Utilizing Galectin 3 as a Marker of Ruptured Vesicles In Vivo

Jonathan London
Loyola University Chicago

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<tr>
<td>AAV</td>
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<td>BBB</td>
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<td>CNS</td>
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<td>CRD</td>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>MSA</td>
<td>Multiple System Atrophy</td>
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<td>NAC</td>
<td>Non-B-amyloid component</td>
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<td>PAGFP</td>
<td>Photoactivatable Green Fluorescent Protein</td>
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<td>PD</td>
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<td>PFAP</td>
<td>Photoactivatable Fluorescent Protein</td>
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<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>SNpc</td>
<td>Substantia Nigra Pars Compacta</td>
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<td>UV</td>
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CHAPTER ONE

LITERATURE REVIEW

Statement of the Problem

Parkinson’s disease (PD) is the second most prevalent neurodegenerative disorder globally. PD patients exhibit multiple debilitating symptoms, notably movement-related disorders, as well as cognitive and behavioral problems[1]. It affects 1-2% of the population over the age of 50 with a current estimation of 1.5 million in the United States alone[2]. The pathological hallmark of PD is the loss of dopaminergic neurons of the substantia nigra pars compacta (SNpc). The loss of these neurons underlies the movement related disorders in PD patients. Current treatments of PD do not affect the pathological mechanisms in which the disease progression occurs [3, 4].

Lewy bodies and Lewy neurites (LB/LN) are found in the cells of the substantia nigra pars compacta. They are considered a histological hallmark of Parkinson’s disease. LB/LN are somewhat spherical structures that displace other cellular components (Fig 1). Mutated/aggregated forms of the protein A-syn have been identified as the major protein component of LB/LN formed during PD progression[5]. Numerous studies have shown that the duplication, triplication and/or mutations of the α-syn gene are the cause of PD development and progression in affected individuals [6-9]. These pathological α-syn proteins are able to transfer between cells thus spreading their pathology [10]. The
pathway in which these $\alpha$-syn entered these uninfected cells has been unknown, until recently.

Figure 1. Lewy Bodies are a histological hallmark of Parkinson’s Disease. Lewy bodies are spherical composites of mutated and aggregated forms of the protein $\alpha$-syn. They are found in the substantia nigra of individuals who suffer from Parkinson’s Disease. This figure is an H & E stain from Ella Bossy-Wetzel (2004) [11].

Additionally, evidence of mitochondrial dysfunction, increases in the production of Reactive Oxygen Species (ROS) and decreases in antioxidant production in PD patients and PD animal models have also been clearly documented[12-14]. These finding
are correlative since mitochondrial dysfunction has been shown to increase ROS production[15]. This increase in ROS in patients with PD is believed to be the underlying cause of cell dysfunction and cell death. It has been theorized that the presence of pathological forms of α-syn are the cause of these physiological signs of mitochondrial dysfunction. Therefore, it was pertinent to identify the pathway in which the pathological forms of α-syn entered uninfected cells.

To determine if pathological α-syn enters uninfected cells through endocytosis, the cytosolic protein Gal3 was utilized to visualize the colocalization of α-syn with ruptured vesicles in vitro [16](Fig 2). Gal3 is a lectin protein present diffusely throughout the cell cytosol that binds the β-galactoside sugars on the inner leaflet of the lysosome [17]. Once the pathological α-syn ruptures the lysosomal vesicle, the β-Galactoside sugars are exposed to the cytosol allowing the cytosolic Gal3 to bind and aggregate on the inner leaflet. Although pathological α-syn has been shown to rupture lysosomes in vitro, its ability to rupture lysosomes or endosomes has yet to be demonstrated in vivo. We hypothesize that the utilization of Gal3 as a marker of ruptured vesicles in vivo will allow us to determine whether pathological forms of α-syn cause the rupture of vesicles in vivo.
Figure 2. Cytosolic Galectin 3 can be utilized as a marker of ruptured vesicles in vitro. Gal3 has a low affinity for the B-galactosides on the inner leaflet of endosomes. When the membrane of the endosome ruptures the cytosolic Gal3 can enter the vesicle and bind the B-galactosides on the inner leaflet. Therefore, when α-syn ruptures the vesicle membrane the colocalization of the Gal3 and α-syn can be observed. This figure was provided by D. Freeman [16].

The Epidemiology of Parkinson’s Disease.

PD poses an increasingly large burden both socially and economically on many societies with aging populations. PD pathogenesis causes the development of crippling
symptoms in affected individuals. The development of the classic motor control related signs of PD is the primary base for diagnosis. Unfortunately, development of these signs indicates the disease has caused significant damage to the dopaminergic neurons of the SNpc[4]. There is currently no approved treatment for PD that targets a component in the etiology of PD. Genetic risk factors for PD development include both inheritable and sporadic gene mutations. These mutations have been the focus of a majority of research pertaining to PD[18].

PD is clearly an age related disease. Epidemiological studies have shown a rare occurrence of PD before 50 years. The prevalence of the disease increases significantly with age. PD was diagnosed in nearly 4% of the highest age group (80yrs+)[19]. The annual economic impact of PD in the United States is estimated at $10.8 billion. The annual direct medical cost to an affected individual is between $10,043 and $12,491. These are more than double the annual amount spent on patients without the disease. Nursing home care accounts for a large portion of the patients total direct medical cost. The development of motor control dysfunctions caused by PD forces many patients to enter nursing home care[20, 21].

PD causes patients to develop multiple behavioral and motor control dysfunctions. Behavioral problems include the development of depression, anxiety, fear or isolation. Dementia develops in about 25-40% of patients with PD[22]. Dementia seems largely responsible for the reduced life expectancy of patients with PD. Motor control dysfunctions are considered the classic signs of a PD development. The
developments of resting tremors, bradykinesia, rigidity, or postural imbalance are the primary signs used to diagnose PD in patients. Further, the physician will also observe the severity of your response to drugs used to treat PD as a diagnostic method. Since the development of these motor control problems is indicative of significant loss to the dopaminergic neurons in the SNpc patients whom are diagnosed are later in the stages of PD progression[4]. This is a common scenario since there aren’t any diagnostic lab tests for PD.

The current approved treatments for PD do not inhibit the mechanism in which PD causes its pathological effects. This is greatly due to the limited amount of knowledge of PD etiology. Most treatments for PD attempt to restore the proper levels of the neurotransmitter dopamine by increasing dopamine levels. L-dopa is commonly given to patients diagnosed with PD. L-dopa is the precursor to dopamine. Dopamine is unable to cross the blood brain barrier (BBB) and enter the brain cavity. L-dopa is able to cross the BBB and enter the brain cavity. Once L-dopa enters the brain cavity it is metabolized to produce dopamine. The effectiveness of this treatment decreases overtime and patients symptoms will continue to worsen after prolonged use[23, 24]. Other drug therapies inhibit the degradation of dopamine and reduce the effects of acetylcholine, a neurotransmitter that can cause a drop in dopamine levels[25]. Deep brain stimulation (DBS) has recently been implemented as a procedure that can provide dramatic improvements in many patients’ symptoms. In this procedure, a wire is placed in a specific location deep within the brain. An electric current is then induced in the wire.
thus stimulating the brain. The mechanism in which DBS works through is not completely understood[26].

Risk factors for developing PD have been extensively researched. These studies have attempted to identify the non-genetic and genetic risk factors for PD. The discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its damaging effects on the dopaminergic neurons in the SNpc led to the hypothesis that exposure environmental toxins may be a risk factor of PD[27]. Evidence consistently points towards a positive association between pesticide exposure and PD risk[28]. Multiple epidemiological studies have shown a reduced risk of PD among cigarette smokers[29]. The biological basis that may underlie this association is still poorly understood. Caffeine consumption was shown to significantly decrease PD risk according to a meta-analysis based on eight case-control studies and five cohort studies. Caffeine is an inhibitor of the adenosine A\textsubscript{2} receptor and improves motor deficits in a mouse model of PD[30]. Studies have also shown that higher intake of polyunsaturated fatty acids significantly reduce PD risk[31].

Genetic risk factors for PD have been extensively researched since the discovery of monogenetic mutations that have been shown to correlate with higher risk for PD. These mutations are considered either inheritable or idiopathic (sporadic). The former mutations clearly operate under Mendelian inheritance. These forms of mutations are estimated to cause about 10% of PD cases. Mutations in the gene for the protein $\alpha$-syn, and other proteins that are involved in cellular protection against oxidative stress, mitochondrial dysfunction are among the mutations known. The sporadic mutations do
not operate under Mendelian inheritance\cite{32}. They are thought to result from complex interactions between environmental and genetic factors. Genes associated with dopamine metabolism, mitochondrial metabolism, detoxification, other neurodegenerative diseases, and familial PD are commonly studied candidates for mutation that cause sporadic PD development in patients.

**Synucleopathies**

**Pathology**

James Parkinson first described a neurodegenerative disease condition he called “shaking palsy” in 1817. He described individuals exhibiting tremors, rigidity, and considerable debilitations in motor control\cite{33}. PD pathology is defined by a loss of dopamine producing neuronal cells in the SNpc and the presence of LB/LN. These dopaminergic neurons of the SNpc indirectly play a critical role in fine motor control and learned responses to stimuli. Dopamine modulates neuronal synaptic activity which is needed for fine and gross motor control. Low dopamine levels in the brain, caused by the dopaminergic neuron death in PD, result in stiffness with reduced and slowed movement\cite{3, 34}.

The LB was first described by Friederich Lewy in 1912 in substantia innominata samples from individuals that exhibited Parkinsonism-like symptom \cite{5}. Its biochemical composition remained unknown until 1997. Soon after the discovery of a point mutation in the \( \alpha \)-syn gene as a cause for inheritable PD, \( \alpha \)-syn was found to be the major component of LB/LN in LB/LN forming synucleopathies such as PD and dementia with
Lewy bodies (DLB)[6-8]. The filamentous glial and neuronal inclusions of multiple system atrophy (MSA) have also been found to be made of α-syn [35].

**α-synuclein**

The first synuclein nucleotide and amino acid sequences were reported by Maroteaux et al. in 1988. The protein received its name due to its localization in the presynaptic nerve terminals and portions of the nuclear envelope[36]. Following studies have failed to confirm a nuclear localization. Two other homologues of this original synuclein have been discovered subsequently[36-38]. These synucleins have thusly been named α-syn, B synuclein, and gamma synuclein. B synuclein and Gamma synuclein have not shown any correlation with neurodegenerative disease to date.

α-syn is an amphipathic protein that has little secondary structure. This structure allows for a high level of heat-stability[39]. Structural research indicates that the intrinsically unstructured α-syn forms an alpha helical confirmation at the N-terminal in the presence of cellular membranes. Unique to α-syn is a central hydrophobic region known as the non-B-amyloid component (NAC) [39-41](Fig 3). Studies pertaining to Alzheimer’s disease indicate this region is essential for α-syn aggregation. Deletion of this region has confirmed the role of the NAC in following studies. α-syn can bind lipid membranes, and selectively inhibits phospholipase D2. Phospholipase D2 localizes to the plasma membrane where it plays a possible role in signal-induced cytoskeletal
regulation and endocytosis[42-45]. These observations lead many to believe α-syn may have a role in regulating vesicular transport processes.

Figure 3. Diagram of Alpha Synuclein. α-syn contains a highly conserved amphipathic region that forms an alpha helical structure in the presence of a lipid membrane (1-60). A-syn also has a hydrophobic non-B-amyloid component (NAC) that is unique to α-syn and facilitates aggregation (61-95). A-syn also has an acidic C-terminal.

Pathological Alpha Synuclein

In 1995, Polymeropoulos et al. established a genetic link between L-Dopa responsive parkinsonism with Lewy body formation in a large Italian family and chromosome 4q21-23. Deletion of these genes proved to cause debilitating mental disfunction and development. In 1997, further investigation discovered a point mutation in the α-syn gene in this family and three greek families that shared a common founder with the Italian family. In this A53T α-syn mutation alanine (A) residue 53 is changed to threonine (T)[6]. The relevance of α-syn within PD development was controversial since many rodents and zebra finch carry this mutation. This was later settled after the
discovery of another point mutation in the α-syn gene in a German family with inheritable PD. In this A30P α-syn mutation A residue 30 is changed to Proline (P)[46]. Since residue 30 is A in all species examined the scientific community agreed on the relevance of α-syn in PD development. Soon after, it was discovered that α-syn was the major component of LB/LN[6-8]. In 2003, Zarranz et al. reported the discovery of the third major point mutation in α-syn in a Spanish family that suffered from autosomal dominant parkinsonism, dementia and visual hallucinations of varying severity. In this E46K mutation glutamic acid (E) residue 46 is changed to lysine (K). Mice expressing these mutant α-syn develop neurological symptoms similar to those of PD[47].

Due to the abundance of α-syn aggregation in the pathology of Parkinson’s disease a number of studies have investigated the amino acids of α-syn that are critical to aggregation in vitro. The central hydrophobic region of α-syn the NAC was found to be essential for α-syn aggregation. The deletion of residues within the NAC region prevented α-syn aggregation in vitro[40, 41]. In a transgenic Drosophila model with this α-syn construct, there was no evidence of large aggregates or oligomeric species of α-syn in these animals and no loss of tyrosine hydroxylase-positive neurons. Transgenic Drosophila models with increased α-syn aggregation exhibited increased neurotoxicity and loss of tyrosine hydroxylase positive neurons[48, 49].

The animal models of PD that have been developed were either neurotoxin induced or through the development of a transgenic animal. These models don’t fully recapitulate the dopminergic neuron degeneration, motor deficits, and the LB/LN
accumulations found in PD pathology[50, 51]. In 2012, Luk et al. reported the initiation of Parkinson-like neurodegeneration in non-transgenic mice given intracranial inoculations with synthetic α-syn fibrils. The fibrils were shown to seed aggregation of endogenous wt α-syn, transmit pathological α-syn to other healthy cells, causes death of dopaminergic neurons and cause motor deficits in mouse model[10].

**Reactive Oxygen Species (ROS)**

In both sporadic and genetic PD, oxidative stress is thought to be the underlying mechanism that leads to cell dysfunction and death. The SNpc of PD patients have shown evidence of increased ROS production and decreased antioxidant activity[12-14]. Tyrosine hydroxylase is a known ROS-generating enzyme. This causes greater susceptibility to cellular damage from oxidative stress in dopaminergic neurons. Additionally nigral dopaminergic neurons contain iron, which catalyzes the Fenton reaction and contributes to further oxidative stress. Since the dopaminergic neurons are highly sensitive to ROS, moderate oxidative stress can result in cell death[13].

Dopamine is normally packaged within cytoplasmic vesicles. When the level of cytosolic dopamine becomes excessive it is oxidized into Dopamine Quinone due to ROS. Dopamine Quinone has been shown to covalently modify proteins whose dysfunction has been linked to PD pathophysiology. A-syn is one of the particular proteins that are modified by Dopamine Quinone. This modification of α-syn promotes the protofibril formation of α-syn. This protofibril form of α-syn is thought to be
pathogenic. Dopamine Quinone also causes mitochondrial dysfunction, as well as the inactivation of DA transporters and tyrosine hydroxylase[52, 53].

Multiple mutations in genes of mitochondrial proteins were discovered in the familial forms of PD[32]. Mitochondrial dysfunction is a common cause of dramatic increases in ROS production in multiple cell types. Neurons utilize mitochondrial aerobic respiration for ATP production. Hydrogen peroxide and super oxide radicals are byproducts formed during oxidative phosphorylation in the mitochondria. The increase in ROS production due to mitochondrial dysfunction can cause oxidative stress which can cause peroxidation of the mitochondria-specific lipid cardiolipin. This results in the release of cytochrome c into the cytosol, which initiates the apoptosis pathway. MPTP was discovered to cause Parkinson’s Disease –like symptoms in individuals by causing mitochondria dysfunction in neurons of the SNpc. It achieves this by causing damage to Complex I of the electron transport chain. This causes leakage of electrons, which thus causes ROS generation. A-syn has been shown to interact with mitochondrial membranes and inhibit Complex I[15] (Fig 4).
Figure 4. MPTP damages Complex I of the electron transport chain. MPTP mimics the effects of Parkinson’s Disease by damaging Complex I of the electron transport chain. This causes a leakage of electrons that in turn leads to increased ROS production.

Loss of neuronal cells due to neuroinflammation is common in organisms with PD. The neuroinflammatory response is controlled by microglia. Microglia are innate immune cells located in the central nervous system. They are the main immune responsive cells in the central nervous system. Microglia are considered to be “resting” when they are not “activated” by chemical stimulants generated due to brain damage and/or the presence of an antigen. Once activated the microglia release free radicals such as nitric oxide and superoxide while functioning as an innate responsive cell. These free radicals contribute to the oxidative stress of the microenvironment. The SNpc of sporadic and familial PD patients have increased levels of activated microglia[54]. It has been
shown that the addition of aggregated α-syn to a primary mesencephalic neuron-glial culture causes activation of microglia and dopaminergic neurodegeneration. This cytotoxicity does not occur when microglia are absent from the culture[55]. Neuron derived α-syn stimulates astrocytes to produce inflammatory modulators which augment microglial chemotaxis, activation, and proliferation[56]. Currently, there is no therapy clinically available that delays the neurodegenerative process.

**Ruptured Vesicles**

Evidence indicates that pathological α-syn has the ability to be transferred from an infected neuronal cell to a healthy neuronal cell[57-60]. It has been hypothesized that the pathological forms of α-syn enter the cells via endocytosis. This theory called into question the method in which α-syn escapes the vesicle in order to enter the cytosol. Previous work with Adenovirus Type 5 has shown that the rupturing of lysosomes in cells caused by adenovirus infection leads to Cathepsin B-Dependent Mitochondrial Stress and the production of ROS[15]. Since similar physiological signs are present in the cells of PD patients, our lab suggested that α-syn enter the cell via endocytosis and ruptures from lysosomes. These hypotheses were later confirmed through experimentation. α-syn aggregates were shown to localize to areas of vesicle rupture in vitro (Fig 2). These ruptured vesicles were shown to be positive for lysosomal associated membrane protein-2 (LAMP2). This indicates that the vast majority of vesicles ruptured by α-syn are lysosomes[16]. Previous studies of the α-syn protein have shown that low pH environments cause α-syn to undergo a conformational change[61]. Therefore, the
low pH environment of the lysosome may cause a conformation change in the α-syn which allows it to rupture the vesicle and escape into the cytoplasm. The rupture of lysosomes has been associated with an increase in cellular ROS in cells due to the release of activated cathepsins contained within the lysosome. Furthermore, α-syn induces a cathepsin B dependent increase in ROS in infected cells[16].

**Galectin 3**

Galectins are a family of evolutionary conserved animal lectins that bind β-galactosides. They are found ubiquitously in mammals, invertebrates and fungi[62]. Galectins have considerable immunoregulatory activities including regulation of immune homeostasis[63]. All galectins exhibit sequence homology in the carbohydrate recognition domain (CRD) but they have different affinities for different saccharide ligands[64]. Gal3 (Gal3) is a structurally unique member of the galectin family and exhibits both extracellular and intracellular functions. It is important to note that Gal3 doesn’t posses a secretion signal peptide that would direct transport through the classical endoplasmic reticulum-Golgi apparatus secretory pathway. Gal3 contains a single CRD and an elongated N-terminus compared to other galectin members (Table 1). This elongated N-terminus plays a role in protein oligomerization and is believed to participate in the interaction with other intercellular proteins. Therefore Gal3 can interact with both carbohydrates and proteins[65, 66]. At low concentrations, Gal3 is a monomer that can potentially form oligomers upon binding to multivalent saccharides. Gal3 has the ability to crosslink cell surface receptors, thus forming a cluster of ligands into a lipid raft micro-
domains[67, 68]. Gal3 ability to bind cell surface receptor has been shown to affect multiple cellular processes including cell-cell adhesion, proliferation, differentiation, and cytokine secretion[69-72]. Additionally, Gal3 has both a pro- and anti- apoptotic activities determined by extracellular or intercellular location[73]. Gal3 is now known to be related the pathophysiology of multiple diseases, including cardiovascular disease, cancer, asthma, prion infection, rheumatoid arthritis and streptococcus pneumonia infections. In vivo, an increase in the extracellular concentration of Gal3 has been measured in the inflammatory setting in animal models. Gal3 levels are currently being used as an indicator of multiple types of infections and disease conditions in clinical medicine settings[74-76]. Gal3 was discovered to be regulated at the protein level in response to IL-1B, and at the mRNA level in response to advanced glycation end products casein[77]. These findings are consistent with the up regulation of Gal3 during innate immune activation.
Table 1. Galectin 3 has a unique structure compared to other members of the Galectin family. Gal3 is the only chimeric type member of the Galectin family. It contains one carbohydrate recognition domain and one dimer binding site. Gal3 has a uniquely elongated N-terminus. This elongated N-terminus is believed to give Gal3 inflammatory regulatory capabilities.

Multiple studies show that Gal3 has a critical role in the process of leukocyte trafficking, activation and cytokine release[78]. Gal3 was discovered to cause Calcium influx in monocytes. This calcium influx involves a pertussis toxin-sensitive pathway, which suggest a G protein-coupled receptor could be a target receptor for Gal3[79].

<table>
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<td>Tandem Repeat Type</td>
<td>Galectin 4,6,8,9,12</td>
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<tr>
<td>Chimeric Type</td>
<td>Galectin 3</td>
<td>CRD</td>
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Table 1. Galectin 3 has a unique structure compared to other members of the Galectin family. Gal3 is the only chimeric type member of the Galectin family. It contains one carbohydrate recognition domain and one dimer binding site. Gal3 has a uniquely elongated N-terminus. This elongated N-terminus is believed to give Gal3 inflammatory regulatory capabilities.
vitro, Gal3 can cause superoxide release from monocytes and promote the uptake of apoptotic neutrophils from monocyte derived macrophages[79-81]. The macrophages of mice deficient in Gal3 showed reduced phagocytosis apoptotic thymocytes in vitro compared to wild type mice. Phagocytic clearance of apoptotic thymocytes by peritoneal macrophages was attenuated in Gal3 deficient mice in vivo[74]. These studies suggest Gal3 is a pro-inflammatory protein. It is important to mention that recent studies pertaining to the role Gal3 plays in salmonella infection and asthma have suggested that Gal3 also acts as an anti-inflammatory protein[82, 83]. These results may not represent the effect of endogenous Gal3. A large number of in vivo and in vitro studies suggest that Gal3 is pro-inflammatory.

Within the last 5 years Gal3 has been utilized as a marker of vacuole lysis by invasive pathogens. Multiple infectious agents, including Shigella bacteria, must evade the immune system while invading the host. Upon internalization into the host cell the bacteria lyses the phagosome before it is fused with a lysosome and escapes into the cytosol[84]. This process was poorly understood since there wasn’t a reliable marker for ruptured vesicles. Paz and company utilized the affinity that Gal3 has for the β-galactosides sugars on the inner leaflet of the ruptured vesicles to identify ruptured vesicles. Once the bacteria lysed the vesicle the β-galactoside sugars were exposed to the cytosol and the cytosolic Gal3. By labeling the Gal3 after bacterial infection Paz and company were able to reliably identify ruptured vesicles which would have clusters of oligomerized Gal3 bound to their inner walls[17].
Photoactivatable Green Fluorescent Protein fused to Galectin 3

The utilization of fluorescent proteins, such as green fluorescent protein (GFP), allows for non-invasive analysis of protein localization and dynamics in living cells[85]. This is achieved by producing a fusion protein with the fluorescent protein and a protein of interest. This fusion protein can then be expressed in a cell or organism and its movements can be tracked via fluorescent microscopy. Innovation developed using these fluorescent proteins include the production of photoactivatable fluorescent proteins (PFAP)[86]. One such protein created by utilizing GFP is photoactivatable GFP (PAGFP). When initially expressed PAGFP emits low intensity light. After exposure to approx. 400 nm wavelength light (UV light) the PAGFP will undergo irreversible photoconversion causing it to emit light with nearly 100 fold greater intensity (Fig 5). This photoconversion involves the decarboxylation of Glu222 followed by chromophore conversion from a neutral to anionic state[86]. PAFP’s introduce a new methodology of kinetic microscopy of living cells, which was traditionally associated with fluorescence recovery after photobleaching (FRAP)[87]. It is worth mentioning that some PAFPs undergo a reversible photoconversion process[88]. These photoactivatable proteins are also useful when overcoming hindrances to resolution that can develop when viewing tissue samples using fluorescent microscopy techniques, such as tissue autoflourescence [89, 90].
Figure 5. Photoactivatable green fluorescent protein increases its light intensity by nearly 100 fold after activation with ≈400 nm light (UV light). The photoconversion of photoactivatable green fluorescent protein is an irreversible process. It is widely used in protein kinetics studies to track proteins in living models. Prior to exposure to UV light the PAGFP will emit a low level of fluorescent light. Following exposure PAGFP will increase its fluorescent light intensity by nearly 100 fold.
CHAPTER TWO
EXPERIMENTAL METHODS

Cell lines and reagents

The human monocyte cell line THP-1 was obtained from the American Type Culture Collection (ATCC). ATP and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich. PMA was used to differentiate Thp-1 monocytes. The THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media, supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 1mg/ml streptomycin, 1mM sodium pyruvate and 2 mM glutamine. The human neuroblastoma cell line, SH-SY5Y was obtained from the American Type Culture Collection (ATCC). SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (FBS)(Hyclone) and 100 IU/mL penicillin, 1mg/mL streptomycin and 10 µg/mL ciprofloxacin. Cells were maintained in a 37°C incubator with 5% CO2. The mCherry-gal3 plasmid was a generous gift from Dr. Christopher Wiethoff. Retroviral vectors and Lentiviral vectors were generated utilizing transfection of 293T cells with the packaging plasmids vesicular stomatitis virus glycoprotein (VSV-g), delta nrf and mCherrygal-3. Transfections were performed using polyethylenimine (PEI) (molecular weight, 25,000; Polysciences). Supernatants containing these recombinant retroviral 38 vectors were
collected 48 hours post-transfection and used to transduce SH-SY5Y and THP-1 cells. Positive transductants were selected with Geneticin/G418.

**Alpha Synuclein**

Full length α-syn were purchased (rPeptide) and the lyophilized protein was rehydrated in PBS with 100 mM NaCl immediately upon arrival to a concentration of 1 mg/ml and was aliquoted and stored at -80°C. In order to generate aggregates, alpha-synuclein was incubated for 3 days at 37°C under constant agitation at 300 revolutions per minute (rpm) followed by storage at -80°C. Aggregates were added at a concentration of 3µg/ml per the protocol provided by Volpicelli-Daley[91].

**Western Blot**

Purified proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis, (SDS-PAGE), proteins were transferred to nitrocellulose membranes and detected by incubation with the primary antibody. The monoclonal mouse anti-human Gal3 antibody from BD Pharmingen was purified from tissue culture supernatant or ascites by affinity chromatography. Secondary antibody conjugated to HRP (Thermo Scientific) was used where necessary, and antibody complexes were detected using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). Chemiluminescence was measured using a BioRad ChemiDoc XRS imaging system.
(BioRad). Gal3 values were quantified using Imagj software to perform densitometry analysis of the western blot bands.

**Animals and Surgery**

Young adult Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were housed two to a cage with ad libitum access to food and water during a 12 h light/dark cycle, according to the rules set by the Research Ethical Committee at Rush University Medical Center. Recombinant adeno associated virus serotype 6 vector encoding human mutant (A30P) α-syn gene (rAAV6-h-A30P) were prepared and titered as described previously (Towne et al., 2008). Under halothane anesthesia, 2 μl of the vector suspension was injected stereotaxically into the right nigral region at 5.3 mm posterior and 2.3 mm lateral to bregma, 7.7 mm ventral to dura. The needle was kept in place for an additional 5 min before slowly being withdrawn. At 6 weeks after injection, animals (rAAV6-h-A30P, n= 10) were perfused through the ascending aorta with physiological saline, followed by 4% ice-cold paraformaldehyde. The brains were post-fixed in the same solution for 2 h, transferred to 10%, 20%, 30% sucrose, and sectioned on a freezing microtome at 40 μm in the coronal plane. All sections were collected and stored in order in a cryoprotectant solution before processing. All animal experiments had prior approval from the Institutional Animal Care and Use Committee at Rush University.
Immunofluorescence Microscopy

For in vitro experiments cells were allowed to adhere to Fibronectin (Sigma-Aldrich) treated glass coverslips and fixed with 3.7% formaldehyde (Polysciences) in 0.1 M piperazine-N, N’bis(2-ethanesulfonic acid) PIPES buffer at pH 6.8 for 15 min.

For experiments utilizing rat brain tissue slides, primary antibodies to Gal3 and α-syn were used in their preparation. The monoclonal mouse anti-human Gal3 antibody from BD Pharmingen was purified from tissue culture supernatant or ascites by affinity chromatography.

Primary antibodies were secondarily labeled with fluorophore-conjugated mouse anti-human antibodies (Jackson ImmunoResearch). Images were collected with a DeltaVision microscope (Applied Precision) equipped with a digital camera (CoolSNAP HQ; photometrics), using a 1.4 numerical aperture (NA) 100X objective lens, and were deconvolved with SoftWoRx deconvolution software (Applied Precision). Structured Illumination images were collected on an OMX microscope (Applied Precision) and deconvolved and reconstructed using SoftWoRx software (Applied Precision). Tiff images and 3-dimensional reconstructions were generated using Imaris software (Bitplane).

Image Analysis

Deconvolved images were analyzed for colocalization of PAGFP Galectin-3 (Gal-3) with mCherry Galectin 3 by use of the Surpass Mode of the Imaris software package.
Statistical Analysis

Results are presented as mean + standard error of the mean. Two-sample t-test was used to compare between different experimental groups. All statistical analyses were performed using InStat 3.0 (GraphPad Software). \( p<0.05 \) was considered statistically significant.

Densitometry Analysis

Western blots bands were analyzed utilizing ImageJ software. Control levels were normalized to a value of 100. Variable band values are expressed in comparison to the normalized control values of 100. Adjustments to Gal3 band values were made in proportion to the differences in their corresponding actin band values compared to the actin bands of the control samples.
CHAPTER THREE

RESULTS

Colocalization of Galectin 3 and α-syn observed In vivo

Gal3 is a lectin protein which binds the β-galactosides on the outer leaflet of the cell membrane and on the inner leaflet of the cellular vesicles. Once a vesicle is ruptured the Gal3 are able to enter the vesicle and bind the β-galactosides on its inner leaflet. The colocalization of Galectin3 and α-syn at the site of ruptured lysosomes has been used to identify part of α-syn ’s pathway of infection. These in vitro data indicate that α-syn enters the cell through endocytosis and then ruptures from the lysosome in order to enter the cytosol. In order to visualize this colocalization in vivo, histology slides were prepared using the brain tissue of rats that were treated with Adeno-Associated-Virus (AAV) vector expressing the pathological α-syn mutant A30P. These tissue samples were then stained with fluorescent anti-bodies to Gal3 and α-syn. The histology slides were examined under a fluorescent microscope. Visualization of α-syn was clear, distinguishable, and isolated within the Cy5 channel. Contrarily, we were unable to isolate Gal3 in the FitC channel due to bleed through from tissue autofluorescence. This tissue autofluorescence hindered us from having clear
resolution of the Gal3. Thus, we weren’t able to visualize with certainty the colocalization of Gal3 and α-syn \textit{in vivo}.

**Galectin 3 levels increase in rat brain tissue when exposed to pathological alpha synuclein mutant (A30P)**

Increases in Gal3 levels have also been well documented during multiple pathogenic infections including streptococcus pneumonia and prion infection. We therefore wanted to determine whether Gal3 levels increased in vivo when a rat brain is exposed to a pathological mutant of the protein α-syn. Utilizing an A30P α-syn expressing AAV vector and fluorescent antibodies to A30P α-syn and Gal3 we were able to visualize and quantify differences in Gal3 levels during infection with pathological α-syn in the rat brain (Figure 6A). We utilized rat brain tissue that was injected with AAV vector that wasn’t expressing the A30P α-syn protein as our control group. The level of fluorescent intensity was indicative of the protein expression levels. Upon evaluation of multiple brain sections, we found that the levels of Gal3 had significantly increased in the brain of rats injected with the AAV vector expressing A30P α-syn compared to the levels in the control rat brains (Figure 6B).
Figure 6. Galectin 3 levels in Rat Brain tissue treated with AAV expressing pathological alpha synuclein (A30P). Rat brain tissue sections from rats receiving right nigral brain injections of AAV expressing pathological \( \alpha \)-syn were stained with fluorescently labeled antibodies to Gal3 (Green) and \( \alpha \)-syn (Red) (A). Gal3 levels in rat brain tissue treated with AAV expressing pathological \( \alpha \)-syn had significantly higher Gal3 levels (B). Two-sample t-test, *\( p < 0.005 \), \( n = 5 \) rat brains with 68 brain regions analyzed.
Galectin 3 levels increase in neuronal cell line (Sy5y) during treatment with pathological alpha synuclein aggregates

The brain is composed of multiple cell types. Two cell types which contribute greatly to the composition of the brain are the Neurons and Glial Cells. The increase in the levels of Gal3 in rat brain tissue following midbrain injection with virus vector expressing A30P α-syn beckoned us to determine which cell type was responsible for this increase. We utilized Sy5y neuroblastoma cells as our neuronal cell line. The Sy5y cells were treated with pathological α-syn aggregates and harvested at 24, and 48 hour time points. Gal3 levels were then determined qualitatively by western blot analysis (Figure 7A) and quantitatively by densitometry analysis (Figure 7B). This allowed us to determine whether neuronal cells contribute to the increase in Gal3 levels. We found that Sy5y neuroblastoma cells showed a ≈ 50% increase in intercellular levels of Gal3 after 48 hours of treatment via western blot analysis. Though increases in Gal3 levels were observed, the basal levels of Gal3 in Sy5y cells were considerably low.
Figure 7. Galectin 3 levels increase after treatment with alpha synuclein oligomers in Sy5y cells. Gal3 levels in SY5Y cells increase by approx. 50% after 48 hours of treatment with α-syn oligomers (B). Cells were harvested at 24 hr and 48 hr time points. Gal3 levels were analyzed by western blot and quantified using Image J software and were normalized to control values (A). Control values normalized to 100.

Galectin 3 levels increase in immune cell line (differentiated THP-1 monocytes) during treatment with pathological alpha synuclein aggregates

With the discovery that our neuronal cell line has low basal levels of Gal3 we then wanted to analyze the change in the levels of Gal3 in glial cells after treatment with pathological aggregates of α-syn. Since microglia have been shown to be activated
during PD progression we wanted to use them as our glial cell model. Differentiated Thp-1 monocytes (macrophages) are known to produce large amounts of Gal3 and are widely utilized in studies of microglia. We utilized differentiated Thp-1 monocytes that were differentiated in macrophages as our representative glial cell line. Differentiated Thp-1 macrophages are widely accepted as a representative cell line of microglial during experimentation. Differentiated Thp-1 cells were treated with pathological α-syn aggregates and harvested at 24, and 48 hour time points. Gal3 levels were then determined qualitatively by western blot analysis and quantitatively by densitometry analysis (Figure 8A). This allowed us to determine whether the glial cells are more likely to be the major contributors to the increase in Gal3 levels than neuronal cell types. We found that the differentiated Thp-1 monocytes showed a nearly 100 percent increase in intercellular levels of Gal3 after 48 hours of treatment (Figure 8B). The basal levels of Gal3 were comparably higher than that of our neuronal cell line making a 100 percent increase in Gal3 levels substantial.
Figure 8. Galectin 3 levels increase after treatment with alpha synuclein oligomers. Gal3 levels in THP-1 cells increase by approx 50% after 48 hours of treatments with α-syn oligomers (A). Cells were harvested at 24 hr and 48 hr time points. Gal3 levels were analyzed by western blot and quantified using Image J software and were normalized to control values (B). THP-1 data is representative of three independent experiments. Control values normalized to 100.

PA-GFP Gal 3 CRD has similar binding properties to fully expressed Galectin 3

In our attempt to use a green fluorescent protein labeled Gal3 (GFP-Gal3) to label ruptured vesicles in vivo, we were unable to resolve GFP-Gal3 puncta due to tissue
autoflourescence. We have since utilized the photoactivatable fluorescent protein (PAFP) ,photoactivatable green fluorescent protein (PAGFP) , in order to correctly resolve the Gal3 in the presence of tissue autoflourescents. This would be done by comparing the pre- and post- activation photos of the tissue sample and determining which source of light is being emitted by the PAGFP-GAL3. In order to utilize Gal3 as a marker of ruptured vesicles in our in vivo studies we want to express the PAGFP-Gal3 protein in the CNS of a transgenic mouse. Since Gal3 is known to function as an inflammatory regulator protein, we want to eliminate its inflammatory mediating function before expressing it in the CNS of a transgenic mouse. We accomplished this by expressing only the CRD region of Gal3. This would allow the fusion protein to have the same binding characteristics as the fully expressed Gal3 while alleviating the potential complications caused by its inflammatory effects. Western blot analysis of the PAGFP-Gal3 CRD (PAGFP-CRD) confirms that the fusion protein is properly expressed at ≈36 kD (expected size at 35 kD) (Figure 9B). The PAGFP-CRD is able to be activated through exposure to ≈400 nm light (Ultra violet light) and undergo photoconversion in vitro thus causing a nearly ten-fold increase in light intensity emission (Figure 9A). Finally, by expressing both fully expressed cherry Gal3 and PAGFP-CRD in the same cell we were able to observe similarities in their binding properties. The data shows that PAGFP-CRD exhibits identical binding properties to fully express cherry Gal3(chGal3) (Figure 9C).
Fig 9. PAGFP labelled Galectin 3 CRD exhibits binding properties similar to Galectin 3. Gal3 CRD binds to β-galactosides in a similar manner as fully expressed Gal3(C). The antibodies used to label Gal3 CRD and PAGFP appear at the same level indicating the fusion protein is being expressed intact (B). The PAGFP fused to the Gal3/CRD emits higher intensity light once activated (A).
CHAPTER FOUR
DISCUSSION

The previous results were gathered while attempting to utilize Gal3 as a marker of ruptured vesicles in vivo. The progress gained in this study will allow us to test the hypothesis that pathological forms of α-syn including mutants and oligomers are able to rupture lysosomes in vivo. This hypothesis was developed using previous work which showed that α-syn aggregates have the ability to rupture lysosomes in vitro[16]. The affinity Gal3 has for the B Galactoside sugars on the inner leaflet of lysosomes/endosomes has been utilized as a tool for determining pathogen etiology. Multiple pathogens enter the host cell during infection. It is necessary for these pathogens to evade the host immune system in order to survive. This is accomplished by the utilization of multiple evasion techniques and mechanism. One known evasion method includes endocytosis of the pathogen by the host cell followed by pathogenic vesicle escape into the cytosol. During the pathogens escape the vesicle is ruptured and the cytosol is able to access the internal space of the vesicle. This allows Gal3 proteins to enter the vesicle and bind its’ receptor[17]. While attempting to use Gal3 as a marker of ruptured vesicles in rat brain tissue that had been exposed to the pathogenic α-syn mutant A30P we were unable to gain effective resolution of Gal3. This failure was due to the abundance of tissue autofluorescence. The visualization of the co-localization of the α-
syn mutant protein and the Gal3 protein is a critical part to the experiment. Since we were unable to accomplish this we had to modify our approach in order to gain enough resolution of the Gal3 protein. Though we failed to visualize co-localization of the two proteins, we were able to quantify the levels of Gal3 in A30P treated tissue by use of fluorescent microscopy. This quantification showed that Gal3 levels were significantly increased in tissue samples that were exposed to the pathological A30P α-syn protein. It is well documented that Gal3 levels are increased in multiple disease states including cardiovascular disease, cancer, asthma, prion infection, rheumatoid arthritis and streptococcus pneumonia infections[74-76]. Increases in Gal3 levels pertaining to synucleiopathies such as parkinson’s disease has yet to be documented. Previous work has indicated that Gal3 is involved in innate immune activation and is regulated at the protein level by IL-1B, an inflammatory cytokine. This observation correlates with previous work which indicated an increase in IL-1B levels in the substantia nigra of PD patients[92, 93]. It also correlates with data that shows α-syn aggregates are able to cause caspase 1 activation in vitro, thus causing the release of IL-1B[16].

Gal3 is a useful marker of ruptured vesicles largely due to its diffuse distribution in a cell’s cytoplasm. In the presence of ruptured vesicles these diffuse proteins aggregate on the inner leaflet of these ruptured vesicles. These aggregates amplify the signal of the fluorescently labeled Gal3 distinguishably from that of the diffuse protein. Therefore, a strong contrast between the signal intensity of the diffuse Gal3 from the aggregated Gal3 proteins is critical to the efficacy of Gal3 as a marker of ruptured vesicles. Consequently,
an increase in the concentration of diffuse cytosolic Gal3 would diminish the effectiveness of Gal3 as a marker of ruptured vesicles.

Neurons and Glial cells are two major types of cells present in the composition of the CNS. Since we are ultimately interested in utilizing Gal3 as a marker of ruptured vesicles in neuronal cell lines we want to determine whether neuronal cells contribute to the increase in Gal3 concentrations and, if so, how large is the relative contribution. We decided to utilize differentiated THP-1 cells as our representative Glial cell line and SH-Sy5y human neuroblastoma cells as our neuronal cell line. We treated SH-Sy5y human neuroblastoma cells and differentiated THP-1 human monocyte cells with α-syn oligomers for up to 48 hours. Western blot analysis showed increases in Gal3 levels in both cell types. The most significant increases were observed at 48 hours. Though the Sy5y neuroblastoma cells did show a nearly 50% increase in Gal3 levels their basal Gal3 levels were significantly lower than those of the differentiated human monocyte THP-1 cells. The THP-1 cells exhibited a nearly 100% increase in Gal3 levels following 48 hours of treatment. These results lead us to believe that the neurons are not the major contributor of Gal3 production in the CNS during pathological α-syn infection.

It is important to note that this α-syn induced increase in Gal3 levels eliminates the use of endogenous Gal3 as a useful marker of ruptured vesicles in vivo. The newly expressed Gal3 is released into the cytosol of the infected cell. If the concentration of Gal3 in the cytosol increases then the contrast between diffuse cytosolic Gal3 and the aggregated vesicular Gal3 will become less distinguishable. These protein dynamics also
renders the capability of our software to quantify and calculate fluorescent data inadequate. The increase in overall Gal3 levels in the cell would cause a considerable amount of variability in the experiment data. This variability would thus cause imprecise analysis to be produced.

In order to utilize Gal3 as a marker of ruptured vesicles in vivo we would have to overcome the loss of resolution due to tissue autofluorescence as well as the increased cytosolic Gal3 concentrations due to pathological α-syn treatments. We elected to accomplish this by fusing Gal3 to the fluorescent protein photoactivatable green fluorescent protein (PAGFP). The fusion protein exhibits expression levels that are not affected due to α-syn pathology. Examination of the PAGFP-Gal3 protein indicates that it has the same binding property of endogenous Gal3. Analysis of the PAGFP-Gal3 protein also confirmed its ability to be fluorescently activated when exposed to approx. 400 nm light. This activation causes the fluorescence of the PAGFP to intensify approximately 100 fold. This change in light intensity allows us to differentiate Gal3 oligomer fluorescence from the autofluorescence of tissue samples.

In order to complete our in vivo studies we will be expressing the PAGFP-Gal3 in the cells of CNS of a transgenic mouse. Gal3 is known to be an inflammatory regulating protein[82]. This function is unique to the Gal3 within the Galectin family of proteins. The uniqueness of the structure of Gal3 is believed to give Gal3 this unique function. Increasing the expression of an inflammatory regulating protein in the CNS of a transgenic mouse can cause lower animal viability and even death. In order to alleviate
this concern, we expressed the homologous CRD region of the Gal3 protein fused to the PAGFP (PAGFP-CRD). This eliminates the unique structure of Gal3 as well as its’ unique inflammatory regulating function. Analysis of the PAGFP-CRD protein indicates that it has similar binding properties to Gal3. Analysis of the PAGFP-CRD protein also confirmed its ability to be fluorescently activated when exposed to approx. 400 nm light.

The PAGFP-CRD will be expressed in the CNS of a transgenic mouse. The transgenic mouse will be treated with pseudovirus vector expressing a pathological mutant of α-syn. The brains will then be harvested and prepared to be analyzed by fluorescent microscopy based on the methods developed by the Kordower lab. We believe this analysis will provide evidence that the pathological α-syn enters the cell through endocytosis and ruptures from the vesicle in order to enter the cytosol.
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VITA

Jonathan London was born on June 9th, 1986 in Frankfurt, Germany at a United States army base hospital to Michael and Eudora London. He returned to the states at the age of one and moved to Colorado Springs, Colorado where he graduated from Mesa Ridge High School. After spending three years working construction Jonathan attended community college at Pikes Peak Community College. After graduating with his Associates of Science in 2009, he attended Colorado State University. At Colorado State University Jonathan majored in Biological Sciences and conducted research on marine diving mammal physiology. He was awarded his bachelor’s degree in 2011.

Jonathan entered the Integrative Cell Biology graduate program in 2012 following his undergraduate education. He joined Dr. Edward Campbell’s lab in January 2013 to begin working on his Master’s thesis. His research focused on the utilization of Galectin 3 as a marker of ruptured vesicles in vivo. While completing his master’s thesis Jonathan attended the Masters of Business Management program at Wake Forest University.

Upon completion of his master’s degrees, Jonathan will be pursuing a career working in the biotechnology industry. He plans to finance his own biotechnology venture in order to increase the quality of the healthcare and agricultural fields.