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- Sraavya Anne

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Dear Readers,

Welcome to the first issue of the third season of the IYNA Journal! We greatly appreciate your readership, continued or new. This year, we have worked hard at producing more high-quality articles for everyone to read and encouraging a growing number of high school students to submit their findings, research, and/or interviews to the journal.

We have been receiving many wonderful articles from you guys. It is clear how much the journal is improving as we progress into the new year. We would just like to thank everyone who have submitted articles to this issue and prior issues alike. Without your dedication and hardwork, we would not be able to spread the word about the amazing diversity in subject matter that neuroscience has to offer. With that being said, here are some previews of the articles in this release:

In the General Neuroscience section, Nidhi Shah discusses electrical brain impulses. In the Disease section, Anaya Aswale gives an overview on Amyotrophic Lateral Sclerosis, Annika Zhou reports on the Fecal Microbiota Transplantation of Parkinson's Disease, Phoebe Wilson explores Pathogenesis and the role of Oxidative Stress in Parkinson's Disease, Marta Luterek talks about the RNA interference in the treatment of neurological conditions, and Aayush Setty explains the role that peroxynitrite and chaperonins play in the molecular pathogenesis of Parkinson's Disease. In the Research section, Sraavya (Aashi) Anne reports on the efficacy of plant extracts in Alzheimer's Disease using Transgenic C. elegans and Kyle Sugita explores using modified B. subtilis to replace Levodopa Medication for Parkinson’s.

We would like to recognize all of our dedicated editors for helping us make this issue the success that it is. You can see all of their names and positions on our Contributors page. If you have any questions, comments, or suggestions for us, please feel free to contact us at info@youthneuro.org. We hope you enjoy reading this issue as much as we enjoyed editing it!

Best Regards,
Sojas Wagle - IYNA Journal Editor-In-Chief
Anita Singh - Managing Editor
Anushka Sarda - Senior Editor
Abstract
In ancient times, dreams have been believed to be a window to the supernatural. Even today, an action we spend almost a third of our lives doing is still a mystery. Dreams are often given this magical aura, but where do dreams really come from? Sleep is the product of many stages of REM (rapid eye movement) and NREM (non-rapid eye movement) sleep. Everyone dreams and rapid eye movement is a telltale sign that an individual is having a dream. Dreaming is one of the many mysteries of the brain and nobody knows exactly how they occur. Nevertheless, many scientists have proposed possible theories as to why people dream. For example, the Psychoanalytic Theory of Dreams suggests that dreams reflect unconscious desires and thoughts. On the other hand, the Activation-Synthesis Model of Dreaming is more factual, proposing that dreaming occurs when the brain is trying to make sense of signals during REM sleep [4]. While there is no consensus on the cause of dreams, they can have many positive and negative effects on cognitive abilities and functions.

REM and NREM Sleep

Sleep includes many important stages from REM to NREM sleep. The first three stages, lasting from 5-15 minutes, are NREM sleep [3]. During the first stage, the eyes are closed, and it is easy to wake up. The second stage is when the heart rate begins to slow down and the body temperature drops; this is also called light sleep. The final stage of NREM sleep is deep sleep, which is when it is difficult to wake up. During this stage, the body repairs tissues, builds bone and muscle, and strengthens the immune system. About 90 minutes after the first stage of NREM sleep, the body goes into REM sleep, during which the stages last longer and eyes convulse. The brain is also the most active during REM sleep, causing more intense dreams. Waking up during this stage can leave one groggy or disoriented [1].

Figure 1: The graph above shows the cycles of REM and NREM sleep. Note that N3, or the third stage of non-REM sleep, takes up the majority of the time during the early hours of sleep, but as one sleeps longer REM sleep, the parts indicated in red, take up majority of the time which is when dreams usually occur [8].
Anatomy of Sleep

The sleep/wake cycle is determined by the suprachiasmatic nucleus within the hypothalamus. Damage to the SCN could lead to erratic sleep patterns, as the SCN is unable to match the person’s circadian rhythm with the light-dark cycle [7].

The brain stem controls the transitions between wake and sleep by communicating to the hypothalamus. In the hypothalamus and the brain stem, sleep-promoting cells produce a chemical called GABA, which reduces the activity of arousal centers. The brain stem also sends signals to certain muscle cells so that people don’t act out their dreams, which is essential in REM sleep [7].

The thalamus relays information from the senses to the cerebral cortex. It is inactive during the NREM stages of sleep in order to help tune out the external world, but it is active during REM sleep in order to send the cortex images, sounds, and other sensations that fill dreams [7].

The pineal gland receives signals from the SCN to produce the hormone melatonin, which helps put a person to sleep once the lights go down. For people who have lost their eyesight, regularly taking small doses of this hormone can stabilize their sleep patterns [7]. The basal forebrain promotes sleep and wakefulness through the release of adenosine, which supports sleep drive. Caffeine counteracts sleepiness by blocking the actions of adenosine [7]. The amygdala is especially active during REM sleep, as it is involved in processing emotions [7].

Enhancing Cognitive Abilities

The necessity of dreams in memory and cognitive abilities is widely debated, although there have been many recent studies showing that dreams do play a critical role in memory consolidation and other cognitive tasks. In a study led by Richard Boyce, the long-term spatial memory of mice was tested by training mice to spot a new object placed in a controlled environment, where two objects of similar shape and volume were standing. As mice tend to spend more time exploring a new object rather than a familiar one, the mice gravitated towards the new object. During REM sleep, the
researchers turned off the mice’s memory-associated neurons through light pulses to determine if this affected their memory consolidation. This proved to be successful when, on the next day, the mice did not succeed on the same spatial memory task learned previously. When compared to the control group, the mice’s memory seemed to be impaired. This was also tested during sleep stages outside of REM sleep, which showed no change to memory consolidation, proving the necessity of neurological activity during REM sleep in memory consolidation [2].

To master anything, daily experiences are hammered into long-term memory during REM sleep. We’ve all heard the expression “sleep on it.” When we sleep, we tend to think about the events from our day during REM sleep so that the next day, we are better prepared. In fact, REM sleep is when we probably attain the most creative state of mind in the entire day. On multiple occasions, skills learned in activities, such as sports, seem to improve on their own because of REM sleep. In a study conducted by Stuart M. Fogel, a controlled group of adults performed either a spatial navigation task or tennis for 30 minutes. They were then asked to mentally rehearse the experience and provide a detailed wake verbal report on their mental rehearsal. During sleep, the subjects underwent polysomnography (PSG) monitoring and were awoken periodically during light NREM sleep to provide a dream report. After the nap, the subjects were tested on the same tasks. The performance after the nap showed improvement in both the tennis and the spatial navigation task [5].

How Can the Benefits of Sleep be Magnified?

Now that we know the benefits of sleep on memory consolidation and cognitive tasks, how can we get the best out of our sleep? First, since sleep/wake cycles are largely affected by our ability to sense light, it is important to sleep with the lights turned off. Dimming lights a little earlier before sleep can help as well. Rather than sleeping in on the weekends and staying up late certain nights, it is important to keep regular sleep cycles so that the body knows when to sleep. Keeping temperatures lower at night also helps to signal to your body that it is time to sleep. When someone is sick, it is usually hard to fall asleep because of the high body temperature. Instead of staying awake in bed when you are having trouble sleeping, it is better to stand up and do something else to train your mind that the bed is only for sleeping. Lastly, consuming caffeine and alcohol before sleeping interferes with sleep and could cause frequent wake-ups during the night [6].

Glossary

1. **Rapid Eye Movement (REM)** - Stage of the sleep cycle in which the eyes twitch and most dreaming occurs

2. **Non-Rapid Eye Movement (NREM)** - Occupies the first 3 stages of the sleep cycle when the brain is generally inactive

3. **Hypothalamus** - A peanut-sized structure located between the thalamus and the brain stem that performs many vital tasks [7]
4. **Suprachiasmatic Nucleus (SCN)** - Clusters of thousands of cells located within the hypothalamus that receive information about light exposure directly from the eyes and control behavioral rhythm [7]

5. **Circadian Rhythm** - 24-hour cycle driving the change between sleepiness and alertness at regular intervals

6. **Brain Stem** - The central trunk of the brain, including structures such as the pons, medulla, and midbrain

7. **Thalamus** - Relays sensory information and acts as the center for pain perception

8. **Cerebral Cortex** - The covering of the brain that interprets and processes information from short- to long-term memory

9. **Pineal Gland** - A structure located within the brain’s two hemispheres that produces the hormone melatonin

10. **Basal Forebrain** - Structures located near the front and bottom of the brain that promote sleep and wakefulness

11. **Amygdala** - Almond-shaped structure involved in processing emotions

12. **Polysomnography (PSG)** - Records brain waves, oxidation level in blood, heart rate, breathing, and eye and leg movements

References


Amyotrophic Lateral Sclerosis: An Overview

Anaya Aswale

Introduction
Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig’s Disease, is a progressive neurodegenerative disease that affects nerve cells in the brain and spinal cord [1]. According to the ALS Association, more than 5,600 people are diagnosed per year and as many as 30,000 Americans currently are affected [2]. Out of those 30,000 Americans, 60% are found to be males and 93% are Caucasian [3]. This article will look into the causes, methods of diagnosis, symptoms, and treatments for Amyotrophic Lateral Sclerosis.

Causes and Risk Factors

ALS is a neuromuscular disease that is characterized by progressive weakness in the muscles, which can eventually lead to paralysis, as a result of the death of motor neurons located throughout the nervous system. The death of motor neurons leads to muscle weakness. Though the cause of Amyotrophic Lateral Sclerosis is not known, scientific evidence from studies suggests that genetic and environmental factors may have an effect on the development of ALS [4].

In 1993, scientists found that mutations in the superoxide dismutase 1 (SOD1) gene were linked to some cases of ALS found in families. In fact the discovery of certain mutations has led scientists to believe that changes in the processing of RNA may cause motor neuron degeneration, since RNA is an important molecule in synthesizing specific proteins and
performing activities relating to gene regulation \[4\]. Researchers are in the process of studying the chemical imbalances in people with ALS; specifically, glutamate, a chemical messenger in the brain which is known to be toxic to nerve cells at elevated levels \[5\]. Researchers are also studying the impact of environmental factors, such as viruses, diets, and more. They have suggested that exposure to certain toxins during war or demanding physical activities may be the reason why veterans and athletes have a higher chance of developing ALS \[4\].

Data shows that ALS is inherited in 5 to 10% of people, but for the rest of the population, the cause is unknown. However, when it comes to factors that may cause ALS, there is a suspicion that heredity, age, sex, and genetics could be risk factors. In addition, smoking, toxin exposure, and military service have also been linked as factors that may trigger ALS \[5\].

**Diagnosis**

ALS is different for every person and therefore there is no one way to diagnose it. However, it is primarily diagnosed based on symptoms recognized by the physician. Since the symptoms are similar to that of Parkinson’s disease and stroke, it can be very difficult to diagnose. It may take several months to know for sure if someone has ALS since the disease gets worse over time and can inhibit eating, swallowing, and even breathing. Though diagnosis can be difficult, there are a few tests that are done to rule out other diseases. One test would be electromyography (EMG), which detects and records the electrical signals that are produced by muscle tissues as they contract. This test sends electric shocks through nerves in order to see if they are damaged. In addition, EMG will show if there is a clear abnormality in the muscles. Some other tests include Nerve Conduction Study (NCS), which measures a nerve’s ability to send a signal to the muscles; X-rays, Magnetic resonance imaging (MRI), spinal tap, and blood and urine evaluations. \[4\][7]

**Symptoms**

Though initial symptoms vary for different people, there are a few generic symptoms that may cause a doctor to suspect ALS. These symptoms include muscle twitching in the arms, leg, shoulder, or tongue, muscle cramps, tight and stiff muscles, muscle weakness affecting an arm, a leg, neck, or diaphragm, slurred and nasal speech, and difficulty chewing or swallowing \[4\]. The symptoms can begin either in the arms and legs or in speech and swallowing \[8\]. Despite where the symptoms begin, muscle weakness spreads to other parts of the body as the disease advances, eventually the disease will progress to a point where the diagnosed person will no longer be able to stand or walk, get in or out of bed on their own, or use their hands and arms \[4\].
Treatments

Though there is no definite cure for ALS, treatments are available through therapy and medication. Medicines can control muscle cramping, difficulty swallowing, pseudobulbar affect (uncontrollable crying or laughing), pain, depression, and any other symptoms that arise as a result of ALS [7][4]. Often, doctors may prescribe Food and Drug Association (FDA) approved drugs like Riluzole and Edaravone to treat ALS. Riluzole is used to reduce damage to motor neurons by decreasing levels of glutamate and has shown to prolong survival by a few months [4]. According to the National Institute of Neurological Disorders and Stroke, “edaravone is believed to slow the decline of clinical assessments of daily functioning in persons with ALS” [4]. In addition, people affected by ALS may benefit from physical therapy. It can enhance a person’s ability to perform gentle and low-impact aerobic exercises and can improve cardiovascular health as well as help them cope with breathing problems, fatigue, and depression. Speech therapy, which can help a person maintain the ability to communicate, can also be beneficial for people with ALS [4].

References

Fecal Microbiota Transplantation for Parkinson’s Disease

Annika Zhou

Abstract

Parkinson’s disease (PD) is a long-term degenerative neurological disorder of the central nervous system that mainly affects the motor system and the control of movement. A primary form of Parkinsonism, a group of disorders where there is progressive loss of motor function due to the degeneration of neurons, the idiopathic disease affects ten million people worldwide with more than sixty-thousand diagnosed cases per year [1]. Parkinson’s disease is known for its high prevalence, especially in the aging population, its eventual resistance to drug treatments, and lack of effective therapy. Recent research has shown, however, that alterations in gut microbiota composition and dysbiosis are linked to a disruption in the gut-brain axis [2]. Experiments have further indicated that the enteric nervous system is involved in PD progression toward the central nervous system and that modulation of gut microbiota has been associated with improvements in neuropsychiatric and neurological disorders [2]. A safe and effective way of manipulating gut microbiota is through fecal microbiota transplants. FMT has been used successfully as a treatment for irritable bowel syndrome (IBS) and autism in addition to numerous other disorders [3]. This article seeks to advocate for clinical trials and experiments with FMT as a treatment option for Parkinson’s disease.

Parkinson’s Motor and Non-Motor Symptoms

Parkinson’s disease, a neurodegenerative disease, is typically diagnosed from physical symptoms, such as tremors. Symptoms consist of tremors of a limb, often beginning in the hands or fingers. Parkinson’s disease is, for the majority of the time, diagnosed based on motor symptoms like the aforementioned tremors. However, non-motor symptoms such as constipation, abdominal pain, IBS, and gastrointestinal dysfunction have drawn little attention in research. While Parkinson’s disease is characterized by cardinal motor impairments, this wide range of non-motor symptoms typically precede the motor symptoms. In a study performed by China Medical University in Taiwan, the prevalence of constipation and abdominal pain in PD are 46.7%-52.5% and approximately 28% respectively [4]. Several studies, such as the Chinese Medical University study,
have also indicated that there is a correlation between IBS and the risk of PD [4]. By following the medical history of 23,875 patients with newly diagnosed IBS and 95,500 subjects without IBS to compare, the incidence of PD was 1.76 times higher in the IBS group than in the non-IBS group; further data suggests that patients with IBS have a 48% higher chance of getting PD than the population without IBS [4]. In the last decade, hypotheses concerning the concept of a gut-brain axis and the idea that Parkinson’s disease begins in the gut have gained much more support [3]. It has been recognized that such gut-brain axis interactions in Parkinson’s disease may be influenced by an imbalance in gut microbiota, which explains why gastrointestinal dysfunction typically precedes the motor symptoms of Parkinson’s disease.

The Gut-Brain Axis

The gut-brain axis or the GBA consists of bidirectional communication between the enteric and central nervous system, linking the emotional and cognitive centres of the brain with peripheral intestinal functions [2]. The bidirectional link is a complex form of communication that not only ensures the maintenance of gastrointestinal homeostasis, but also affects the mood, motivation, and higher cognitive functions of an individual [2]. The maintenance of homeostasis is theorized to be an important part of maintaining a healthy mental state. Dysbiosis, a microbial imbalance, is highly associated with mood disorders and is linked to a disruption of the gut-brain axis [2]. Current treatments like levodopa are based on restoring dopaminergic neurotransmission and alleviating motor symptoms like tremors but leave non-motor symptoms untreated. Accumulating evidence suggests that the enteric nervous system is involved in Parkinson’s disease progression toward the central nervous system. In this sense, perhaps by altering an individuals dietary components or through fecal transplants, we may influence the gut-brain axis by altering the microbial composition of the enteric nervous system.

Past Usage of Fecal Transplantation in Other Diseases

Figure 1. A diagram illustrating the gut-brain axis connection [6].
In the past, fecal transplants have been tested and researched in patients of several other neurological disorders and gastrointestinal diseases. While commonly used as a treatment for gastrointestinal diseases, such as *Clostridium difficile* infection, Crohn’s disease, and ulcerative colitis, the application of fecal transplants has been experimentally used in the treatment of metabolic and autoimmune disorders as well [7]. Case reports also exist showing that fecal transplants have been effective in treating multiple sclerosis, chronic fatigue syndrome, and IBS. The implementation of fecal transplants is not only cheap and reliable, unlike other forms of treatment for Parkinson’s disease, such as long-term Levodopa use or invasive surgery, but also tends to maintain its effect over long periods of time (usually multiple years). The long-term effect of fecal transplants is visible in usage for numerous gastrointestinal disorders and diseases. According to Linda Lee, Director of the Johns Hopkins Integrative Medicine and Digestive Center, the procedure of fecal transplantation is simple, and in treatment for *C. difficile*, for example, fecal transplants are effective in 90% of patients [8]. Lee further reports that “70% of people who receive fecal transplants for *C. difficile* report an improvement of symptoms within just three days” and that fecal transplants have the potential to “[improve] the quality of life [8].” Studies regarding *C. difficile* infections have shown that 30% of patients relapse when treated with rounds of antibiotics, which means that patients are spending significant amounts of money for repeated courses of antibiotics and are not getting better; in contrast, fecal transplants are not only effective, but also cost-friendly in treating gut infections, making it a better option [8]. If a single fecal transplant treatment can be effective for numerous years, it can easily replace the standard drug treatment of Levodopa.

**Limitations of Fecal Transplantation**

While fecal transplantation has shown potential in becoming a treatment for Parkinson’s disease among many other diseases and disorders, such an innovative and new treatment is not without any risk. Lee compares the beginning of fecal transplantation use to “the early days of blood transfusion,” reminding readers that researchers could be unaware of deadly microbiota like they had been unaware of hepatitis C at the time [8]. Scientists have not yet discovered all the organisms or the full composition of humans’ gut microbiota, so if the theory that gut bacteria play an important role in diseases is true, fecal transplantation could have unanticipated dangers and consequences. Long-term effects of fecal transplantation cannot be predicted at this time; however, treatments have so far been effective with only minor side effects like constipation and bloating [8].

**The Application of Fecal Transplants in Neurological and Neuropsychiatric Disorders**

Promising advancements in autism research involve the gut-brain axis and gut microbiome, the collection of microbes that live in our intestines and helps digest food and prevent the overgrowth of bacteria. Similar to Parkinson’s disease, roughly 30% to 50% of autistic individuals experience gastrointestinal problems, such as constipation and diarrhea [3]. These statistics further reinforce earlier statements regarding the connection between neurological disorders and dysbiosis. Research performed on children with autism with gastrointestinal problems have indicated that children with worse gastrointestinal problems also have worse autism-related symptoms [3]. When gastrointestinal problems are relieved, behaviour improves. Research conducted has further shown
long-term beneficial effects for children diagnosed with ASD treated through Microbiota Transfer Therapy (MTT), a specific type of fecal transplant [3]. Fecal transplants in the experiment eventually lead to improvements in gut health and symptoms that persisted for years following the initial treatment [3]. Two years post-treatment, most initial improvements in gut symptoms remained, and ASD symptoms steadily reduced over the next two years; professional evaluators indicated a 45% reduction in core ASD symptoms in terms of language, social interaction, and behaviour [3]. In this case study, by transferring healthy microbiota to individuals lacking certain gut bacteria, it was possible to create a more diverse population of bacteria in a patient and improve gut and neurological health. Besides ASD, chronic fatigue syndrome and multiple sclerosis are other examples of neuropsychiatric and neurological diseases and illnesses that have been effectively treated with fecal transplants [7]. Considering the similarity in symptoms and the similar effects of the GBA in PD and autism specifically, it can be inferred that fecal transplants have the potential to alleviate Parkinson’s symptoms or be used as a preventative treatment or a method of early diagnosis.

Conclusion

The gut, according to Patrik Brundin MD, PhD, Van Andel Research Institute and J. William Langston MD, Stanford Udall Center, Department of Pathology, Stanford University, both Editors-in-Chief of the Journal of Parkinson’s Disease, “has emerged as one of the new frontiers in PD research [5].” The gut-brain axis among other research developments has demonstrated the possibility and great potential of fecal transplants as a treatment for Parkinson’s disease. Fecal transplantation has demonstrated its potential impact in neurological disorders, as it has demonstrated its ability to ameliorate symptoms in gastrointestinal diseases related to PD and other neurological disorders, such as multiple sclerosis and autism. Given the similarities in symptoms and the similar gut-brain axis connection in the two diseases, it is reasonable to predict that fecal transplants can not only be used as a preventative treatment or method of early diagnosis, but can also be used to relieve motor symptoms in Parkinson’s disease.

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Pathogenesis and the Role of Oxidative Stress in Parkinson’s Disease

Phoebe Wilson

Abstract

Parkinson’s Disease (PD) is a cortical degenerative disease characterised by a progressive deficiency in dopaminergic neurons, resulting in passive limb movements, tremors, and posture and gait impairments. Despite the pathology of the disease being apparent, the etiology of the gradual loss of dopamine within the substantia nigra (SN) remains unexplained. Of the many pathogenic processes speculated to be instrumental in the deterioration of dopaminergic cells, oxidative stress has long been implicated as a top contender. Interferences have been observed in the oxidation-reduction conservation within neurons, which consequently impede a number of assorted biological processes, leading to neuronal cell death.

Etiology of Parkinson’s Disease

While Parkinson’s Disease remains idiopathic, since the underlying processes accountable for neuronal degradation have yet to be determined, its cardinal motor symptoms have indisputably been linked to the deprivation of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [1]. Substantial advances have been made in terms of pinpointing the genetic and environmental constituents that lead us to becoming more susceptible to developing Parkinson’s. Pesticide exposure is still a hot topic with associations being made between certain agrochemicals and nigral dopaminergic cell death [2]. Additionally, the levels of basal oxidative stress normally observed in healthy humans have been noted to rise in Parkinson’s patients [3]. Collective efforts in research have led to the acquisition of evidence to suggest that oxidative damage is a direct contributing factor to the noticeable neuronal dopamine decline [5].

The Biochemistry of Oxidative Stress

Oxidative stress is a state our body enters when overwhelmed by the presence of free radicals.
unstable molecules) that we are unable to counteract due to a reduction in our antioxidant defenses. Thus, a disequilibrium occurs between the levels of reactive oxygen species (ROS) and the antioxidant response. Due to the substantial amount of reactive oxygen species that are produced in the brain, their involvement in Parkinson’s Disease is considered to be significant. This is due to the brain utilising 20% of the entire body’s oxygen supply, of which a large amount is allocated to producing ROS, thus rendering the brain incredibly vulnerable to oxidative stress. This physiological disturbance has been linked to over 200 critical health conditions and can lead to further DNA and tissue damage, inflammation, and apoptosis (programmed cell death) [5]. For quite some time, it has been theorised that oxidative stress is linked to a number of central nervous system (CNS) conditions. Though contrasting greatly on a pathological level, Huntington’s Disease, Alzheimer’s Disease, and Amyotrophic Lateral Sclerosis have all been linked to oxidative stress, supporting the postulation that it plays an instrumental role in overall neurodegeneration [1].

Further evidence was found in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson’s Disease, a widely used prototype for mimicking aspects of the disease through the employment of dopaminergic neurotoxins [6].

Figure 2. Immunostaining of tyrosine hydroxylase (TH)–positive neurons in the substantia nigra pars compacta (SNpc) and terminals in the striatum. Mice were intoxicated with MPTP and tissues were immunostained for TH following standard protocol. (a) Illustration of the effect of MPTP on TH immunostaining in both the mouse SNpc and striatum at 7 days after injection. In a saline-injected control, there is a dense TH-positive network of cell bodies and fibres in the SNpc and striatum. After the MPTP injections, there is a dramatic reduction in TH immunoreactivity at the levels of the SNpc and the striatum. Ventral midbrain sections are counterstained with Nissl (blue) for anatomical reference. Panel (b) shows the loss of SNpc neurons in MPTP-dosed mice counted by stereology and (c) shows the loss of striatal fibres in MPTP-dosed mice assessed by optical density. Bars represent means ± standard error of the mean (SEM) of 5 mice per group. Statistical analyses were carried out using the unpaired student’s t-test. *Lower than saline controls.“ Adapted from Jackson-Lewis & Przedborski (2007) [6].

This has been conducive to researchers theorizing that dopaminergic neurons could be eradicated as a consequence of early-onset oxidative stress [8]. The discovery of neuromelanin in dopaminergic cells led to further observations of molecular species being formed through the autoxidation and polymerization of dopamine. This in turn fueled the notion that the higher basal levels of oxidative stress detected in the substantia nigra were a result of dopaminergic metabolism [2].

Postmortem Findings in Relation to Oxidative Stress

Postmortem examinations and autopsies have consistently attributed oxidative stress to be an indicator of nigral dopaminergic cell degeneration [1-3, 8-11]. Autopsy tissue obtained from Parkinsonian patients’ brains revealed additional oxidative damage to lipids, proteins, and DNA [9]. Determining which factors lead to oxidative damage and tissue dysfunction can be complex, as
reactive oxygen species (ROS) are capable of effectively harming all biological macromolecules [8]. Relating back to the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model in Figure 2., individuals subjected to exposure of the aforementioned neurotoxin exhibited inflammatory responses [10]. These were produced through the activation of microglia and astrocytes, which consequently promote neuronal cell deterioration. In vitro and in vivo studies conducted on rodents exposed to inflamagens observed the activation of microglia and formation of free radicals. These events were sufficient enough to prompt nigral dopaminergic decline [11]. Additionally, two groups of neurotoxins that resembled MPTP were detected in postmortem studies: isoquinolines (IQs) and β-carbolines [12]. These compounds were linked to precipitating apoptotic cell death, analogous to MPTP.

**Conclusion**

Our brains possess poor capacities to handle oxidative stress and exhibit little regenerative capacity. The substantia nigra in particular is prone to a substantial amount of oxidative damage, though this has yet to be fully elucidated [3]. Plenty has yet to be discovered in terms of determining the specific causes of Parkinson’s, and this article has sought to present a standpoint on only a select few aspects. The research described herein suggests that oxidative stress and free radical formation hold notable roles in the pathogenesis of dopaminergic cell deprivation in Parkinson’s Disease. An earlier study suggested potentially incorporating extracerebral tissues into future research in order to fuel the debate on whether Parkinson’s may be a more generalised disease [13]. Other studies have proposed delving deeper into the neuroinflammatory reactions of nigral dopaminergic neurons [10, 11]. For now, Parkinson’s remains incurable, and we can merely continue to endeavour in uncovering new knowledge that can assist us on a clinical level to prevent the debilitating nigral dopaminergic loss that characterises this disease.

**References**


RNA Interference in Treatment of Neurological Disorders

Marta Luterek

Introduction

RNA interference (RNAi) is one means of reducing gene expression. Interference effects take place after the transcription stage by degrading a corresponding sequence of messenger RNA (mRNA). Since the discovery of RNAi, for which A. Fire and C.C. Mello were awarded the Nobel Prize, it has become evident that RNAi holds great potential to be used as a treatment for many types of diseases, including those of neurological origin. In this article, research is presented on the topic of RNAi in treatment of Huntington’s disease, spinocerebellar ataxia, Parkinson’s disease, and Alzheimer’s disease. Moreover, a discussion on both the potential and risks of using RNA interference as a therapeutic agent is included.

The Discovery of RNA Interference

RNA interference (RNAi) is a means of silencing a gene after its sequence has been transcribed from DNA into mRNA. It was discovered by Fire and Mello, who were awarded the 2006 Nobel Prize in Physiology or Medicine for their findings [1]. They injected double-stranded RNA (dsRNA) directly into the body of their model organism – the nematode worm Caenorhabditis elegans. The dsRNA they used corresponded to a 742-nucleotide sequence of the unc22 gene, which encodes an abundant but nonessential myofilament protein. The decrease in unc22 activity leads to a phenotype of severe twitching movements [2].

The injected nematodes showed strong twitching, while the control group and the worms injected with single-stranded RNA remained phenotypically normal. This led them to conclude that injection of dsRNA inhibited expression of the unc22 gene while single strands of RNA (sense or antisense) did not inhibit genes with the same efficiency. They also stated that the
silencing effect was specific for the mRNA sequence homologous to the dsRNA because other mRNA sequences were not affected. In a follow-up paper, they stated that interference by dsRNA causes no changes in the original DNA sequence [3]. This was surprising at the time because interference effects were hypothesized to result from a simple mechanism of hybridization between the injected single-stranded RNA and the mRNA within the cell [1]. Since the discovery of RNAi and its regulatory potentials, it has become evident that RNAi technology is precise, efficient, and stable and that it has potential in inhibiting expression of desired genes [4].

The Mechanism of RNAi

Protein synthesis requires the cell to perform transcription – the copying of the DNA sequence into mRNA, which is then transported into the cytoplasm. After post-transcriptional modifications, the mRNA sequence is translated into a protein. RNAi interrupts gene expression after the transcription stage. The process of RNAi is initiated by double-stranded RNA molecules (dsRNA). They can be either exogenous – introduced into the cell via vectors or by laboratory manipulations – or endogenous – in the form of microRNAs coming from introns – genes that are not translated into proteins [5].

When the dsRNA is in the cell, it is recognized by a protein called Dicer, which functions as a ribonuclease – an enzyme that catalyzes the degradation of RNA into smaller fragments [5]. The dsRNA is cleaved into small pieces (20-25 nucleotides). These fragments are called small interfering RNAs (siRNAs). They are then separated into single strands and integrated with proteins to form the RNA-induced silencing complex (RISC) [6]. The single strand of RNA integrated with the complex binds to a complementary sequence in the mRNA and degrades it [6]. The mRNA is disabled and no protein is made. Thus, the expression of a gene is silenced.

The Therapeutic Potential of RNAi

Since the discovery of RNAi, the idea of RNAi therapeutics has developed into a creative and competitive field that has attracted intense interest and is one of the most highly investigated fields in biotechnology research [7]. Understanding the mechanism of RNAi leads to a conclusion that due to its specificity and effectiveness, RNAi holds the potential to be used as a therapeutic agent for dominantly inherited disorders and disorders in which a protein is a key element to its pathogenesis. The possibility of engineering siRNA to silence specific sequences of a mutant allele without interfering with the expression of the normal allele has been demonstrated in multiple studies [8-12]. Diseases with a genetic component that could potentially be treated using RNAi include many neurological disorders, such as Huntington’s disease (HD), Alzheimer’s disease (AD), Parkinson’s disease (PD) and spinocerebellar ataxia (SCA) [13].
RNAi in Treatment of Huntington’s Disease

Neurogenic diseases caused by nucleotide repeat expansion are often fatal. Such diseases include Huntington’s disease and spinocerebellar ataxia, which are induced by a repetition of CAG sequences, coding for the amino acid glutamine [14].

Huntington’s disease is an autosomal dominant disorder, which means that a person needs to inherit only one copy of the defective gene to develop the disorder [15]. However, the offspring of a person affected by HD may have more rapid progression or severity of symptoms due to the relative increase in the length of the CAG repeat during meiosis and the subsequent expression of proteins with longer polyglutamine tracts [14]. The molecular defect that causes the disease is a somatic mutation found on chromosome 4 in a gene that codes for a protein called huntingtin [15]. The disease causes neurons to die in various areas of the brain, such as the neostriatum, substantia nigra, cerebral cortex, and hippocampus [15]. The hallmark symptom of HD is uncontrolled movement of the arms, legs, face, and upper body [16]. Psychological symptoms include changes in behavior, emotion, judgement, and cognition [15]. Treatments available for HD patients are only able to lessen symptoms of involuntary movements and psychiatric disorders using antipsychotic drugs and antidepressants [15]. However, there is currently no cure for HD and no way to slow progression [16].

RNAi in Treatment of Spinocerebellar Ataxia

Spinocerebellar ataxia is a term referring to a group of ataxias that are characterized by neurodegeneration in the cerebellum and the spinal cord – areas involved in movement control [17]. Most types of SCA are caused by trinucleotide CAG repeat expansions [17]. Like in HD, the severity of symptoms depends on the genetic recombination of the expanded CAG repeats.

Symptoms of SCA include loss of balance, poor coordination, and slurred speech, though patients usually retain full mental capacity [18]. There is currently no way to fully cure SCA. The clinical care of patients focuses on managing the symptoms through physiotherapy, occupational therapy, and speech therapy [18].

There is considerable debate about how the proteins with expanded CAG sequences cause disease, what causes the selective neurodegeneration by mutant proteins, and how their expression causes the specific symptoms of each nucleotide repeat disease [14]. However, there is no doubt that each of the CAG-induced diseases is caused by expression of the mutant gene [14]. This leads to a conclusion that RNAi could serve as a method of inhibiting expression of the mutant genes in some of the diseases, thus inhibiting onset of the disease. However, it has been demonstrated that expanded CAG repeats, although accessible to RNAi, are not preferential targets for silencing [18]. Nevertheless, an associated single-nucleotide polymorphism can be exploited to achieve silencing provided that it is placed centrally in the siRNA. Researchers were successful in inhibiting the SCA3 gene using this method, and it is thought that this method should extend to HD [18].
RNAi in Treatment of Alzheimer’s Disease

RNAi is also researched as a possible treatment for Alzheimer’s Disease (AD). AD is the most popular type of elderly dementia characterized by irreversible neurodegeneration. Symptoms of AD include trouble recalling very recent events, loss of verbal memory, and decline in cognitive function. Doctors are currently unable to cure AD, but research in this field continues while patients receive treatments meant to slow the progression of symptoms and improve their quality of life [17]. There are two key proteins in the pathogenesis of AD: tau protein and amyloid precursor protein (APP). Developing ways to inhibit accumulation of these proteins is of great interest when it comes to developing potential treatments for AD. Researchers were successful in developing siRNAs that allele-specifically suppressed the most widely studied tau protein and APP mutations, and they delivered the siRNAs into cells via short hairpin (shRNA) plasmids [19]. They also found that successful engineering of effective siRNAs required mutations to be placed centrally in the guide strand of the siRNA [19]. Thus, they concluded that in mammalian cells, it is possible to silence a single disease allele without activating pathways that result in the spread of silencing signals (such pathways are present in plants and worms). However, delivering the siRNA to the correct target cells in the brain still poses a major challenge. Viral vectors have shown potential in delivering genes into neurons of the central nervous system [19]. Nevertheless, the long-term consequences of triggering the RNAi pathway are still unknown, but future RNAi studies in transgenic animal models of AD and other diseases may help to answer these questions [19].

RNAi in Treatment of Parkinson’s Disease

Parkinson’s disease is a neurodegenerative disorder that predominantly affects dopaminergic (dopamine-producing) neurons in the substantia nigra – a basal ganglia structure located in the midbrain [21]. Symptoms of PD usually develop slowly and differ among patients but generally include tremor (involuntary shaking of the hands, arms, legs, or jaw), bradykinesia (slowed movement), rigid muscles, speech problems, and balance problems [21]. As for previously mentioned diseases, PD cannot currently be fully cured, and treatment is aimed at controlling the symptoms.

A rare, inherited form of PD is connected with a mutation of the alpha-synuclein protein, which is a component of Lewy bodies – abnormal structures composed of protein that develop inside nerve cells [22]. Alpha-synuclein is encoded by the SNCA gene and overexpression of the gene as well as nucleotide variations appear to be involved in the pathogenesis of PD [23]. Scientists developed a RNAi-based treatment method called ExCont-RNAi – expression-control RNAi – which they used to moderately silence the overexpressed SNCA genes to return to a normal level [22]. They introduced the siRNA into the fibroblasts from a PD patient in whose cells SNCA was expressed approximately twofold more than in normal fibroblasts [23]. Fibroblasts treated with the siRNA reduced the expression of SNCA approximately by half, similar to the level found in healthy fibroblasts [23]. These findings demonstrated that normalization of overexpressed SNCA by RNAi is possible in PD cells. Furthermore, no significant off-target effects of the therapy were found.

Conclusion
The discovery of RNAi expanded the possibilities of gene therapy for many diseases and changed the way we think about inhibiting gene function. As discussed in this article, RNAi holds the potential to be used in therapy of multiple neurological disorders. Since the discovery of RNAi, genetic therapy has become one of the most quickly advancing fields in medicine. Companies devoted solely to developing RNAi-based medications have emerged, such as Alnylam Pharmaceuticals. However, despite the promising potential, the long-term consequences of chronically triggering the RNAi pathway \textit{in vivo}, as may be required to treat neurodegenerative conditions, are unknown \cite{10}. Another problem connected with RNAi as a therapeutic agent is that data has shown that genes can differ widely in their susceptibility to inhibition with no obvious sequence features predicting the success or failure of a given siRNA. For example, every siRNA designed against ataxin-3 displayed significant activity (7/7, 100%), whereas tau proved to be more difficult to inhibit with only a single region centered on the V337M mutation yielding effective siRNAs (3/7, 43%) \cite{11}. Furthermore, it is difficult to directly predict the siRNA concentration required for therapeutic effects \textit{in vivo}, where the disease protein is expressed at much lower levels than in experimental systems used in the studies and siRNA delivery could be less efficient \cite{17}. Despite the uncertainties connected with RNAi therapeutics, it is definitely an area with great therapeutic potential, so it continues to be studied as a means to develop ways for use as a treatment for neurological diseases of genetic origin or with a genetic component.

References


Peroxynitrite and Chaperonins in the Molecular Pathogenesis of Parkinson’s Disease

Aayush Setty

Abstract

Parkinson’s Disease is a common neurodegenerative motor disease, a neurodegenerative disease that affects normal movement, that is prevalent in the elder population. It is characterized by the selective neurodegeneration of dopaminergic neurons in the substantia nigra pars compacta of the human brain. This disease is idiopathic, meaning its root causes are unknown and the mechanisms behind its molecular pathogenesis are not very well understood. As a result, treatments to the disease, such as Levodopa, mainly serve as symptomatic treatments or aim to slow down dopaminergic neuronal death. Over the years, there have been many proposals as to what fundamental molecular cascades lead to the development of selective neuronal death, but there has been no definitive mechanism outlined. Recent studies involving chaperone proteins and oxidative stress coupled with high throughput drosophila fruit fly studies have shed light on new possible molecular pathways leading to the pathogenesis of Parkinson’s Disease[1].

Apparent Pathology in Parkinson’s Disease

Although Parkinson’s Disease (PD) is idiopathic, the pathological markers of the disease are very well characterized. In contrast with other neurodegenerative diseases, neuronal loss in Parkinson’s is confined mainly to dopaminergic neurons in the substantia nigra pars compacta(SNpc), a structure located in the midbrain[2]. Dopaminergic neurons in the SNpc contain large amounts of neuromelanin, a dark polymer pigment similar to melanin, which gives a healthy substantia nigra a characteristic dark color[3]. In post-mortem analysis of Parkinson’s patients, the SNpc was found to be devoid of the dark coloration that neuromelanin possesses suggesting the neuronal loss of dopaminergic neurons in the SNpc (Figure 1).

Figure 1. Panel A shows a section of the midbrain from a control patient with a dark SNpc. Panel B shows a section of the midbrain from a patient that has PD and thus has a light SNpc.
due to dopaminergic neuronal death. Adapted from Lima, et al (2012)[4].

On a cellular level, dopaminergic neurons in the SNpc of Parkinson’s patients show widespread accumulations of protein aggregates known as Lewy Bodies (LBs), which are mainly made of a relatively common soluble presynaptic protein called α-synuclein[5][6]. These aggregates form in two stages. First, they form soluble circular oligomers, otherwise known as protofibrils, and form insoluble fibrils in the second stage. These insoluble fibrils are thought to be the main constituents of LBs[7]. The role these LBs play in the pathogenesis of PD is unknown; although, some evidence cites it as being a neuroprotective mechanism, whereas other evidence shows it as a toxic structure [8].

![Figure 2. LBs in the brainstem SNpc of PD patients are visualized through anti-synuclein antibody immunohistochemistry. These LBs are insoluble and mostly made of misfolded α-synuclein. Adapted from Baba, et al. (9).](image)

Pathophysiology of Parkinson’s Disease

PD affects mostly the motor functions of patients. Symptoms such as the classic parkinsonian gait (short, shuffling steps resulting in a loss of balance), bradykinesia (slowed movement), and hand tremors serve as hallmark features in the diagnosis of PD [10]. The pathophysiology behind the development of these symptoms is well known and is isolated to one brain system: the basal ganglia. The basal ganglia and the cerebellum are important brain structures involved in the modulation of movement. The basal ganglia itself is very important in the proper initiation of movement and the proper regulation of voluntary movement. The SNpc is a crucial component of the basal ganglia system due to its role in supplying the circuit with the neurotransmitter dopamine. Dopamine itself is an excitatory neurotransmitter that serves as the primary means in which the basal ganglia initiates a movement [11].

In a properly functioning basal ganglia, the system will take in cortical inputs, process those inputs through two pathways, the direct and indirect pathway, and ultimately ends with a thalamic output to the VA/VL nucleus. In a properly functioning basal ganglia, the processing segment of the circuit, as previously mentioned, has a direct and indirect pathway. The direct pathway promotes the initiation of movement, whereas the indirect pathway inhibits the initiation of movement. Thus, a balance between these two pathways leads to a comprehensive movement regulation system. Dopamine supplied by the SNpc directly increases the activity of the direct pathway and inhibits the activity of the indirect pathway. The basal ganglia is able to have variability in the effects of
dopamine due to the different receptors at the synapse of neurons in the direct versus the indirect pathway. The neurons in the direct pathway contain D1 dopamine receptors, which promote excitatory postsynaptic potentials (EPSPs) in the postsynaptic neurons and activate it. On the other hand, neurons in the indirect pathway contain D2 dopamine receptors, which promote inhibitory postsynaptic potentials (IPSPs) in the postsynaptic neurons and its activity[12].

Thus, the loss of dopaminergic neurons in the SNpc deprives the basal ganglia of dopamine, which is crucial in proper movement initiation. Without dopamine, the direct pathway would be under activated while the indirect pathway would be overactivated which results in difficulty of coordinated movements, bradykinesia, hand tremors, and shuffling gait. Patients have even reported that at late stages of PD they have to focus very hard on the movements they want to perform before their muscles actually perform that action. This symptom serves as a perfect example of how the pathophysiology of the disease in the basal ganglia leads to the manifestation of symptoms related to movement initiation.

The activation of movement through the direct pathway is mediated through a system of disinhibition. In essence, as seen in Figure 3, the internal globus pallidus (Gpi) and substantia nigra pars reticulata (SNr) send continuous inhibitive signals to the VA/VL nucleus of the thalamus. This, in turn, inhibits movement. The direct pathway inhibits the Gpi and SNr with the purpose of deactivating the constant inhibition that it provides. By inhibiting the inhibition, the direct pathway allows for the initiation of movement. The indirect pathway sends excitatory signals to the Gpi and SNr which magnify the inhibition that the Gpi and SNr already enforce on the VA/VL nucleus[13]. This results in further inhibition of movement.

Figure 3. The basal ganglia has two main pathways, the direct pathway and the indirect pathway. The excitatory signals (white arrows) and inhibitory signals (black arrows) are organized in a unique way for there to be an efficient pathway for movement initiation through the disinhibition of the thalamus through the direct pathway. Adapted from Bunner and Rebec (2016) [12].
Mitochondrial dysfunction in many diseased states leads to the release of highly reactive free radicals and reactive oxygen species (ROS) into the cytoplasm. These free radicals and ROS tend to react with proteins and have the ability to change a protein’s primary, secondary, and tertiary structures, which can lead to widespread protein misfolding and aggregation or early proteolysis [14]. These free radicals and ROS pose no severe threat on their own due to the fact that cells possess cellular mechanisms to deactivate and dispose of ROS and free radicals with relative ease. Proteins such as superoxide dismutase (SOD) react with ROS and convert them into relatively harmless compounds. The cell is only put under oxidative stress (OS) if the cell’s internal anti-oxidant mechanisms are unable to reduce ROS concentration. This can either be due to an unusually high concentration of ROS and free radicals in the cytoplasm or if a cell’s protective mechanisms are degraded or are not functioning properly. In both scenarios, ROS will ultimately interfere with the normal functioning of proteins, thus impeding the proper functioning of the cell.

Lewy bodies and α-synuclein aggregates have been long suspected to form as a result of OS in the SNpc dopaminergic neurons, however, the theory seemed unlikely due to the selective nature of neuronal loss in PD. This selectivity can most likely be explained through the finding that baseline ROS concentration in the SNpc is almost twice as much as other basal ganglia components, thus making the SNpc much more susceptible to OS than other structures [16].

It is widely accepted that OS plays a role in the progression of PD, but it is unclear if it is the direct cause or a later consequence of a larger cascade of molecular events. Regardless of the magnitude of the role that OS plays in PD progression, the presence of ROS, such as superoxide (O2•−), has been shown to lead to the formation of Lewy bodies and α-synuclein aggregates. Primate
and mice models of PD have been induced with the use of pharmacological agents, such as MPTP and paraquat, to induce mitochondrial dysfunction, which leads to the production of free radicals [17][18]. These mitochondrial complex 1 inhibitors effectively recreate the conditions seen in real PD patients due to the fact that in post-mortem analysis there is proof that patients had up to a 30% reduction of complex 1 activity and widespread oxidative damage [19][20].

Ultimately high levels of OS can trigger the activation of caspase 3 expression, which leads to apoptosis, programmed cell death.

**Nitrative Toxicity**

Mitochondrial dysfunction itself is solely responsible for the creation of more ROS, but not for cell death. The most common ROS created is superoxide, which is a byproduct of the electron transport chain in the mitochondria, but it is not toxic to the cell in most cases due to the presence of antioxidant proteins such as SOD and glutathione (GSH) [21][22]. However, when superoxide reacts with nitric oxide (NO), a common retrograde neurotransmitter, it forms a compound known as peroxynitrite (ONOO'). Peroxynitrite is a highly toxic reactive nitrogen species (RNS) and can cause lipid peroxidation and protein nitration. Along with peroxynitrite, nitrogen dioxide (NO$_2$) is also a powerful nitrative species[23].

In PD, ONOO' and NO$_2$ in SNpc neurons have been suspected of causing widespread protein nitration and oxidative damage. Both have been shown to nitrate the tyrosine residues on proteins and free tyrosine in the cell. This, in turn, reduces their enzymatic activity, which could have widespread effects on countless molecular pathways. Nitrated tyrosine residues are known as nitrotyrosine molecules and are able to be stained for in PD models with LBs. It has been noted that nitrotyrosines are heavily present in the center of LBs which may mean that they are crucial in the initial formation of these LBs[24]. One protein that is particularly susceptible to tyrosine nitration is tyrosine hydroxylase (TH): a crucial enzyme in the formation of dopamine in SNpc dopaminergic neurons. Although this is the case, dopamine has been shown to prevent TH nitration outside the cellular environment. However, dopamine does not prevent a reduction in TH activity [25]. This is due to the fact that dopamine becomes the main target for oxidative damage by the nitrative species and oxidatively damaged dopamine molecules have been shown to decrease TH activity through cysteine modifications [26]. Thus eventually, there is a depletion of dopamine through the combined factors a reduction in the rate of dopamine formation and the constant oxidative damage to existing dopamine molecules. After this depletion, nitrative species are able to nitrate the tyrosine residues on TH molecules and form nitrotyrosine.

In addition to this cascade, SOD has been shown to paradoxically catalyze the reaction between ONOO' and tyrosine residues to form nitrotyrosine [27]. Both this catalyzation and depletion of dopamine protection can lead to widespread tyrosine nitration and reduced TH activity and this reduces dopamine production. This reaction leaves SOD damaged and reduces its ability to dismutate O$_2^-$ into H$_2$O$_2$[28].
Another factor that may lead to increased peroxynitrite concentration and toxicity is low uric acid levels. PD patients have decreased uric acid levels compared to controls. Uric acid is a powerful peroxynitrite scavenger and, thus, reduces peroxynitrite concentration. A reduction of uric acid in the body can lead to the unrestrained build up of peroxynitrite in cells [29].

![Figure 5. The interactions between RNS, TH, and dopamine leads to a robust chain reaction that results in a reduction of dopamine production due to the nitration of tyrosine hydroxylase. Uric acid has been seen to scavenge for peroxynitrite, but PD patients have abnormally low levels of it.](image)

The Role of NO and radical - radical recombination

Although NO is crucial for peroxynitrite formation, excess nitric oxide (NO) is able to prevent the generation of excess nitrative radicals through radical - radical recombination reactions. NO is able to combine with both peroxynitrite (ONOO⁻) and nitrogen dioxide (NO₂) to form nitrogen trioxide (N₂O₃) which is a much less nitrative compound [30][31]. This validates another finding: PD patients have been shown to have a deficiency of tetrahydrobiopterin. A deficiency in this compound has the effects of increasing superoxide formation and decreasing NO formation from nitric oxide synthase, the enzyme that forms NO [32].

α-synuclein Aggregation and Lewy Body Formation as a Neuroprotective Process

α-synuclein aggregate formation is a key feature in all PD cases and one of the hallmark markers of the disease. Although there is a strong general correlation between aggregate formation and cell death, it has not been formally concluded that the aggregates are a direct cause of cell death. Studies have shown that α-synuclein, in its normal form, may have a neuroprotective role and has
been shown to protect neurons against OS-induced apoptosis [33]. The mechanisms and reasons for α-synuclein aggregation are largely unknown.

The specific presence of dopamine has been shown to increase the sensitivity of a cell to form α-synuclein plaques. The in vitro expression of excess α-synuclein in dopaminergic cells leads to quick apoptosis but no formation of α-synuclein plaques. In contrast, the same expression of α-synuclein in non-dopaminergic HCN cells had no signs of neurotoxicity or plaque formation [34]. This is most likely due to a lack of oxidatively damaged dopamine molecules in the HCN cells. The neurotoxicity seen in the dopaminergic cells were seen to be in response to soluble damaged α-synuclein products. The damaged α-synuclein did not form plaques but still caused apoptosis. Another study showed that the addition of normal dopamine to α-synuclein resulted in the inhibition of insoluble fibril formation, but resulted in toxic soluble protofibril accumulation. When antioxidants were added, the effects were relieved which suggests that dopamine oxidation was responsible [35]. In addition to this, post-mortem analyses of PD patients have found that cells with more plaques more often survive in comparison to cells that do not form plaques [36]. This supports the idea that plaque formation is actually a neuroprotective mechanism designed to sequester neurotoxic soluble α-synuclein products.

Lipid peroxidation of polyunsaturated fats in the cell, a common effect of high levels of ROS and nitrative species, has also been shown to decrease oligomer and aggregate formation of toxic damaged α-synuclein. 4-hydroxy-2-nonenal, a product of lipid peroxidation, has been shown to prevent α-synuclein fibril formation and support toxic soluble α-synuclein [37][38].

Selective Apoptosis Through the Activation of the JNK pathway

Studies have shown that both patients with PD and animal models of PD have phosphorylated, and thus activated, Jun N-terminal Kinase (JNK) pathway proteins [39]. The JNK pathway, which is mediated by many factors in the cell, is responsible for many functions related to survival signaling and apoptosis. JNK-mediated apoptosis is carried out through the activation of the pro-apoptotic p53 protein which in turn activates caspase 3 proteins [40][41]. These caspase 3 proteins initiate apoptosis in any given cell. P53 knockout mice were shown not to have any neurotoxicity after being treated with MPTP, a common compound used to simulate PD, suggesting that the JNK pathway plays a major role in PD neurotoxicity [42].

The JNK apoptotic pathway is activated by a variety of cellular stressors, but in the context of PD, it has been shown to initiate apoptosis in the presence of excess ROS and oxidative damage to a cell [43]. Dopaminergic neurons in the SNpc seem to be at heightened risk of activation of the JNK pathway due to their baseline higher oxidative load than other dopaminergic neurons. In addition, oxidatively damaged dopamine compounds also show a possibility of directly influencing the activation of this pathway, but even more than this, its effects on α-synuclein show a greater role. Many studies have shown that α-synuclein plays a neuroprotective role not only against ROS, but also specifically in the context of the JNK pathway [44]. Thus, the dopamine-mediated formation of α-synuclein protofibrils not only decreases the neuroprotection that α-synuclein was once giving but also generates the toxic protofibrils. Not only do they pose risk through influencing the JNK
pathway directly, but the generation of these protofibrils have been seen in correlation with Golgi fragmentation, which is an early stage of cell apoptosis and degeneration [45].

The Role of Chaperonins in the Pathogenesis of Parkinson’s Disease

Chaperonins are a class of proteins that catalyze the process of correctly folding a protein after translation or even after denaturation. The heat shock group of proteins have been seen to play a major role in the progression of neurotoxicity in PD models and have been seen in high concentrations in the SNpc cells of PD patients. A drosophila study of the effects of heat shock protein 70 (HSP70) on PD models observed that coexpression of HSP70 and α-synuclein lead to a decrease in neurotoxicity caused by the α-synuclein expression. Although this was the case, there was not a noticeable decrease in fibril and aggregate formation, but on the other hand, there was evidence that HSP70 became parts of the aggregate itself [46].

Other studies have shown that high concentrations of in vitro HSP70 concentrations have even been able to ameliorate α-synuclein aggregation. This amelioration of aggregate formation, although seen to be unnecessary for a reduction in neurotoxicity, has been seen to be a result of the preferential binding of HSP70 to prefibrillar protofibrils. In a controlled in vitro environment without proteasomes or lysosomes of any kind, these protofibrils stayed as soluble compounds and failed to form insoluble fibrils but paradoxically stayed cytotoxic [47]. Similar studies in vivo have shown, conversely, complete amelioration of the neurotoxicity associated with α-synuclein which leads to the suggestion that HSP70 is not solely responsible for the reduced neurotoxicity [48]. This leads to the suggestion that HSP70 allows other cellular mechanisms, such as proteasome systems, to process and destroy toxic α-synuclein protofibrils.

HSP70 and HSP60 have also been shown to reduce RNS concentration, specifically peroxynitrite, in brain stem cells in the presence of a stressor. The chaperonins regulate NO production by interacting with NOS I and NOS II, the main producers of NO, to reduce NO production in the cells. The study showed that HSP70 and HSP60 were able to depress an OS mediated mitochondrial apoptotic cascade in brain stem cells through this decrease of NO concentration, which in turn reduced ONOO− concentrations which ultimately reduced OS [49]. Another study also showed that HSP70 was able to inhibit cell death by inhibiting the JNK pathway and eventual caspase-3 activation by reducing OS [50].

A Potential Pathway Describing the Molecular Pathogenesis of Parkinson’s Disease

All of these factors can be taken together to generate a potential molecular pathway to be used for further research to investigate the intricacies in the molecular pathway that governs PD pathogenesis. Although the initial stimulus for disease onset is unknown, the molecular cascade that occurs after it can be studied and understood with the eventual goal for better therapeutic targets. The pathway below contains all of the molecular mechanisms discussed in the article and proposes a partial pathway responsible for selective dopaminergic loss in the SNpc, α-synuclein aggregation, and various sources of neurotoxicity in PD.
Figure 6. A potential pathway for the molecular pathogenesis of Parkinson's Disease. Tetrahydrobiopterin and Uric Acid are marked as decreased with the red line next to them. The effects of both compounds have been marked as if they were in normal quantities, so their role in the pathogenesis of the PD is opposite to the effects shown because they are decreased.
Conclusion

PD is a highly complex disease with many different environmental and genetic factors that may go into its initiation in the human brain. Its selectivity to dopaminergic neurons in the SNpc makes it a very intriguing disease from a molecular pathology perspective. The limited ability to study the molecular mechanisms behind PD pathogenesis in human patients has been a major bottleneck in PD research but has been worked around through the generation of a variety of reliable animal models. Drosophila and mouse models have made it possible to study PD on a much more specific level and will eventually allow for a very detailed outline of the molecular interactions that ultimately lead to dopaminergic cell death seen to cause Parkinsonian symptoms.

References


Efficacy of Plant Extracts in Alzheimer’s Disease Using Transgenic C. elegans

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Abstract

Alzheimer’s disease (AD) is an irreversible progressive neurodegenerative disease that slowly destroys the human memory. There is an unfulfilled need to develop new treatments for AD, as the existing treatments only offer symptomatic benefits. In this experiment, the effect of four plant extracts: turmeric, ashwagandha, bitter melon, and gotu kola were tested using transgenic models of the nematode Caenorhabditis elegans (C. elegans). Specifically, CL4176 and CL2006 strains that express the Amyloid-β peptide in their muscles and the CL2355 strain that has pan-neuronal expression were used. The CL4176 and CL2006 strains were also made cuticle-deficient by crossing them over with DC19 bus-5 worms for better absorption of the compounds. The effects of these herbs were assessed in CL2355 using a chemotaxis assay and a starvation assay to test associative learning. A paralysis assay was performed in CL4176 to compare the efficacy of these extracts. In addition, CL2006 worms were observed under a fluorescent microscope to quantify the amount of amyloid-beta aggregation in the body wall muscles of these worms. For all the assays, ginkgo biloba extract was used as a positive control, and the results were also compared with wild-type N2 worms. In all the assays conducted, turmeric and ashwagandha performed superior to ginkgo and achieved statistically significant results (N = 50, p-value < 0.05) over bitter melon, gotu kola, and untreated worms. Further studies into the mechanism of action, safety, and efficacy studies of these compounds in higher organisms could translate these into viable human treatments for AD.

Research

Alzheimer’s disease is a chronic neurodegenerative disorder and is the most common cause of dementia among older adults. Microscopic examination of the brain tissues from AD patients show intracellular neurofibrillary tangles of tau protein and extracellular amyloid-beta plaques [1]. The amyloid hypothesis states that amyloid-beta accumulation starts a series of downstream events exacerbating oxidative stress and damage to the mitochondria, thus leading to neuronal dysfunction
and cell death. Oxidative damage and neuroinflammation are also found to play a role in the pathology of AD.

C. elegans is a transparent, 1-mm-long roundworm. With its rapid reproduction of offspring and a short life span of 23 days, C. elegans is a good model to study age-related diseases. Strains CL4176 and CL2006 express amyloid-beta peptides in their muscles. CL2355 has pan-neuronal expression of amyloid-beta protein.

Turmeric is known to have antioxidant and anti-inflammatory properties, and both characteristics can slow the progression of AD [10]. Its anti-inflammatory effects lead to the inhibition of cyclooxygenase-2 enzymes (COX2), thus reducing the chronic inflammation that occurs in AD. Curcumin, the active ingredient in turmeric, is also known to decrease the formation of free radicals and increase the activity of superoxide dismutase, an enzyme that helps remove harmful free radicals from accumulating in cells [9]. Ashwagandha has been previously found to have neuroprotective effects through its ability to increase cholinergic activity and stimulate dendrite formation [10]. Gotu kola is thought to improve attention span and increase cognitive function as shown through studies in rats. Gotu kola exerts neuroprotective effects by potentiating naturally occurring enzymes that work as antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase [6]. Bitter melon is highly researched as having hypoglycemic properties but has also been found to protect against oxidative stress [8]. Due to the various attributes of these natural substances as investigated by previous studies, they were proposed as ways to alleviate symptoms of AD.

Methods

Crossover: CL4176 and CL2006 worms were crossed with DC19 worms to create new strains of cuticle-deficient C. elegans. A PCR/gel electrophoresis was performed to check for the presence of the mutation.

Plate Preparation: Plant extracts were diluted to a concentration of 0.5 mg/ml and pipetted onto NGM agar plates. A OP50 E. coli liquid culture was pipetted onto the plates, and the worms were grown on these plates. [2]

Paralysis Assay: The expression of amyloid-beta in CL4176 (smg-1dvls2 - bus-5) depends on a temperature upshift from 15 °C to 25 °C. CL4176 was maintained at 15 °C on plates containing vehicle or drug. Then, the worms were egg-synchronized and were allowed to grow for 18 hours at 15 °C (n = 50). After 18 hours, the temperature was upshifted to 25 °C to induce amyloid-beta expression. Paralysis was scored at 1-hour intervals until all worms were paralyzed. A worm was considered paralyzed when it did not respond to a gentle touch from a platinum wire or if the worm was only able to move its head.

Fluorescent Staining: CL2006 (dvls27 - bus-5) worms were egg-synchronized and plated onto seeded plates containing either drug or vehicle and propagated at 15 °C for 72 hours. L4 stage worms
were transferred to NGM plates with extract or vehicle and incubated at 25 °C for 48 hours. The worms are then incubated in Methoxy X04 dye solution (1 mM) for 4 hours. They are allowed to destain for 12 hours on seeded NGM plates with no vehicle or drug, picked onto agar pads with sodium azide to be mounted, and observed under a fluorescent microscope. The mean numbers of amyloid-beta staining per head area were quantified using ImageJ software. [5]

Benzaldehyde Chemotaxis Assay: CL2355 worms that exhibit pan-neuronal expression of amyloid-beta protein has an impaired chemotaxis response. Age-synchronized L4 worms were cultured on plates containing vehicle or drug and pipetted into M9 buffer. The solution of worms was centrifuged and aspirated for a total of four times. Benzaldehyde, a well known chemoattractant, was used to test the worms’ chemosensation abilities. Benzaldehyde and water were placed into the appropriate quadrants of the plate, and the worm solution was pipetted in the center. A chemotaxis index was calculated for the worms. [2]

NaCl Associative Learning Assay [11]: Age-synchronized CL2355 worms in the L4 stage were cultured on NGM plates with extracts or vehicle then pipetted into M9 buffer. The worms were centrifuged, the supernatant was pipetted out, and the worms were placed into an unseeded (no food source) NGM plate containing NaCl and allowed to crawl for four hours. In the naive chemotaxis assay performed prior to this assay, it was found that NaCl by itself was neither an attractant nor a repellant. However, when NaCl is associated with starvation, N2 worms tend to be repelled by it. A chemotaxis assay was performed (as described for benzaldehyde) to test associative learning.

Results

For all graphs shown, standard error bars were calculated using a 95% confidence interval.
Paralysis Assay: Turmeric- and ashwagandha-treated worms had the most delayed onset of paralysis compared to other treatments, indicating that they were the most successful in this assay.

Benzaldehyde Chemotaxis Assay: All treatments showed a significantly stronger chemoattractant response. Turmeric- and ashwagandha-treated worms had a chemotaxis index that was significantly higher than all other treatments.

NaCl Associative Learning Assay: All treatments showed a significantly stronger associative learning response. Turmeric- and ashwagandha-treated worms had a chemotaxis index that was significantly higher than all other treatments.
NaCl Associative Learning Assay: Standard error bars as well as p-values with an alpha of 0.05 were used to establish significance in this assay. All treatments led to significant improvement in associative learning. Turmeric and ashwagandha performed superior, although they were not significantly better than all other treatments. Both ashwagandha- and turmeric-treated worms were not significantly different from N2 worms, showing how they were best able to restore the associative learning response.

Fluorescent Microscopy Assay: All treatments significantly decreased the number of plaques in the head muscle of the worms. Turmeric and ashwagandha were the most effective extracts, as they had significantly fewer plaques than worms grown on other extracts.

Conclusion

Among all the extracts tested, turmeric and ashwagandha performed superior across all assays. This further warrants a possibility for these extracts to be tested in higher organisms for developing viable human treatments.

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References


Modified B. subtilis Strain to Replace Levodopa Medication for Parkinson’s

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Abstract
This paper proposes a medication which would safely and effectively replace levodopa (LD) treatment for patients with Parkinson’s disease (PD), thus eliminating levodopa-induced dyskinesia (LID) in the progression of the disease. At present, the most effective treatment for the motor symptoms of PD uses LD combined with carbidopa (CD) to prevent the breakdown of LD in the gut and avoid the associated nausea. CDLD, however, is taken in periodic doses, for example 25 mg three times a day, causing spikes in dopamine levels and thus damaging dopamine receptors. This inability to accurately read dopamine levels causes hyperkinetic movements, otherwise known as LID. Thus, the continued use of CDLD is directly related to the development of LID. This paper identifies the most effective alternative to CDLD as a continual source of dopamine in the brain. The proposed solution is oral ingestion of dopamine-producing B. subtilis bacteria, with the norepinephrine-producing genome removed, combined with the NanA surface protein from pneumococcus to allow transgression of the blood-brain barrier (BBB). B. Subtilis is nonpathogenic and takes on the endospore morphology under stressful conditions, including the gut, and would thus eliminate the need for carbidopa. The only potential complication involves hypoglycemia, as B. subtilis requires glucose to survive. In rare cases, the bacteria may cause low glucose levels, which can be remedied with glucagon injections. The proposed medication would eliminate the threat of LID by removing intermittent dopamine spikes, as well as the need for CD. Patients would only need one medication, administered once.

Current Treatment for Parkinson’s Disease

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Bradykinesia, rigidity, and other motor problems in patients with Parkinson's disease (PD) is presently treated with levodopa (LD), usually combined with carbidopa (CD) to prevent decarboxylase enzymes from decomposing LD in the stomach and inducing nausea [1]. However, CDLD is a temporary fix which tends to treat motor issues only in the early stages of Parkinson's [2] and, as the disease progresses and use of CDLD continues, patients develop new movement disorders that are a direct result of prolonged CDLD use.

These side effects, most notably levodopa-induced dyskinesia (LID), are a result of CDLD being taken in periodic, controlled-release doses every few hours [3]. The peaks and troughs in dopamine levels that this produces causes complications for PD patients because the pulsatile stimulation of dopamine damages corresponding dopamine receptors [4]. Patients develop LID, or uncontrollable movements, when these damaged receptors periodically perceive “peak doses” of dopamine and later have difficulty reading the amount of dopamine actually present in the brain [5], resulting in uncontrollable movement. While extended-release levodopa is prescribed to lengthen the effect of one dose, it still produces periodic highs and lows, just less frequently, meaning LID still poses a threat to patients, simply later in the disease’s progression.

The goal of this paper is to propose a theoretical treatment which would safely and continually produce dopamine in the brain and thus eliminate the periodic stimulation of dopamine receptors currently experienced under CDLD treatment, effectively eliminating the threat of LID in Parkinson’s patients.

**Development of Theoretical Treatment**

Initially, one might seek to remedy the side effects of CDLD with further, counter-balancing medication. Neurologists could prescribe the glutamate inhibitor amantadine [23], MAOB inhibitors which prevent breakdown of LD by MAOB enzymes, and COMT inhibitors which inactivate dopamine so it will remain in the brain longer [6]. However, to do so is adding more medication to a treatment which will always incite this pulsatile stimulation, meaning the peaks and troughs will always be there, only less often given different medication. No matter what patients use to extend the presence of dopamine in the brain, there will still exist a “pendulum”, where neurologists try to swing from no movement to normal movement, and potentially overshoot to have patients experience too much movement under LID [6].

With the root of the problem being intermittent spikes of dopamine, the most feasible approach seems to lie in producing the exact opposite: a constant source of dopamine. A living organism which ceaselessly releases dopamine is, in this paper, theorized to be a solution to the peaks and troughs in dopamine levels that Parkinson’s patients experience in taking CDLD and which ultimately leads to LID.

Strains of the *bacillus* bacteria have been studied and are known to produce norepinephrine and dopamine [7]. With the norepinephrine-producing genome removed, this bacteria could be the
constant source of dopamine needed. Additionally, the problem of LD being broken down in the gut by DOPA decarboxylase [8] and tyrosine decarboxylase [9], currently combated with CD which defends levodopa long enough for it to reach the brain, will be remedied by the ability of bacillus bacteria to adopt the endospore morphology, by which stressful environmental conditions cause the bacteria to take on the most durable cell type in nature and become capable of withstanding the ravaging conditions of the gut [10]. Using bacillus bacteria would no longer pose the threat of nausea, as bacillus in the gut is both in the endospore form and biologically inactive, therefore not producing the dopamine that is to instead be produced in the brain [11].

Figure #1. Scanning electron microscopic image of B. subtilis 168 [22]

Bacillus has a reputation for being the cause of food poisoning, but this is specifically the B. cereus strain [12]. It happens to be the case that another strain of bacillus, B. subtilis, is renowned for being a non-pathogenic [13], benign organism [11] and is currently used in other medical studies. B. subtilis has its own reputation for being one of the safest strains of bacteria for testing in the human body [14], and thus B. subtilis was selected as the strain of bacteria to be used in this study.

Thus far, it is understood that the proposed microorganism would consist of B. subtilis with the norepinephrine-producing genome removed. It had been established that, under the conditions of the gut, B. subtilis would exist in the endospore morphology, meaning it would not produce dopamine for decarboxylase enzymes to break down, eliminating the threat of nausea in patients and the need for additional medications like carbidopa. The B. subtilis would be absorbed into the bloodstream and transported to the brain, and here it must cross the blood-brain barrier (BBB).

Dopamine alone can not cross the BBB [15], this is a function only of levodopa [16], thus an entire bacteria would not be able to cross the same structure meant primarily to keep pathogenic bacteria out. The solution lies in the bacteria Streptococcus pneumoniae, better known as pneumococcus, which has a surface protein called NanA that allows the bacteria to transgress the BBB and cause bacterial meningitis [17]. With this NanA protein removed from the surface of pneumococcus and combined with the modified B. subtilis, it would be possible for the dopamine-producing bacteria to cross into the brain.
The concern might be raised that bacteria in the brain ought to be detrimental to the patient. A recent study has established the presence of bacteria entering and residing in the brain, including the substantia nigra which lacks dopamine under PD, without inflammation or damage to structures [18]. The study additionally supports the presence of gut bacteria in the brain. In this token, the modified *B. subtilis*, which is already a non-pathogenic and benign bacteria, would pose no pathological threat to the brain.

**Potential Issues**

Possible complications do exist in the bacteria’s means of staying alive long enough to divide and continue producing dopamine. While research has shown that *B. subtilis* will adapt to the gastrointestinal tract (GIT) as its natural habitat and easily germinate and sporulate [26], in the brain *B. subtilis* will require glucose to continue reproduction [19]. The associated low glucose levels in the brain is called hypoglycemia and can, in very rare and unlikely situations, cause functional brain failure [20]. Should patients develop hypoglycemia during use of the *B. subtilis* medication, their condition can be remedied by the well-established use of glucagon injections into the bloodstream to raise plasma glucose levels [21]. In any case, if a patient is at risk of developing hypoglycemia, perhaps from already low glucose levels in the body, the patient should be prescribed glucagon injections or be considered a poor candidate for the *B. subtilis* medication and explore different treatment such as carbidopa-levodopa, dopamine agonists, or deep-brain stimulation (DBS) surgery which has proven to be extremely effective in addressing symptoms of Parkinson’s.

The concern may also be raised that the immune response to the introduction of *B. subtilis* bacteria could reflect that of bacterial infections such as salmonella, pneumonia, or tuberculosis. Past and ongoing studies have established that *B. subtilis* is not only safe to use as food supplements and probiotics [25], but additionally improves such bodily functions as intestinal homeostasis, natural growth process, immune response and the body’s ability to resist disease [24]. As a matter of necessity, it has also been discovered that *B. subtilis* plays a central role in the healthy development of gut-associated lymphoid tissue (GALT) and the buildup of necessary antibody repertoires [26]. Thus, research into the effects of *B. subtilis* ingestion has established the contrary to that which might be assumed of the effect of *B. subtilis* bacteria in the body. The bacteria is not simply safe to ingest, but additionally boosts and fortifies a variety of necessary bodily functions such as development and immune response.

**Conclusion**

After thorough and extensive research to find the safest and most effective solution to LID in Parkinson’s patients, the proposed treatment is composed as follows. The medication would include *Bacillus subtilis* (with the norepinephrine-producing genome removed) combined with the NanA surface protein from meningitis-inducing pneumococcus (which will allow *B. subtilis* to cross the blood-brain barrier) ingested once, in a form similar to that of probiotic pills. The new microorganism will endure the conditions of the gut while in the endospore state, be absorbed into
the bloodstream, travel to the brain, cross the BBB with the help of NanA, and travel to the substantia nigra where it will safely and constantly produce dopamine. Should physicians evaluate a potential user of this treatment and find that the patient is at risk of hypoglycemia from low levels of glucose, the patient should either be allowed glucagon injections or be advised against taking the new medication and instead consider CDLD, dopamine agonists, or DBS. Introduction of the modified bacteria will not trigger negative immune responses as *B. subtilis* plays an inherent role in certain bodily functions such as gut tissue development and additionally enhances others functions in regards to growth and immune strength.

While there is no literature that states explicitly why such an approach to Parkinson’s treatment has not been taken, it can be inferred that current treatment (primarily carbidopa-levodopa) does a good enough job for now. In enough cases, carbidopa-levodopa will ease the symptoms of Parkinson’s disease, and ongoing research simply strives to improve old medication and develop new medication that counteracts the dyskinesia that arises as a side effect of the treatment. This paper proposes a solution which will go farther back in the disease’s progression and, rather than applying short-term remedies to the problem, address Parkinson’s at its source (a lack of dopamine in the brain). The goal is to keep patients from embarking on an endless cycle of pill-taking and instead “nib it in the bud” such that it is impossible for side effects to begin, because there will be no medication taken from which side effects can manifest.

References


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