nNOS activity is decreased in the striatum of L-DOPA-treated Parkinson’s disease model

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Abstract

Parkinson’s disease (PD), resulting from the loss of dopaminergic neurons in the basal ganglia, is a devastating neurodegenerative disease that impairs voluntary movement. Currently, the standard treatment for PD aims to replace dopamine via L-DOPA administration. However, this treatment often causes equally debilitating symptoms, termed dyskinesias. cGMP, a messenger molecule that plays a role in neuronal excitability, has recently been noted as a possible target for therapy since levels of cGMP, controlled through degradation by phosphodiesterases, and synthesis by nitric oxide (NO) signaling, are depressed during dyskinesias. This study sought to measure the activity of neuronal nitric oxide synthase (nNOS) in a rat model of PD and dyskinesia to determine whether the decrease in cGMP is due to decreased NO-dependent-synthesis or increased degradation. We found decreased levels of NO in dyskinetic animals, indicating a decrease in nNOS activity correlating to the decrease in cGMP reporting in other studies.

Introduction

Neurodegenerative Diseases

The human brain is often compared to a hardwired computer: made up of functional circuits that are critical for making decisive and correct calculations. Correspondingly, the brain is made up of many circuit-like pathways involving trillions of neurons, each making thousands of connections, and innumerable signaling permutations. These complex sets of interconnecting pathways that make up our brains not only integrate all the sights, smells, sounds, tastes, and mechanical forces we perceive, but also orchestrate our perceptions, thoughts, and emotions. However, just as when a computer is wired incorrectly, the pathways of the brain can malfunction. The resulting changes in the pathways of the brain and nervous system often cause disorders such as schizophrenia, epilepsy, multiple sclerosis, and various neurodegenerative conditions.

The neurological and psychiatric disorders that result from rewiring, damage, and dysfunction account for approximately 1% of deaths and 11% of total disease burden (World Health Organization). While all of these diseases are debilitating, a specific family of disorders, called neurodegenerative diseases, is particularly devastating. Common neurodegenerative diseases include Alzheimer’s, Parkinson’s, Huntington’s, and Lou Gehrig’s disease (i.e., Atrophic Lateral Sclerosis). Neurodegenerative diseases become increasing prevalent with age, have no known cure, and are difficult to diagnosis (World Health Organization). While these diseases have treatments for symptoms, they are limited in ability to stop or even slow disease progression. None of the current available treatments reverse the damage already caused by the time diagnosis takes place. To exacerbate the problem, advances in health care and medical treatment allow people to live to more advanced ages, increasing the size of the population most susceptible to these devastating diseases.

Each neurodegenerative disease is characterized by the gradual degradation of specific brain regions and the buildup of specific proteins (Figure 1). Diseases associated with accumulation and aggregation of proteins are termed proteinopathies. They are differentiated and diagnosed by the resulting motor and cognitive impairments. For example, the buildup of Tau and β-Amyloid paired with the degeneration of the cortex and hippocampus (involved in cognition and memory, respectively) in Alzheimer’s disease (AD) leads to impaired cognition and memory (Alzheimer’s Association). Aggregates of the protein huntingtin and the degeneration of the cortex and basal ganglia (voluntary motor function) are characteristic of Huntington’s disease (André, Cepeda, & Levine, 2010). Likewise, α-synuclein accumulation in the substantia nigra, a component of the basal ganglia, occurs in Parkinson’s disease (PTD; Parkinson’s Disease Foundation). The loss of neurons ultimately causes the main symptoms of each of these diseases by disrupting key signaling pathways within the brain. The focus of my research will be on PD and the disrupted signaling that results from the loss of dopaminergic neurons and subsequent treatment with L-DOPA.

Signaling in the Brain

The brain is composed of many subpopulations of cells. Some of these cells, namely neurons, give rise to the characteristic signaling circuits while others, glia, provide nourishment, structural support, and protection. Neurons pass electrical signals along their axons to terminals innervating densely branched dendrites of neighboring neurons. These signals pass along the long, narrow appendages of the neuron by a self-propagating electrical charge called the action potential. Neurons are discontinuous with one another, but form synaptic connections at these junctions. Therefore, neurons must send a signal from the presynaptic terminal of the axon to the postsynaptic specialization of the dendrite. The production and release of chemical messengers, called neurotransmitters, enables this signaling to occur (Purves, 2008).

Neurotransmitters can exert either excitatory or inhibitory influences on the post-synaptic cell. Excitatory neurotransmitters, such as glutamate, further propagate neuronal signaling by increasing the chance of an action potential in the postsynaptic neuron. Conversely, inhibitory neurotransmitters, such as gamma aminobutyric acid (GABA), lower the chance of action potential generation in the postsynaptic cell, and therefore, decrease the likelihood of the electrical signal continuing. Additionally, some neurons release neuromodulators, such as dopamine and nitric oxide (NO). Dopamine can either facilitate or impede action potential generation depending on the receptor that it binds to on the postsynaptic neuron. Dopamine binding to D1 dopamine receptors facilitates action potentials, whereas binding to D2 dopamine receptors impedes the neuron from generating a signal (Purves, 2008; Jenner, 2008). My project will seek to understand how the disruption of signaling molecules in PD and its treatment lead to disease progression.

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alter the ubiquitin levels of α protein degradation via the lysosome and proteosome (Cuervo et al., 2004; Devi et al., 2006).

exocytosis and endoplasmic reticulum also been shown to impair vesicle transport by altering the activation of other proteins (Ahn et al., 2002). Additionally, α-synuclein knockout models of PD show deficits in dopamine transmission (Abeliovich et al., 2000). Each of these mechanisms has been implicated in the pathogenesis of PD and all cause loss of dopaminergic neurons, resulting in disrupted dopamine transmission from the SN to the striatum.

Factors and Phenotypes of PD
Looking at the origin of the disrupted dopamine signal from the SN, Foster and Lewy (1912) observed, via a light microscope, cells exhibiting large clumps of protein. These Lewy Bodies (Figure 2A) were later found to be largely comprised of the protein alpha-synuclein (Spillantini et al., 1998). Although PD comes in two forms, familial and sporadic, alpha-synuclein and Lewy Bodies are common to all cases of PD. The familial forms of PD are caused by genetic mutations while sporadic forms are caused by environmental, non-heritable idiosyncratic factors. Although genetics do not cause sporadic PD, they may predispose individuals to risk factors contributing to the development of the disease.

Sporadic PD, which accounts for about 90% of all cases, is largely impacted by environmental factors such as pesticide exposure, infection, and mitochondrial damage (Altschuler, 1996; Dawson & Dawson, 2003; Ascherio et al., 2006). Each of these factors play a role in the pathology of sporadic PD and are affected by aging. This suggests that advancing age is one of the greatest risk factors of PD.

Familial PD, although making up only 5-10% of PD cases, is better understood because specific genetic mutations can be traced to the factors that contribute to pathophysiological mechanisms of PD. Familial factors include PARK2, DJ-1, and PINK1 which play a role in protein disposal, cellular oxidative stress response, and cellular stress response, respectively (Kitada et al., 1998; Bonfati et al., 2003; Valente et al., 2004). Additionally, familial PD can be caused by dominant genetic factors, particularly mutations in the gene SNCA, encoding alpha-synuclein, and LRRK2. The SNCA gene, when duplicated or mutated, produces either too much or misfolded alpha-synuclein which can lead to PD. The discovery of these genetic mutations and their connection to PD has allowed researchers to study the link between the effects of mutations and the resulting pathology. Additional genes associated with PD are listed in Figure 2B.

Two key characteristics of α-synuclein regulation are thought to lead to PD: the protein's localization to dopaminergic pre-synaptic terminals and the flexibility of the protein’s shape (Maroteaux & Scheller, 1991; Polymerosopoulos et al., 1997; Glasson, 1999). Since the SN...
has many dopaminergic neurons projecting to the basal ganglia, α-synuclein build-up in nigrostriatal neurons affects this connection considerably. Additionally, the flexibility of the protein allows it to easily misfold and aggregate, forming the characteristic Lewy Bodies seen in PD. Although α-synuclein is commonly linked to cell toxicity, it is unclear whether the misfolded protein or the aggregation process is toxic (Glasson, 1999; Uvesky & Eliezer, 2009). While both familial and sporadic factors can cause α-synuclein aggregation, the outcome remains the same: cell loss in the SN and alteration of the basal ganglia pathways that control motor activity.

Current Treatments for PD
Although neither sporadic nor familial PD has a cure, there are currently several treatments used to reduce the symptoms and progression of the disease. Several popular treatments are listed in Figure 3. The main form of treatment focuses on replacing dopamine. L-DOPA, distributed under the commercial name levodopa, crosses the blood-brain barrier and is converted into dopamine by the enzyme aromatic L-amino acid decarboxylase (Poewe et al., 2010). The intermittent administration of levodopa, however, does not reproduce the continuous stimulation that normally occurs in the striatum, and this abnormal phasic dopamine transmission leads to side effects. Another method of treatment is the drug melevodopa, a form of L-DOPA that has higher water solubility and provides a more continuous administration of L-DOPA to the brain. A further method of increasing dopamine in the brain is through inhibition of monoamine oxidase B (MAO-B). Blocking MAO-B prevents the degradation of dopamine in neuronal synapses and allows dopamine to produce a stronger effect. MAO-b inhibitors also slow disease progression, but when used on its own for therapy, does not decrease the severity of symptoms to the extent of other options. Several types of receptors located in the basal ganglia have also been targeted. Dopamine receptor agonists simulate the effects of dopamine, while Adenosine A2A receptor antagonists regulate output signals from the indirect pathway of the basal ganglia, and blockade of these receptors is a promising new approach for treating PD (Jenner, 2003; Pinna et al., 2007; Hickey & Stacy, 2011).

Beyond pharmacological approaches, deep brain stimulation targeted to the subthalamic nucleus or the GPi of the basal ganglia provides an improvement of symptoms in a subpopulation of patients. While this treatment is advantageous and often used for advanced PD, the mechanism by which it is beneficial is not yet completely understood (Hickey & Stacy, 2011). Gene therapy also offers hope for both reducing symptoms and slowing progression of PD. While this method shows promise in animal models of PD, clinical trials in humans are still in progress. Each of these therapies, while providing some benefits, has side-effects as well, ranging from hallucinations to motor impairments. Despite the plethora of treatments already in existence, additional therapeutic strategies and targets need to be identified to better understand the pathophysiology of PD and to provide improved treatment to patients.

Unfortunately, the above-mentioned therapies have negative side effects as well: chronic treatment with L-DOPA can lead to another class of movement disorders called L-DOPA-induced dyskinesias. These movement disorders result from chronic, intermittent L-DOPA treatment and are often exacerbated by subsequent L-DOPA administration (Jenner, 2003). The symptoms of L-DOPA-induced dyskinesias include uncontrolled movement of the face and extremities as well as dystonia, an uncontrollable torsion of the body or limbs (Winkler et al., 2002; Yamamoto & Soghomonian, 2009).

Although L-DOPA treatment leads to dyskinesia in about one third of patients after just two years, the onset latency of these dyskinesias depends on several factors, including the amount of denervation, or loss of dopaminergic neurons, and the dose of L-DOPA used for treatment (i.e. greater denervation or higher doses of L-DOPA will cause dyskinesias to occur sooner; Chase, 1998; Poewe et al., 2010). These symptoms eventually develop in about eighty percent of patients after ten years of treatment with some cases severe enough to counter all benefits of taking L-DOPA (Poewe et al., 2010). Although the standard treatments have been used for several decades, novel treatments are required to attenuate the medical and social burden of both PD and dyskinesia. The unwanted side effects of treatment may be a result of L-DOPA augmenting dopamine levels in a non-physiological manner, while ignoring several other signaling pathways that remain disrupted (Hickey & Stacy, 2011). The topic of my research will be to further our understanding of the pathophysiological mechanisms leading to dyskinesias through disrupted pathways in the basal ganglia.

The Basal Ganglia
The basal Ganglia (Figure 4) are a set of highly interconnected forebrain and midbrain nuclei that control voluntary initiation and coordination of movement. The basal ganglia consist of the striatum (caudate and putamen), the internal and external segments of the globus pallidus (GPI &
increased abnormal movements. Decreased inhibition of the thalamus, and therefore, nearly the opposite effect occurs in the basal ganglia as well. Between symptoms, some researchers from chronic L-DOPA treatment. Because of this relationship between symptoms, some researchers have proposed that nearly the opposite effect occurs in the basal ganglia as well. This would imply that, following L-DOPA administration, the direct pathway would be over-stimulated while the indirect pathway would be over-inhibited, together resulting in the decreased inhibition of the thalamus, and therefore, increased abnormal movements.

NO Signaling and MSN regulation
In addition to the influences of dopamine from the SNpc and glutamate from the cortex, nitric oxide (NO) can regulate MSN activity. NO was discovered in 1987 as the endothelial derived relaxation factor in peripheral blood vessels functioning to relax smooth muscles (Palmer et al., 1987; Garthwaite et al., 1988; Bradt & Snyder, 1990). NO is released by the reaction of L-arginine and molecular oxygen, mediated by the enzyme nitric oxide synthase (NOS; Stuehr et al., 1991; Griffith & Stuehr, 1995). Three isoforms of NOS exist: neuronal (nNOS), endothelial, and inducible NOS (Nathan & Xie, 1994; Dawson & Dawson, 1996; Brennan & Bradt, 1997; Alderton et al., 2001).

Particularly relevant to PD and dyskinesia, nNOS is found throughout the brain and is activated by transient elevations in calcium levels, mediated by glutamate N-methyl-D-aspartic Acid (NMDA) receptor activation (Garthwaite et al., 1988). This pathway is described in Figure 5. When the frontal cortex is electrically stimulated, NMDA receptors in the striatum activate and, in turn, activate nNOS (Sammut et al., 2007a, b; Park & West, 2009). Alternatively, if dopamine D2 receptors are activated, the activity of nNOS is suppressed, decreasing NO signaling (Hoque et al., 2011; West & Tseng, 2011).

Since NO is a gaseous molecule, it is able to diffuse across cell membranes of the interneurons into MSNs where it can then bind to its primary target receptor, soluble guanylyl cyclase (sGC), which is found most abundantly in the striatum (Hofmann et al., 1977; Matsuoka et al., 1992; Boehning & Snyder, 2003). This enzyme is found in the dendrites of MSNs and ultimately activates the thalamic stimulation of the motor cortices to allow purposeful movement to occur. Meanwhile, the indirect, or striatopallidal, pathway is involved with inhibition of thalamus originates in the GPi/SNpr and is mainly GABAergic and thus inhibitory (Steiner & Tseng, 2010).

The direct, or striatonigral pathway, is thought to play a key role in disinhibition of movements (i.e. the accelerator) and contains a high proportion of D1 dopamine receptor-expressing MSNs (Gerfen et al., 1990; Onn, West, & Grace, 2000). D1 receptors facilitate glutamate-driven excitatory responses in MSNs and ultimately enable the thalamic stimulation of the motor cortices to allow purposeful movement to occur. Meanwhile, the indirect, or striatopallidal, pathway is involved with inhibition of unwanted movements (Reiner et al., 2003). In contrast to the direct pathway, the outcome of activation of the indirect pathway results in the inhibition of movement (i.e. the brake).

As described earlier, PD is characterized by the loss of dopamine-producing neurons in the SN (Olanow & Tatton, 1999). This loss of dopaminergic input has an overall hypokinetic effect due to its alteration of basal ganglia output, culminating in over-suppression of normal movements. This suppression is the basis for the characteristic symptoms of PD: rigidity, difficulty initiating movement, tremor, and postural dysfunction.

The symptoms resulting from the loss of dopamine are nearly the opposite of the symptoms of dyskinesias resulting from chronic L-DOPA treatment. Because of this relationship between symptoms, some researchers have proposed that nearly the opposite effect occurs in the basal ganglia as well. This would imply that, following L-DOPA administration, the direct pathway would be over-stimulated while the indirect pathway would be over-inhibited, together resulting in the decreased inhibition of the thalamus, and therefore, increased abnormal movements.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Levodopa</th>
<th>Melevodopa</th>
<th>MAO-b inhibitors</th>
<th>DA agonists</th>
<th>DBS</th>
<th>Gene Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benefits</td>
<td>Replete dopamine</td>
<td>Replete dopamine more consistently</td>
<td>Reduce degradation of dopamine</td>
<td>Mimics dopamine, slows dyskinesia onset</td>
<td>Reduce Symptoms in Advanced PD</td>
<td>Many targets</td>
</tr>
<tr>
<td>Deficits</td>
<td>Dyskinesias</td>
<td>Still in clinical Trials</td>
<td>Not as effective in late stages</td>
<td>Hallucinations, edema</td>
<td>Invasive surgery</td>
<td>Still in clinical trials</td>
</tr>
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Figure 3: Current therapies for Parkinson’s disease: Common treatments listed along with the benefits and deficits associated with each therapy (Jenner, 2003; Pinna et al., 2007; Poewe et al., 2010; Hickey & Stacy, 2011).
by a family of cyclic nucleotide degradation enzymes called phosphodiesterases (PDE). PDEs are found throughout the body and exist in several forms (Hebb & Robertson, 2007). The different forms of PDEs are dependent on their location, the type of cyclic nucleotide they break down, and how they are regulated (Hebb & Robertson, 2007). The cellular role of PDE is to metabolize, or break down, cyclic nucleotides such as cAMP and cGMP by cleaving the phosphodiester bond of the cyclic ring and rendering the molecule inactive (Conti & Jin, 1999).

cGMP disruption in PD and dyskinesia
Parkinson's disease is characterized by a loss of dopaminergic cells in the nigrostriatal pathway (Hornykiewicz, 1975). This loss of dopamine being delivered to the striatum results in many physiological changes. For instance, D1 receptors become supersensitive and are downregulated, while D2 receptors are upregulated (Gerfen et al., 1990). Ultimately, the reduction in dopamine leads to indirect pathway hyperactivity and direct pathway hypoactivity (Marsden, 1982; Albin et al., 1989; Alexander et al., 1990; Hirsch et al., 2000). In further support of this, Kravitz and colleagues (2010) showed that the activation of the indirect pathway MSNs induced PD-like symptoms in mice and that activation of the direct pathway helped to reduce these symptoms (Kravitz et al., 2010). Additionally, cortical oscillations are observed due to lack of D2 receptor-mediated suppression of MSN spike activity, resulting in increased sensitivity in the indirect pathway (Murer et al., 2007). Changes in synaptic plasticity have also been shown to support these findings: long-term depression in D1 receptors and long-term potentiation in D2 receptors provide more evidence that the direct and indirect pathways are altered to ultimately reduce voluntary movements (Day, Wokosin, Plotkin, Tian, & Surmeier, 2008).

While dopamine is known to aid in the regulation of NO signaling, the role of the NO signaling cascade in striatal function is not fully understood (West & Tseng, 2011). This pathway has an influence on pathology as multiple pharmacological studies have shown (West et al., 2002; Del Bel et al., 2005; Sammut et al., 2010). The inhibition of sGC or nNOS results in decreased locomotion in animals with intact dopaminergic neurons (Stewart et al., 1994; Del Bel et al., 2004). Thus, the NO-sGC-cGMP pathway likely plays an important role in movement control in the basal ganglia (West & Tseng, 2011). However, studies measuring levels of cGMP in the striatum of dopamine-depleted rodents have produced conflicting results (De Vente et al., 2000; Chalimoniuk et al., 2004; Sancesario et al., 2004; Chalimoniuk & Langfort, 2007; Giorgi et al., 2008). Other studies have reported that PDE levels are increased, suggesting a compensatory mechanism for augmented production of cGMP by NO signaling (West & Tseng, 2011).

To further complicate this pathway, independent studies have found that both nNOS inhibitors and PD medications can alter the levels of cGMP in the striatum. However, the mechanism leading to this decrease is not yet fully understood. While both types of cyclic nucleotides are metabolized by PDEs, cAMP is synthesized by adenylyl cyclase through activation of D1 receptors and cGMP is synthesized by NO stimulation of sGC. Thus, it would be useful to know if nNOS activity also affects the levels of cGMP in the striatum during dyskinesias. This study will focus on the NO regulation of cGMP and will seek to address the following questions: 1) How is the activity of nNOS altered in dyskinetic rats, and 2) how do behavioral changes associated with dyskinesias correlate with changes in nNOS activity. Understanding the changes in the nNOS/cGMP pathway due to chronic dopamine depletion and L-DOPA treatment in correlation with behavioral changes will provide insight into the pathophysiology and treatment of dyskinesias and PD.

Hypothesis and Aim
We hypothesized that nNOS activity will decrease after chronic L-DOPA administration as measured at the peak of dyskinesias. Specifically, we predict that cortically-evoked NO efflux will decrease at 30 minutes post-L-DOPA treatment in dyskinetic, but not non-dyskinetic, animals. NO change is predicted to occur to some extent in sham-
operated, saline-treated lesioned control, and non-dyskinetic rats. A decrease in NO would be consistent with the observed decrease in cGMP tissue levels during the peak of dyskinesias, as was shown in previous work by Giorgi et al. (2008).

Figure 5: NO and cGMP regulation: Glutamate and dopamine from the cortex and substantia nigra, respectively, are released to nNOS interneurons, activating NMDA and D1 receptors and promoting nNOS to catalyze the reaction of L-arginine into L-citrulline and nitric oxide (NO). NO then diffuses from the interneurons to medium spiny neurons where it activates soluble guanylyl cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP). cGMP can then go on to facilitate excitation of the neuron or activate protein kinases. Additionally, certain phosphodiesterases (PDE) degrade cGMP, while other PDEs are activated by cGMP. Adapted from West & Tseng (2011).

The aim of our study was to elucidate the mechanism causing a change in the levels of cGMP and understand the pathophysiology of both dyskinesias and PD. We completed this aim through electrochemical recordings of NO in the striatum of Sprague-Dawley adult rats. Since nNOS catalyzes the reaction producing NO, measuring NO efflux is an indirect method of measuring nNOS activity. Rats were placed in one of three chronically treated groups: 1) sham lesion with saline administration, 2) 6-OHDA lesion with saline administration, and 3) 6-OHDA lesion with L-DOPA administration. Group 1 acted as a surgical control to ensure that surgery alone did not cause changes in NO levels. Group 2 acted as a control for lesioned animals to track changes in NO levels after dopamine depletion. Group 3 was the experimental group, which provided NO recordings in dyskinetic animals. Figure 6 summarizes the expected outcomes of our study.

Findings
Our study provided evidence that cortically-evoked NO efflux decreases in both saline-treated 6-OHDA lesioned and L-DOPA treated 6-OHDA lesioned rats, suggesting that nNOS activity may decrease in PD and dyskinesias. NO recordings also suggested that acute L-DOPA administration may further decrease nNOS activity 50-60 minutes post-injection in dyskinetic animals; however, this result was not statistically significant. Continued experimentation is required to clarify these trends seen in this preliminary data set.