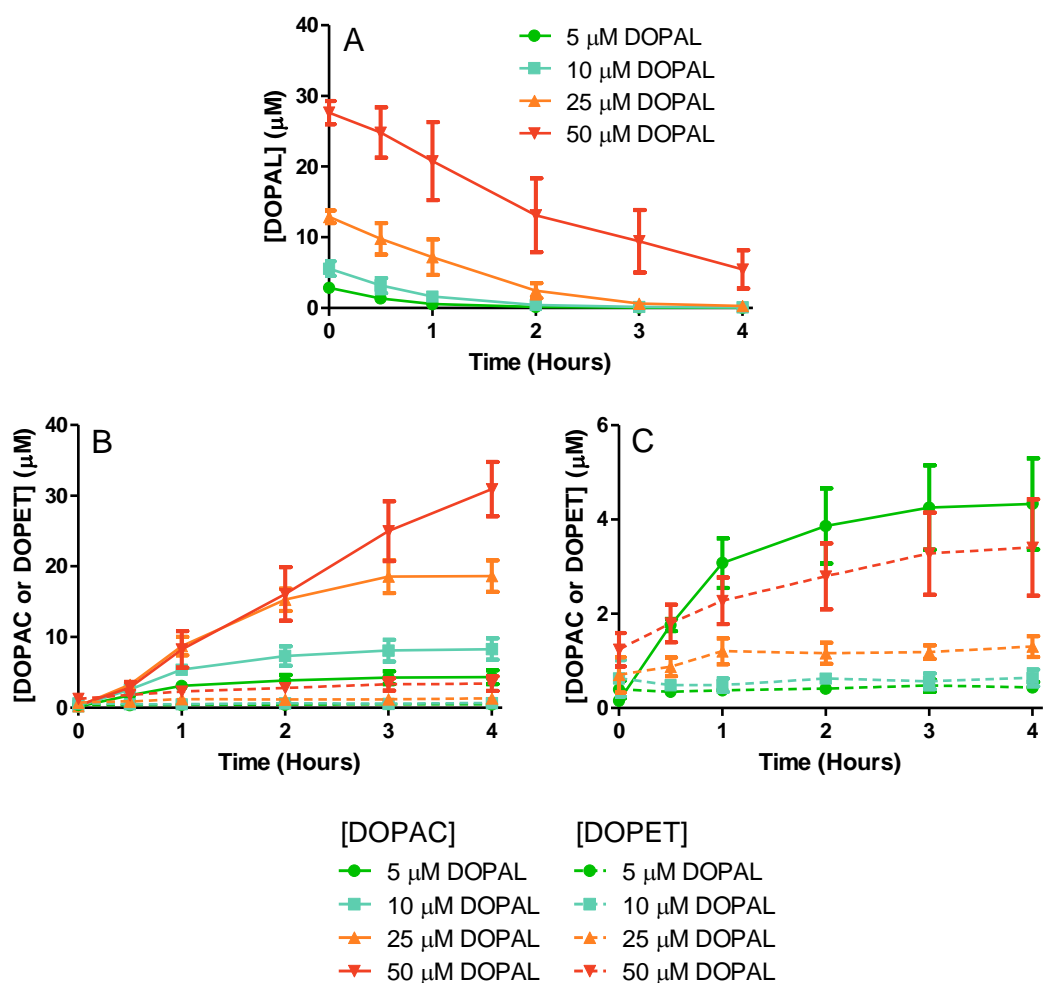
Figure 4.2   DOPAL is metabolized by BV-2 microglia over 4 hours (A) to form DOPAC and DOPET (B & C). The results shown indicate a dose-dependent trend. DOPAC is the predominant metabolite (B). An expansion of the lower region of the graph can be seen in panel C. Nearly complete metabolism of DOPAL to DOPAC and DOPET is observed. (n=5, in triplicate ± SEM)

Catabolism of DOPAL in the Presence of MDA

MDA is a gold standard product of lipid peroxidation also known to inhibit ALDH and aldehyde reductases.[43, 45, 46] Research demonstrating this inhibition was performed in a model dopaminergic cell line and has never been assessed in microglia.[43]
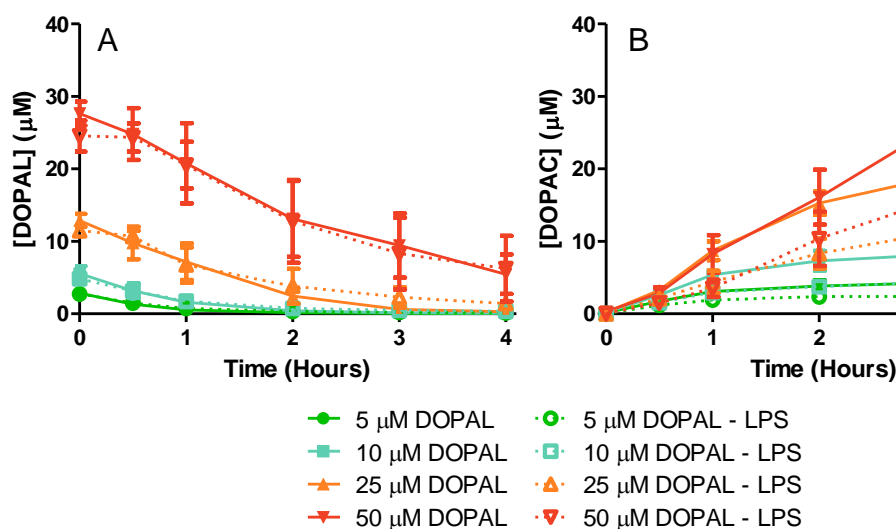
Figure 4.3   Levels of extracellular DOPAL decrease over four hours, similar to control, in BV-2 microglia pre-treated overnight with LPS to induce activation (A). Extracellular DOPAC produced is, on average, decreased at all doses of DOPAL by 40% in activated microglia (B). Control results (generated in non-activated cells), identical to 1A &B, are depicted with a solid line and solid symbols. (*n*=5, in triplicate ± SEM) Results from activated microglia are depicted with dashed lines and open symbols. (*n*=3, in triplicate ± SEM)

BV-2 microglia were pretreated with 50 μM DOPAL for 15 minutes prior to addition of varying concentrations of MDA (1, 10, 25, and 50 μM). MDA caused a dose-dependent inhibition of DOPAL catabolism. Decreased levels of DOPAL consumption were detected at MDA concentrations as low as 1 μM (Figure 4.4, panel A). At 50 μM MDA, there was very little change in extracellular DOPAL levels, even after 4 hours (4.4, panel A). Inhibition of ALDH as evidenced by a decrease in DOPAC production was also observed at 1 μM MDA and dose-dependently with increasing MDA concentrations (4.4, panel B). Treatment with 50 μM MDA exhibited a substantial decrease in DOPAC production.
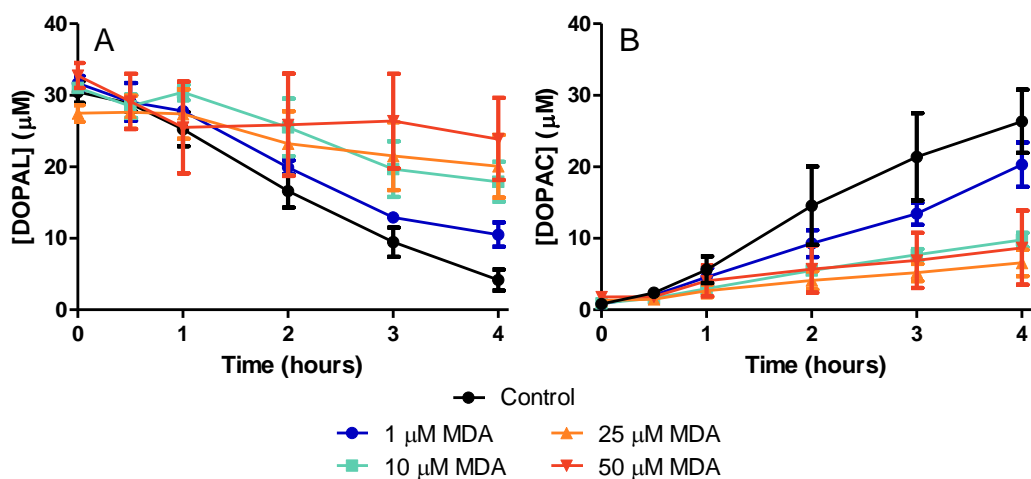
Figure 4.4   Treatment with MDA, a lipid peroxidation product and endogenous ALDH inhibitor, leads to a decrease in DOPAL consumption (A) and DOPAC production (B) over 4 hours. Changes in extracellular DOPAL and DOPAC after MDA treatment were dose-dependent. Cells treated with 50 µM DOPAL alone are indicated as control with a black line. (n=3, in triplicate ± SEM)

Catabolism of DOPAL in the Presence of 4HNE

An additional product of lipid peroxidation is 4-hydroxynonenal (4HNE), which has previously been demonstrated to inhibit ALDH. This inhibition was also shown within a dopaminergic cell line and has not been measured in microglia.[43] The BV-2 microglia were treated with 50 µM DOPAL for 15 minutes prior to addition of varying concentrations of 4HNE (1, 10, 25, and 50 µM). A dose-dependent decrease in DOPAL catabolism was observed with increased concentration of 4HNE present over 4 hours (Figure 4.5, panel A). As DOPAL consumption decreased, DOPAC production also decreased (4.5, panel B). Treatment with 1 µM 4HNE decreased the production of

DOPAC minimally, while 10 µM 4HNE led to a very potent inhibition of DOPAC

production, after one hour and out to four hours.
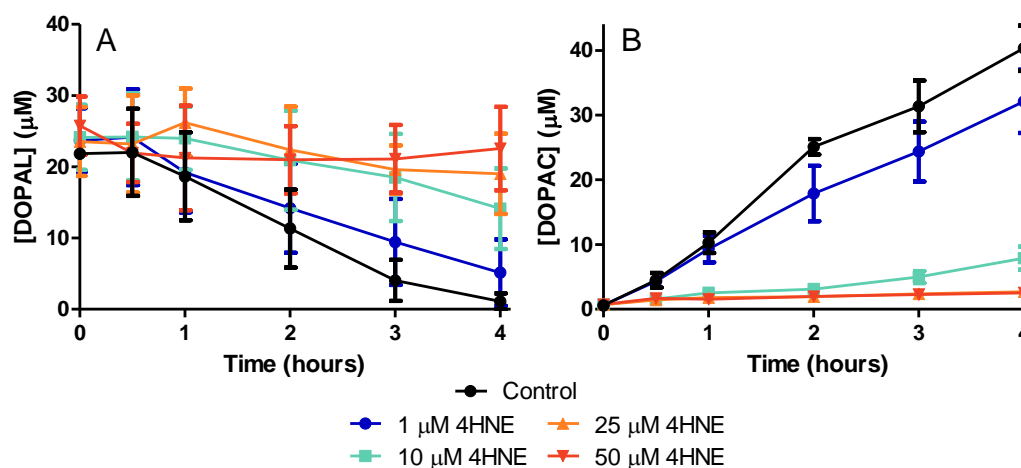
Figure 4.5   Treatment with lipid peroxidation product 4HNE, an endogenous
ALDH inhibitor, leads to a dose-dependent decrease in DOPAL
consumption (A) and production of DOPAC (B) over 4 hours.
Data represented as control are indicative of cells treated with 50
µM DOPAL alone. (*n*=3, in triplicate or duplicate ± SEM)

Cytotoxicity of DOPAL

DOPAL has been demonstrated to be a highly toxic molecule to dopaminergic

cells, both in vitro and in vivo,[53, 55] but no studies have examined its cytotoxicity toward

glial cells. Measurements of lactate dehydrogenase release (LDH assay) and

mitochondrial activity (MTT assay) were used to determine cell viability after four hours

of treatment with varying concentrations of DOPAL (Figure 4.6). The LDH assay is used

to determine whole cell viability, based on permeability of the cell membrane. This assay

demonstrated little DOPAL toxicity to the BV-2 microglia after 4 hours of exposure and

at concentrations up to 50 µM (4.6, panel A). The MTT assay is a determination of cell

viability by the level of functional mitochondrial reductases. DOPAL also exhibited little

toxicity to the BV-2 microglia after 4 hours by the MTT assay (4.6, panel B). The MTT

assay can also be used for determination of cell proliferation, and it is anticipated the

slight increase over control observed in the MTT assay may be due to BV-2 activation

upon DOPAL exposure, which will be further examined in Chapter 5.
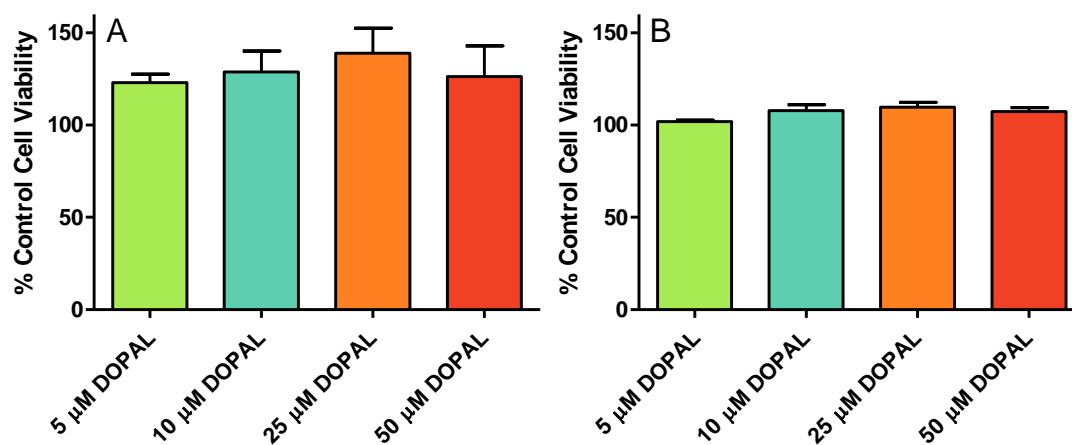
Figure 4.6   DOPAL is not toxic to BV-2 microglia over 4 hours, as determined by mitochondrial viability (MTT assay, A) and lactate dehydrogenase release (LDH assay, B). Concentrations of DOPAL shown (5-50 µM) are known to be significantly toxic to dopaminergic cells. Results shown are determined as % untreated control. ($n=4$, in triplicate ± SEM)

Cytotoxicity of DA

A prevailing hypothesis in the PD field is that the toxicity of DA itself plays a role in the progression of the disease. DA toxicity has been observed *in vitro* and *in vivo* in neuronal and non-neuronal cells,[49, 78] so it was important to examine DA toxicity in the microglia. BV-2 microglia were treated with varying concentrations of DA and the toxicity was observed by both the LDH and MTT assays (Figure 4.7). DA was not observed to be toxic in either assay at concentrations from 5 to 50 μM.
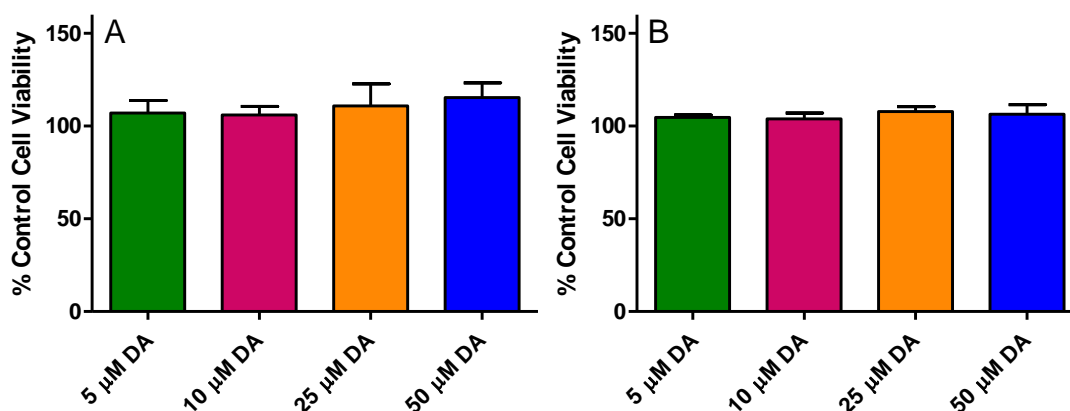
Figure 4.7   DA is not toxic to BV-2 cells over 4 hours. Toxicity was determined by MTT (A) and LDH (B) assays. Results shown are determined as % untreated control. (*n*=3, in triplicate ± SEM)

Endogenous Aldehyde Toxicity in Microglia

Previous work demonstrated MDA and 4HNE to be non-toxic to dopaminergic cells at concentrations as high as 50 μM.[43] However, the toxicity of these aldehydes to glial cells has never been explored. MDA interfered with ALDH catabolism of DOPAL

to DOPAC, the predominant product of DA catabolism. Additionally, MDA

demonstrated significant, dose-dependent toxicity to the BV-2 cells as compared to

control, at concentrations non-toxic to dopaminergic cells at 10 μM and above (Figure

4.8, panel A). A similar trend was observed following treatment with 4HNE, which also

inhibited ALDH catabolism of DOPAL to DOPAC. Significant toxicity was observed at

both 25 and 50 μM 4HNE for the microglia (Figure 4.8, panel B). These concentrations

of 4HNE were previously shown to be non-toxic to dopaminergic cells by the MTT

assay.[43]
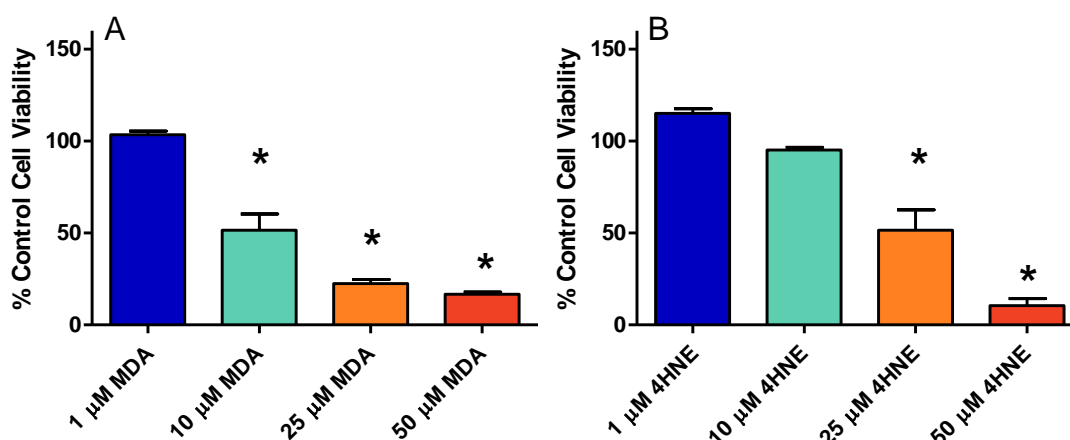
Figure 4.8   Gold standard products of lipid peroxidation MDA (A) and 4HNE
(B) are significantly toxic to BV-2 microglia, at concentrations
lower than those toxic to dopaminergic cells, as determined by the
MTT assay. Results shown are determined as % untreated control.
*, significantly different from control ($p<0.0001$), based on one-
way ANOVA with Tukey post-test. ($n=3$, in triplicate ± SEM)

<u>Discussion</u>

Measurement of DA or DOPAL catabolism in microglia has not previously been documented. While it might be expected that microglial exposure to DOPAL would be limited, prior research has demonstrated extracellular DOPAL levels to be considerably higher than intracellular levels in dopaminergic cells.[43] Increased extracellular DOPAL would come in contact with other cells within the nervous system, including microglia. DA catabolism to DOPAC by BV-2 microglia was lower than anticipated. A number of explanations could attempt to explain these results, e.g. a lack of DA transporter in the microglia and decreased expression of MAO in this cell line. To this author's knowledge, expression of MAO and DA transporter in BV-2 microglia has not been conclusively demonstrated. As DA contains a free amine, it would be positively charged at physiological pH. This charge would prevent simple diffusion across the cellular membrane, therefore transport would be required to allow DA entry into the cell. Also, while MAO-B is typically attributed to 'glial' expression, often the glia described are astrocytes rather than microglia.[65, 115, 116] The BV-2 microglia did exhibit effective catabolism of DOPAL to DOPAC and DOPET, with DOPAC being a major product. This clearance of DOPAL would reduce levels of DOPAL present to kill dopaminergic cells, however, other pathological changes in the glial environment could alter that metabolism. DOPAL levels decrease in the presence of activated microglia, but DOPAC production is significantly inhibited. This altered metabolism warrants further investigation in the future. One factor that may play a role in decreased DOPAC production is the increased expression of COX-2 that takes place upon glial activation. Previous work has shown COX-2 to be capable of DOPAL oxidation, which would

account for the decrease in the DOPAL concentration over time and a lack of DOPAC production.[47] Additionally, as protein levels change within an activated glial cell, it is possible levels of proteins susceptible to DOPAL modification increase, and DOPAL that enters the cell never reaches the ALDH to be oxidized to DOPAC.

Changes in microglial DOPAL catabolism were also observed in the presence of products of lipid peroxidation; 4HNE and MDA. There is established evidence for increased levels of 4HNE and MDA in patients with PD.[41, 42] These increased levels would lead to decreased DOPAL catabolism, meaning an increased concentration of DOPAL would exist in the extracellular space, which could, in turn, increase dopaminergic cell death.

DOPAL has been observed to be highly cytotoxic *in vitro* and *in* vivo to dopaminergic cells and regions of the substantia nigra, respectively.[53-55, 59, 83] DOPAL was not observed to be toxic to the BV-2 microglia at physiologically-relevant, low μM levels shown to cause significant toxicity to dopaminergic cells. Previous work has shown an increase in cell number to correlate to an increase in the value determined by the MTT assay.[117] The observance of greater than 100% of control cell viability could be an indicator of glial proliferation, a known result of glial activation. This result could be a preliminary indicator that DOPAL causes glial activation. DA was also observed to be non-toxic to the microglia. MDA and 4HNE were significantly toxic at concentrations known to cause no significant toxicity to dopaminergic cells.[43] The difference between DOPAL and these products of oxidative stress that leads to a difference in observed cytotoxicity merits continued exploration. All three molecules contain reactive aldehyde moieties, with DOPAL additionally containing a reactive catechol. The presence of both

the aldehyde and the catechol is necessary for DOPAL to elicit its protein-crosslinking and protein-modification capabilities,[62] but appears to also be necessary for its lack of toxicity in microglia.

The initial insult leading to development of PD remains elusive, but microglial activation has been observed in PD patients. This activation is becoming an increasingly popular component in the discussion of PD initiation and progression. The increased ratios of DOPAL to DA and DOPAL to DOPAC observed in PD patients indicate the potential for glia to encounter DOPAL at higher concentrations than they normally experience. Additionally, products of oxidative stress, like 4HNE and MDA, could provide for even greater elevation of DOPAL as they prevent its metabolism to DOPAC. Further investigation into microglial metabolism of DOPAL, and even activation by DOPAL, could allow for elucidation of a mechanism of PD pathogenesis or progression, allowing for generation of a treatment for this devastating disease.

CHAPTER FIVE

PARKINSON'S DISEASE AND DOPAL: ACTIVATION OF BV-2 MICROGLIA BY

DOPAL AND IMPLICATIONS FOR NEURONS

Introduction

While the etiology of Parkinson's disease (PD) has yet to be elucidated, an increasingly popular hypothesis for the initiation and progression of this neurodegenerative disease involves the activation of microglia.[76, 111] Activated microglia have been detected in the brains of PD patients, and proinflammatory cytokines have been observed both in the brain and cerebrospinal fluid of these patients.[72, 73, 77] Unfortunately, the mechanism for this microglial activation remains unknown. Research indicates activated microglia are capable of causing degeneration of dopaminergic neurons through release of cytokines, nitric oxide, and other reactive oxygen species.[71, 111, 118]

Microglia are the specialized immune cells of the brain that are responsible for clearing toxicants via phagocytosis to protect neurons from damage locally.[74, 111] When these cells encounter injury or immunologic stimulus, they undergo a process called activation. Prior to activation, the microglia are known to exist in a resting or ramified state. Upon activation, the cells rapidly undergo morphological changes to an active, ameboid state, a representation of which can be seen in Figure 5.1.[69, 74, 119-121] These changes allow for a more rapid motility toward the site of injury. Along with morphological changes, activated microglia upregulate major histocompatibility complex molecules, increase expression and release of cytokines, and increase expression of other inducible proteins, including cyclooxygenase 2 (COX-2).

Resting, Ramified   Active, Ameboid
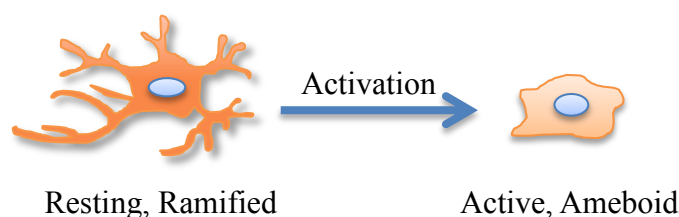
Figure 5.1   Microglia undergo morphological changes upon activation from resting, ramified structures, with long branched processes, to active, ameboid-shaped cells able to move rapidly to a site of injury or toxicity.

A number of hypotheses exist for the mediators that generate microglial activation in PD. These include infection by bacteria,[74] exposure to viral infection,[122] closed head injury,[123, 124] and exposure to pesticides.[125, 126] Also, it is thought that neuronal damage can, in turn, lead to microglial activation, which could then lead to further cell death.[71, 74] Furthermore, evidence exists for dopamine (DA) quinone-modified cell membranes to generate glial activation *in vitro*.[52] *In vivo* experiments involving upregulation of monoamine oxidase (MAO) in mouse brain astrocytes also demonstrated glial inflammation.[65] Finally, direct injection of 3,4-dihydroxyphenylacetaldehyde (DOPAL), the cytotoxic MAO metabolite of DA, into the substantia nigra led to increased glial proliferation, a sign of activation, at sub-cytotoxic concentrations.[55]

As PD selectively affects the dopaminergic cells within the substantia nigra, recent work has suggested DA itself could be the toxin responsible for the disease etiology. However, the MAO metabolite of DA, DOPAL, is significantly more toxic than DA itself (i.e. 100-fold) and has been implicated in PD for a variety of reasons. In addition to its cytotoxicity,[54, 55, 57, 59] DOPAL has been found to be capable of protein

modification and crosslinking (Chapter 3),[61, 62, 107] and able to inhibit key enzymes, including the rate-limiting enzyme in DA biosynthesis, tyrosine hydroxylase.[53, 64]

Prior to the work presented in Chapter 4, the direct interaction of DOPAL and non-neuronal cells remained unexplored. Understanding of the metabolism and toxicity of DOPAL in microglial cells described in Chapter 4 was critical for a preliminary understanding of the interaction between DOPAL and glia. The definitive experiments for studies with microglia required exploration of activation and the consequences of this activation for dopaminergic cells, which will be discussed in this chapter. BV-2 cells, which are an immortalized, murine microglial cell line, were used for this work, along with the dopaminergic, neuronal murine MN9D cell line. The use of the neuronal cell line allowed for examining of the ramifications of DOPAL-mediated microglia activation for neurons.

Experimental Procedures

Materials

DOPAL was obtained via either a previously described biosynthetic method, involving isolated rat or mouse liver MAO, with DA as the starting material, or a previously described synthetic method with epinephrine as the starting material.[47, 91, 92] Concentration of DOPAL was determined by an ALDH assay with nicotinamide adenine dinucleotide (NAD) and high-performance liquid chromatography (HPLC) analysis, which also were both previously established.[62, 93] DA, epinephrine, NAD, and all other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich.

Cell Culture

BV-2 microglia were obtained via a generous gift from Dr. John Hong, Head, Neuropharmacology Section, NIEHS, NIH and were cultured as previously described.[114] Briefly, the cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen) at 37°C, 5% CO_2 in a humidified atmosphere. Cells were plated at 4×10^4 per well in 6-well plates and incubated for two days prior to experiment with one media change.

MN9D dopaminergic cells were originally generated by Dr. Lisa Won and Dr. Alfred Heller, and were received as a generous gift from Dr. Michael Zigmond at the University of Pittsburgh. This cell line was cultured as previously described.[127] In short, cells were grown on poly-L-lysine coated plates in DMEM supplemented with 10% Fetal Clone III serum (Hyclone), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 5% CO_2, 37°C in a humidified environment. Cells were plated 1×10^5 per well in 6-well plates and incubated for five days prior to experiment in differentiating media containing 1 mM sodium *n*-butyrate.

Treatment of BV-2 Cells with LPS,

DA, and DA Metabolites

BV-2 cells were treated for 4 hours in Krebs-Ringer-HEPES buffer (131 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 0.4 mM KH_2PO_4, 6 mM glucose and 20 mM HEPES, pH 7.4) to prevent sequestering of DOPAL by proteins in normal media. Cells were first rinsed with a small volume of Krebs-Ringer-HEPES buffer and then allowed to equilibrate for 15 minutes at 37°C, 5% CO_2, prior to addition of 4 μg/mL

lipopolysaccharide (LPS, a known positive control for glial activation), DA, DOPAL or DOPAC at concentrations shown for each experiment. At four hours, pictures were taken of the cells via digital camera and light microscope. After four hours, an additional volume of DMEM (containing 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin) was added in a ratio of 1:1 to the Krebs-Ringer-HEPES buffer. The cells were incubated for 20 additional hours prior to collection of the extracellular media and lysis, described below. Cells used for ELISA analysis of the extracellular cytokines were treated in DMEM containing 2% FBS for 24 hours with the above-listed compounds, after which time the extracellular media was collected and stored at -70°C. This media (BV-2 conditioned media) was also used for toxicity experiments with the MN9D cells as described below.

<div align="center">TNF-α and IL-6 Sandwich ELISA</div>

Microglial release of exogenous cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), is a known indicator for activation. TNF-α and IL-6 release were measured via a sandwich ELISA kit, following manufacturer's instructions (BioLegend).

<div align="center">Lysis of Cells for Western Blot Analysis</div>

Following 24-hour exposure to LPS, DA, DOPAL, or DOPAC, BV-2 cells were lysed as previously described for analysis by COX-2 Western blot.[128] Following removal of the extracellular media, cells were treated for 10 minutes with lysis buffer (2% SDS (w/v), 2% glycerol (v/v), and 62.5 mM Tris, pH 6.8). Lysate was then heated for 10 minutes at 95°C. Protein concentration was determined by the BCA assay (Pierce) prior to Western blot analysis.

COX-2 Western Blot of BV-2 Cell Lysate

Lysate samples collected following 24-hour experiments (5 μg/lane) were subjected to protein fractionation via SDS-PAGE, using a 7.5% acrylamide gel. Proteins were then transferred to nitrocellulose. Western blots were blocked overnight with 5% milk in TBS-T (w/v) at 4°C. Primary antibody (goat anti-COX-2, Santa Cruz Biotechnology, 1:200) was added and membranes were incubated 2 hours at room temperature. Membranes were then rinsed in TBS-T and incubated for 1.5 hours at room temperature with secondary antibody (bovine anti-goat, Santa Cruz Biotechnology, 1:10,000). Following TBS-T rinses, membranes were incubated with 3 mL ECL Prime (GE/Amersham) for 5 minutes. Film was exposed to the membranes and developed.

Incubation of MN9D Cells with DOPAL

or BV-2 Conditioned Media

Four-hour metabolism and toxicity experiments were carried out in Krebs-Ringer-HEPES buffer, as described above for BV-2 experiments. Cells were rinsed with the buffer and allowed to equilibrate for 15 minutes at 37°C, 5% CO_2. DOPAL was added at time 0 at the indicated concentrations. Aliquots of the extracellular media were collected at times 0, 30, 60, 120, 180, and 240 minutes into 6% perchloric acid to precipitate proteins, spun at 10,000xg, and analyzed via HPLC. After 4 hours, cell viability was determined by the MTT assay, described below. MN9D cells were also treated for 4 hours with BV-2 conditioned media as described above. Following the 4 hour treatment, cell viability was measured by the MTT assay. Control wells for this experiment were treated with fresh DMEM containing 2% FBS.

Quantification of DOPAL and Metabolites by HPLC

Extracellular DOPAL and DOPAC were quantified via HPLC analysis as previously described.[43, 62] Proteins were precipitated and centrifugation carried out as described above, and then samples were injected on an Agilent 1200 Series Capillary HPLC system. Separation was accomplished with a Phenomenex C18 Luna microbore column (1x150 mm, 100Å) using isocratic conditions consisting of 0.1% trifluoroacetic acid in water with 6% acetonitrile (v/v) at a flow rate of 50 μL/min. Metabolites were detected with a photodiode array at 202 and 280 nm absorbance. Area under the curve was determined for DOPAL and DOPAC. Concentration of metabolites was generated from standard curve measurements.

Cell Viability Assays

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay for the MN9D cells. The MTT assay evaluates cell viability by the ability of mitochondrial reductases to reduce the yellow tetrazolium salt into a purple formazan dye. Following 4-hour exposure to DOPAL or BV-2 conditioned media, as described above, MN9D cells were incubated with MTT (0.5 mg/mL) for 1.5 hours at 37°C. Following this incubation, the extracellular medium was removed and centrifuged at 10,000xg for 5 minutes to pellet any formazan dye in floating cells. The pellet of dye and dye within each well were dissolved in DMSO. Absorbance of each product was measured using a Molecular Devices Spectra-Max plate reader at 650 nm and 570 nm. The absorbance at 650 nm was subtracted from the absorbance at 570 nm and then compared to the untreated control values to determine percent of control cytotoxicity.

<u>Results</u>

Morphological Changes in BV-2 Cells

Following DOPAL Treatment

As mentioned in the introduction and indicated in Figure 5.1, microglia undergo

morphological changes upon activation.[69, 120] The images displayed in Figure 5.2 exhibit

these changes occurring in the cells after only four hours. The control cells (5.2, panel A)

have resting processes, as do some of those treated with LPS, a known positive control

for glial activation (5.2, B). Some of the LPS-treated cells have begun to retract their

processes, indicating the beginnings of activation. The microglia treated with 10 μM

DOPAL (5.2, C) have begun process retraction, while the cells treated with 100 μM

DOPAL (5.2, D) have completely drawn in their processes and show blebbing,

demonstrating apoptosis (indicated with the arrowheads). These data strongly

demonstrate microglial activation occurs rapidly (within 4 hours) at high levels of

DOPAL.

Release of TNF-α by BV-2 Microglia

Following Treatment with DA Metabolites

TNF-α levels in the extracellular medium of BV-2 microglia were measured after

a 24-hour incubation with 4 μg/mL LPS as positive control or varying concentrations of

DA metabolites by sandwich ELISA to determine the extent of activation of these cells

(Figure 5.3). Increased release of TNF-α, an inflammatory cytokine, is known to occur

upon microglial activation and be an indicative endpoint for activation.[71, 111, 129] TNF-α

levels were significantly elevated upon treatment with 10, 25 and 50 μM DOPAL, which

are physiologically relevant concentrations for aberrant DOPAL metabolism. DOPAL

has been detected at levels of around 2 μM in non-diseased human brain.[90] There was no

significant release of TNF-α observed after treatment with DA or DOPAC.
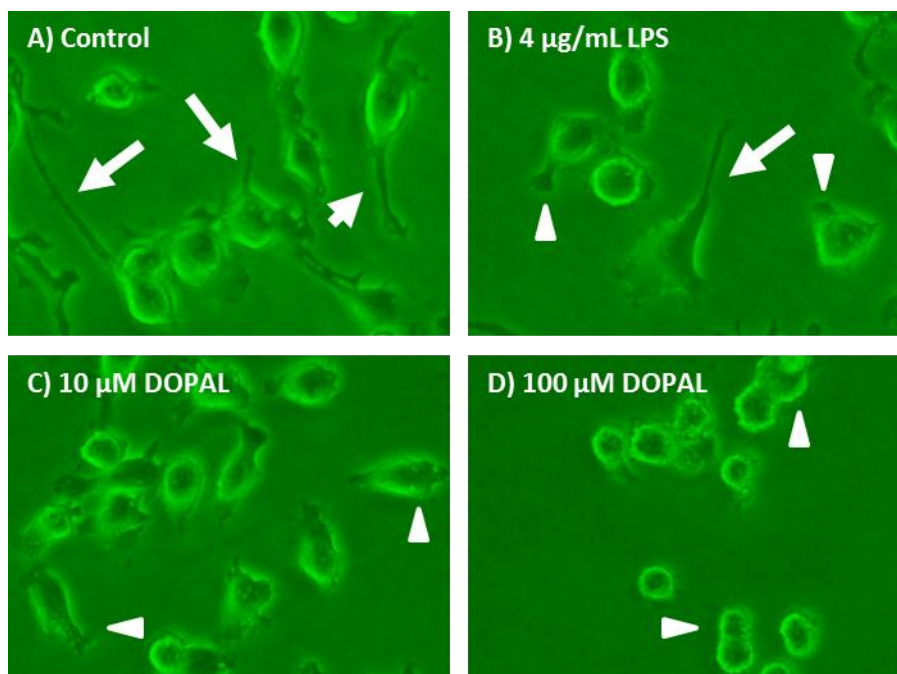
Figure 5.2   Morphological characteristics of BV-2 microglia after 4-hour
incubation: A) Control, B) 4 μg/mL LPS, C) 10 μM DOPAL, D)
100 μM DOPAL. Arrows indicate microglial processes,
arrowheads indicate retracting processes and blebbing.

IL-6 Release by BV-2 Microglia

Following Treatment with DA Metabolites

Microglia release a wide array of cytokines upon activation. While it is thought

that TNF-α may play a significant role in mediating neuronal death generated by

activated microglia,[71] a number of other cytokines are known to be upregulated and

released by microglia which could lead to a neurotoxic environment. IL-6 has been

shown to be released upon glial activation and was measured by sandwich ELISA.[128]

Results shown in Figure 5.4 are indicative of IL-6 release following treatment with

4 µg/mL LPS, or 50 µM DA, DOPAL or DOPAC. DOPAL caused significant activation,

as measured by IL-6 release, while DA and DOPAC did not.
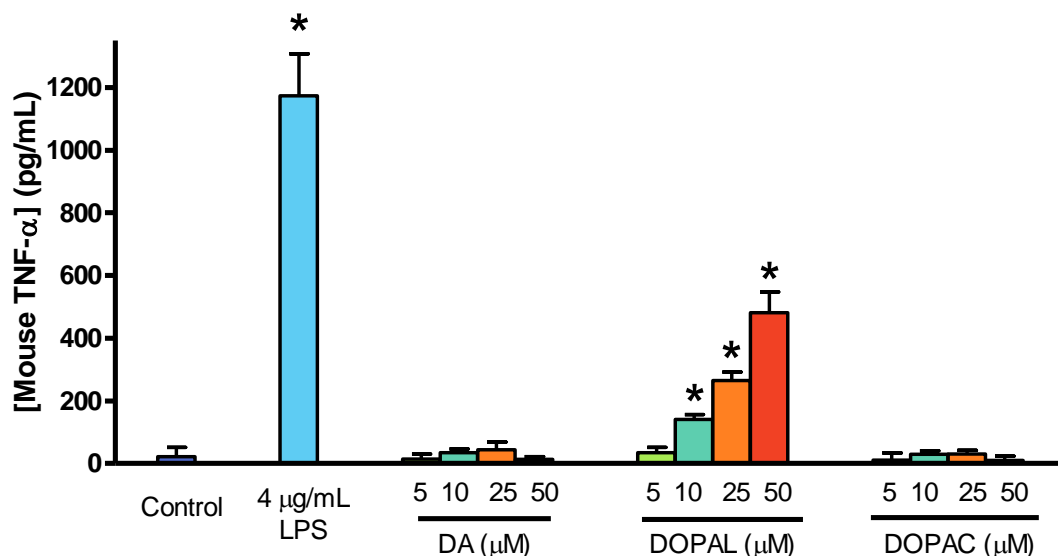
Figure 5.3   DOPAL, but not DA or DOPAC, significantly activates BV-2
microglia at physiologically relevant concentrations as measured
by TNF-α release.   (Results shown are the average from an
experiment on one day in three individual plates ± SD). The
control and LPS bars were generated as the average of experiments
on 4 days, each in three individual plates. *, significantly greater
than control, ($p<0.0001$), based on two-tailed t-test.

Activation of Microglia by DOPAL

Measured by Expression of COX-2

Thus far, evidence of microglial activation by DOPAL has been presented

through morphological changes and extracellular observations of cytokine release. In

addition to changes in cytokine expression and release, changes in expression of

intracellular proteins also take place upon microglial activation. One frequently

measured protein is the inducible COX-2.[128, 129] COX-2 expression was measured by
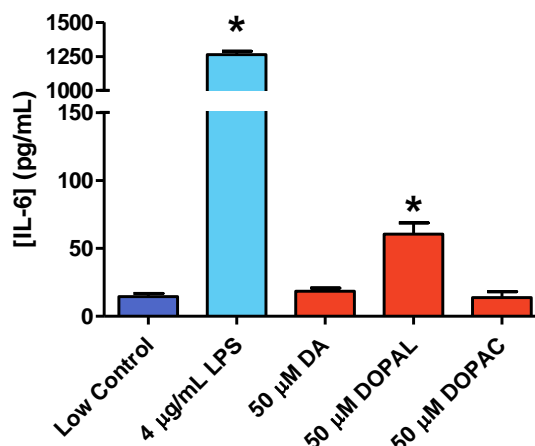
Figure 5.4   Activation of BV-2 microglia as observed by release of IL-6 upon treatment with 4 μg/mL LPS, or 50 μM DA, DOPAL or DOPAC. Only DOPAL caused significant activation. (Results shown are the average from an experiment on one day in three individual plates ± SD). *, significantly greater than control, ($p<0.05$), based on one-way ANOVA with Tukey's post-test.

Western blot of lysate from BV-2 cells treated with increasing concentrations of DOPAL (5, 10, 25, 50 μM) or 4 μg/mL LPS for 24 hours. Results are displayed in Figure 5.5, where panel A shows a representative Western blot at 70 kDa. The graph in panel B indicates the average densitometry from three Western blots generated from experiments on two different days, with each individual Western blot having been run with lysate from individual plates. The bars in the graph in Figure 5.5, panel B, represent the fold increase in band intensity over the control band.

COX-2 Expression in BV-2 Microglia

Treated with DA, DOPAL or DOPAC

While increased COX-2 expression was observed with increasing doses of DOPAL, it was important to compare COX-2 expression following treatment with DA or DOPAC to the expression induced by treatment with DOPAL. Cells were treated with
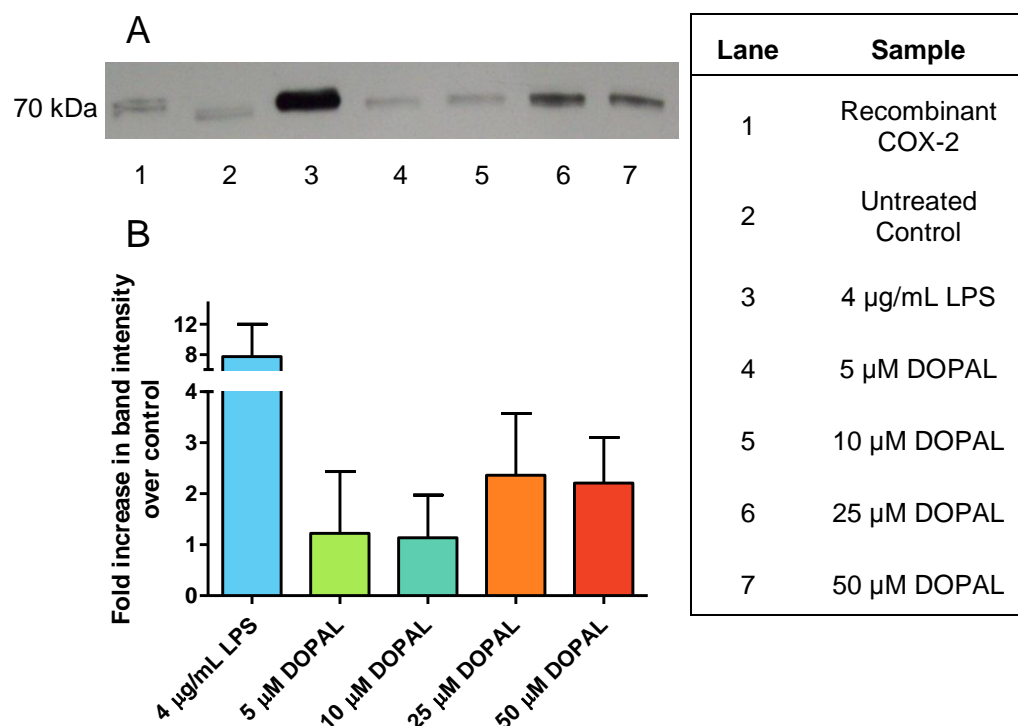
Figure 5.5   COX-2 expression increases in BV-2 microglia following treatment with increasing concentrations of DOPAL. Panel A contains a representative COX-2 Western blot at 70 kDa. The graph in panel B is the average ± SD of the fold increase in band intensity over the control well from 3 Western blots generated from 3 individual 6-well plates on 2 separate days.

50 µM of each compound or 4 µg/mL LPS for 24 hours, and lysate from each individual

plate was collected separately. Incubation with DA or DOPAC produced very little

change in COX-2 expression, compared to control (Figure 5.6). Panel A is a

representative COX-2 Western blot at 70 kDa. Densitometry values for the control lanes

(1 and 2) were averaged for the control value used for generation of the graphs in panel

B. Values were produced for the graph from densitometry of two blots generated by two

individual experimental plates.
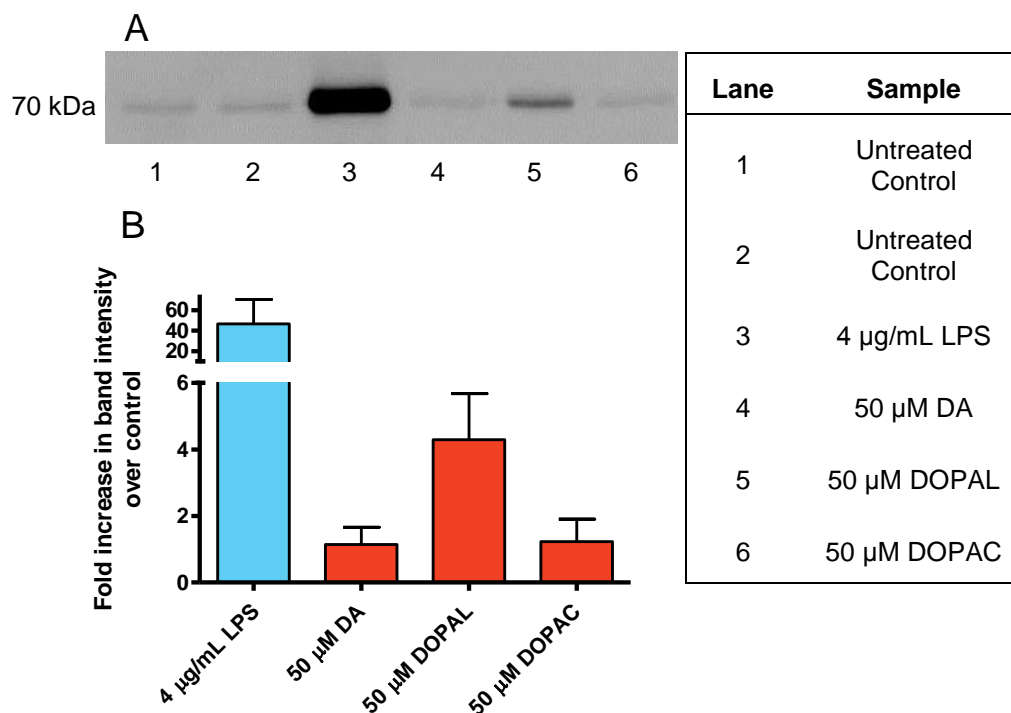
Figure 5.6   Changes in COX-2 expression are limited to treatment with DOPAL. Neither DA nor DOPAC treatment elicited change in expression from that of control. A representative COX-2 Western blot is presented in panel A. Panel B contains graphical representation of the average ± SD of the fold increase in band intensity over the control well from 2 Western blots generated from 2 individual 6-well plates.

DOPAL Catabolism in MN9D Dopaminergic Cells

The MN9D dopaminergic cell line was produced by somatic cell fusion of murine embryonic neurons from the rostral mesencephalic tegmentum with a neuroblastoma cell line (N18TG2). First publication of this cell line came from the lab of Dr. Alfred Heller at The University of Chicago in 1991.[127] The cells were presented as capable of DA biosynthesis and as an improved model for dopaminergic neurons over the previously established dopaminergic PC12 line isolated from a rat adrenal medullary tumor. This

cell line was selected for work in this project as it is murine-derived like the BV-2 microglia, which would facilitate the most physiologically relevant environment for examining glial-mediated toxicity of neurons. Previously published work with BV-2 microglia and neurons used the human-derived SH-SY5Y cells to explore microglia-neuron interactions.[129-131] Before beginning MN9D experiments with BV-2 conditioned media, it was important to examine the metabolism and toxicity of DOPAL in this cell line, which was previously unknown. Differentiated MN9D cells were treated with increasing concentrations of DOPAL and aliquots of the extracellular media were collected over 4 hours. Samples were analyzed by HPLC to determine levels of DOPAL and DOPAC (3,4-dihydroxyphenylacetic acid, the ALDH metabolite of DOPAL) present. In Figure 5.7, levels of DOPAL (panel A) and DOPAC (panel B) in the MN9D media measured over 4 hours are shown. DOPAL was metabolized dose- and time-dependently to DOPAC, with the exception of treatment with 50 μM DOPAL. Treatment with 50 μM DOPAL produced roughly equal concentrations of DOPAC as that of treatment with 25 μM DOPAC.

<div align="center">Toxicity of DOPAL in MN9D Cells</div>

The toxicity generated by DOPAL in differentiated MN9D cells was determined by the MTT assay which measures cell viability by mitochondrial reductase activity. DOPAL was less toxic to the MN9D cells after 4 hours than previously reported in PC6-3 cells after only 2 hours.[53] Significant toxicity was observed in the PC6-3 cells by the MTT assay and the trypan blue method at concentrations as low as 10 μM at 2 hours. Results from the MTT assay for MN9D cells treated with DOPAL are in Figure 5.8. Significant toxicity was only observed at 50 μM DOPAL.
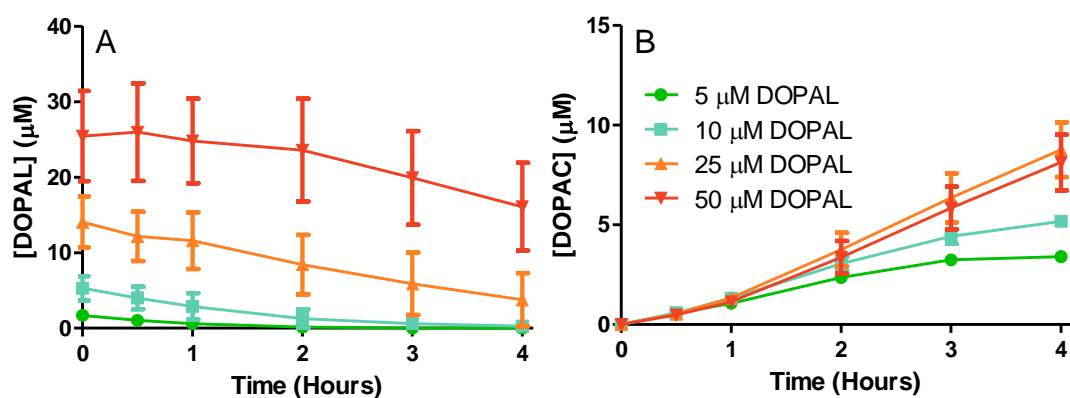
Figure 5.7   DOPAL (panel A) is metabolized by differentiated MN9D
              dopaminergic cells to DOPAC (panel B) over 4 hours. DOPAC
              produced following treatment with 50 μM DOPAL is similar to
              that produced following treatment with 25 μM DOPAL. ($n=3$, in
              triplicate ± SEM)
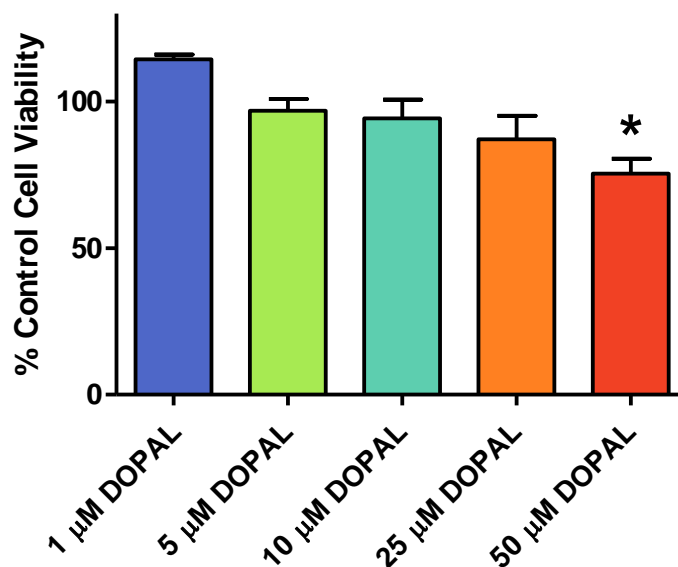
Figure 5.8   DOPAL is not significantly toxic to differentiated MN9D cells
              after 4 hours, except at 50 μM, as determined by the MTT assay,
              an indicator of mitochondrial viability. Results shown are
              determined as % control. *, significantly different from control
              ($p<0.05$), based on one-way ANOVA with Tukey post-test. ($n=3$,
              in triplicate ± SEM)

Treatment of MN9D Cells with BV-2 Conditioned Media

Glial-mediated toxicity of dopaminergic cells is thought to be due to release of

cytokines and other molecules, such as nitric oxide, that occurs upon microglial

activation. BV-2 media that was collected following 24 hours of incubation with

4 μg/mL LPS or 50 μM DA, DOPAL or DOPAC and analyzed for TNF-α and IL-6

release was added to differentiated MN9D cells. Following a 4-hour incubation with the

BV-2 conditioned media, the MTT assay was performed on the MN9D cells (Figure 5.9).

Results shown are percent control, where the control was treated with fresh DMEM

containing 2% FBS. No statistical significance was found for these results.
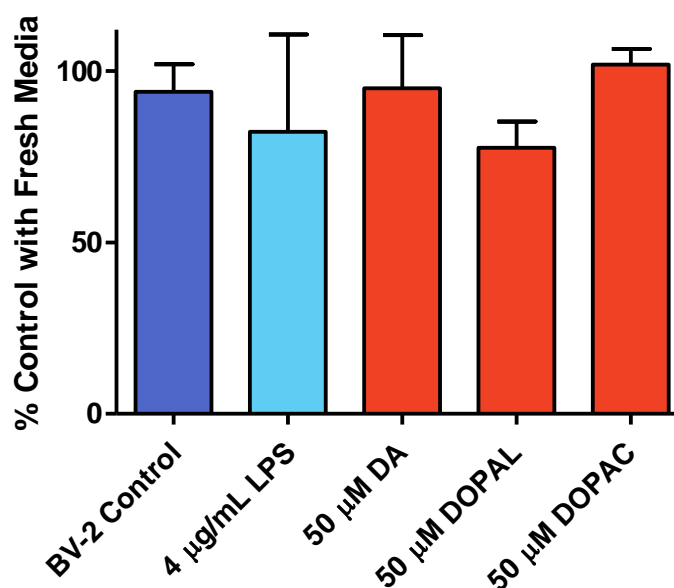
Figure 5.9   BV-2 conditioned media generated by incubation with 50 μM
DOPAL is more toxic to differentiated MN9D cells than that
produced by 50 μM DA or DOPAC after 4 hours. Bars represent
the average (± SD) of 3 wells, each from an individual 6-well
plate, each treated with conditioned media from 1 individual BV-2
plate.

<u>Discussion</u>

Activated microglia have been observed in the brains of patients with PD. This activation is frequently presented as localized to the substantia nigra, the dopaminergic region of the brain most affected in PD.[72, 76] This localized activation, along with evidence of the ability of microglia to cause damage and even death to nearby neurons,[74, 111, 118] makes gaining understanding of potential mechanisms for activation important in studying PD. While a number of mechanisms of activation have been proposed (e.g. bacterial or viral infection, pesticide exposure, oxidative stress),[74, 122-126] DOPAL has also been shown to cause glial proliferation upon direct injection into the substantia nigra.[55] Glial activation was also observed in an animal model with upregulated astrocytic MAO-B.[65]

Examination of the interaction between microglia and DOPAL is important, as there is increased chance of microglia encountering DOPAL in the brains of PD patients for a number of reasons. First, Fornai et al. observed extracellular DOPAL *in vivo* in rodents by microdialysis HPLC analysis, indicating that even in a healthy brain, some level of extracellular DOPAL exists.[132] Second, work with products of lipid peroxidation caused potent inhibition of ALDH in dopaminergic cells pre-treated with DA, which led to increased levels of DOPAL. The extracellular concentration of DOPAL was found to be substantially higher than the intracellular concentration, indicating DOPAL was moving across the cell membrane against a concentration gradient.[43] These results, in addition to the upregulation of MAO that occurs with age, point to increased extracellular levels of DOPAL.[112] Goldstein et al. recently observed levels of DA, DOPAL, and DOPAC in the brains of PD patients post-mortem and compared them to age-matched

controls. Ratios of DOPAL to DA and DOPAL to DOPAC were four-fold greater in the brains of PD patients than those of control subjects.

Work presented in this chapter examined the microglial response in BV-2 microglia to treatment with DOPAL and compared it to that for DA and DOPAC. Results presented in Chapter 4, Figure 4.6, panel A provided a preliminary indicator of microglial activation upon incubation of DOPAL by values for % cell viability greater than 100%. Values greater than 100% are indicative of proliferation,[117] which is a marker of glial activation. The next indicator of glial activation presented in this work was the morphological changes observed in the BV-2 cells following 4-hour exposure to DOPAL. The retraction of ramified processes and change of cell shape to a more ameboid-like structure are indicative of glial activation, and were observed at both 10 and 100 μM DOPAL, similar to the positive control (LPS treatment).

Release of neurotoxic cytokines is thought to be a mechanism for microglial-mediated neuron death, like that observed in PD.[71, 74, 118] The catechol and aldehyde groups present in the structure of DOPAL do appear to be necessary to elicit activation in BV-2 microglia, as DOPAL caused statistically significant activation, as measured by release of both TNF-α and IL-6, while DA and DOPAC did not. As DOPAL is nearly completely metabolized to DOPAC in 4 hours by these microglia, it is important to note that only DOPAL and not DOPAC stimulated activation. Release of TNF-α generated by DOPAL treatment was dose-dependent, indicating that as levels of DOPAL increase with progression of PD, likely the amount of neurotoxic cytokines released would also increase. However, the physiological relevance of the release of TNF-α and IL-6 shown in this work is not currently known.

Microglial activation causes many changes in protein expression, including expression of the inducible enzyme COX-2. Expression of COX-2 was increased dose-dependently in the presence of DOPAL. Additionally, DA and DOPAC did not generate any significant changes in levels of COX-2 expressed as compared to untreated microglia. These changes again point to DOPAL as the mediator of microglial activation. Additionally, increased expression of COX-2 has distinct ramifications within this system, as recent work by Anderson et al. demonstrated the ability of COX-2 to oxidize DOPAL to oxidized products, likely including the more reactive semi-quinone radical and quinone.[47] This oxidation of DOPAL could lead to further cellular damage for dopaminergic cells by protein modification or even increased microglial activation.

Upon establishing DOPAL's ability to cause activation of the BV-2 cells, the metabolism and toxicity of DOPAL in MN9D dopaminergic cells was investigated, with the purpose of understanding the response of these cells to DOPAL before exploring the ability of BV-2 cells to mediate death of dopaminergic cells. MN9D cells were found to metabolize DOPAL to DOPAC over four hours. This production was not complete at 25 or 50 μM DOPAL, and a similar concentration of DOPAC (~10 μM) was produced following incubation with 25 or 50 μM DOPAL. A number of factors could be at play in this result, including differences in movement of DOPAL or DOPAC across the cell membrane for this cell line. It is possible the mechanism of transport is saturated for one metabolite or the other preventing measurement of these levels extracellularly. Likewise, a level of saturation for ALDH metabolism of DOPAL could be the cause of this result. It would be prudent to investigate levels of ALDH within this cell line to confirm the less-than-complete metabolism of DOPAL is not due to decreased ALDH expression.

DOPAL was found to be significantly toxic to this cell line at 50 μM. Some toxicity was observed at lower concentrations, but the results were not statistically significant.

While glial activation in and of itself is highly relevant in a PD paradigm, establishing the ability of DOPAL-generated activation in microglia to cause toxicity to dopaminergic neurons was able to bring the research in this dissertation full-circle. MN9D cells were treated with BV-2 conditioned media previously generated by incubation with LPS, DA, DOPAL or DOPAC. Greatest mean toxicity was observed for the BV-2 conditioned media generated by incubation with DOPAL, followed by the BV-2 media generated by incubation with LPS. Toxicity observed for BV-2 conditioned media generated by incubation with DA or DOPAC was equal to or less than that observed for the BV-2 conditioned media control.

While the definitive cause of the increased glial activation observed in PD and resulting toxicity for dopaminergic neurons has not yet been established, these results indicate DOPAL could play a role in the process. Microglial activation by DOPAL could lead to PD-hallmark toxicity for dopaminergic cells. Further work is required to establish the exact mechanism for this DOPAL and glial-mediated toxicity. A greater understanding of the part DOPAL plays in PD glial activation and neurotoxicity will hopefully allow for discovery of disease biomarkers and pharmacological agents that could help slow or halt the progression of this degenerative disease.

CHAPTER SIX

RESEARCH SUMMARY

<u>Restatement of Hypothesis</u>

Parkinson's disease (PD) is a selective neurodegenerative disorder that affects the dopaminergic neurons within the substantia nigra. Incidence of the disease increases with age, and over 1 million Americans currently suffer from PD.[11] The etiology of this debilitating disease, in which patients exhibit a loss of motor control, is still unknown. A wide array of research is currently taking place in this field to elucidate a mechanism for PD initiation and progression. While genetic anomalies are thought to make up a small percentage of the cases of PD (10%), researchers are looking to uncover further underlying genetic mechanisms.[22-24, 26] Evidence has shown the primary protein present in the PD-hallmark aggregate Lewy bodies, α-synuclein, aggregates faster following a single amino acid mutation due to a genetic mutation. Due to a high incidence of PD in populations living near areas of elevated pesticide application or bioaccumulation, scientists have discovered some correlation between pesticide exposure and PD incidence, leading to further research in this realm.[32-35, 37] Treatment of microglia with pesticides *in vitro* has been indicated to lead to increased levels of oxidative stress.[125]

Oxidative stress has been implicated as a significant contributor in PD. Increased transition metal levels have been measured in the brains of PD patients, and these metals are capable of participating in the Fenton reaction (Scheme 1.2), which produces a free hydroxyl radical.[29] Increased reactive oxygen species have been shown to lead to increased lipid peroxidation. Hallmark products of lipid peroxidation, 4-hydroxynonenal (4HNE) and malondialdehyde (MDA), have been detected at elevated concentrations in

the brains of PD patients.[41, 42] An additional contributor to increased oxidative stress

within the substantia nigra is dopamine (DA). As a result of its catchol moiety, DA is

able to auto-oxidize through a one- or two-electron mechanism to a semi-qunone radical

and ortho-quinone, respectively.[47, 48] Oxidation of DA generates reactive oxygen species

that can elicit further cellular damage. The neurotoxic, aldehyde metabolite of

monoamine oxidase (MAO) activity on DA, 3,4-dihydroxyphenylacetaldehyde

(DOPAL), has also been shown to be able to oxidize through a one- or two-electron

mechanism to produce free radicals and reactive oxygen species.[47]

DOPAL has been shown to be 100-fold more toxic than DA, and has been

implicated in PD for a variety of reasons.[54, 55, 57] DOPAL is capable of covalent protein

modification and protein crosslinking, including aggregation of α-synuclein.[63]

Additionally, it is capable of inhibiting key enzymes in DA biosynthesis.[53, 64] The ratios

of DOPAL to DA and DOPAL to DOPAC were observed to be 4-fold higher in the

brains of PD patients as compared to controls, which further indicates DOPAL is a highly

relevant molecule for continued study.[58]

An additional component of PD under investigation currently is the presence of

activated microglia within the substantia nigra. Activated microglia are capable of

releasing a host of neurotoxic cytokines and causing damage to dopaminergic neurons

upon activation.[71, 74] The mechanism that generates the activated microglia in PD

patients remains unknown. DA-quinone modified dopaminergic cell membranes have

been shown to be capable of producing microglial activation, but little work has been

done to explore the interaction of DA itself and its metabolites with microglia. This body

of work addressed the disparity in this realm by testing the following hypothesis:

DOPAL, a reactive metabolite of dopamine, causes sub-cellular and cellular changes relevant to Parkinson's disease, including protein aggregation and microglial activation.

Discussion of Specific Aims

Specific Aim 1: Ascertain the Reactivity of DOPAL

Toward Amino Acids, Peptides,

and Proteins Relevant to PD.

As illustrated in Figures 3.1 and 3.2, DOPAL reacts with a determinable rate constant with lysine and arginine. This reaction was determined to be covalent through mass spectrometry of peptides, shown in Tables 3.1 and 3.2. Additionally, the reaction observed in the presence of DOPAL and peptide requires an environment in which oxidation can occur (Figure 3.3), as incubation of the peptide and DOPAL with ascorbate led to formation of a single adduct, rather than multiple DOPAL adducts. An additional confirmation of modification of a protein by DOPAL was observed with the aid of nitroblue tetrazolium dye which detects catechols.[97] Figure 3.4 illustrates the ability of DOPAL to bind bovine serum albumin, even in the presence of a structurally analogous metabolite, 3-methoxy-4-hydroxyphenylacetaldehyde (MOPAL). Additional proof of the necessity for the catechol moiety of DOPAL can be observed in Figure 3.5, where DOPAL produced crosslinking of glyceraldehyde-3-phosphate dehydrogenase, but MOPAL, which does not contain a catechol, did not.

The work in this chapter continued with exploration of the role of DOPAL in aggregation of the main component of PD-hallmark Lewy bodies, α-synuclein. DOPAL, but not DA or DOPAC, caused aggregation of α-synuclein after four hours (Figure 3.6).

α-Synuclein oligomerization caused by DOPAL was both dose- and time-dependent, as illustrated by the Western blots in Figures 3.7 and 3.8. These Western blots confirm and strengthen the results previously published by Burke et al. indicating aggregation of α-synuclein by DOPAL. The incubations performed in this publication used tris buffer, which likely skewed the data, as tris contains a free amine, with which DOPAL would readily react.[63] Additionally, these results show aggregation of α-synuclein (10 μM) after incubation with even 1 μM DOPAL, while Burke showed aggregation of 2 μM α-synuclein after incubation with 15 μM DOPAL. While the incubation shown in Figure 3.7 was four hours, and that of the Burke paper was one hour, the results in Figure 3.8 indicate aggregation of 10 μM α-synuclein does occur even after 1 hour with 10 μM DOPAL (a 1:1 ratio protein:DOPAL, vs. 1:15). Aggregation of α-synuclein by DOPAL required an oxidative environment, as antioxidants ascorbate and N-acetyl cysteine prevented oligomerization at very low concentrations (Figures 3.9 and 3.10). Prevention of this aggregation by antioxidants could be inhibited in PD, as levels of an endogenous reducing agent, glutathione, are significantly lower.[39, 110] These results further point toward the ability of DOPAL to cause α-synuclein aggregation *in vivo*. Further results of DOPAL perturbation of α-synuclein in solution were observed through circular dichroism (Figure 3.11). Changes observed (a decrease in α-helix-type structure and increase in β-strand-type structure) upon incubation with DOPAL mirror results observed that indicate formation of amyloid fibrils.[101, 102] Final confirmation of DOPAL modification was observed by mass spectrometry (Figure 3.12). The results in this chapter indicate DOPAL is able to cause protein aggregation that is relevant to the progression of PD as hypothesized.

Specific Aim 2: Determine the Metabolism

and Toxicity of Dopamine Metabolites,

Specifically DOPAL, in Microglia.

BV-2 microglia were employed to explore the direct interaction of DOPAL with

non-neuronal cells. It was found that these cells metabolize DA at low levels to

3,4-dihydroxyphenylacetaldehyde (DOPAC), the acid product of aldehyde

dehydrogenase (ALDH) metabolism of DOPAL (Figure 4.1). The metabolism of

DOPAL was far more robust, with nearly complete conversion of DOPAL to DOPAC

observed after four hours (Figure 4.2). Activation of the microglia prior to the

metabolism experiment with DOPAL decreased the metabolism of DOPAL to DOPAC

by 40% at all doses, as shown in Figure 4.3. While DOPAL levels did decrease similarly

to those in control microglia, the lack of DOPAC production needs further investigation.

As seen in Figures 4.4, 4.5, and 4.8, products of oxidative stress, 4HNE and MDA, both

caused a dose-dependent inhibition of DOPAL catabolism to DOPAC, and were also

found to be toxic to microglia. The observed toxicity was significantly greater than that

observed for dopaminergic cells in previous work.[43] Despite the toxicity of these

endogenous aldehydes, DOPAL itself was found to be non-toxic to the BV-2 microglia at

concentrations up to 50 μM after four hours. Significant toxicity of DOPAL has been

observed in dopaminergic cells at concentrations as low as 10 μM.[53] This lack of

observed toxicity led way to the work presented under Specific Aim 3.

Specific Aim 3: Define the Microglial Response to

Aberrant Levels of DOPAL and Elucidate

the Functional Consequence of Microglial Activation

Generated by DOPAL Treatment

for Dopaminergic Neurons.

The presence of microglial activation is well-established in PD, but the cause of this activation remains unknown.[76] The DOPAL to DA ratio in the substantia nigra is significantly greater in patients with PD,[58] and increased levels of DOPAL have been observed outside dopaminergic cells against a concentration gradient.[43] This increase in extracellular DOPAL would allow for interaction with non-neuronal cells, making examination of the ability of DOPAL to elicit microglial activation important. DOPAL caused morphological changes in BV-2 microglia after 4 hours, as observed in Figure 5.2. This change in morphology was indicative of activation. These results were corroborated with the dose-dependent release of TNF-α observed following treatment with DOPAL, but not DA or DOPAC (Figure 5.3). Activation was also demonstrated through IL-6 release by cells treated with DOPAL, but not DA or DOPAC in Figure 5.4. The lack of activation by DOPAC after 24 hours is of note, as these cells metabolize DOPAL to DOPAC nearly completely after 4 hours. A final measure of activation, shown in Figure 5.5, involved Western blot for expression of the inducible protein, cyclooxygenase-2 (COX-2), which was upregulated upon treatment with DOPAL. Changes in protein expression were not observed after incubation with DA or DOPAC (Figure 5.6).

Exploration of the interaction between the MN9D dopaminergic cell line and DOPAL was important, prior to the addition of BV-2 conditioned media that could

contain some residual DOPAL. The MN9D cells metabolized DOPAL to DOPAC over four hours, Figure 5.7. Saturation of this metabolism was observed at production of ~10 μM DOPAL. Significant toxicity was observed only at 50 μM DOPAL (Figure 5.8), indicating these cells are more resistant to toxicity than the dopaminergic PC6-3 cells.[53] The culminating experiment for this project, shown in Figure 5.9, demonstrated the greatest toxicity to MN9D cells by BV-2 conditioned media generated by DOPAL treatment. Treatment with DA- and DOPAC-BV-2 conditioned media exhibited little to no toxicity. The results presented throughout these two aims indicated DOPAL is capable of mediating neuronal toxicity through microglial activation, as hypothesized.

<div align="center">Conclusions and Implications for Parkinson's Disease</div>

The debilitating, neurodegenerative disease, first described by James Parkinson, is characterized by the selective loss of dopaminergic neurons within the substantia nigra, and the inclusion of protein aggregates known as Lewy bodies within these neurons. Oxidative stress, activated microglia, and DOPAL have all been implicated as contributors to the still unknown etiology for PD.[40, 55, 76] The ability of DOPAL to modify proteins was previously established, but results presented in Chapter 3 indicate DOPAL to play a significant role in the aggregation of α-synuclein, the main component of Lewy bodies. Aggregation took place at ratios of α-synuclein to DOPAL as low as 10:1. The prevention of this aggregation by antioxidants indicates that in levels of high oxidative stress and the absence of reduced GSH seen in PD, DOPAL-mediated aggregation of α-synuclein could occur rapidly and indiscriminately. Additionally, aggregated α-synuclein has been shown to cause microglial activation.[75]

The microglial response to the presence of DOPAL was previously unknown. Microglia were shown to be able to metabolize DOPAL to DOPAC but this metabolism was significantly reduced in activated microglia, Furthermore, this metabolism was inhibited in the presence of products of lipid peroxidation known to be elevated in PD. Elevated levels of DOPAL have been shown to cause inhibition of tyrosine hydroxylase, the rate-limiting enzyme of DA biosynthesis.[53, 64] Inhibition of this enzyme alone could lead to deleterious effects within dopaminergic cells, including depletion of DA.

While DOPAL was not toxic to microglia, further investigation in Chapter 5 demonstrated microglial activation. Prior to this work, glial activation had been observed in PD, and the mediator of this activation was unknown. The results of the current work suggest an endogenous reactive metabolite, such as DOPAL. The activation generated by DOPAL could have a number of ramifications for the progression of PD. Conditioned media from the BV-2 cells treated with DOPAL caused toxicity to the MN9D dopaminergic cells, indicating the mechanism of neurotoxicity by DOPAL-mediated glial activation is a viable mechanism for the hallmark death of dopaminergic cells in PD. An illustration of these results and the implications for PD is shown in Scheme 6.1. Research presented here implicates DOPAL as a significant neurotoxin that mediates neuronal death through a variety of mechanisms, including microglial activation. Further understanding of the role DOPAL plays in PD initiation and progression is most certainly required. However, these findings indicate continued, in-depth exploration of patient treatment with antioxidants, MAO inhibitors, and anti-inflammatory agents would be highly beneficial. Reduction of the elevated levels of DOPAL seen in PD, regardless of the mechanism, would be prudent to prevent further cellular damage.
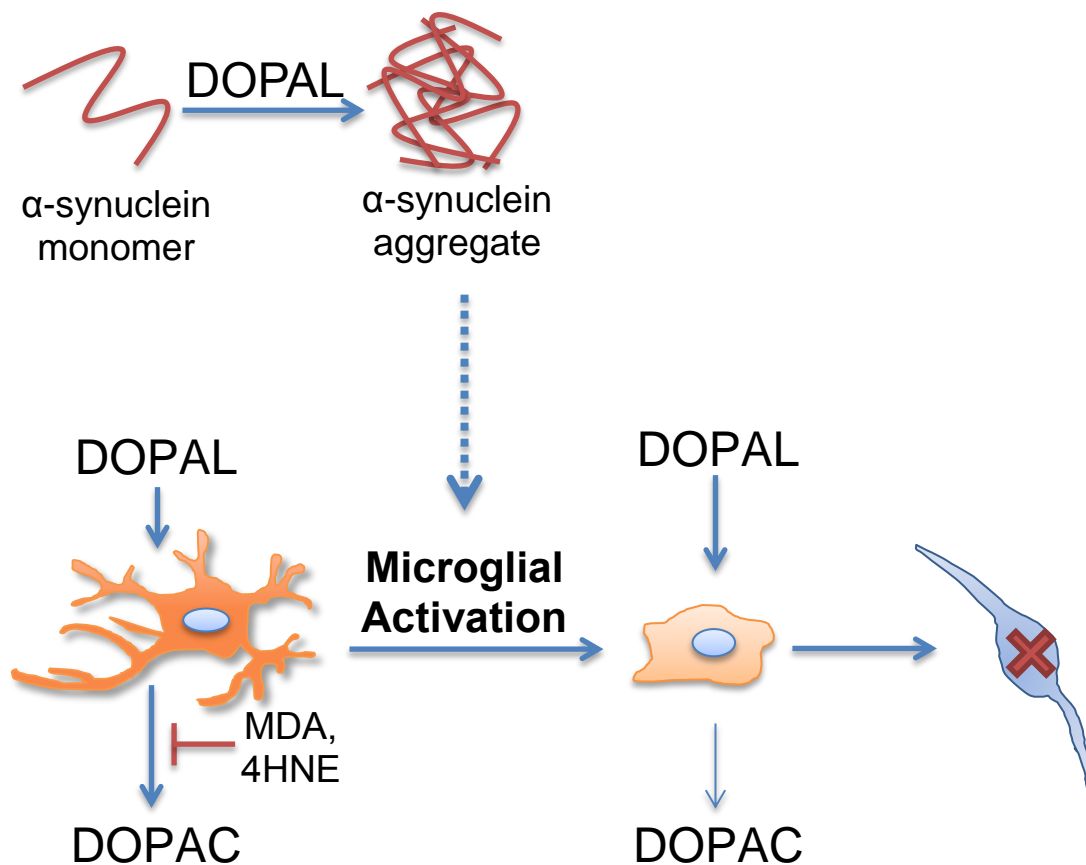
Scheme 6.1   Summary of findings in this work and their implications for PD. DOPAL causes aggregation of α-synuclein. Not shown in this work, but previously established; α-synuclein causes microglial activation. DOPAL is metabolized by microglia to DOPAC. This metabolism is inhibited by products of lipid peroxidation, 4HNE and MDA and decreased in activated microglia. DOPAL causes microglial activation, which, in turn, leads to dopaminergic cell death.

Future Directions

α-Synuclein Aggregation

Further investigation into DOPAL-mediated α-synuclein aggregation will strengthen the hypothesis that DOPAL leads to Lewy body aggregates of α-synuclein, and a greater understanding of the mechanism through which this happens. Analysis of the whole protein following DOPAL incubation by mass spectrometry will allow for confirmation of the exact number of DOPAL adducts present on the protein. Additionally, MS-MS analysis will provide confirmation of the residues subject to DOPAL modification. Alternate established methods of examining α-synuclein aggregation via fluorescent agents (i.e. Thioflavin-T and 8-anilino-1-naphthalenesulfonic acid (ANS)) would also be valuable.[25, 101, 102, 133] These methods would provide further information regarding rate of α-synuclein aggregation and structural changes caused by DOPAL.

Exploration of aggregation of α-synuclein within dopaminergic cells with depleted levels of GSH to mimic the environment of a PD brain would also be a relevant and logical next step for this work.

Investigation of the ability of DOPAL to facilitate aggregation of α-synuclein containing the A53T or A30P mutations would be an additional step in further understanding the role DOPAL may play in forming Lewy bodies in PD.

Metabolism of DOPAL in Activated Microglia

An altered metabolism of DOPAL was observed in BV-2 microglia that were activated with LPS prior to experiment (Figure 4.3). While DOPAL levels decreased over time almost identically to control cells, DOPAC production was depleted by 40% at

all doses of DOPAL. The mechanism for this decreased level of extracellular DOPAC deserves further investigation. The altered metabolism could be due to increased protein expression, which would increase the number of proteins available for DOPAL modification. Additionally, increased oxidative stress observed upon glial activation could generate products of lipid peroxidation which have been established to inhibit DOPAL catabolism (Figures 4.4 and 4.5), although additional mechanisms must be in play to explain the loss of DOPAL similar to control. Activation of microglia could also lead to changes in the fashion in which DOPAL and DOPAC move across the cell membrane. Preliminary work to tease out the mechanisms at play in the changes in metabolism should involve measuring the intracellular levels of DOPAL and DOPAC via lysis and HPLC analysis. If levels of intracellular DOPAL and DOPAC do not account for the loss of DOPAL without DOPAC production, two experimental approaches could be employed to look for DOPAL-modified proteins. Previously established work has indicated aminophenylboronic acid resin is able to trap DOPAL-modified proteins through formation of a boronic ester with the catechol.[53] SDS-PAGE of the wash and release fractions following this procedure would indicate the presence of DOPAL-bound proteins, which could also be compared to lysate from cells treated with DOPAL that were not previously activated. Further analysis by mass spectrometry would allow for identification of the proteins modified. A second approach would involve use of an antibody that recognizes DOPAL-modified proteins for immunoprecipitation and/or Western blotting.

Mechanism of Microglial Activation by DOPAL

Microglial activation is frequently discussed as an on/off-type change. Microglia are often considered either resting or activated. However, scientists have proposed more of a 'continuum' of activation that involves a number of different mechanisms and resulting phenotypes.[68, 134] Further exploration of microglial activation by DOPAL along this continuum would be important for greater understanding of DOPAL's role in activation and ultimately PD. The preliminary experiments for this work would require probing the mechanism by which DOPAL elicits activation. This could take place through a wide array of experiments, including incubation of DOPAL with cells from which various receptors have been prevented by antibody incubation or genetic modification.[134] It is possible that while some activation by DOPAL was observed through these experiments, the cytokines examined were not those produced at the highest levels upon incubation with DOPAL. An alternate method for probing a greater number of cytokines with greater efficiency would include use of the well-established Luminex bead-based flow cytometry system.[135]

Microglial Activation by Structural Analogs

Results presented in Chapter 5 illustrated DOPAL produced activation through a number of observed endpoints, but DA and DOPAC did not cause activation. It would be valuable to further confirm that both the catechol and aldehyde moieties are necessary for activation through activation studies by structural analogs to DOPAL, as has previously been performed to demonstrate DOPAL reactivity (similar to the research in Chapter 3) and DOPAL-mediated inhibition of tyrosine hydroxylase.[62, 64]

Microglial Activation by DOPAL-Aggregated α-Synuclein

Aggregated α-synuclein has been implicated in the activation of microglia,[75] and DA-quinone-modified cell membranes have also been implicated in microglial activation.[52] Study of the activation of microglia by DOPAL-aggregated α-synuclein would be a very important next step in examining an additional role DOPAL could play in mediating microglial activation, and ultimately dopaminergic cell death.

Microglia-Neuron Co-Culture

Results shown in Figure 5.9 indicate that DOPAL is able to cause dopaminergic cell death through microglial activation. This discovery could be pursued to a greater, and more physiologically-relevant extent by use of a co-culture/media sharing system between the MN9D cells and BV-2 cells. Treatment of the MN9D cells with DOPAL in the presence of 4HNE or MDA would presumably lead to elevated extracellular DOPAL which would cause microglial activation in the BV-2 cells suspended in a trans-well system over the neurons. The cytokines produced by the BV-2 cells would be released into the media shared with the MN9D cells and generate a toxic environment, similar to that observed in the substantia nigra of PD patients.

Ultimately, all of the above proposed experiments would allow for greater understanding of the role DOPAL plays with proteins, microglia, and neurons in PD. This increased understanding would provide further avenues to study and work toward development of therapeutic interventions to prevent and slow progression of the dopaminergic degeneration caused by PD.

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