Cannabinoid Modulation of Microglial Activation in Multiple Sclerosis

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SENIOR THESIS APPROVAL

This Honors thesis entitled

"Cannabinoid Modulation of Microglial Activation in Multiple Sclerosis"

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and submitted in partial fulfillment of the requirements for completion of the Carl Goodson Honors Program meets the criteria for acceptance and has been approved by the undersigned readers.

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Abstract:

Multiple Sclerosis (MS) is a neurodegenerative disease that is estimated to affect 400,000 Americans. There is no cure for MS and some treatments at this point are toxic to patients. Dr. Lori Hensley’s lab is researching two drugs that could potentially be used to treat MS without toxic effects. Previous work in the lab shows ajulemic acid, a synthetic molecule similar to metabolites from the cannabis plant, selectively suppresses markers of the inflammatory response. I worked on the mechanism of ajulemic acid’s action by investigating the roles of several candidate receptors in mediating these observed effects.

Introduction:

Immune Response

The human body under normal circumstances has an efficient immune system that protects and heals the body. There are two main divisions of the immune response, innate immunity and the adaptive specific immune response. Innate immunity is a constant part of the body that has a defense mechanism against foreign cells such as pathogens. It is not specific to one type of pathogen; therefore, it is usually followed by the adaptive specific immune response. Adaptive immunity is started by the recognition of an antigen by a lymphocyte. An antigen is a marker of a cell that binds with antibodies. It marks the cell as foreign or self. There are two types of lymphocytes that are active during the adaptive response. First, B lymphocytes, which after a process, become plasma cells are responsible for secreting antibodies. Second, T lymphocytes are responsible for the actual recognition and killing of foreign cells (Charles A. Janeway and Travers 1994).

In normal production of lymphocytes, these cells go through a process called self tolerance. Self tolerance is the ability not to respond to self antigens. This occurs in the developmental stages of
lymphocyte production. If during lymphocyte development, the lymphocyte binds to a self antigen, it is programmed to die. In autoimmune diseases like MS, something has gone wrong. According to Janeway and Travers, an autoimmune response occurs when a “specific adaptive immune response is mounted against self.” Specifically, in autoimmune diseases, T cells recognize the self cell as foreign and elicit a response accordingly (Charles A. Janeway and Travers 1994).

**Microglia**

Microglia are cells in the central nervous system (CNS) that act as part of the immune system of the body. They are responsible for removing waste from the CNS (Kalat 2007). For this function to occur, they are able to develop into phagocytic cells (Young, Lowe et al. 2006). Phagocytic cells ingest and destroy foreign material. Microglia normally control the number of T cells in the CNS and are involved in the perpetration and termination of the inflammatory response. This process is defective in MS patients and accounts for the chronic inflammation caused by the T cell presence. In MS, microglia are found in the lesions that are actively demyelinating (Sanders and DeKeyser 2007). Our experimental studies were performed on highly aggressive proliferating immortalized (HAPI) microglia cells. This cell line has been immortalized, allowing the division of cells to continue without having to use new cells for each experiment. Because HAPI cells do have some differences from primary microglia cells, we duplicated our experiments in primary microglia. HAPI cells do not produce the cytokine IL1-B that is an important cytokine in the study of the inflammatory response. Most of the data presented in this text will be from HAPI microglia cells with a few exceptions.

**Astrocytes**

Astrocytes are star-shaped cells in the brain that help with the synchronizing of axons (Kalat 2007). They have several roles including mechanical support and transfer of metabolites. For our
study, the two main functions of astrocytes are the production of the blood-brain barrier and their role in CNS tissue repair after damage (Young, Lowe et al. 2006). Due to these roles, it is thought that when astrocytes are activated in the inflammatory response, they create leakage in the blood-brain barrier allowing the self-reactive T cells to get into the brain.

Multiple Sclerosis

Multiple Sclerosis is an autoimmune disease. In MS, the central nervous system (CNS) is the site of the problem. Autoreactive T cells attack the myelin sheath of neurons, and inflammatory cytokines are released by these T cells as well as microglia and astrocytes (Lori Hensley 2006). Cytokines are proteins produced by one cell that affect other cellular behavior. Cytokines released by T cells and other lymphocytes are called interleukins (IL). Interleukins are released and act on cells that have been identified as foreign and program them for apoptosis, or cell death (Charles A. Janeway and Travers 1994).

The myelin that is lost through this autoimmune attack is replaced by sclerosis or scar tissue. The destruction of the myelin affects the ability of the nerve fibers to conduct impulses throughout the CNS (2006). The inability of the myelin to conduct impulses is what causes the symptoms of MS. It is very difficult to give a list of symptoms because they vary from situation to situation. The symptoms include bladder and bowel dysfunction; problems with memory, attention, and problem solving; vertigo; dizziness; emotional changes; fatigue; balance and coordination problems; numbness; pain; spasticity; sexual dysfunction; vision problems; headache; hearing loss; itching; seizing; speech disorders and tremors (2006).

Because of the wide range of symptoms, MS is difficult to diagnose. What makes it even harder to diagnose is that there is not just one specific diagnostic test. Despite the difference in diagnostic tools, they all must confirm two signs for a positive MS diagnosis. First, there must be signs of disease in different parts of the nervous system. Second, the patient must have experienced at least
two flare-ups of symptoms. A complete medical history is the first step in starting to diagnose a patient because there are some genetic factors involved in the disease. The next step is determining the level at which the nervous system is functioning. Three different tests may be used to determine this level. A magnetic resonance imaging (MRI) is used to view the brain and look for lesions (2006). There are two problems with this; not everyone with MS has lesions and not all patients can have an MRI done (Lori Hensley 2006). An evoked potential test is also used. It measures the level at which the nervous system is working and can show demyelination by measuring the response time and accuracy of a person’s nervous system to certain stimuli. The third test used is a spinal tap, otherwise known as a lumbar puncture (2006). The spinal tap is specifically looking for an increase in immune response proteins (Lori Hensley 2006).

Once a patient is diagnosed with MS, a physician determines which of the four types of MS they have. At the time of initial diagnosis, the most common form is relapsing-remitting. The patient with this type has periods of flare-ups, also called relapses, attacks, or exacerbations, where the symptoms worsen, but they also have times of partial or complete recovery. A rare form of MS is the primary-progressive. These patients have a continuing worsening of their disease from the onset. Half of the people with relapsing-remitting MS will develop the third type, secondary progressive, within ten years. This form combines relapsing-remitting and primary progressive. The patient has an initial period of relapsing-remitting, and then the disease gets steadily worse. The fourth and final form is progressive-relapsing. Patients with this form have a steadily worsening disease, but also have relapses with or without recovery (2006).

With such a wide range of symptoms, diagnostic tools, and forms, there is no cure and limited treatment. The goal of the treatment is to make the quality of life better by treating the symptoms and slowing the progression of the disease. Different types of therapy and drugs are used. The drug treatments approved by the FDA are Betaseron, Avonex, Rebif, Tysabri, Copaxone and Novantrone. Novantrone is the company that produces mitoxantrone, which is a form of chemotherapy (2006).
problem with mitoxantrone is not only does it have beneficial effects on the patient but negative as well (Lori Hensley 2006). The major beneficial effect is the progression of demyelination is slowed (Tull 2006). The primary negative effect of mitoxantrone is it can only be used for the relapsing-remitting and secondary progressive forms of MS. Also, patients can only take it for two years of their lives due to its cardiotoxic affects. The specific cardiotoxic affects include a higher possibility of the patient developing congestive heart failure (Goodin, Arnason et al. 2003). Mitoxantrone is the only treatment currently available for progressive forms of the disease.

Cannabis

One drug that has been debated for medical use for years is cannabis, otherwise known as marijuana. THC (tetrahydrocannabinol) is a psychoactive drug produced from the cured flowers and the trichomes of the female cannabis plant. THC has both psychoactive and medicinal properties. Cannabis is usually taken in by either smoking or ingestion. Cannabis has been shown to help in limb muscle spasms, migraines, pain, cramps, asthma, and several other symptoms of diseases (Baker, Pryce et al. 2003). One trial showed improvement in walking and patient perception of spasticity, muscle spasms, pain, and sleep. Although some of the tests results did not show as great a change as hoped, they did support the patient's claims of improvement. It seemed the longer the trial went, the better the drug worked. The same trial also showed reduction in relapses that required hospital stays (Zajicek, Sanders et al. 2005). One source showed that during a medical trial using cannabis on MS patients, the cytokine levels, specifically interferon-\( \gamma \), interleukin-10, and interleukin-12, were measured. However, no differences were found between treated and control patients (Katona, Kaminski et al. 2005). Cytokine levels reflect the amount of inflammation in the CNS. Due to the nature of cannabis, there are not as many trials being run as there are on other drugs.

The medicinal use of cannabis has been around for thousands of years, but came to North America in the late 1800s or early 1900s. Since its entrance into the medical scene, cannabis has been
debated. The government fears the use of it for medicinal purposes will spiral out of control, but they too have gone back and forth on the use of *cannabis*. The government first dealt with this issue in 1854 when *cannabis* was on the US Dispensary lists as a remedy for neuralgia, depression, hemorrhage, pain relief, and muscle spasm. In 1870 it was on the list of US Pharmacopoeia as a medicine. The government’s standing shifted in August of 1937 with the Marijuana Tax Act that put a tax on transactions dealing with marijuana. This act did not outlaw marijuana, but made it too expensive for physicians to prescribe to their patients. In 1941, *cannabis* was removed from the US Pharmacopoeia list. However, the Common Law Doctrine of Necessity gave Robert Randall the right to use marijuana to treat his glaucoma. In 1978, two years after Randall was given this right, the National Institute of Drug Abuse (NIDA) started. The NIDA allowed patients to receive marijuana from the government. However, this policy was reversed in 1992. Another setback to the medicinal use of *cannabis* includes former Presidents Gerald Ford, Jimmy Carter, and George Bush publishing letters in 1996 opposing the legalization of marijuana for the reason it would “undermine youth drug prevention programs” (History). The above government actions are just a few of the actions that have taken place involving the medicinal legalization of *cannabis*. Other debates about *cannabis* include the morality of its use and the side effects that seem to occur from use over time.

*Ajulemic Acid*

Because *cannabis* is such a debated drug, researchers are currently looking for a drug that has similar effects but is less controversial. One of these drugs is ajulemic acid (AJA). AJA is a cannabinoid-derived structure that has no evidence of the psychotropic actions common with *cannabis* (Burstein 2005). In other words, it should have the positive effects of *cannabis* without the negative.

AJA when tested with rats showed relief of pain and inflammation. It also prevented destruction of inflamed joints. Because AJA does not have the psychoactive properties of *cannabis*, it
has low dependence liability. Rats were given AJA for 14 days and then observed for opiate withdrawal effects; none were observed. After the 14-day trial, tests were run for the level of carcinogenicity. AJA was found to have none (Burstein 2005).

Another problem that most anti-inflammatory medications have is production of ulcers. Rats given high doses of AJA (up to 1000 mg/kg) had no ulcers form; this could be due to the lack of inhibition of cyclooxygenase-1 (Cox-1) in AJA. Cox-1 is a protein that acts as an enzyme to help protect the inner stomach. It does this by speeding up production of chemical messengers called prostaglandins that work in cells responsible for inflammation to protect them. After tested in rats, a phase 2 human trial was run with chronic, neuropathic pain patients, and the data supported AJA as a safe, well-tolerated drug with no adverse effects (Burstein 2005). The only side effects observed were dry mouth and tiredness (Burstein, Karst et al. 2004).

In theory, AJA would be the answer to everyone’s problems with MS treatment. It would help MS patients with symptoms and progression without a time limit on usage. Christians and people with moral dilemmas should have no problem with AJA because it does not have psychoactive effects, which would also relieve the fears of the message being sent to young people. Also, the government would be able to approve the drug without fear of it being a gateway drug to other illegal substances.

Materials and Methods

Basic Procedure:

Throughout my research, the protocol of experiments changed depending on the type of treatment being tested on our HAPI cells, but the basic process was the same. After growing cells in flasks for several days, they were placed into well plates for a day to adhere to the bottom of the plate. Once the cells adhered, treatment was performed. The treatment usually started with the antagonist of choice, followed by the agonist of choice 30 minutes later. Once the agonist and antagonist had time to react and bind to the receptor sites, lipopolysaccharide (LPS) was added to induce the inflammatory
response from the cells. The cells were incubated for a day and analyses were performed. The first analysis ran was a nitrite assay to determine the level of nitric oxide released by the cells. The higher the nitrite levels, the more inflammatory response the cells experienced. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay was run in addition to the nitrite assay as an internal control. If the cells showed similar viability in all experimental conditions, then our experimental data was accurate, but if the cells varied significantly in viability, our data was skewed. Both the nitrite and MTT analyses were inexpensive and easy to perform; therefore, they were done first. If good results were obtained, a number of ELISAs could be performed with other cytokines such as IL-6, TNF-α, MCP-1, IL-1B and IL-12p40.

**Primary Cell Culture**

Cerebral cortices from one- to three-day-old C57BL/6 mice were excised, meninges removed, and cortices minced into small pieces. Cells were separated by trypsinization followed by trituration of cortical tissue. The cell suspension was filtered through a 70-μm cell strainer to remove debris. Cells were centrifuged at 153×g for 5 min at 4 °C, resuspended in Dulbecco’s modification of Eagle’s medium (DMEM) containing 10% FBS, 1.4 mM l-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, OPI medium supplement, and 0.5 ng/mL recombinant mouse GM-CSF, and plated into tissue culture flasks. Cells were allowed to grow to confluence (7–10 days) at 37 °C/5% CO₂. Flasks were then shaken overnight (200 rpm at 37 °C) in a temperature-controlled shaker to loosen microglia and oligodendrocytes from the more adherent astrocytes. These less adherent cells were plated for 2–3 hours and then lightly shaken to separate oligodendrocytes from the more adherent microglia. Microglia were seeded in 96-well plates (4 × 10⁴ cells/well) and incubated overnight at 37 °C/5% CO₂. Astrocytes were recovered by trypsinization and seeded into 96-well plates (1 x 10⁵ cells/well).
Cell Culture

HAPI microglia cells were cultured at 37°C and 5.0% CO₂ in DMEM with Earle’s salts containing a final concentration of 10% fetal bovine serum and 1.38 mM L-glutamine.

Determination of Cell Viability

Cell viability was measured with an MTT assay, measuring mitochondrial activity. MTT solution was prepared by diluting MTT 1:50 with culture medium. Cell culture media was removed from cells and replaced with 500 µl MTT solution and incubated at 37 °C for one hour. MTT solution was removed and cells were lysed by adding 500 µl DMSO/well. Plates were rocked for 30 minutes and read on a plate reader at 570 nm.

Determination of Nitrite Production

Cells were plated in a 24-well plate at 3.68 x 10⁵ cells/well and incubated for 24 hours before treatment with the antagonist of interest. Cells were then incubated for 30 minutes, and the agonist of interest was added. Cells were then incubated an additional 1-4 hours before inducing activation of HAPI microglial cells with LPS. Media was harvested from wells after 24 hours; 50 µl of each sample was added to a 96-well plate with 50 µl Gries reagent and read on a plate reader at 550 nm.

ELISA Assays

Media was harvested from cells treated as above and used in enzyme-linked immunosorbent assays (ELISA) according to manufacturer’s recommendations (BD Biosciences-Pharmingen).
Results

Prior to my research, Dr. Hensley's lab found AJA does suppress the inflammatory response of cultured microglia without being toxic to the cells. This finding was determined by inducing an inflammatory response, using LPS (a substance found in bacterial cell walls) in microglial cells that were pre-treated with AJA. Figure 1 shows the MTT assay illustrating that AJA was not toxic to the cells. AJA has shown to be a specific suppressor in the fact that it suppresses some cytokines, doesn’t suppress some, and enhances others. In the HAPI system, AJA suppressed nitric oxide (figure 2). In the primary microglia, AJA showed signs of suppressing MCP-1 and IL-1B (figure 3). MCP-1 in HAPI cells showed no suppression and there was a small hint of enhancement (figure 4). IL-1B is a cytokine that is not released by HAPI cells and therefore must be tested in primary microglia. It is suppressed in both primary astrocytes and microglia. In the HAPI system, AJA does not suppress IL-6 (figure 5). AJA showed no effect on the response of TNF-α in HAPI cells (figure 6), but showed enhancement in primary astrocytes (data not shown). Since TNF-α is needed for repair this could be a good sign that AJA suppresses the worst cytokines and enhance the cytokines with repair mechanisms.
FIG 1. Ajulemic acid does not affect the viability of HAPI microglial cells induced by LPS. Cells were pre-treated for 4 hours with the indicated concentrations of ajulemic acid. LPS was added at 0.5 mg/ml and 24 hours later cell viability was determined. Values represent the average for triplicate cultures and standard errors are indicated.
FIG 2. Ajulemic acid inhibits LPS induction of nitrite in HAPI microglia cells. Cells were pre-treated for 4 hours with the indicated concentrations of ajulemic acid. LPS was added at 0.5 mg/ml and 24 hours later the concentration of nitrite in the culture media was determined. Values represent the average for triplicate cultures and standard errors are indicated. Comparisons were made to LPS-only treated cultures. Asterisks indicate statistical significance of $p<.05$. 
FIG 3. Ajulemic acid inhibits LPS induction of IL1-B in primary microglia. Cells were pre-treated for 4 hours with the indicated concentrations of ajulemic acid. LPS was added at a final concentration of 0.5 mg/ml and 24 hours later the concentration of IL-6 in the culture media was determined. Values represent the average for triplicate cultures and standard errors are indicated. Comparisons were made to LPS-only treated cultures. Asterisks indicate statistical significance of $p < .05$. 
FIG 4. Ajulemic Acid shows no inhibition of LPS induction of MCP-1 in HAPI microglia cells.

Cells were pre-treated for 4 hours with the indicated concentrations of ajulemic acid. LPS was added at a final concentration of 0.5mg/ml and 24 hours later the concentration of MCP-1 in the culture media was determined. Values represent the average for triplicate cultures and standard errors are indicated. Comparisons were made to LPS-only treated cultures. Asterisks indicate statistical significance of $p < .05$. 
FIG 5. Ajulemic acid does not inhibit LPS induction of IL-6 in HAPI microglia cells. Cells were pre-treated for 4 hours with the indicated concentrations of ajulemic acid. LPS was added at a final concentration of 0.5 mg/ml and 24 hours later the concentration of IL-6 in the culture media was determined. Values represent the average for triplicate cultures and standard errors are indicated. Comparisons were made to LPS-only treated cultures. Asterisks indicate statistical significance of $p<.05$. 
FIG 6. Ajulemic acid shows no effect on LPS induction of TNF-α in HAPI microglia cells. Cells were pre-treated for 4 hours with the indicated concentrations of ajulemic acid. LPS was added at a final concentration of 0.5 mg/ml and 24 hours later the concentration of TNF-α in the culture media was determined. Values represent the average for triplicate cultures and standard errors are indicated. Comparisons were made to LPS-only treated cultures. Asterisks indicate statistical significance of $p<0.05$. AJA shows enhancement of TNF – α in primary astrocytes (data not shown).
Based on these findings, the search for how AJA works began. When I started working on AJA, we knew that AJA worked to selectively suppress the inflammatory response, but we wanted to know how or through what receptor. Our hypothesis was that AJA was working through the PPAR-γ receptor. AJA has been shown to bind to the ligand domain of the human PPAR-γ in previous experiments (Liu, Li et al. 2003; Ambrosio, Dias et al. 2007). The problem with this hypothesis is that it is very difficult to test this specific receptor. The first item on the agenda was to rule out the cannabinoid receptors, which would give more support to the hypothesis. There are two cannabinoid receptors, CB1 and CB2. CB1 is known to be the receptor mediating psychotropic effects while CB2 is thought to mediate immune responses; therefore, we wanted to look at CB2. We had to set up a positive control using known CB2 agonists and antagonist, which would be used to compare AJA’s effects on the cells. Agonists are compounds that are known to bind to and activate the specific receptor being examined. Antagonists are compounds that are known to bind to the same receptor competitively and block the effects of the agonist. Three different agonists (L-759,633, GW 405,833, CP 55,940) were used, but none behaved in a manner that would serve as a good positive control. One of the systems we tried was the agonist L-759,633 and the antagonist AM630. After the concentration was found at which L-759,633 was effective to suppress LPS-induced nitrite release (figure 7), it was tested with AM630 concentrations (figure 8). The problem with these concentrations was that AM630 was found to be toxic at concentrations above 10 μM and L-759,633 was not effective until 120 μM. Because the agonist’s concentration was so much larger than the antagonist concentration, the agonists overwhelmed and bound to the sites despite the presence of the antagonist. A positive control was needed for comparative purposes. If we merely used the CB2 antagonist AM630 with AJA and did not abrogate the suppression of nitrite we normally observed, we could assume these effects were not mediated by CB2. However, it could be that no effect was seen because something in the design of our experiment was not optimal. A positive control would have provided validity to a negative result.
FIG 7. Concentrations of L-759,633 were tested to find the minimal concentration necessary for suppression of LPS-induced nitrite release. Cells were pre-treated for 3 hours with the indicated concentrations of L-759,633. LPS was added at a final concentration of 1 μg/ml and 24 hours later the concentration of nitrite in the culture media was determined. Values represent the average for triplicate cultures and standard errors are indicated. Comparisons were made to LPS-only treated cultures. Asterisks indicate statistical significance of $p<.05$. Later experiments showed a dosage of 120 μM to be optimal for both repression of nitrite and viability.
FIG 8. AM630 did not abrogate the suppression effects of L-759,633. Cells were pre-treated for 30 minutes with the indicated concentrations of AM630 before adding 120 μM of L-759,633 for an additional 3 hours. LPS was added at a final concentration of 1 μg/ml and 24 hours later the concentration of nitrite in the culture media was determined. Values represent the average for triplicate cultures and standard errors are indicated. Comparisons were made to LPS-only treated cultures. Asterisks indicate statistical significance of $p<.05$. 
One problem that was brought to our attention was that lipopolysaccharide (LPS), which we used to induce the inflammatory response in our cells may down-regulate the CB2 receptor (Lee, Newton et al. 2002). If this was true, it would explain why we were not able to set up a positive control with LPS. We switched to using TNF-α and IFN-γ to induce the inflammatory response, but the results showed no difference between it and LPS. We would have expected that the TNF- α and IFN- γ results would show more suppression of cytokines if LPS down-regulated CB2 and our effects were mediated through this receptor, but it showed no signs of this (figure 9).

![Graph](image)

**FIG 9.** TNF- α and IFN- γ induced inflammatory response instead of LPS induction did not alter suppression of nitrite by AJA. Cells were pre-treated for 3 hours with the indicated concentrations of AJA. TNF- α and IFN- γ was added at a final concentration of 100 units/ml of IFN – γ and 500 units/ml of TNF- α and 24 hours later the concentration of nitrite in the culture media was determined. Values represent the average for triplicate cultures and standard errors are indicated. Comparisons were made to TNF- α and IFN- γ -only treated cultures.
Discussion

Despite the struggles and disappointments of our positive control testing, we still have some support for AJA working through PPAR-\(\gamma\). CB receptors are known to work at nanomolar amounts, and we have only seen suppression at micromolar amounts. This supports that our effects were neither CB1 nor CB2 mediated. A problem mentioned earlier, about LPS down-regulating CB2, was not supported by our data because when TNF-\(\alpha\) and IFN-\(\gamma\) were used to induce the inflammatory response there was no increase in suppression by AJA seen. Although none of these results were what we expected, they still support our original idea.

The research field for cannabinoid derivatives is still wide open. For example, ajulemic acid is currently being used to kill neural derived cells with anti-tumor properties. This could lead to alternate treatments or cures for some types of cancer. AJA is also being used in clinical trials for neuropathic pain and muscle spasms. Canabidiol, another cannabinoid, is currently being used in pain trials and is showing some of the same positive effects of AJA(Rog, Nurmikko et al. 2007). Other cannabinoid receptors such as GPR55 are being found as well(Baker, Pryce et al. 2006). We may not have been able to find the receptor AJA mediated its response through because it has not yet been found.

The good news for MS patients is that research is continuing and expanding. Currently in the lab, research is still being done on AJA and another potential treatment, resveratrol. Research is being conducted on the neuroprotection capabilities of these drugs. Although it might seem our research ended the opposite of what we expected, it did not end hopelessly. Research is an ongoing process where all experiments move us one step closer to understanding the disease and potential treatments. The more knowledge that researchers have, the more likely we will see advancement in the treatment of multiple sclerosis.
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