ISSN 2616-5791 Vol.4, Issue 1: 25-33, DOI: 10.25236/AJMHS.2023.040105

The formation mechanism of lewy body at DMV related to parkinson's disease

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Abstract: *The purpose of this experiment is to demonstrate the mechanism of Lewy body aggregation and formation in DMV by gene modification and injection of exogenous preformed fibrils, and how this relates to Parkinson's disease. Through a series of work such as cardiac perfusion, frozen section, and immunohistochemical staining, the existence of cholinergic neurons and Lewy bodies at the DMV was finally confirmed by 63x laser confocal microscopy, which explained the symptoms of the digestive system during the early stage of Parkinson's disease.*

Keywords: *Lewy body, Parkinson's disease, DMV, Alpha-synuclein*

1. Introduction

A neurodegenerative disease that is commonly seen in the elderly, Parkinson's disease is characterized by unintentional movements, for example, resting tremors, muscle rigidity, balance impairments, instability, Parkinson's gait etc., as well as cognitive issues, such as dementia and depression. Patients with Parkinson's disease usually suffer from slowly developing symptoms that worsen with age. [1] One percent of the population over 60 has Parkinson's disease.[2] Research on Parkinson's disease is imperative, not only because it could have a fatal effect on a multitude of people, but also because it cannot be fully cured. In spite of the fact that nearly anyone could be at risk of developing Parkinson's disease, some studies have identified certain risk factors can increase the possibility of the disease like age, sex, heredity, exposure to toxins, etc. [3]

Although the underlying causes and treatments for Parkinson's are still unknown, many researches have been conducted in the 200 years since Parkinson was first discovered by a British doctor James Parkinson in 1817 and a lot has been accomplished concerning the pathogenesis and potential treatments. [4] Treatment with the drug levodopa, which is converted into dopamine in the brain, was successful in the 1960s. Other drugs have been developed since the discovery of levodopa, with the effect of either enhancing dopamine's functions , inhibiting its breakdown, or prolonging its duration. [5] Lewy bodies, a pathogenesis of Parkinson's disease, were also discussed. A Lewy body is an eosinophilic cytoplasmic inclusion that resembles a spherical mass in the cytoplasm. It has a dense core that is encircled by a halo of radiating fibrils that are 10 nm wide. It is an aberrant α-synuclein aggregate. [6]

α-synuclein is a major protein associated in neurodegenerative disorders such as Lewy Body dementia and Parkinson's disease, which are referred to as synucleinopathies. α-synuclein assembles into larger aggregates known as Lewy bodies in these diseases. α-synuclein is a 14 kilodalton protein discovered in neurons' presynaptic terminals and the substantia nigra region of the brain. [7] Its purpose is not entirely clear. The non-amyloid component, or NAC domain of α-synuclein has a hydrophobic middle section that is essential for aggregation. Numerous oligomeric species can be created by the combination of α-synuclein monomers. Aggregation continues as the oligomers develop into soluble protofibrils or filaments. The structure of filaments can evolve from an alpha helix to a beta sheet and then to insoluble fibrils. The accumulation of these insoluble fibrils forms Lewy bodies. [8] During the early stage of Parkinson's disease, Lewy bodies were most prominent in the DMV(dorsal motor nucleus of the vagus) and anterior olfactory bulb, causing resting tremors, stomach pain and loss of smell. [9] As the disease progresses, Lewy bodies spread to other areas of the brain through synaptic transmission between neurons in the circuits.

My study was done by observing at brain slices stained using immunohistochemistry and DAPI at DMV of a CHAT-cre, LSL-A53T-GFP gene-edited mouse which is stereotactically injected in the DMV with PFF(preformed fibrils). This enables us to use optical microscopy and confocal laser scanning microscope(CLSM) to see the presence and specific locations of Lewy bodies in the DMV, so as to prove

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the formation mechanism of Lewy bodies in relation to Parkinson's disease.

2. Materials and Methods

2.1. Animal Modelling

A living non-human animal model mimics aspects of a biological process or disease found in humans through the use of genetic engineering or other interventions. [10] It is necessary to use animal models to study human diseases in order to gain a better understanding of the disease and avoid the risk of harming real people.

In this experiment, a mouse model will be used to simulate the formation and spread of Lewy bodies within the mouse brain. The genes of humans are similar to those of mice, and the differences between individuals are not significant. Mice are inexpensive, easy to raise, and have a fast reproductive cycle.

To simulate the formation and spread of Lewy bodies, we needed to cross two gene-edited mice to achieve the A53T mutation similar to that of Parkinson's patients, and inject PFF to give the mouse physiological characteristics similar to those of Parkinson's patients.

2.1.1. Genetic Engineering

The mouse used in this experiment is crossbred from a CHAT-cre mouse and a floxed mouse carrying the LSL-A53T-GFP gene sequence. Through CHAT-Cre, the floxed allele is recombined within neurons to achieve gene knockout and drive the expression of the floxed transgene. LoxP-Stop-LoxP cassettes enable Cre-mediated conditional expression of genes in mammalian cells and animals. [11] The LSL expression cassette contains three repeats of SV40 polyA with LoxP sites at both ends of the sequence. In the absence of Cre recombinase, the expression cassette completely blocks the normal expression of the target gene.

When LSL-carrying transgenic animals are bred with transgenic animals that express Cre on the cholinergic neurons, the offspring will only express Cre in cholinergic neurons, deleting the stop sequence in the floxed mouse and allowing the A53T -GFP gene sequence to be transcribed.

The A53T mutation was discovered in 1997 in the α-synuclein gene, which codes for a presynaptic protein considered to be important in neural plasticity. [12] GFP codes for green fluorescent protein which enable cre target cells to be clearly distinguished under the microscope when exposed to light in the blue to ultraviolet range.

2.1.2. Stereotactic Injection

The stereotactic injection allows researchers to precisely target deep structures within the brain utilizing a stereotactic atlas, which gives the 3D coordinates of each area in relation to anatomical landmarks on the skull. [13] Anesthetized animals are placed on a stereotaxic frame once the skull has been exposed. This device enables the accurate placement of experimental equipment at the predetermined coordinates.

By precisely injecting PFF into the mouse's DMV using this method, we can witness how the fibrils change into Lewy bodies. Because they seed and drastically speed up the aggregation of alpha synuclein monomers, PFFs are effective in developing disease models. [14] Injection of PFFs into rodent brains or addition of PFFs to primary neurons both cause aggregation and pathology of alpha synuclein. This Lewy body pathology spreads from the injection site and can be identified with antibodies.

Pathology can be induced in animals roughly 10–15 times faster by injecting PFFs than by using transgenic models. Additionally, α-synuclein PFFs can be attached to fluorescent dyes for in vitro tracking.

2.2. Obtaining the Brain Slices

In order to observe the formation of Lewy bodies in the DMV area of the mouse’s brain, I need to cut the brain into slices with a thickness of 40 micrometers through transcardiac perfusion, brain dissection, cultivation and frozen section, allowing for microscope examination.

2.2.1. Transcardiac Perfusion

Transcardiac perfusion, which uses the vasculature to accomplish systemic fixative distribution in the

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living animal, is a widely used standard for tissue fixation. [15]

To remove blood and preserve brain tissue for immunostaining or in situ hybridization, transcardiac perfusion with saline and 4% paraformaldehyde (PFA) is frequently utilized. The vascular system should be flushed with saline to remove the blood. Blood contains a variety of components, including protein, blood cells, sugar, etc., which may interfere with the subsequent procedure. PFA decomposes into formaldehyde in solution, which crosslinks DNA and protein molecules to maintain the structural integrity of cells and tissues.

I first prepared saline and PFA and placed them in a suitable environment with the proper temperature (saline in 37 degrees Celsius, PFA in zero degrees Celsius). Mice were weighed and given urethane anesthesia prior to proceeding the surgery. Pinching the tail to ensure it is entirely anesthetized before putting it on a surgical plane. If a DNA sample is required, I also need to cut off the tip of the tail for about 2 millimeters. I made the initial incision in the abdomen, then cut through the midline to the xiphoid process. Carefully cut the ribs on both sides of the sternum to open the thoracic cavity and reveal the heart. Insert the needle into the left ventricle while the infusion tube is submerged in saline at 37°C. In order to allow for blood depletion, a portion of the liver was cut. I transferred the tubing from saline to PFA for tissue fixation after 10 to 15 minutes. 4% PFA can stiffen tissue and facilitate sectioning. It effectively preserves proteins in tissues and cells. Additionally, it preserves antigens for immunohistochemistry, preventing their inactivation or diffusion.

2.2.2. Brain Dissection and Cultivation

I used scissors to decapitate the mouse's head. First, a midline cut in the skin from the neck to the nose is made in order to reveal the skull. Using tweezers or scissors, I gradually removed all hair, skin, and tissue from the skull. Following delicate scraping along the skull's sagittal and lambdoid lines using scalpel, I carefully lifted the skull along the score using forceps, and peel off the skull. Avoid damaging the brain's tissue during operation.

To dry and maintain the integrity of the brain for subsequent frozen sectioning, I stored the brain in PFA and then transferred it into a 30% sucrose solution for further fixation and dehydration.

2.2.3. Frozen Section

Frozen sectioning is a technique that involves fast cooling the tissue with an embedding medium OCT at a low temperature before cutting it into pieces. The cryostat is a crucial piece of equipment for cryosectioning.

In order to mount the dehydrated brain on the chuck, I first cut a plane into the frontal region close to the olfactory bulb. OCT embedding medium was used to freeze the brain. Fix the chuck on the cryotome when the embedding medium has frozen and turned white, and then rotate the handle to direct the cryotome to slice the brain into 40-micron slices. The slices should be put in a Petri dish with phosphate-buffered saline (PBS). It can be terminated after finishing cutting the DMV area that needs to be observed. Wrap the petri dish with the slices in foil and store in the refrigerator.

2.3. Staining

In order to distinguish the seemingly uniform or identical regions of the brain and to investigate the cellular, structural, and molecular composition of tissues and organs, I used immunohistochemical staining in the experiments in order to observe the antigen that the antibody was targeting; I also used DAPI staining to observe the presence of nerve cells.

2.3.1. Immunohistochemistry (IHC)

Immunohistochemistry is the application of the principle of specific antigen and antibody combination to identify the antigen in tissue cells. [16]

We need to immerse the brain slices in a blocking buffer prior to immunohistochemistry staining. A mixture of goat serum and PBST (PBS+triton-x-100) serves as the blocking buffer. It induces many interfering proteins to bind to non-specific antibodies in serum first. As a result, background interference is decreased, binding sensitivity is improved, and the primary antibody is more likely to attach specifically to the target antigens.

Two primary antibodies were employed in this experiment: anti-p-α-synuclein from rabbits and anti-ChAT from goats. Anti-p-α-synuclein antibodies produced from rabbits can bind to phosphorylated α-synuclein as well as PFF and Lewy bodies in the mouse brain. To promote the combination of antigen

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and antibody, anti-p-α-synuclein must be diluted with PBST, which is done by adding Triton-x-100 to PBS. The ratio of dilution is 1:2000. The second primary antibody is goat anti-ChAT, which is an antibody that can bind to cholinergic neurons in the brain. Anti-ChAT also needs to be diluted with PBST, the dilution ratio is 1:500. After dilution, DMV-specific brain slices were selected, immersed in the solution that contains PBST, anti-p-α-synuclein, and anti-ChAT and then shaken for six hours using a shaker. The excessive primary antibody should be removed using PBST, shaken for ten minutes at room temperature using a shaker. This procedure needs to be repeated three times.

After incubating and washing off the primary antibodies, I used two secondary antibodies which can combine with the primary antibodies. The secondary antibodies are marked with fluorescent dyes that transform short wavelengths of excitation light into longer wavelengths of emission light, a visible, bright-colored fluorescent light after absorption of a particular wavelength of light. Consequently, it enables us to locate the antigen and witness the presence of Lewy bodies and cholinergic neurons that the primary antibodies bind to under a microscope. Donkey anti-rabbit 550 and donkey anti-goat 633 were the two secondary antibodies utilized in the experiment. They must be diluted in PBST at a ratio of 1:500 as well.

2.3.2. DAPI Staining

DAPI is a fluorescent dye that can attach to the AT base pair of double-stranded DNA across the cell membrane. A DAPI molecule can take up three base pairs' spaces. [17] Under a microscope, nerve cells can be detected by fluorescence because double-stranded DNA increases the fluorescence intensity of DAPI molecules tied to it by a factor of roughly 20. [18] The fluorescence intensity can also be used to calculate the amount of DNA present. When DAPI dye is stimulated by UV light for fluorescence microscope observation, the wavelength range of the emission light spans from blue to turquoise.

In the experiment, PBS was used to dilute DAPI in the ratio of 1:5000. I submerged the brain slices in the DAPI and PBS solution and then placed it on a shaker for 15 minutes at room temperature. After incubating DAPI, brain slices are soaked in PBS and shaken at room temperature for 15 minutes to remove any DAPI excess.

3. Results and Analysis

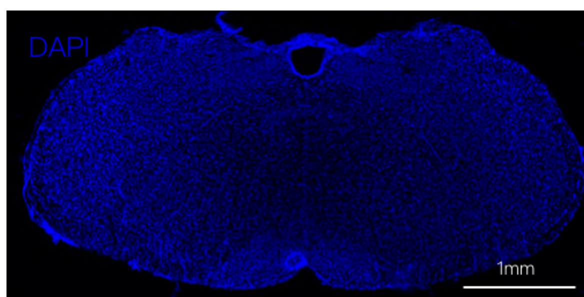

Figure 1: DAPI stained brain under 10x microscope

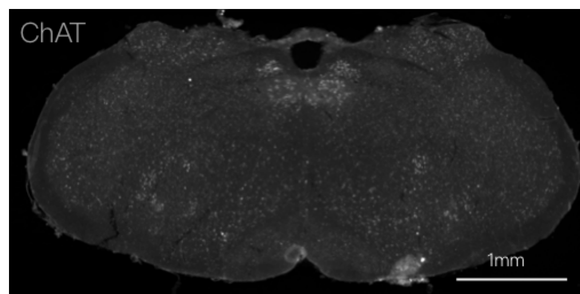

Figure 2: Immunochemistry of cholinergic neurons under 10x microscope

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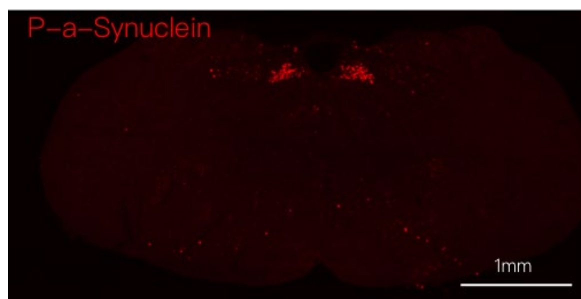

Figure 3: Immunochemistry of p-α-synuclein under 10x microscope

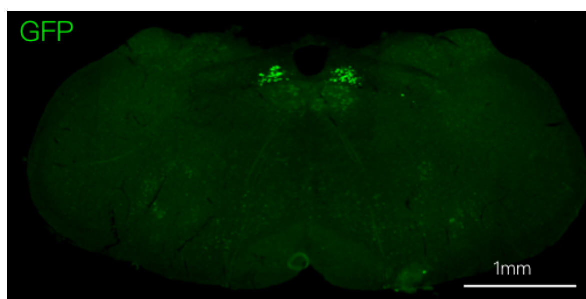

Figure 4: Immunochemistry of green fluorescent protein under 10x microscope

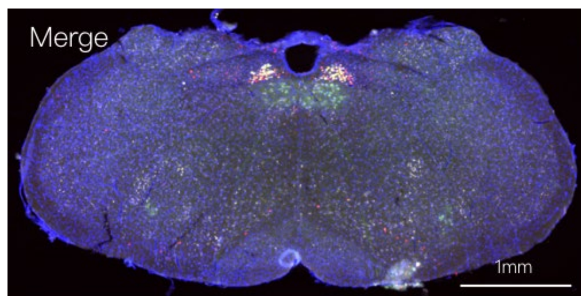

Figure 5: Merge image of figure 1 to figure 4

The images in these five figures were scanned using a 10x microscope (figures 1 to 5). Multiplexing fluorescence imaging allows us to see the sample's different components.

Figure 1 is an image of all cells in a brain slice marked by DAPI. We can see that there are many fine dots, those are the nuclei stained with DAPI.

Figure 2 is an image of cholinergic neurons stained using immunohistochemical technique with the combination of secondary antibody donkey-anti-goat 633 and primary antibody goat-anti-ChAT, and the combination of primary antibody and antigen. Here we can observe that the area of the DMV is relatively whitish. This represents the presence of cholinergic neurons in the DMV region.

Figure 3 is an image of phosphorylated a-synuclein using the combination of secondary antibody donkey-anti-rabbit 550 and primary antibody rabbit-anti-p-α-synuclein. In this image we can see that most of the red fluorescence is concentrated in the DMV region. This indicates the existence of phosphorylated a-synuclein in the DMV region, perhaps in the form of fibrils, oligomers, or Lewy bodies, which we could not observe and identify under the 10x microscope.

Figure 4 represents cholinergic neurons in which GFP (green fluorescent protein) and A53T sequences are present. Cre recombinase in these cells deletes the stop sequence, enabling expression of GFP and A53T mutations. It can be seen that green fluorescence is in the DMV region in the image, and there is also some fainter fluorescence in the hypoglossal nucleus. This indicates that there are cholinergic neurons with the A53T sequence in these regions.

Figure 5 is the overlay image of all figures. It can be seen that there is yellow color in the DMV area, which is the colocalization of anti-p-α-synuclein and GFP, indicating that there are acetylcholine neurons with phosphorylated a-synuclein in the DMV. There is also colocalization of GFP and anti-ChAT in the hypoglossal nucleus, indicating the presence of A53T choline neurons in these places.

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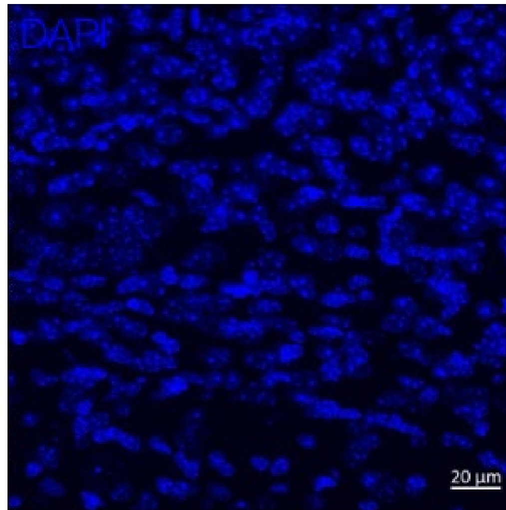

Figure 6: DAPI stained neurons under 63x confocal microscope

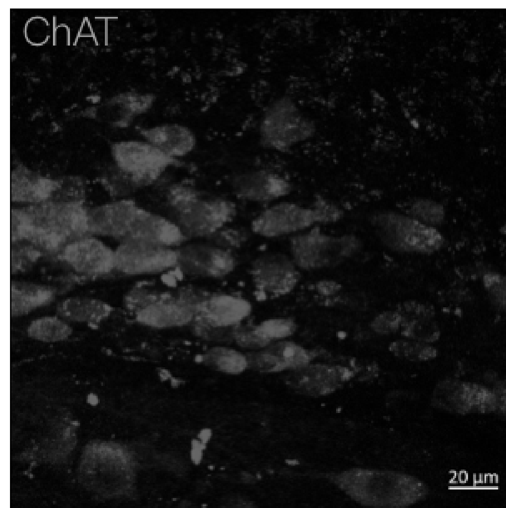

Figure 7: Immunochemistry of cholinergic neurons under 63x confocal microscope

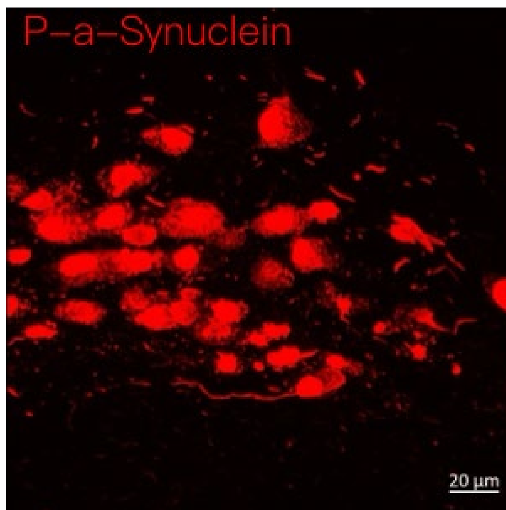

Figure 8: Immunochemistry of p-α-synuclein under 63x confocal microscope

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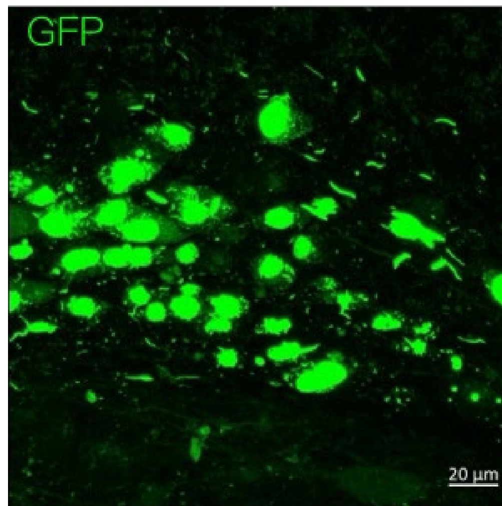

Figure 9: Immunochemistry of green fluorescent protein under 63x confocal microscope

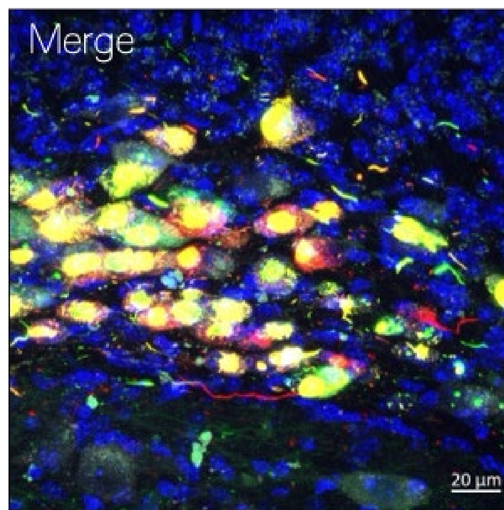

Figure 10: Merge image of figure 6 to figure 9

These five figures are images captured by a 63x laser confocal microscope (Figure 6-10). The DMV is the area being scanned.

Figure 6 shows neurons marked with DAPI staining. It is evident that this one can see more details than the 10x image scanning. You can see that some cells have more intense fluorescence, the DNA is more abundant in these nuclei.

Figure 7 is an enlarged image of choline neurons stained with goat-anti-ChAT and donkey-anti-goat 633 antibodies at the DMV.

Figure 8 shows an image of phosphorylated α-synuclein stained by rabbit-anti-p-α-synuclein and donkey-anti-rabbit 550. The fibrous, thread-like α-synuclein, and clump-like Lewy bodies—a circular core surrounded by yarn-like filaments—are clearly visible in this image. We need to look at other images for further confirmation.

Figure 9 is a green fluorescent protein targeted cholinergic cell containing the A53T gene sequence. This figure has many similarities with Figure 8. Along with numerous fibrous masses like Lewy bodies, this picture also contains a few scattered particles that resemble threads.

Figure 10 is an overlay image of all the pictures. There are a multitude of colocalizations on this graph. Many cholinergic neurons containing phosphorylated a-synuclein and some a-synuclein fibers can be clearly seen. These colocalized clusters of α-synuclein can be identified as Lewy bodies.

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4. Discussion and Conclusion

The results of this experiment demonstrate how Lewy bodies form in the mouse brain. The insertion mutant gene sequence and the exogenous preformed fibrils were used to create an animal model for the investigation of Parkinson's disease pathogenesis. Preformed fibrils introduced exogenously cause endogenous alpha-synuclein to misfold without the overexpression of alpha-synuclein. Preformed fibrils trigger the creation of new fibers, causing the fibers to rearrange and aggregate into the inclusion structure—Lewy body.

The experiment shows that phosphorylated alpha-synuclein co-localized with cholinergic cells in DMV brain areas. This suggests that cholinergic nerves exist in the DMV brain region. More than 80% of the neurons in the DMV project to the stomach, and the majority of them are cholinergic in nature, according to the paper "Spatial organization of neurons in the dorsal motor nucleus of the vagus synapsing with intragastric cholinergic and nitric oxide/VIP neurons in the rat." [19] Both gastric contraction and relaxation can be mediated via the vagus nerve pathway to the stomach. This explains why early-stage Parkinson's patients experience digestive symptoms such constipation, nausea, and stomach pain.

References

[1] Jankovic J. (2008). Parkinson's disease: clinical features and diagnosis. Journal of neurology, neurosurgery, and psychiatry, 79(4), 368–376. https://doi.org/10.1136/jnnp.2007.131045

[2] Obeso, J. A., Stamelou, M., Goetz, C. G., Poewe, W., Lang, A. E., Weintraub, D., Burn, D., Halliday, G. M., Bezard, E., Przedborski, S., Lehericy, S., Brooks, D. J., Rothwell, J. C., Hallett, M., DeLong, M. R., Marras, C., Tanner, C. M., Ross, G. W., Langston, J. W., Klein, C., … Stoessl, A. J. (2017). Past, present, and future of Parkinson's disease: A special essay on the 200th Anniversary of the Shaking Palsy. Movement disorders: official journal of the Movement Disorder Society, 32(9), 1264–1310. https://doi.org/10.1002/mds.27115

[3] Ascherio, A., & Schwarzschild, M. A. (2016). The epidemiology of Parkinson's disease: risk factors and prevention. The Lancet. Neurology, 15(12), 1257–1272. https://doi.org/10.1016/S1474-4422(16)30230-7

[4] Beitz J. M. (2014). Parkinson's disease: a review. Frontiers in bioscience (Scholar edition), 6(1), 65–74. https://doi.org/10.2741/s415

[5] Fahn S. (2008). The history of dopamine and levodopa in the treatment of Parkinson's disease. Movement disorders: official journal of the Movement Disorder Society, 23 Suppl 3, S497–S508. https://doi.org/10.1002/mds.22028

[6] Wakabayashi, K., Tanji, K., Odagiri, S., Miki, Y., Mori, F., & Takahashi, H. (2013). The Lewy body in Parkinson's disease and related neurodegenerative disorders. Molecular neurobiology, 47(2), 495–508. https://doi.org/10.1007/s12035-012-8280-y

[7] Burré, J., Sharma, M., & Südhof, T. C. (2018). Cell Biology and Pathophysiology of α-Synuclein. Cold Spring Harbor perspectives in medicine, 8(3), a024091. https: // doi. org/ 10. 1101/ cshperspect. a024091

[8] Mehra, S., Sahay, S., & Maji, S. K. (2019). α-Synuclein misfolding and aggregation: Implications in Parkinson's disease pathogenesis. Biochimica et biophysica acta. Proteins and proteomics, 1867(10), 890–908. https://doi.org/10.1016/j.bbapap.2019.03.001

[9] Braak, H., Del Tredici, K., Rüb, U., de Vos, R. A., Jansen Steur, E. N., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. Neurobiology of aging, 24(2), 197–211. https://doi.org/10.1016/s0197-4580(02)00065-9

[10] Kin, K., Yasuhara, T., Kameda, M., & Date, I. (2019). Animal Models for Parkinson's Disease Research: Trends in the 2000s. International journal of molecular sciences, 20(21), 5402. https://doi.org/10.3390/ijms20215402

[11] Nagy A. (2000). Cre recombinase: the universal reagent for genome tailoring. Genesis (New York, N.Y.: 2000), 26(2), 99–109.

[12] Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., & Nussbaum, R. L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science (New York, N.Y.), 276(5321), 2045–2047. https://doi.org/10.1126/science.276.5321.2045

[13] McSweeney, C., & Mao, Y. (2015). Applying stereotactic injection technique to study genetic effects on animal behaviors. Journal of visualized experiments: JoVE, (99), e52653. https: // doi. org/ 10.

ISSN 2616-5791 Vol.4, Issue 1: 25-33, DOI: 10.25236/AJMHS.2023.040105

3791/52653

[14] Volpicelli-Daley, L. A., Luk, K. C., & Lee, V. M. (2014). Addition of exogenous α-synuclein preformed fibrils to primary neuronal cultures to seed recruitment of endogenous α-synuclein to Lewy body and Lewy neurite-like aggregates. Nature protocols, 9(9), 2135–2146. https: // doi. org/ 10. 1038/ nprot. 2014.143

[15] Gage, G. J., Kipke, D. R., & Shain, W. (2012). Whole animal perfusion fixation for rodents. Journal of visualized experiments: JoVE, (65), 3564. https://doi.org/10.3791/3564

[16] Magaki, S., Hojat, S. A., Wei, B., So, A., & Yong, W. H. (2019). An Introduction to the Performance of Immunohistochemistry. Methods in molecular biology (Clifton, N.J.), 1897, 289–298. https: // doi. org/ 10.1007/978-1-4939-8935-5_25

[17] Kapuscinski J. (1995). DAPI: a DNA-specific fluorescent probe. Biotechnic & histochemistry: official publication of the Biological Stain Commission, 70(5), 220–233. https: // doi. org/ 10. 3109/ 10520299509108199

[18] Chazotte B. (2011). Labeling nuclear DNA using DAPI. Cold Spring Harbor protocols, 2011(1), pdb. prot5556. https://doi.org/10.1101/pdb.prot5556

[19] Zhou, S. Y., Lu, Y. X., Yao, H., & Owyang, C. (2008). Spatial organization of neurons in the dorsal motor nucleus of the vagus synapsing with intragastric cholinergic and nitric oxide/VIP neurons in the rat. American journal of physiology. Gastrointestinal and liver physiology, 294(5), G1201–G1209. https://doi.org/10.1152/ajpgi.00309.2006