



The Activation of Microglia and the Generation of Reactive Oxygen Species Induced by Medial Forebrain Bundle Axotomy

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ABSTRACT

Background: We endeavored to examine the impact of reactive oxygen species (ROS) on the degeneration of dopaminergic neurons by employing the medial forebrain bundle (MFB) axotomy model, a recognized animal model of Parkinson's disease.

Methods: The MFB of Wistar rats was lesioned and animals were sacrificed at 7, 14 and 28 days post-lesion for immunofluorescent staining. Additionally, in some experimental animals, hydroethidine was injected into the abdominal cavity 30 minutes before sacrifice to examine the presence of ROS within the brain tissue and characterize the cell types responsible for their generation throughout the pathological progression.

Result: At 7 days post-surgery, an excessive accumulation of activated microglia was observed in the ipsilateral substantia nigra (SN) using antibodies OX42 and OX6, indicating their strong immunopositive reaction. Although the reactivity decreased somewhat in the 14-day and 28-day groups compared to the 7-day group, a significant number of activated microglia still congregated. Additionally, these cells exhibited strong immunopositive reactions to ED1, indicating their active phagocytic activity. Furthermore, many activated microglia showing positive reactions in OX6 immunofluorescent staining simultaneously exhibited ethidium fluorescence activity, indicating their vigorous generation and secretion of ROS.

Key words: Medial forebrain bundle (MFB) axotomy, Reactive oxygen species (ROS).

INTRODUCTION

Reactive oxygen species (ROS) are a chemically defined group of reactive molecules derived from molecular oxygen. The term ROS refers to a group of molecules that contain at least one oxygen atom and exhibit higher reactivity than molecular oxygen (O₂) (Herb and Schramm, 2021). ROS, produced by various biochemical and physiological oxidative processes in the body, are associated with numerous physiological and pathophysiological processes. They play a major role in the pathogenesis of various human diseases. At low concentrations, ROS exhibit beneficial effects by regulating intracellular signaling and homeostasis; however, at high levels, they cause significant damage to proteins, lipids and DNA (Acharya *et al.*, 2010; Xue Tong, 2020).

ROS are produced intracellularly as byproducts by mitochondria and other cellular elements and exogenously by pollutants, tobacco smoke, drugs, xenobiotics and radiation (Prasad *et al.*, 2017).

In this study, the researcher aimed to induce the gradual death of midbrain dopaminergic neurons by performing medial forebrain bundle (MFB) axotomy in Wistar rats and to examine the extent of microglial activation using immunofluorescent techniques. Additionally, by identifying the presence of ROS and the characteristics of the cells producing them in the substantia nigra (SN), the researcher aimed to suggest that excessively activated microglia could negatively impact the survival of nigral dopaminergic neurons.

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MATERIALS AND METHODS

Preparation of experimental animals

All experimental procedures were approved by the Animal Review Board of Eulji University in accordance with the National Institutes of Health (NIH Publication No. 8023, revised 1996). The study was carried out during the period of January 2021 to December 2023, at the Department of Biomedical Laboratory Science at Eulji University in Daejeon. Throughout the entire experimental process, every measure was taken to minimize the suffering of the animals and the use of more animals than necessary was avoided. Male Wistar rats weighing approximately 250-300

grams (Charles River Lab., NC, USA) were used in this study. All experimental animals were housed with free access to food and water in a controlled environment maintained at 20-22°C with consistent humidity and a 12-hour light-dark cycle. Food was withheld from the animals from the night before surgery until the day of surgery, but water was continuously provided *ad libitum*.

The experimental animals were deeply anesthetized by intraperitoneal injection of a ketamine (70 mg/kg) and xylazine (8 mg/kg) mixture, then secured in a stereotaxic apparatus (Stoelting Co., IL, USA). A 3 cm midline incision was made in the scalp and the periosteum was separated from the skull using cotton swabs to expose the bregma and lambda landmarks. The bite bar of the stereotaxic apparatus was adjusted to ensure that the bregma and lambda were on the same horizontal plane. Using the bregma as a reference point, a small hole was drilled into the skull at a point 2.8 mm posterior and 3.0 mm to the right using a dental drill. Through this hole, a cannula containing a retractable wire knife (Scouten knife; David Kopf Instruments, CA, USA) was inserted to a depth of 9.0 mm ventral to the dura mater. The wire knife was then extended 2.2 mm medially towards the midline, moved 3.0 mm dorsally, held in place for approximately one minute and then moved 3.0 mm ventrally to completely transect the right MFB (Yang *et al.*, 2018). The wire knife was then retracted and the cannula was slowly removed. The incision in the scalp was immediately sutured. A schematic representation of the MFB axotomy method is shown in Fig 1 (Fig 1A, B).

After the surgery, the experimental animals were kept in a 37°C warming environment until they fully recovered from anesthesia. No specific abnormal behaviors due to the surgery were observed in the animals. The animals were sacrificed at 7, 14 and 28 days post-axotomy. The contralateral brain, which did not undergo axotomy, was used as a control.

The animals were sacrificed at 7, 14 and 28 days post-surgery for use in the study. Following the same method, the experimental animals were deeply anesthetized and a catheter was inserted into the ascending aorta. The animals were perfused with 100 mL of 0.1 M phosphate buffer (PB, pH 7.4) followed by 400 mL of 4% paraformaldehyde for perfusion fixation. The brain was extracted and sectioned using a brain matrix (Stoelting Co.) to include the midbrain. The sectioned brain tissue was post-fixed in the same fixative solution used for perfusion fixation for 12 hours, followed by immersion in 30% sucrose solution at 4°C overnight. The following day, after confirming that the brain tissue had completely settled in the sucrose solution, the tissue was embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Tokyo, Japan), rapidly frozen using dry ice and then sectioned into continuous coronal sections of 40 µm thickness using a Cryostat Microtome (Leica Microsystems Inc., Wetzlar, Germany). The produced consecutive sections were sequentially distributed into 12 tissue wells containing 0.1 M PB solution, spaced approximately 480 µm apart, to allow for the collection of tissues forming a single staining unit. After sectioning, the tissue sections were transferred in sequence to tissue wells containing cryo-protection solution (30% glycerol, 30% ethylene glycol, 0.2 M NaOH in 0.1 M PB) and stored at -20°C until further use.

In some experimental animals, to verify the proper establishment of the animal model, the entire brain was dissected along the longitudinal fissure into left and right hemispheres. Each brain region was then frozen and sectioned into 40 µm thickness using the same method as described above.

Detection of ROS using hydroethidine

Hydroethidine (HET) is a substance that reacts with superoxide to produce a fluorescent compound called

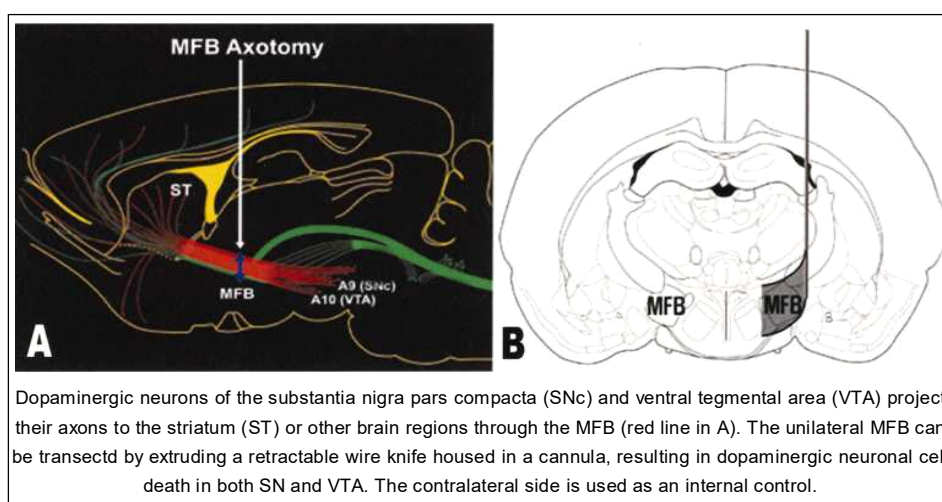


Fig 1: Sagittal (A) and coronal (B) view of the adult rat brain illustrating how the medial forebrain bundle (MFB) axotomy is conducted.

ethidium, making it highly sensitive for detecting oxidative reactions occurring within the body. In particular, in its HET state, hydroethidine can freely penetrate cell membranes. Once oxidized within the cell to ethidium, however, ethidium cannot exit the cell, making it highly useful for studying the extent and location of ROS generation within the body (Zorov *et al.*, 2014).

Thirty minutes prior to sacrificing the experimental animals, 500 μ l of 1 mg/ml concentration of hydroethidine (HET, Molecular Probes, CA, USA) was injected into the abdominal cavity. After 30 minutes of administration, the animals were sacrificed using the same method as described above. The brains were then extracted, fixed and frozen, followed by creating consecutive 40 μ m-thick coronal sections using the same method. Subsequently, immunofluorescent staining using other antibodies as needed was performed. The location and extent of ROS generation within the tissue were assessed by observing ethidium, which was oxidatively generated, under a fluorescence microscope (excitation, 510 nm; emission, 580 nm).

Immunofluorescence

The tissue sections were rinsed three times for 10 minutes each in 0.1 M PBS, followed by pre-treatment with 5% normal donkey serum for 60 minutes. Subsequently, they were incubated with mouse monoclonal anti-TH antibody (1:100; Chemicon) at 4°C for 12 hours. The sections were rinsed three times for 10 minutes each in 0.1 M PBS, followed by incubation with AMCA-conjugated donkey anti-mouse IgG (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 2 hours. Afterward, they were rinsed three times for 10 minutes each in 0.1 M PBS, followed by pre-treatment with 5% normal goat serum for 60 minutes. OX6 (1:50) primary antibody was then incubated with the sections at 4°C for 12 hours. After rinsing three times for 10 minutes each in 0.1 M PBS, the sections were sequentially treated with biotinylated goat anti-rabbit IgG (1:200; Vector) and FITC-conjugated Avidin D (1:50; Vector) at room temperature for 2 hours and 1 hour, respectively. Following fluorescence staining, all tissues were rinsed three times for 10 minutes each in 0.1 M PBS, mounted on slides coated with Vectabond and coverslipped using Vectashield Mounting Medium (Vector).

Taking photographs of experimental results

The tissue samples following immunofluorescence staining were observed using an Olympus microscope (AX70; Olympus Inc, Tokyo, Japan) and images were captured with a Nikon digital camera (DXM 1200; Nikon Inc, Tokyo, Japan). The captured images were processed using Adobe Photoshop software (version 7.0; Adobe Systems Inc, San Jose, CA, USA) to enhance image quality.

RESULTS AND DISCUSSION

Establishment of the animal model

To confirm the successful establishment of the Parkinson's

disease animal model induced by MFB axotomy, the brains of experimental animals were extracted 7 days post-axotomy and sectioned sagittally. Hematoxylin and eosin (H&E) staining and TH immunohistochemical staining were performed on both the ipsilateral (surgery side) and contralateral (control side) tissues.

In the H&E staining of the contralateral side, the MFB area extending from the midbrain SN to the striatum exhibited a lighter eosin staining compared to the surrounding tissues, making it distinctly identifiable. Additionally, TH immunohistochemical staining showed strong immunoreactivity in the midbrain SN, MFB and striatum. Some nuclei in the olfactory bulb, cerebellum, pons and medulla also displayed positive immunoreactivity (Fig 2A,C). In contrast, on the ipsilateral side, it was confirmed that the MFB between the SN and the striatum was precisely severed (indicated by arrows in Fig 2B, D). Consequently, tyrosine hydroxylase, produced in the midbrain dopaminergic neurons and normally transported through the MFB, was observed to accumulate at the distal end of the severed area, unable to proceed further (indicated by an asterisk in Fig 3D). These findings confirm the successful establishment of the MFB axotomy animal model.

Generation of ROS in activated microglia

To verify the generation of ROS in activated microglia induced by MFB axotomy, hydroethidine was injected intraperitoneally 30 minutes before sacrificing the experimental animals. Observation of ethidium fluorescence in the SN revealed numerous fluorescent signals in the ipsilateral SN. The distribution of these signals closely corresponded to the distribution of microglia as identified by OX42, OX6 and ED1 immunostaining (Fig 3A-D).

To identify the characteristics of cells containing ethidium fluorescence, TH/OX6 double immunofluorescence staining was performed. The results showed that many of the activated microglial cells, which were immunopositive for OX6, also contained ethidium fluorescence (Fig 3E-H).

Regulation of ROS

ROS are beneficial for biological systems, acting as signaling molecules and enhancing immune defense. However, they also have harmful effects, such as causing tissue and organ damage. Maintaining the balance of ROS is crucial for minimizing oxidative damage and meeting energy requirements. Relatively high levels of ROS can lead to oxidative damage or induce apoptosis during immune responses or under pathological conditions (Yang and Lian, 2020).

The widespread distribution of ROS grants them a fundamental role in biological systems. Although ROS play an important role in pathogen resistance and cellular signaling, they are also widely recognized as harmful reactive particles, as they damage intracellular proteins, lipids and nucleic acids (Yang and Lian, 2020). Under these conditions, excessively produced ROS cause oxidative damage to both the body and pathogens. ROS are widely

involved in fundamental mechanisms and pathways. They not only impair cells and tissues through oxidative damage but also play an important role in many homeostasis processes, including metabolism, immunity, growth and differentiation (Shadel and Horvath, 2015).

ROS impact diseases primarily through their role as signaling molecules and oxidants that affect cell survival and oxidative damage (Yang and Lian, 2020). Neurons are crucial cells that regulate sensory organs and the muscular system. Damage to these cells can result in neuropathy and movement disorders. Due to their relatively low antioxidant activity, neurons are particularly susceptible to oxidative damage. Mitochondrial defects can increase ROS production, which in turn can activate JNK and sterol-regulatory element-binding proteins (SREBP) in neurons. This activation leads to neurodegeneration by promoting the accumulation of lipid droplets (Liu *et al.*, 2015). Elevated ROS levels in the SN pars compacta result in the apoptosis of dopaminergic neurons, contributing to neurodegeneration in both Down syndrome and Parkinson's disease (Tieu *et al.*, 2003).

To accurately understand the pathology of Parkinson's disease and explore treatments that can alleviate clinical symptoms, as well as to scientifically prove the safety and efficacy of newly discovered drugs before clinical application, the foremost prerequisite is to develop an appropriate animal model of Parkinson's disease that can replicate the pathological phenomena observed in humans (Beal, 2001).

Compared to Parkinson's disease animal models using MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Mustapha and Mat Taib, 2021) or 6-OHDA (6-hydroxydopamine) (Guimarães *et al.*, 2021), the MFB axotomy has the

advantage of inducing the death of dopaminergic neurons in the adult central nervous system more chronically over a relatively extended period.

In this study, the researcher aimed to investigate the activation of microglia induced by the selective death of dopaminergic neurons, the subsequent generation ROS and the relationship between activated microglia and dopaminergic neurons. From this perspective, it was considered that the MFB axotomy model is more suitable for this study than acute degeneration induction models such as 6-OHDA or MPTP administration. Additionally, it offers the advantage of using the non-treated contralateral hemisphere as a control, enhancing its utility (Song *et al.*, 2008). No behavioral abnormalities were observed in the experimental animals following surgery and approximately three days post-operation, all animals exhibited normal behavioral patterns. However, one animal out of the entire cohort showed a significant decrease in food intake and excessive weight loss after surgery. This animal was euthanized and excluded from the study. Since the surgical procedure targets the region where the MFB passes through the hypothalamus and its subregions, it is hypothesized that the observed phenomenon in this animal, characterized by damage to the feeding center located in the hypothalamus, is attributable to the surgery.

Activation of microglia following dopaminergic neuron degeneration

Microglia have the characteristic ability to bind to various lectin substances such as Griffonia Simplicifolia Agglutinin I, B4, Ricinus Communis Agglutinin, Mistletoe Agglutinin and complement receptor 3 (CR3), even in their resting state. Upon activation, they strongly express major histocompatibility

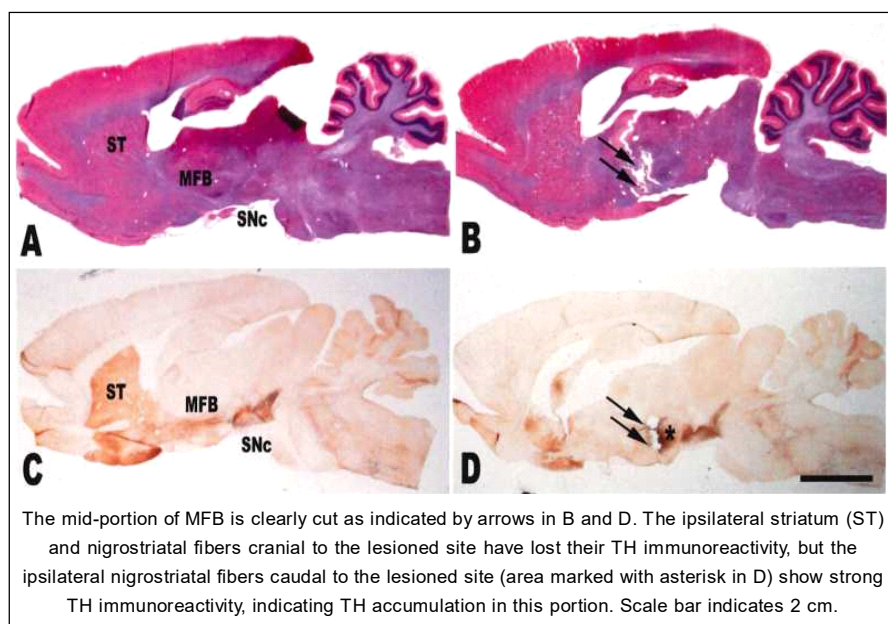


Fig 2: Para-sagittal views of contralateral (A and C) and ipsilateral (B and D) rat brain labeled with H & E (A and B) and TH antibody (C and D) at 7 days post lesion.

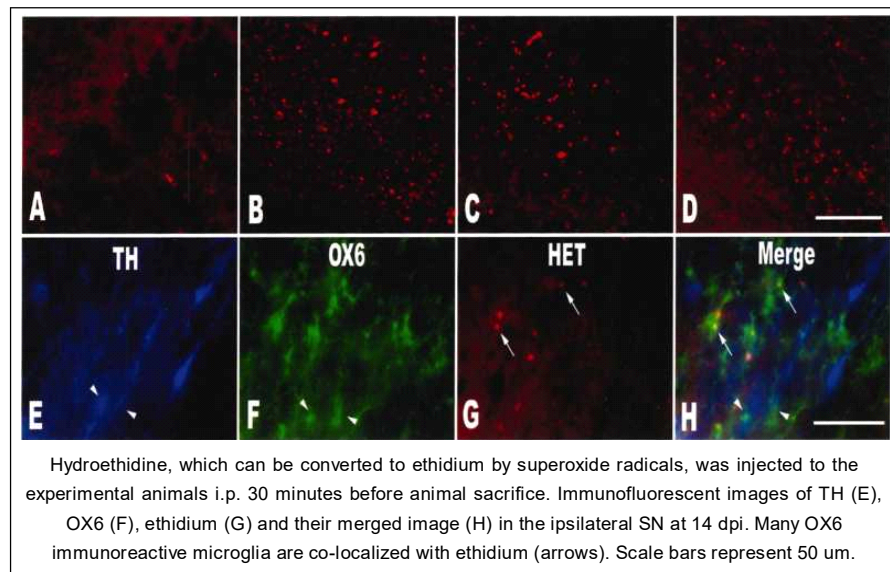


Fig 3: Representative ethidium fluorescence in SN of the contralateral (A) and ipsilateral side at 7 dpi (B), 14 dpi (C) and 28 dpi (D).

complex (MHC) class I and class II molecules (Sawada *et al.*, 2006). Additionally, activated microglia in rats are known to actively synthesize various phagocytosis-related proteins and utilize them in lysosomal phagocytosis, which can be identified using the ED1 antibody. Furthermore, studies on various neurodegenerative diseases have revealed that microglia showing immunopositive reactions to ED1 are actively engaged in phagocytosis (Cho *et al.*, 2003; Gyu-Ri Kim, 2023).

Seven days after MFB axotomy, a strong immunopositive reaction to OX42 was observed in the microglia gathered in the SN. These microglia also showed a strong immunopositive reaction to ED1 and OX6. Furthermore, these activated microglia exhibited the morphology of activated ramified microglia. (Stence *et al.*, 2001) reported through hippocampal tissue culture experiments that activated ramified microglia do not perform phagocytic activity on surrounding tissues. However, in this study, it was confirmed that the activated ramified microglia, which were activated due to the death of dopaminergic neurons, also showed immunopositive reactions to the ED1 antibody. This demonstrates that activated ramified microglia are also actively engaged in phagocytic activity.

Activated microglia and ROS

One of the key pathological findings in the brains of Parkinson's disease patients is the localized accumulation of activated microglia in the SN (Mandel *et al.*, 2004). This microglial activation in the SN has also been observed in Parkinson's disease animal models induced by MPTP administration (Cheng *et al.*, 2021; Ozkan, 2016). Additionally, increased microglial activation in the striatum and SN of Parkinson's disease patients has been revealed through *in vivo* positron emission tomography (PET)

imaging using the specific ligand for activated microglia, 11C-PK11195 (Cicchetti *et al.*, 2002). This suggests that activated microglia play an important role in the onset and progression of Parkinson's disease.

In this study, the researcher used hydroethidine to confirm that the death of dopaminergic neurons induced by MFB axotomy leads to the activation of surrounding microglia, which actively produce and secrete ROS. This suggests that microglia activated by certain factors actively secrete ROS, potentially exerting negative effects on the surrounding healthy neurons. After administering naloxone, which is known to have microglia-activation inhibitory effects and then inducing an inflammatory response with lipopolysaccharide (LPS), it was found that artificially suppressing microglia activation provided neuroprotective effects when compared to the control group (Chandana Choudhury Barua, 2022; Liu *et al.*, 2000). In other words, activated microglia can negatively impact surrounding neurons through the ROS they produce.

CONCLUSION

Based on these findings, it has been confirmed that following the induced degeneration of dopaminergic neurons by MFB axotomy, microglia are activated. These activated microglia vigorously produce and secrete ROS, potentially adversely affecting the survival of surrounding neurons. Therefore, it is hypothesized that suppressing excessive activation of microglia to reduce the generation of ROS at an appropriate level could potentially delay neuronal demise and promote the survival of surrounding healthy neurons in Parkinson's disease.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Animal Review Board of Eulji University in accordance with the National Institutes of Health (NIH Publication No. 8023, revised 1996) and handling techniques were approved by the Animal Review Board of Eulji University in accordance with the National Institutes of Health (NIH Publication No. 8023, revised 1996).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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