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Improvement of Depressive-like Behaviors in Hemi-parkinsonian Rats with Non-invasive Peripheral Focused Ultrasound Modulation

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Improvement of Depressive-like Behaviors in Hemi-Parkinsonian Rats with Non-Invasive Peripheral Focused Ultrasound Modulation

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Abstract

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disease with marked loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). In PD, there are motor and non-motor symptoms. Depression, a non-motor symptom, is seen in 40% of the patients with PD which decreases their quality of life. For treatment-resistant depression, vagus nerve stimulation (VNS) has been shown to improve depression. Here, non-invasive focused ultrasound (FUS) is investigated as a therapeutic to improve depression-like behavior by targeting the celiac plexus since it is innervated by the vagus nerve. FUS was chosen due to its parallel mechanism to VNS. Male Sprague Dawley rats were used and made hemi-Parkinsonian ("PD") with craniotomy surgery which delivered unilateral six-hydroxydopamine (6-OHDA) in the brain to lesion dopaminergic neurons in the right medial forebrain bundle (MFB). Sham (n=23) and Hemi-Parkinsonian (n=40) were evaluated using LATs to determine their forepaw movement. They underwent a sucrose preference test (SPT) to assess the rats' level of anhedonia, or their inability to experience pleasure, which is one behavioral test that evaluates depression-like behavior in rats. FUS was delivered to the rats 21 days post-lesion for five consecutive days. Post-mortem analysis was performed using TH analysis. It was shown that hemi-Parkinsonian rats displayed akinesia in their left forepaw and a >90% reduction in dopaminergic neurons on the lesion side. It was found that hemi-Parkinsonian rats exhibited anhedonia which was improved with FUS treatment. The TH analysis displayed no statistical difference between the different treatment groups. In all, FUS improved anhedonia in hemi-Parkinsonian and sham rats which may be a credible therapeutic for future patients. While further research must be done, FUS may be viable therapeutics for patients with PD experiencing depression.

Acknowledgments

I would like to acknowledge and thank my research advisor, Damian Shin, for granting me the opportunity to participate in his research lab. I would like to thank my mentor, Rachael Herlihy who is a PHD/MD student at Albany Medical College. Her patience and guidance allowed me to grow as a student and researcher. Additionally, I want to thank my family and friends who have also been supportive through my academic journey.

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1. PARKINSON'S DISEASE

1.1 Background and Epidemiology

Parkinson's disease (PD) is the second most common neurodegenerative disease more commonly present in the elderly population (Orayj et al., 2021). PD was named after James Parkinson who discovered this disease which he described as "the shaking Palsy" when he clinically observed tremors, abnormal gait, and a flexed posture. Jean-Martin Charcot suggested this disease be named Parkinson's disease years later and named additional symptoms as criteria for this disease (Marsili et al., 2018). PD impacts over six million individuals globally and is increasing over time (Tolosa et al., 2022). It is impacting roughly three percent of the world's population of those who are 65 years old and approximately five percent of individuals above the age of 85 (Cerri et al., 2019).

While it is mostly seen in the elderly population, PD may present itself in the younger population. Early-onset PD, while it is rare and consists of roughly 3-5% of PD cases, occurs before the age of 40 years old. (Radhakrishnan and Goyal, 2018). It is two times as common in the male's population than the female. These sex differences may be attributed to estrogen which is neuroprotective. A rare form of PD, familial, may be inherited due to genetic mutation. If one has an early onset, it is more likely that the patient has a genetic basis for this disease (Radhakrishnan and Goyal, 2018). Familiar PD may be due to the mutation located in the leucine-rich repeat kinase two (LRRK2), concentrated in the striatum (Iannotta et al., 2020). Furthermore, although PD may be treated, it is a progressive and chronic disease (Abyad and Himmami, 2020). PD is characterized by a loss of dopaminergic neurons which release dopamine throughout the central nervous system. Dopamine is a neurotransmitter that is synthesized from tyrosine by tyrosine hydroxylase and then converted to levodopa by DOPA decarboxylase. Dopamine plays a pivotal role in the nervous

system such as its role in movement and emotion in the central nervous systems along with the immune, renal and digestive system (Haddad et al., 2017).

1.2 Mechanism

Neurodegeneration, of dopaminergic neurons, is seen in the substantia nigra pars compacta (SNc) which project to other crucial regions in the brain; these projections lead to the basal ganglia which follow through to the corpus striatum which is responsible for vital functions. While the dopaminergic system is a vital player in PD, studies have acknowledged the influence of the noradrenergic, cholinergic, and serotonergic systems (Hirano, 2021). Additionally, the presence of Lewy bodies in remaining dopaminergic neurons is thought to contribute to the pathology of this disease (Cerri et al., 2019). There are numerous factors that lead to the onset of PD, including environmental and genetic factors (Abyad, 2020).

1.3 Symptoms

There are motor and non-motor symptoms that must be differentiated. Motor symptoms may appear with 60 to 80 percent of dopaminergic neurons lost (Abyad, 2020). Motor symptoms are largely due to the loss of dopaminergic neurons. The motor circuit in the brain is altered due to dopaminergic loss in the striatum. The motor circuits consist of a direct and an indirect pathway which are controlled by dopamine; the direct pathway consists of neuronal connection in the globus pallidus internus (Gpi) and the substantia nigra pars reticulata involving GABAergic neurons and dopamine D1 receptor. The indirect pathway consists of neurons, which contain D2 receptors that project to the globus pallidus externus (Gpe) and the Gpi through the subthalamic nucleus (Radhakrishnan and Goyal, 2018). In patients with PD, these pathways are interrupted due to the loss of striatal dopaminergic neurons which regulate the GABAergic neurons in the basal ganglia. This results in a decrease in D1 receptor-mediated signaling in the direct circuit and increased D2 receptor-mediated signaling in the indirect circuit thereby subsequently increasing

GABAergic inhibition of the motor cortex (Radhakrishnan and Goyal, 2018). Common motor systems include slowed movement (bradykinesia), or loss of movement (akinesia) initiation, abnormal gait, repetitive movement (dystonia), tremors, and muscle rigidity. Tremors in PD are due to the alterations in the interaction between the basal ganglia and cerebellum. The abnormal gait and balance are due to the impairments of the outputs from the basal ganglia which project to the locomotor area in the midbrain (Radhakrishnan and Goyal, 2018).

While many associate motor symptoms with this disease, numerous non-motor symptoms manifest in patients with PD (Abyad, 2020). Roughly 90% of patients have non-motor symptoms that do not diminish with dopamine treatment. The non-motor symptoms may originate prior to motor symptoms by years. Numerous non-motor symptoms may be due to impairment of the brain-gut-microbiota axis (Radhakrishnan and Goyal, 2018). Some non-motor symptoms are due to neuronal loss external to the SNc along with the disruption of varying neurotransmitters (Abyad, 2020). Non-motor symptoms may be compromised by gastrointestinal issues, difficulty swallowing (dysphagia), cognitive impairment, anxiety, sleep abnormality, and depression. Additionally, research has shown that males with PD have a greater risk for developing dementia (Hirano, 2021). In all, there are numerous motor and non-motor symptoms that are clinically observed with PD.

1.4 Diagnosis

For diagnosing PD, it relies on clinical observation focusing on motor and non-motor features. Unfortunately, there is no singular test that can confirm a PD diagnosis (Jankovic, 2008). Since it is only confirmed neuropathically, there are numerous guidelines for physicians in aiding in diagnosing patients (Marsili et al., 2018). A large aspect of the diagnostic process, a physical exam is necessary to evaluate the clinical presentation that coincides with PD with observed

responsiveness of Levodopa administration and improved motor function. PD is clinically presented by the occurrence of bradykinesia along with other demonstrations such as tremors, abnormal gait, or muscular rigidity. The motor symptoms would appear to manifest asymmetrically and unilaterally (Radhakrishnan and Goyal, 2018). Research should continue to be done to further the knowledge of potential biomarkers for PD (Jankovic, 2008). Identifying specific biomarkers can aid in the diagnosis process preventing misdiagnosis.

1.5 Treatment

The American Academy of Neurology advises that once a functional disability appears that pharmacological treatment should begin (Radhakrishnan and Goyal, 2018). The conventional treatment for PD is generating dopamine pharmacologically via dopamine agonists. (Church, 2021). Levodopa, dopamine agonist, and monoamine oxidase-B (MAO-B) inhibitors are commonly prescribed to improve one's motor symptoms. Levodopa, taken orally, was introduced as a dopamine replacement therapy and has a similar chemical formula as dopamine. Long-term usage of levodopa can produce dyskinesia (Radhakrishnan and Goyal, 2018). Dopamine agonists, molecules mimicking dopamine and their function by binding dopamine receptors, are used as well such as apomorphine and pramipexole. Other medications, such as MAO-B inhibitors, may be used to preserve dopaminergic release by neurons by preventing the breakdown of dopamine. Additional treatment for motor symptoms may include physical therapy and occupational therapy (Church, 2021). There are other forms of pharmacological treatments that do not involve dopamine due to the lack of effectiveness of dopaminergic treatment later in the disease progression. It is hypothesized that this can be due to other neurological transmitters influencing the disease. Medications responding to acetylcholine deficiency such as rivastigmine, a cholinesterase inhibitor, which is used for dementia (Radhakrishnan and Goyal, 2018). Medication is also used for non-motor symptoms; Benzodiazepines, such as diazepam and clonazepam, may be prescribed

to treat anxiety and decease panic and worry in patients. To treat depression, anti-depressants such as SSRI SNRI's and tricyclic compounds are prescribed (Church, 2021).

2. PARKINSON'S DISEASE: DEPRESSION

2.1 Overview

Depression in PD (DPD) is a common non-motor symptom, often overlooked, impacting up to 40% of those who are diagnosed with PD. Depression impacts one's quality of life. Unfortunately, due to the overlapping symptoms of PD, depression in this population may go undiagnosed (Timmer et al., 2017); the cognitive alterations and physical grievances from depression may be dismissed as symptoms from PD, such as fatigue, loss of energy, and hard of concentration (Laux, 2022). As compared to the general population, depression in patients with PD is five times more prevalent than in the general population (Abyad and Hammami, 2020). Additionally, depression represents itself as clinically different from the general population. Patients with PD are more likely to experience extreme exhaustion, anhedonia, a decrease in energy, increased irritability, and pessimism while it is more likely for the general population to develop feelings of worthlessness (Laux, 2022). Furthermore, anhedonia is prevalent, ranging from 10-40%, and a prime symptom of apathy and depression in patients with PD (Assogna et al., 2020). Notably, depression may occur throughout any stage of PD (Prange et al., 2022). It is also a risk factor to have depression in the early stage of PD for having endured worse motor and overall symptoms. Numerous late-stage complications may occur, such as falls and dementia, that have been associated with depression in those patients (Assogna et al., 2020). Depression in the early stages of PD is a risk factor for the patient to have worse motor symptoms (Prange et al., 2022). There is more research to be done to establish a more comprehensive understanding of depression as a non-motor symptom of PD and to develop a more efficient treatment.

2.2 Pathophysiology of DPD

The pathology of depression in those who have PD is complex and influenced by numerous neurotransmitters and brain regions not limited to dopamine from the SNc (Bang et al., 2021). There are numerous studies to illustrate potential mechanisms responsible for depression in PD. Dysfunction in the mesolimbic pathway, a dopaminergic system that incorporates the ventral striatum and anterior cingulate cortex, may result in depression since it influences motivation and reward (Hirano, 2021). Using a single photon emission computed tomography and PET imaging throughout PD stages highlighted presynaptic terminal dysfunction in the mesolimbic system (Prange et al., 2022). Cognitive impairment coincides with numerous anatomical changes in the brain including the basal ganglia, the cerebellum, the thalamus, and changes in the brain volume (Lotankar et al., 2017). Emotional dysfunction may be attributed to alterations in the limbic system in individuals with PD, which may lead to a decline in grey matter, combined with the loss of dopaminergic neurons (Lotankar et al., 2017). A study by Wen highlighted neuroimaging as a means of further analyzing the pathology of depression in Parkinson's Disease. Most patients whose neural activity was analyzed via PET and SPECT had a decrease in neuronal metabolic activity compared to patients who had PD without depression. This was empathized in the following brain regions: frontal lobe, striatum, sub-cortical or limbic regions region including the amygdala, thalamus, hippocampus, insula, and anterior cingulate cortex (Wen et al., 2016). Moreover, there have been numerous studies that conclude the involvement of neuroinflammation in PD and its probable link to depression as a symptom. A study by Lindqvist and his colleagues in 2013 (as cited in Tran et al., 2021) completed post-mortem analysis regarding inflammatory markers in cerebrospinal fluid in a patient's brain. The presence of inflammatory markers was observed to correlate to the severity of non-motor symptoms in patients with PD, including depression (Tran et al., 2021).

In addition to dopaminergic neurons being altered in PD, serotonergic and noradrenergic neurons are influenced as well. These neurotransmitters impact mood regulation and reward which participate in depression (Marsh, 2013). Due to the correlation between depression and worsening motor impairments, the dopaminergic loss may suggest the involvement of dopaminergic loss as a potential pathology of depression (Prange et al., 2022). A study by Wei et al. in 2019 (as cited in Bang et al., 2021), stated that there is a correlation between mesocorticolimbic dopaminergic dysfunction and the level of severity of depression in the patient. Furthermore, the article by Mayberg and Solomon (as cited in Marsh, 2013) states that neurodegeneration of dopaminergic neurons in the mesocorticolimbic region results in dysfunction in the orbitofrontal cortex in the prefrontal cortex. This disrupts serotonergic neurons located in the dorsal raphe (Marsh, 2013) The serotonergic system comprising the limbic area such as the hippocampus, temporal cortex, and the raphe nuclei are modified in patients with depression and PD (Assogna et al., 2020). Furthermore, a study by Boileau in 2008 (as cited in Assogna et al., 2020), used serotonin transporter radioligand to examine tracer bindings outside the striatum via PET scans. This proposed a potential mechanism in which an increase in transported is produced, increasing the reuptake of serotonin. Furthermore, an additional study by Ballanger in 2021 (as cited in Assogna et al., 2020) used PET scans to exhibit a decrease in postsynaptic serotonin receptors, such as 5-HT1A.

2.3 Treatment for DPD

The current treatments for depression with those with PD are those used for depression in the general population (Slaughter, 2001). DPD is often treated with cognitive behavioral therapy and anti-depressants. Popular anti-depressant medication prescribes increase selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs), and tricyclics (TCAs). SSRIs and TCA are the most studied drug regarding the treatment of depression in patients with PD (Assogna et al., 2020). Unfortunately, medication can produce adverse effects for those

with DPD. TCA side effects for this population may include impairment of memory and delirium (Slaughter, 2001). Additionally, SSRIs may worsen motor symptoms including tremors. Furthermore, this treatment is only effective for 20-40% of patients with PD (Laux, 2022).

2.4 Vagus nerve stimulation

The vagus nerve, the tenth cranial nerve, consists of the afferent and efferent neuronal network in the nervous system (Johnson and Wilson, 2018). It is composed of 80% afferent fibers transmitting signals from the peripheral to the central system and 20% of efferent neuron fibers which carry impulses from the central nervous system to the peripheral nervous system. These afferent neurons carry information via the Nucleus Tractus Solitarius (NTS) which can then relay these signals to other brain regions (George, 2000). Important projections from the NTS include medullary motor nuclei, the locus coeruleus (LC), the dorsal raphe nucleus (DRN), to the hypothalamus, the bed nucleus of the stria terminalis (BNST), and the amygdala. Since there are projections from the LC and DRN to the limbic region of the brain, these regions are presumed to influence depression (Carreno and Frazer, 2017) A large sum of data regarding the vagus nerve projections have seen identifies using track tracing in rat models which are thought to be similar in humans. All the innervations by the vagus nerve are not yet known (Johnson and Wilson, 2018).

Vagus nerve stimulation (VNS) has been applied for potential innovative treatment for disorders such as depression. The Food and Drug Administration approved VNS in patients who have tried four or more antidepressant treatments over the age of 18 with major depressive disorder (Carreno and Frazer, 2017). Treatment parameters for depression may differ, however, it is common to use a frequency of 20-30Hz, a pulse duration of 500 microseconds, and stimulation for 30-90 seconds followed by 5 minutes without stimulation (Johnson and Wilson, 2018). A study performed by Nahas et al. demonstrated patients with major depressive disorder or chronic

depressive disorder being treated with VNS had long-term beneficial effects (as cited by Johnson and Wilson, 2018).

2.5 Non-invasive focused ultrasound

Using focused ultrasound as a therapeutic approach can be a resolution for non-invasive treatment. Furthermore, this method is more affordable and transportable than other treatment alternatives. (Rivens et al., 2007). Using ultrasound as a therapeutic approach has been seen as early as the 1980s. This method is aimed to adjust the propagation of action potentials (Puleo and Cotero, 2020). Many of the earlier studies consisted of using ultrasound ex vivo concurrently with an implanted electrode. This method has continued to be used in other studies as a non-invasive option to directly stimulate tissue (Puleo and Cotero, 2020). While research must continue to further our knowledge of ultrasound as a non-invasive treatment, ultrasound should be considered a viable treatment option that is not invasive.

3. OUR PROJECT

This project aimed to examine the effect of focused ultrasound as a potential treatment for hemi-Parkinsonian rats displaying depressive-like behaviors. White Sprague Dawley male rats were used for these experiments. Hemi-Parkinsonian rats were generated via unilateral six-hydroxydopamine (6-OHDA) micro-infusion surgery on the right hemisphere of the brain which influenced the left side of their body. The 6-OHDA lesion destroyed dopaminergic neurons in the medial forebrain bundle near the SNc. A control group was created to discount potential confounding variables. The stereotaxic surgery was performed with a saline injection, rather than 6-OHDA (Slack et al., 2010). A behavioral test, limb-use asymmetry test (LAT), was done to analyze if the surgery was successful with animals exhibiting left-sided motor impairment. This is done by counting how many times their paws tap the cylinder glass they are placed in (Cenci and

Lundblad, 2005). Since their instinct is to explore their environment, the expected behavior would be to touch their front paws on the cylinder wall. If the hemi-parkinsonian surgery was done well, the rat would use their left paw less than 20% of their taps (Cenci and Lundblad, 2005). To then analyze anhedonia in the rats, another behavioral test is done. A sucrose preference test was executed since if the rat displayed depressive-like symptoms, anhedonia, it would have no preference between sucrose solution or water. This study's focal point was to analyze whether focused ultrasound (FUS) treatment improved depressive-like symptoms in hemi-Parkinsonian rats. FUS was used due to its parallel mechanism with the VNS which has been used for treating depression (George, 2000). To eliminate confounding variables, an additional control group was created. This control group underwent the same procedure for the FUS treatment. However, the ultrasound was not turned on, they only received isoflurane; this group was known as the isoflurane control group. Peripheral FUS is less invasive than VNS since no surgery is needed yet has a similar mechanism. Targeting the celiac ganglion, which is innervated by the vagus nerve, can be a new novel treatment.

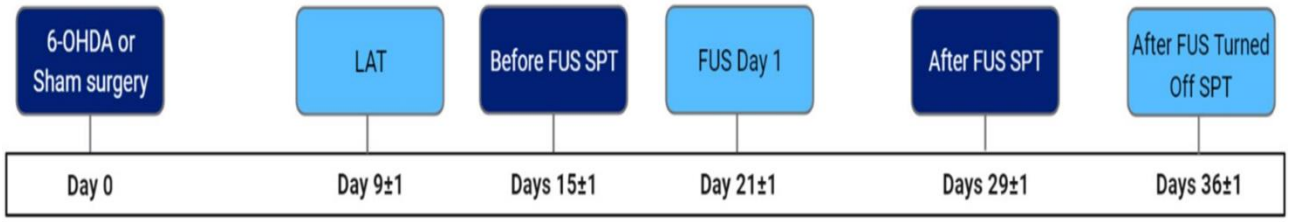


Figure 1. Experimental Schematic Design

The rats underwent 6-OHDA or sham surgery, which is represented at day 0. By day 9±1 days post-surgery, LATs were performed. On day 15±1, SPT is performed prior to FUS treatment. By day 21±1, FUS is performed in the morning and afternoon for the next five days. On day 29±1, SPT is performed after treatment. On day 36±1, SPT was perfomred after FUS was turned off one week to assess long-term effects of FUS.

4. MATERIALS AND METHODS

4.1 Craniotomy surgery

Hemi-Parkinsonian rats were created via the 6-OHDA (six-hydroxydopamine) surgery. All three groups underwent this procedure. The 6-OHDA was injected near the SNc to promote dopaminergic neuron loss in that region. the control group, referred to as shams, receive an identical surgery to eliminate additional confounding variables. However, they are injected with saline, rather than 6-OHDA. In a sterile environment, ear bars, a scalpel, forceps, bulldog clips, and tweezers were prepared while the rat was contained in a chamber flooded with 5% isoflurane, a general anesthetic. Once the rat was removed, an intraperitoneal injection of 1mL of Desipramine and Pargyline was administered. It was then placed in the apparatus while receiving 2% isoflurane through a nose cone. 0.1mL of bupivacaine was injected into the incision site to numb the region. The incision is completed while ensuring the visibility of bregma, where the coronal and sagittal sutures align with the parietal and frontal bone. The coordination for the injection was minus 4.4 rostral to caudal and minus 1.5 medial to lateral from bregma. The drill was then aligned to the coordinate, and the injection pump, containing the 6-OHDA or saline, the syringe needle lowered 7.5 in the dorsal to the ventral position.

4.2 Limb-use Asymmetry Test (LAT)

The limb-use asymmetry was performed a week post-surgery to evaluate the rat's mobility or akinesia. The LAT was performed to behaviorally examine if there was motor impairment due to the injection of 6-OHDA. To increase the rat's activity, it was done with the lights turned off but with red lighting since rats are most active in the darkness. The rat, being in an unfamiliar environment, was left to explore the cylinder and its surroundings. The rat was recorded while it is placed in a transparent cylinder glass. This procedure was done with a maximin and ten minutes and a minimum of five minutes and must sum to 20 taps (Centi and Lundblad, 2005). If the rat

failed to tap 20 times, another LAT was necessary to determine whether the rat can be identified as hemi-Parkinsonian or sham. The recording was examined to establish with the rat was a sham or hemi-Parkinsonian. To do so, the right and left taps were calculated. The right taps were divided by the left taps and multiplied by one hundred to produce a percentage of right taps. If it scored 80% or higher on right forepaw touches, it was assumed to be hemi-Parkinsonian (Cenci and Lundblad, 2005). If the rat displayed impairment in their movement by touching mostly with their right forepaw, they would be behaviorally classified as hemi-Parkinsonian. One cannot definitively determine if a rat is truly hemi-Parkinsonian until post-mortem analysis is performed.

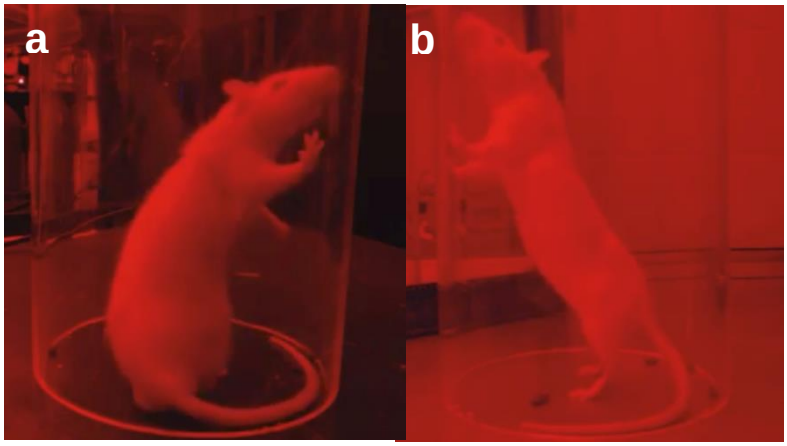


Figure 2. LAT Depiction in Hemi-Parkinsonian and Sham Rats

(a) This depicts the tapping pattern of hemi-Parkinsonian rats during LATs primarily tapping with their right forepaw due to the immobility of the left forepaw. (b) This displays the pattern of sham during LATs tapping with the right and left forepaw.

4.3 Sucrose Preference Test (SPT)

The sucrose preference test was done to measure the level of anhedonia as a tool to determine if the rat displayed depressive-like symptoms (Santiago et al., 2014). SFT was performed three times; once roughly two-week post-surgery, a week post-treatment, and two weeks post-treatment for cohorts in group one. This was composed of two phases: a 24-hour acclimation phase and a 48-hour test phase. The glass bottles were weighed before and afterplaced into the cage to record

how much the rat drank. The acclimation phase required two large glass water bottles with identical stoppers, one labeled W1 and the other W2. To prevent leakage, the stoppers were wrapped with parafilm. This would allow the rats to grow accustomed to having two bottles in their cage. Once the 24 hours were complete, the bottles were weighed and recorded. One glass bottle filled with water (W1) and one with sucrose solution (W2) was placed into the cage to launch the test phase. Once the bottles were weighed, the water and sucrose solution were placed in the cage. W1 was placed on the opposing side from the acclimation period. Once 24 hours passed, the bottles were weighed and switched their position to account for any side biasness. After another 24 hours, the bottles are weighed once more to finalize the test phase.

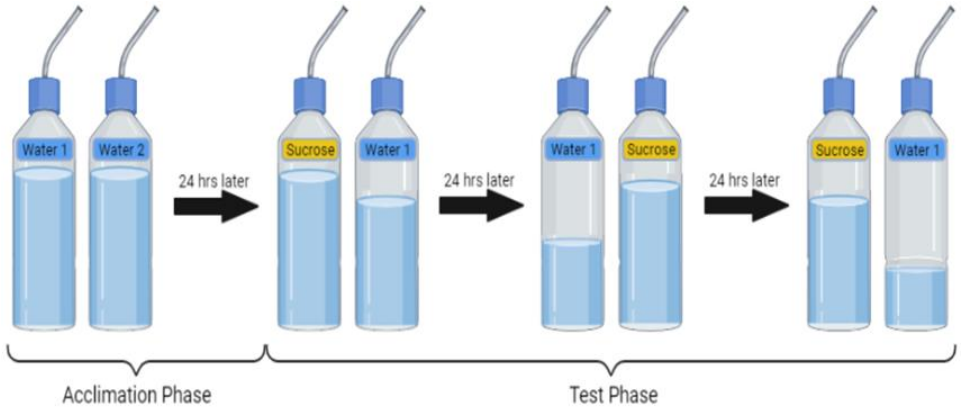


Figure 3. Sucrose Preference Test

The acclimation period consisted of two water bottles for a period of 24 hours. The test phase consisted of a single water and sucrose solution for a period of 48 hours. Once the first 48 hours passed, the bottles swapped sides in the cause to eliminate size preference as a confounding variable.

4.4 Focused Ultrasound Treatment (FUS)

Focused ultrasound treatment was used as the primary source to reduce the depressive-like behaviors in hemi-Parkinsonian rats. The FUS treatment was performed in the morning and afternoon for five consecutive days. The ultrasound was set to a frequency of 2.5MHz, bursts of 300 cycles, and output of 300mV, and burst period of 200ms (Akhtar et al., 2021). The rat was placed in an isoflurane chamber while receiving oxygen. Once the rat became unresponsive, it was

placed by the apparatus, continuously receiving isoflurane and oxygen. Its abdomen was thoroughly shaved since hair can interfere with the accuracy of the ultrasound. The ultrasound gel was applied where the probe is later placed between the xiphoid process and lower left ribcage to target the celiac plexus. This procedure was conducted for three minutes. An isoflurane control group was included to limit confounding variables to ensure that the isoflurane was not responsible for altering the depressive-like treatment. Therefore, the same procedure was done to the isoflurane group, except the ultrasound machine was not turned on during the three-minute session.

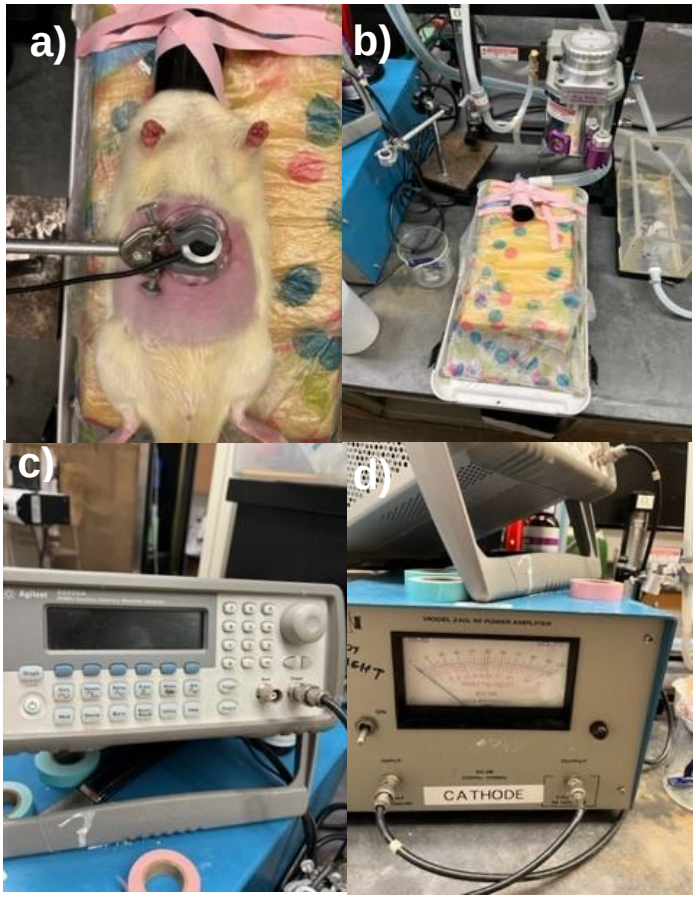


Figure 4. Non-Invasive Focused Ultrasound FUS target the celiac plexus. (A) The transducer was placed by the xiphloid process at an angle to allow the FUS to target the celiac plexus. (B) (C) The function generator is depicted where the setting for sine, burst, and output are controlled. (D) The RF power amplifier is shown.

4.5 Trans-cardiac perfusion

The trans-cardiac perfusion was done to preserve post-mortem brain tissue for immunostaining. Urethane was injected into the rat as a form of anesthesia. The incision was made below the ribcage to uncover the thorax and heart (Mendez et al., 2018). Once the heart was exposed, the right atrium was clipped to permit blood out flow. To prevent the blood from clotting, 60mL of heparin was injected into the left ventricle. Once the heparin was fully administered, 60mL of paraformaldehyde fixates the brain. The brain was removed from the skull and later sectioned using a cryostat.

4.6 Tyrosine Hydoxylase Immunostaining

Tyrosine hydroxylase (TH) staining was performed as a dopamine marker to visualize dopaminergic neuronal loss in the striatum, SNc, and VTA (ventral tegmental area). After the rat's brain was sectioned at 50-60 microns using the cryostat, they were transferred into Eppendorf tubes and then into individual wells, each containing two striatum, two to three SNc, and two VTA slices per rat. The staining comprised of two days. On the first day, the slices were first washed with 0.1M phosphate-buffered saline (PBS) and 3% hydrogen peroxide. After the washing, the slices transferred into 1.5mL Eppendorf tubes consisting of PBS and normal goad serum. The primary antibody, Novus anti-TH, was then added per Eppendorf tube. During the second day, the slices were washed in the wells and placed into a 1.5mL Eppendorf tube containing PBS and normal goat serum. Horseradish peroxide (HRP)-conjugated goat-anti-rabbit antibody was added as the secondary antibody. Once completed, 3,3'-Diaminobenzidine (DAB) solution was added to stain the slice in the striatum, and SNc.

4.7 Tyrosine Hydroxylase Analysis

TH analysis was completed once the stained slices were mounted and cover slipped. These slides were scanned and uploaded to the computer to be examined using the ImageJ software. The

striatum, VTA, and SNc were analyzed, and the right lesion side was compared to the left-lesion side. The densities were acquired with the ImageJ software by dividing the right lesion side by the left un-lesioned side and multiplied by ten for the percentage (Tucker et al., 2021). For the striatum, an area of 300*300 was used for all these regions to ensure consistent among the samples. For the VTAs, an area of 80*80 was used. Regarding the SNc, due to its unique shape, the region was highlighted using the drawing tool on the right lesion side and inverted when analyzing the left un-lesioned side to remain consistent within each sample. This method would allow for insight into the degree of dopaminergic neuronal degeneration.

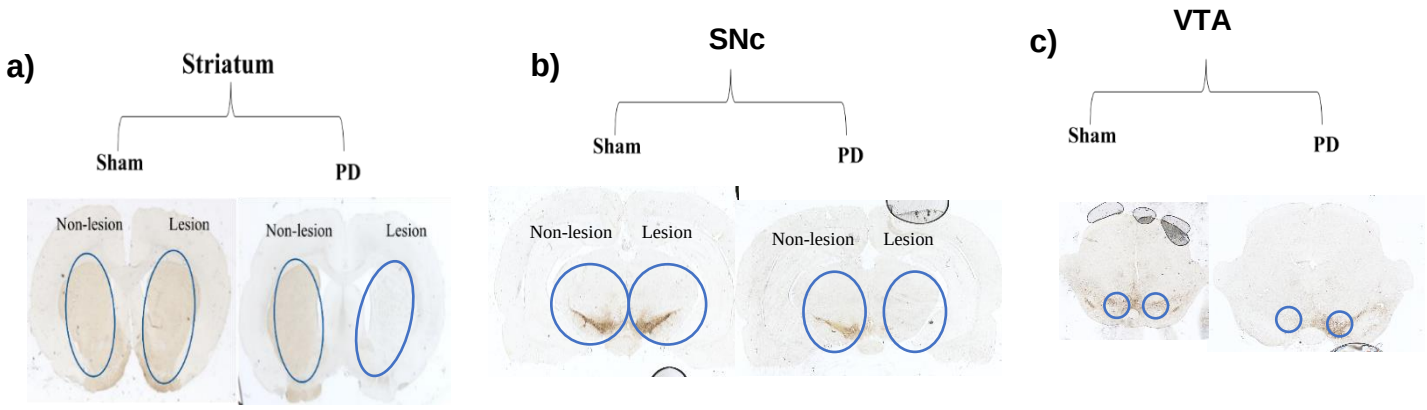


Figure 5. Tyrosine Hydroxylase Staining Comparing the Lesion and Unlesion Side.

(a) The striatum in a sham vs. hemi-Parkinsonian rat is stained. (b) The SNc in a sham vs. hemi-Parkinsonian rat is stained. (c) The VTA in a sham vs. hemi-Parkinsonian rat is stained comparing the lesion to the Unlesion side.

5. CHEMICALS AND DRUGS

Chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Items for immunochemistry, such as antibodies for the TH staining, were received from Novus Biologicals (Littleton, CO, USA), Santa Cruz Biotechnology (CA,USA), or Jackson ImmunoResearch (West Grove, PA, USA.)

6. DATA ANALYSIS AND STATISTICS

Statistical analysis was done using the GraphPad Prism software. A two-way ANOVA with Bonferroni post-hoc multiple comparisons were used for SPT in sham and hemi-Parkinsonian rats. An unpaired T-test was used to analyze LAT results from sham and hemi-Parkinsonian rats. A one-way ANOVA was used for TH analysis. Values were considered statistically significant with a p value of $p<0.05$.

7. RESULTS

7.1 LATs

For our study, 63 rats were analyzed comprising of 23 sham rats and 40 hemi-Parkinson rats. They were grouped into these two groups using the LAT behavioral test. The sham rats had a minimum of 36.54%, a maximum of 68.81%, and a mean of 52.82% for right forepaw touches. Hemi-Parkinsonian rats touched with their right forepaw a minimum of 79.96%, a maximum of 100%, and a mean of 93.91%. This data displays that the sham rats have no preference between tapping between their right and left forepaw. However, the hemi-Parkinsonian favored touching with their right paw emphasizing that their left forepaw mobility was impaired from the 6-OHDA surgery. There was a statically significant difference between the sham and hemi-Parkinsonian rats ($p<0.001$) (fig. 5). It is important to note that one cannot confirm with a rat is a sham or hemi-Parkinsonian rat until post-mortem analysis is done. This was confirmed using tyrosine hydroxylase staining and analysis.

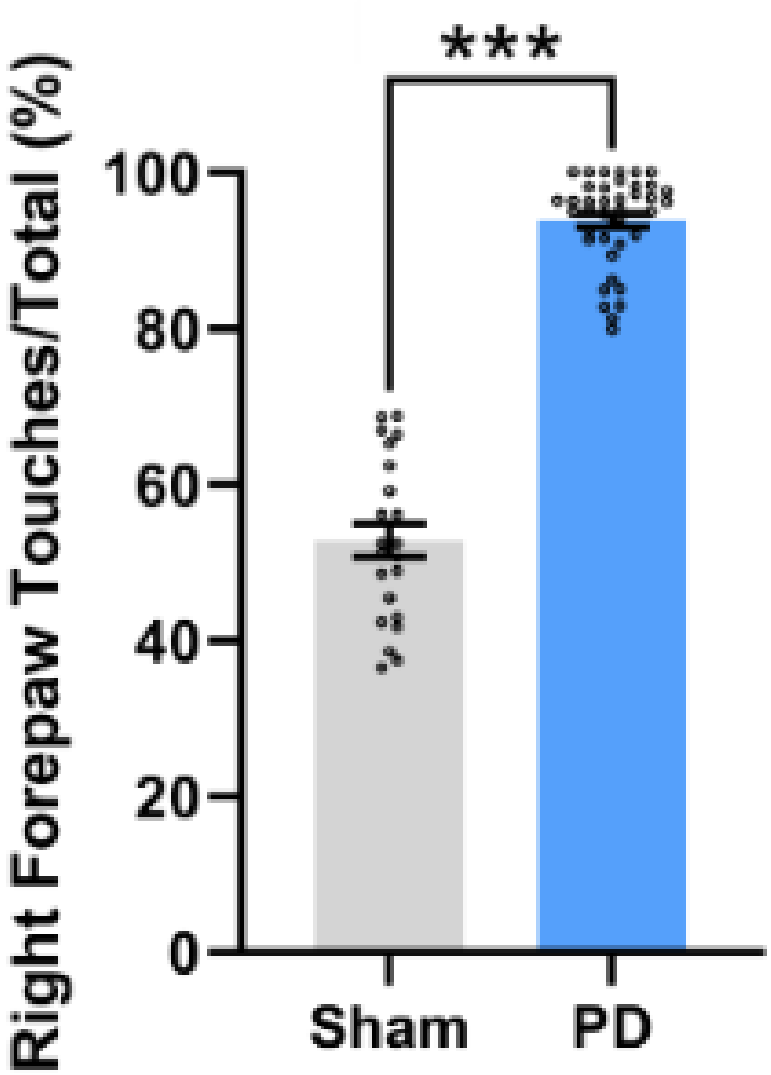


Figure 5. Hemi-Parkinsonian Rats Unilateral Forepaw Tapping

A limb-asymmetry test was performed to analyze if the 6-OHDA lesion in the right forebrain bundle caused immobility in the left forepaw. An unpaired t test was performed analyzing sham rats (n=23 shams) and hemi-Parkinsonian rats (n=40). The right forepaw touches were divided by the total amount of taps to determine the percentage of right forepaw taps. This graph displays the mean and standard error of the mean. There was a statistical significance between sham and hemi-Parkinsonian rats in their percentage of right forepaw touches ($p<0.001$).

7.2 Sucrose Preference Test

To analyze depressive-like behaviors in the rats, the sucrose preference test was used to determine the level of anhedonia in sham and hemi-Parkinsonian rats. This behavioral test

considered the percent of sucrose consumed during the test phase. Figure 6 demonstrates the results of the SPT regarding sucrose consumption (g) and sucrose preference (%). Four groups including sham, hemi-Parkinsonian, sham who received FUS treatment (sham+FUS), and hemi-Parkinsonian rats who received FUS treatment (PD+FUS), were analyzed. SPT was performed before FUS treatment (baseline data), a week after the FUS treatment (treatment), and a week after FUS is turned off (recovery) (figure 6a. At baseline, before FUS the PD+FUS group had a statically significantly lower preference for sucrose than the sham group ($p<0.01$). PD rats with FUS consumed more sucrose solution than without FUS even 1 week after FUS was turned off. This suggests that the PD rats that underwent FUS exhibited less anhedonia. Figure 6b displayed the amount of sucrose solution that these four groups consumed in grams using a two-way ANOVA test. There was a statical difference between the sham and PD, and PD+FUS ($p<0.05$). There was a statistical significance for PD+FUS at baseline compared to treatment ($p<0.05$) and recovery ($p<0.05$).

7.3 Weight

Furthermore, the weight of each rat in the four groups was recorded throughout the experiment (fig. 7). This was analyzed using a two-way ANOVA. They were weighed at the time of the surgery, when undergoing the three SPT, and throughout the five days during the FUS treatment. The weights were seen to be influenced by the FUS treatment. During the five days of treatment, both the sham and hemi-Parkinsonian rats had a decrease in weight ($p<0.001$).

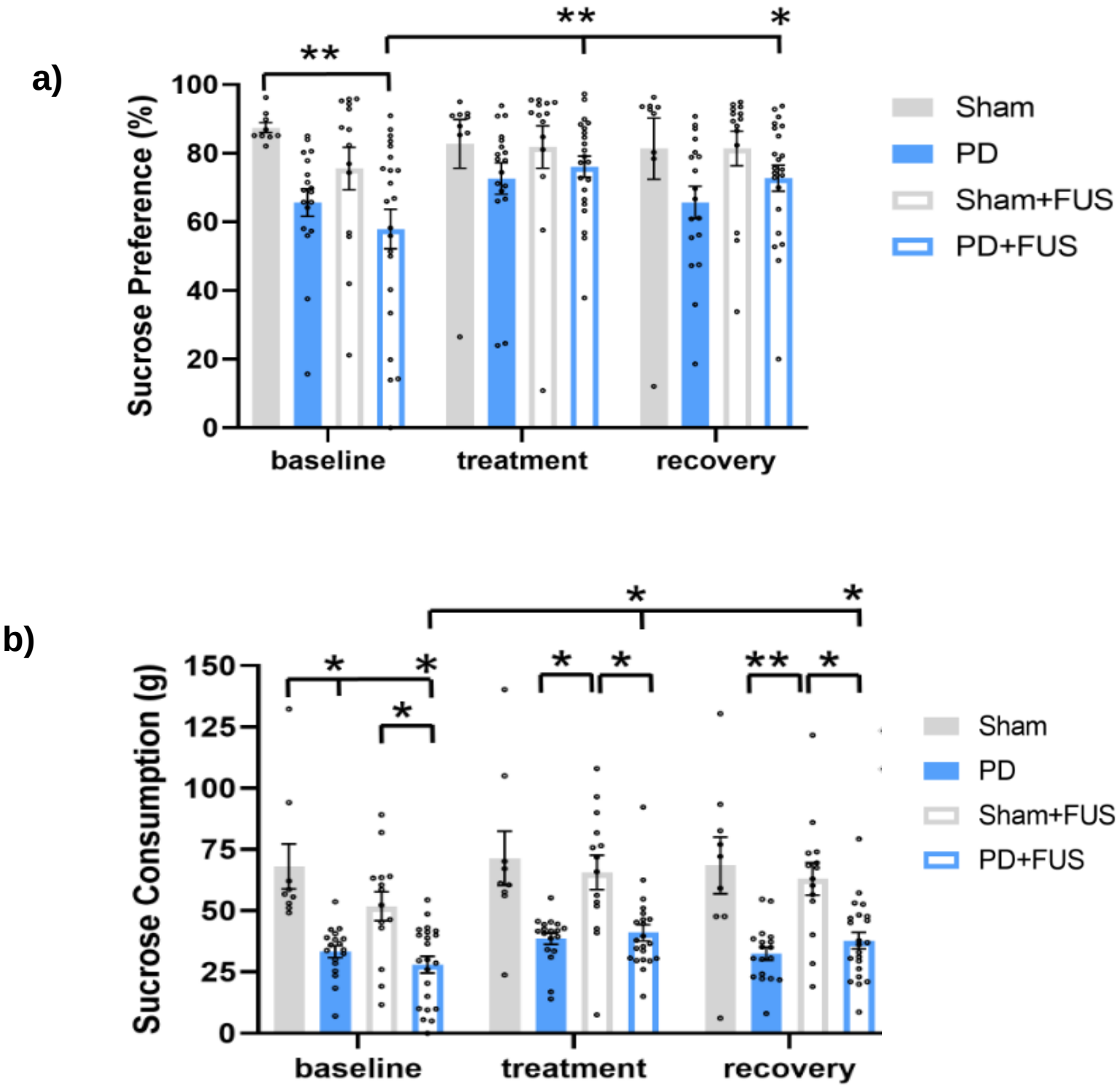


Figure 6. Hemi-Parkinsonian Rats Experienced Anhedonia

(A) The SPT was used to analyze the level of anhedonia in the rats. The sucrose preference percentage was calculated in sham (n=9), PD (n=18), sham+FUS (n=14), and PD+FUS (n=22). The SFT was done prior to FUS treatment (baseline), one week (treatment) and two weeks (recovery) after FUS treatment. Prior to treatment, there a statical significance between the sham and PD+FUS group ($p<0.01$). A statistical significance between baseline PD+FUS and treatment PD+FUS ($p<0.01$) and recovery PD+FUS ($p<0.05$). This data represents the mean and standard error of the mean. A two-way ANOVA followed by the Bonferroni post hoc test was done. (B) Using the sucrose preference test, sucrose consumption (g) was analyzed. Four groups were analyzed, sham (n=9) PD (n=18), sham+FUS (n=14), and PD+FUS (n=22), at baseline, treatment, and recovery. There was a statical difference between the sham and PD, and PD+FUS ($p<0.05$). there was a statistical significance for PD+FUS at baseline compared to treatment ($p<0.05$) and recovery ($p<0.05$). This figure displays the mean and standard error of the mean. A two-war ANOVA was done followed by the Bonferroni post hoc test.

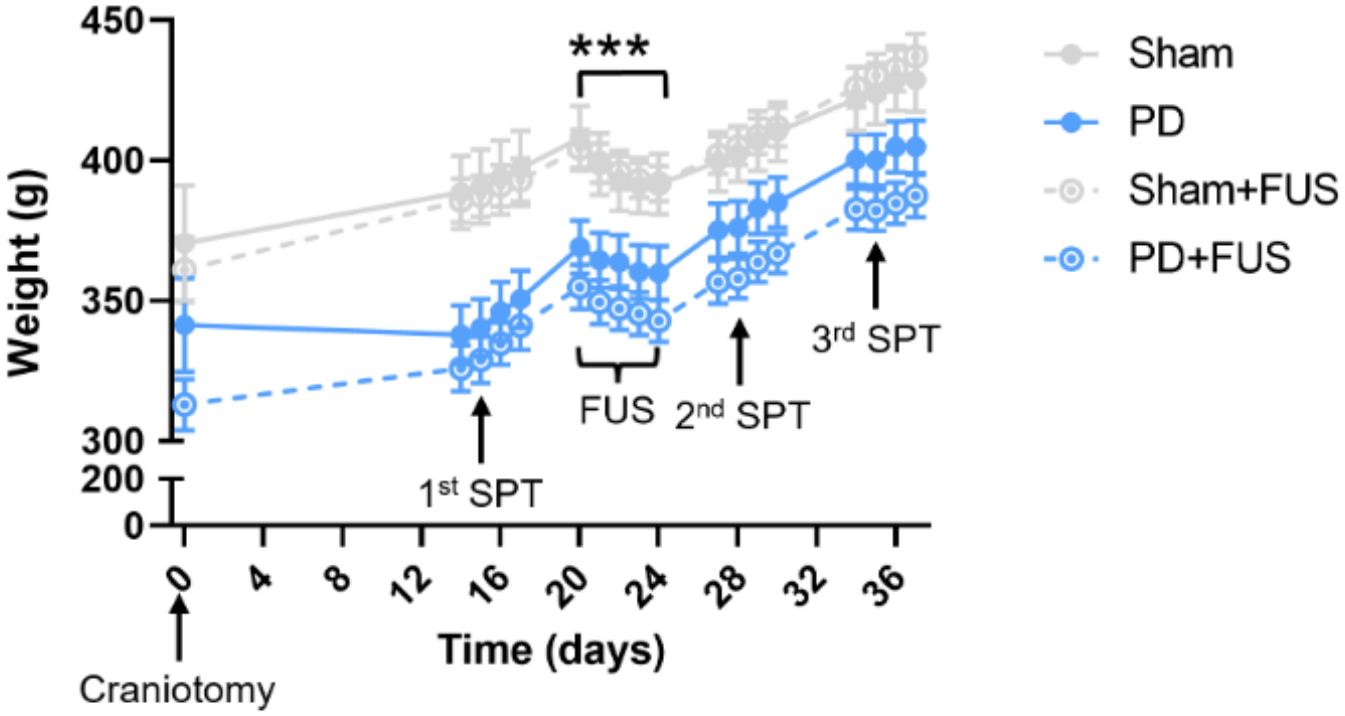


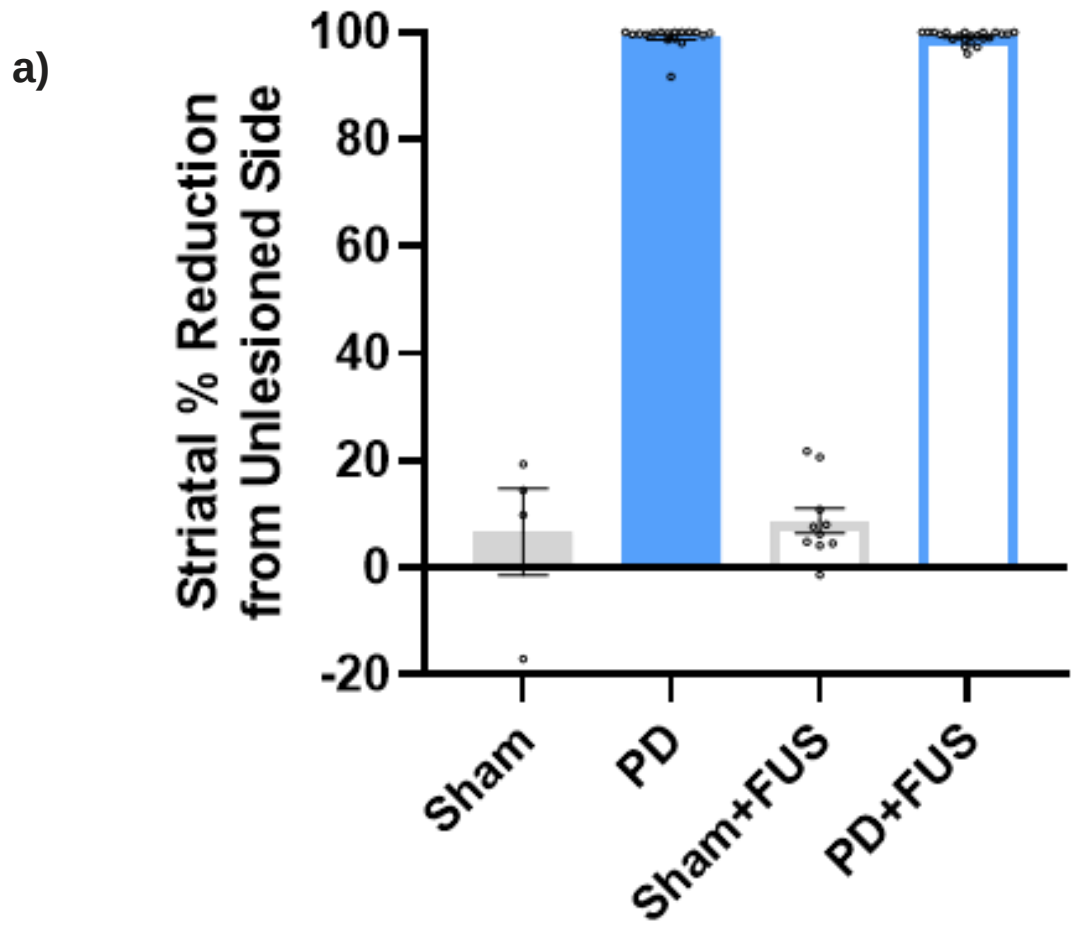
Figure 7. Sham and Hemi-Parkinsonian Have a Decrease in Weight with FUS Treatment

The weight of the rats includes sham (n=9), PD(n=18), Sham+FUS (n=14), and PD+FUS (n=22). For both sham and PD rats, each day of FUS treatment decreased the weight of the animals. Day 0 represents the day of surgery for both sham (injection of saline) and PD (injection of 6-OHDA). The first sucrose preference test (SPT) was done around day 15 ±1, focused ultrasound (FUS) was completed around day 20, with the second SPT complete around day 28 and the third SPT completed around day 36. There was a statistical significance between the first day of treatment (day 20) and day 24 in the four groups ($p<0.001$). This data represents the mean and standard error of mean. A Two-way ANOVA was done followed by the Bonferroni post hoc test.

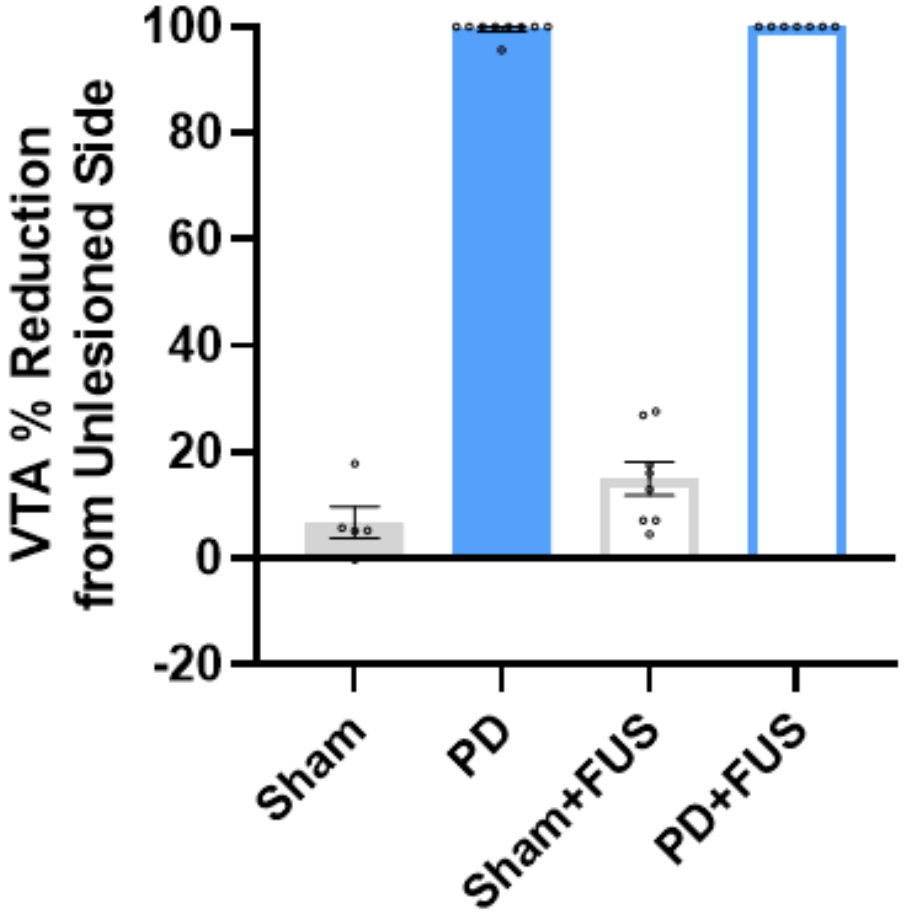
7.4 Tyrosine Hydroxylase Analysis

TH analysis was performed on three brain regions: striatum, VTA, SNc. We investigated the dopaminergic expression in the different treatment groups (sham, PD, sham+FUS, and PD+FUS). This was done to analyze the dopaminergic axons in these regions of interest to decipher the level of degeneration. Figure 8 exhibits the results for TH analysis of the three regions of interests. For the PD and PD+FUS rats, their lesion side had a substantial reduction in TH staining compared to the un-lesioned side due to the loss of dopaminergic axons. In contrast, the sham and sham+FUS rats, the lesion side did not have a substantial reduction in dopaminergic

axons. For the striatum, the percent reduction from the un-lesioned side in the sham rats was 6.67%, sham+FUS rats was 8.75%, PD rats was 99.14%, and the PD+FUS rats was 99.15%. There was no statistical difference between the different treatment groups (fig. 8a). Regarding the VTA, the percent reduction from the un-lesioned side in the sham rats was 6.74%, sham+FUS rats was 14.98%, PD rats was 99.52%, and the PD+FUS rats was 100.0%. There was no statistical difference between the different treatment groups (fig. 8b). For the SNc, the percent reduction from the un-lesioned side in the sham rats was 10.41%, sham+FUS rats was 17.16%, PD rats was 100.0%, and the PD+FUS rats was 99.80%. Again, there was no statistical difference between the different treatment groups (fig. 8c).



b)


VTA % Reduction from Unlesioned Side
100
80
60
40
20
0
-20
Sham
PD
Sham+FUS
PD+FUS

C)

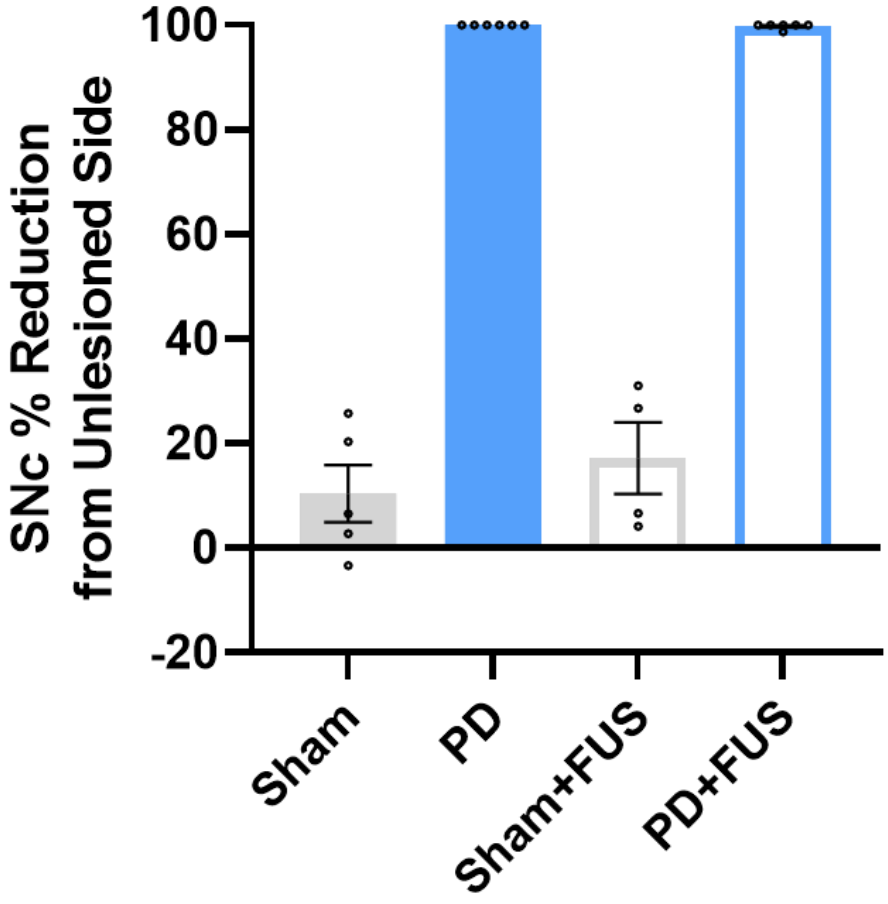

SNc % Reduction from Unlesioned Side
100
80
60
40
20
0
-20
Sham
PD
Sham+FUS
PD+FUS

Figure 8. There was no Statistical Difference Between the Different Treatment Group

The staining in the striatum, VTA, and SNc were analyzed in the different treatment groups (sham, PD, Sham+FUS, and PD+FUS). (a) There was not statistical difference between the different treatment groups in the striatum percent reduction from the un-lesion side. (b) The comparison between the VTA in the different treatment groups did not display any statistical differences. (c) In the SNc, there was no statistical differences between the different treatment groups in the percent reduction of the un-lesion side. This was analyzed using an ordinary one-way ANOVA was done followed by the Bonferroni post hoc test. This figure displays the mean and standard error of the mean.

8. DISCUSSION

Animal models are a great alternative in studying a variety of diseases including PD. Through stereotaxic surgery, hemi-Parkinsonian rats can be created via an injection of 6-OHDA to the right MFB. This surgery is effective in generating symptoms in the rats that correspond to patients with PD such as mobility issues, gastrointestinal tract issue, and depressive-like behaviors. Therefore, treating these rats and their symptoms can translate to treating human patients. In our experiment, rats that underwent the 6-OHDA injection surgery, unlike the saline injection surgery, displayed mobile impairment to the left forepaw which was demonstrated with the LAT (fig. 5). It was found that hemi-Parkinsonian rats that displayed depressive-like symptoms, anhedonia, that were treated with FUS, displayed a decrease in anhedonia. This was not seen in the hemi-Parkinsonian rats that were in the isoflurane alone groups. The FUS did cause rats to decrease their weight which is something to keep in mind when progressing with this research. Therefore, it was evident that the FUS treatment itself was decreasing the depressive-like symptoms.

While depressive-like symptoms in these rats were improved, depression as a non-motor symptom in patients with Parkinson's disease is complex and must be further studied. Since current treatment for human depression is not too effective for this population (Laux, 2022), more research to establish a new therapeutic is necessary. Therefore, it is vital to introduce alternative therapeutics to treat these symptoms. Treatment for depression in human PD has not been

extensively studied. However, there have been studies that have looked into establishing alternative therapeutics for this population. For example, a study by Wu and colleagues in 2017 investigated the effectiveness of physical exercise to treat depressive symptoms in patients with PD. They included primary research from Databases Scopus, PubMed, ProQuest, CINAHL, Cochrance, and Psycho-info and completed a systematic review. They reviewed 769 primary research, but only 11 of those were in their review. The analysis consisted of 342 patients and 17 types of physical activities. It was found that patients' scores on the Quality of Life, Unified Parkinson's Disease Rating Scale, and the Becks Depression Inventory were improved with aerobic exercise (Wu et al., 2017). While this study and other studies with similar results posit that exercise can improve these symptoms, as PD progresses, movement becomes more difficult and might not be feasible for all patients. Therefore, it is beneficial to continue to work toward new treatment options.

There has been research regarding electroconvulsive therapy to treat depression in these patients. A systematic review in 2016 by Borisovskaya and colleagues (as cited in Chikatimalla et al., 2022) analyzed the use of ECT for patients with PD who suffer from depression. This review consisted of 13 case series, two retrospective chart reviews, one retrospective case study, and 27 single case reports. They found that 93% of patients had an improvement in their depressive symptoms. ECT was also seen to improve 83% of patients' motor function for those that described it. It is important to note that some studies report a high chance of adverse effects (Chikatimalla et al., 2022). For example, a study by Tom and Cummings (as cited in Slaughter, 2001), emphasized that ECT may produce adverse side effects such as delirium. Therefore, other non-invasive therapies should be considered.

Referring to animal models, there have been other studies that considered a variety of ultrasound treatments to treat depression. A study by Zhang and their colleagues in 2021 investigated the use of low-intensity-pulsed ultrasound (LIPUS) as a therapeutic for depression. In this study, rats were exposed to unpredictable chronic stress which resulted in depressive-like behaviors. These rats were then treated with LIPUS, rather than high-intensity focused ultrasound (HIPUS) to prevent neuronal damage to the ventromedial prefrontal cortex for four weeks. Compared to our study, which targeted the vagus nerve, their treatment was directly targeted at the region of interest rather than stimulating the periphery to propagate signals to the central nervous system and region of interest. Additionally, during treatment, the animals were awake and moving without constraint. That was an effective method since it eliminated confounding variables that were added due to the process of inducing anesthesia (Zhang et al., 2021). That is why our study included a control group that received isoflurane without the treatments to ensure that the isoflurane did not have an impact on the results. Regarding clinical use, the ultrasound procedure for patients would likely be done without the use of anesthesia like the study by Zhang. While the study by Zhang did not encompass rats that were hemi-Parkinsonian, it did portray that a form of ultrasound treatment did improve depressive-like symptoms in rats.

While our studies and the studies that were mentioned focused on animal models. There has been research on human patients. Transcranial ultrasound stimulation (TUS) has been studied as a non-invasive treatment option, rather than just a diagnostic tool. In the past few years, there have been studies on TUS in humans (Sarica et al., 2022). A study by Sarica and colleagues in 2022, analyzed TUS in a systematic review of human patients. This review included focused and unfocused ultrasound in 35 studies with a total of 677 subjects; they included healthy participants, who had dementia, chronic pain, epilepsy, traumatic brain injury, and depression. They highlight

that studies on drug-resistant epilepsy and depression are shown to be safe. There was no adverse effect in the studies included, while some reported symptoms in 3.4% such as dizziness, mood deterioration, anxiety, muscle twitches, and headache (Sarica et al., 2022). This study cites an article by Reznik and colleagues that strictly focused on patients with depression who were treated with TUS or a placebo TUS (Reznik et al., 2020). The participants had mild to moderate depression in 24 college students. They received either active TUS or TUS that was not turned on which acted as a placebo group. The treatment consisted of five sessions of the TUS over seven days. After treatment, worry was decreased and happiness increased in the patients with the active TUS as opposed to the placebo group (Reznik et al., 2020. Further research into the long-term effects of ultrasound on humans as a therapeutic must be further researched. Ultrasound as a therapeutic model has been in a large sum of studies that have been shown in animal models and human patients. Non-invasive FUS may be a great alternative to treat depression in patients with PD since we know that many of the medications, such as antidepressants, are not effective for members of this population. The studies above provide evidence that FUS treatment for patients can be a viable option as a therapeutic for patients with PD with depression.

Our study emphasized that depressive-like symptoms in hemi-Parkinsonian rats are improved by FUS vagus nerve treatment as a non-invasive therapeutic approach. The sham rats did have a statistically significant difference between the varying timelines of the SPT. The FUS treatment displayed long-term effects since their sucrose preference increased from baseline a week after the FUS was turned-off. Therefore, it was shown that non-invasive FUS treatment in hemi-Parkinsonian rats improves depressive-like behavior having lasting effects. When performing the FUS treatment to target the vagus nerve, we believe that the motor nucleus of the vagus nerve (DMV) or the nucleus tractus solitarius (NTS) can carry impulses to the dorsal raphe

nucleus (DRN) and the locus coeruleus (LC). The specific mechanism of targeting the celiac ganglia to innervate the vagus nerve must be further examined.

9. FUTURE DIRECTION

We believe the DMV or NTS is involved in carrying impulses to the Dorsal Raphe (5-HT) and locus coeruleus (NE) via the FUS treatment. This is thought since VNS for persistent depression possesses similar mechanisms. This target would be beneficial for patients with PD since it has been shown that degeneration occurs in noradrenergic and serotonergic neurons which may be a potential aspect for depression in these patients. There is a large sum of serotonergic neurons in the DRN and a large sum of noradrenergic neurons in the LC which relay to other brain regions which correspond to depression. (Miguelez et al., 2011). Therefore, FUS, which encompass similar mechanism to VNS, can be a feasible treatment option. Further research must be done to understand the neurological pathway involved in depression in PD. Identifying the precise mechanisms that underlies depression in the PD population will enhance potential therapeutics.

10. CONCLUSION

It was shown that anhedonia decreased with the FUS treatment of the vagus nerve. This effect had long-term effects since the decrease in anhedonia remained after a week after FUS was turned off. We also noted that the weight of the sham and hemi-Parkinsonian rats decreased with FUS treatment which is an aspect to consider as further research continues. Finally, the sham and hemi-Parkinsonian rat's dopamine loss were confirmed using TH analysis. There was no statistical difference between the different treatment groups in the striatum, VTA, and SNc regarding the reduction of the un-lesion side.

11. LIMITATIONS

While there was an improvement in depressive like behaviors in hemi-Parkinsonian rats, the mechanism is not well understood. More research must be conducted to reveal how FUS decreased depressive-like symptoms. Additionally, this study was done using an animal model and the results may differ in a clinical setting.

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