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# Medial Forebrain Bundle Axotomy Induced Dopaminergic Neuronal Degeneration is Accompanied with Microglial Activation

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## ABSTRACT

**Background:** We aimed to investigate the role of microglia in the degeneration of dopaminergic neurons by using the medial forebrain bundle (MFB) axotomy model, one of the animal models of Parkinson's disease.

**Methods:** We performed immunohistochemical staining, immunofluorescence staining, and Western blot assays on rats sacrificed 7, 14 and 28 days after MFB asotomy to study tissue immune responses. Animal sacrifice was performed 30 minutes prior to injection of hydroethidine into the abdominal cavity.

**Result:** Using OX42 and OX6 antibodies, excessive activation of microglia occurred in the surgical site of the substantia nigra on 7 days after the procedure. Activated microglia were still present and clustered even in the 14-day and 28-day groups, although their reactivity had slightly decreased compared to the 7-day group. These cells showed strong immunopositive reactions to ED1, indicating that they were actively engaged in phagocytic activity.

**Key words:** Dopaminergic Neurons, MFB Axotomy, Microglia.

## INTRODUCTION

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease, with over one million reported cases in North America alone. Parkinson's disease is characterized by the selective and sustained loss of dopaminergic neurons in the substantia nigra, a region located in the midbrain (Lenka *et al.*, 2022).

In the normal state, microglia serve as the sole immune surveillance cells in the central nervous system and participate in the host defense mechanism against external antigens and tissue repair. It is expected that microglia will also play a role in mitigating neuronal cell death through various pathways in Parkinson's disease. Firstly, microglia can perform a positive role in the survival of neurons by removing toxic substances secreted by dying dopamine neurons. Secondly, microglia are known to support the survival of dopaminergic neurons by generating and secreting various neurotrophic factors. The glial-derived neurotrophic factor (GDNF), which is known to be expressed in activated microglia, has been shown in vitro experiments to play a central role in the survival of developing dopamine neurons (Burke *et al.*, 1998). Several studies have shown that increasing the expression of GDNF specifically in the nigrostriatal pathway using recombinant gene technology, such as viral vectors, in animal models of Parkinson's disease induced by MPTP administration in mature mice or primates, leads to statistically significant inhibition of dopaminergic neuronal death compared to control groups (Eberhardt *et al.*, 2000). While the protective effects of GDNF on dopaminergic neurons have not yet been demonstrated in humans (Kordower *et al.*, 1999), the series of research results presented above indicate that microglia can function as a key neuroprotective factor in Parkinson's disease.

Microglia are intimately related to neurons as the sole immune cells of the central nervous system. Therefore, understanding the pathological response of microglia accompanying neuronal cell death is equally important as understanding the mechanism of neuronal cell death itself. The researchers aimed to investigate the degree of microglial activation following the progressive demise of midbrain dopamine neurons by MFB axotomy, using various immunohistochemical and immunofluorescent techniques as well as Western blot analysis.

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## MATERIALS AND METHODS

### Animal management

All the use and management of experimental animals were conducted in accordance with the standards set by the

National Institutes of Health (NIH) in the United States (NIH Publication No. 8023, revised 1996). The study was carried out during the period of January 2019 to December 2022, at the Department of Biomedical Laboratory Science at Eulji University in Daejeon. Particularly, all measures were taken throughout the entire experimental process to minimize the pain and suffering of the experimental animals and unnecessary use of experimental animals was avoided. Male Wistar rats (Charles River Lab., NC, USA) weighing approximately 250-300 g were used in the experiments and all experimental animals were housed in a room with a temperature and humidity maintained at 20-22°C and a regular 12-hour light-dark cycle, with free access to food and water.

**Medial forebrain bundle (MFB) axotomy**

The animals were anesthetized deeply with a ketamine (70 mg/kg) and xylazine (8 mg/kg) mixture injected into the intraperitoneal cavity and then secured to a stereotaxic apparatus (Stoelting Co., Il, USA) for brain surgery. The scalp of the head was incised along the midline for about 3 cm and the outer periosteum was separated from the two bones using a cotton swab to confirm the bregma and lambda points. The bite bar of the stereotaxic apparatus was adjusted to align the bregma and lambda in the same plane. A small hole was drilled in the skull using a dental drill, 2.8 mm posterior and 3.0 mm to the right of the bregma as a reference point. Through the hole, a retractable wire knife (Scouten knife; David Kopf Instruments, CA, USA) with a cannula was inserted to a depth of 9.0 mm from the dura mater towards the ventral side. The wire knife was retracted inward toward the midline to a length of 2.2 mm, then moved dorsally 3.0 mm and left to rest for about a minute. It was then moved ventrally again 3.0 mm to completely sever the right MFB (Kirby *et al.*, 2012). Afterwards, the wire knife was reinserted and the cannula was slowly removed and the incision in the skin was immediately aseptically sutured.

After the surgery, the experimental animals were kept under 37°C warming until they fully recovered from anesthesia and no abnormal behavior due to the surgery was observed in the experimental animals. The animals were sacrificed 7, 14 and 28 days after the MFB axotomy and the contralateral hemisphere without the axotomy was used as a control group.

**Immunohistochemistry**

Immunohistochemical staining was performed using the free floating method. The tissue sections were washed three times for 10 minutes in 0.1 M phosphate-buffered saline (PBST, pH 7.4) containing 0.1% Triton X-100. To block endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide ($H_2O_2$) for 15 minutes. After washing three times with PBST for 10 minutes each, 5% normal serum from the same species as the host animal used for producing the secondary antibody was incubated for 1 hour to block nonspecific binding. The slides were then incubated with the primary antibody at 4°C for 12 hours. The primary antibodies used in this study included mouse monoclonal anti-rat CDIlb antibody (OX-42; 1:500; Serotec, Oxford, UK) to label microglia and rabbit monoclonal anti-tyrosine hydroxylase antibody (TH; 1:1,000; Chemicon) to label dopaminergic neurons. In addition, mouse monoclonal anti-rat major histocompatibility complex (MHC) class II antibody (clone OX6; 1:500; Serotec) and mouse monoclonal anti-mouse lysosomal enzyme antibody (clone ED1; 1:500; Serotec) were used to discriminate active microglia.

The tissue sections that underwent primary antibody treatment were washed three times with PBST for 10 minutes each. Subsequently, biotinylated secondary antibodies were applied at a 1:200 dilution for each species of primary antibody generated in rabbits, mice and goats, using biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., CA, USA), biotinylated horse anti-mouse IgG (Vector) and biotinylated rabbit anti-goat IgG (Vector), respectively. The sections were then allowed to react for 2 hours at room temperature. After washing the tissue sections three times with PBST for 10 minutes each, they were incubated with avidin-biotin-peroxidase complex (ABC; Vector) for 1 hour at room temperature. Subsequently, the sections were stained for 3-5 minutes with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Korea) containing 0.01% hydrogen peroxide. The stained tissues were mounted onto Vectabond-coated slides, air-dried for one day, dehydrated through a series of alcohol solutions, cleared in xylene and mounted with permount.

**TH/OX6 Double immunohistochemical staining**

To investigate the pathological relationship between activated microglia and dopaminergic neurons, double immunohistochemical staining for TH/OX6 was performed. Using the same method, primary immunohistochemical staining with TH antibody was first performed and visualized with DAB staining. To prevent nonspecific immune reactions of avidin and biotin, 1:50 dilutions of avidin and biotin were separately applied to the tissue sections for 15 minutes each, using the Avidin-Biotin blocking kit (Vector). After the blocking, the tissue sections were washed three times with PBST for 10 minutes each and then the second immunohistochemical staining was performed using OX6 antibody. The chromogen for the second reaction was developed using nickel DAB, which was prepared by adding 0.02% nickel ammonium sulfate to 0.05% DAB containing 0.01% hydrogen peroxide.

**Immunofluorescence**

The tissue sections were washed three times for 10 minutes in 0.1 M PBS, followed by pre-treatment with 5% normal donkey serum for 60 minutes. The sections were then incubated with mouse monoclonal anti-TH antibody (1:100; Chemicon) at 4°C for 12 hours. The tissue sections were rinsed three times with 0.1 M PBS for 10 minutes each and

then pre-treated with 5% normal donkey serum for 60 minutes. Mouse monoclonal anti-TH antibody (1:100; Chemicon) was applied to the sections and allowed to react at 4°C overnight. After rinsing the sections three times with 0.1 M PBS for 10 minutes each, the sections were incubated with AMCA-conjugated donkey anti-mouse IgG (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 2 hours. After completion of the fluorescent staining, all tissues were washed three times with 0.1 M PBS for 10 minutes each and then mounted onto Vectabond-coated slides and sealed with Vectashield Mounting Medium (Vector).

### Taking pictures of tissue samples

The tissue specimens that underwent immunohistochemistry and immunofluorescence staining were observed using an Olympus microscope (AX70; Olympus Inc, Tokyo, Japan) and images were captured using a Nikon digital camera (DXM 1200; Nikon Inc, Tokyo, Japan).

### Western blot assay

The dissected tissue samples were homogenized using Ringer's solution (128 mM NaCl, 1.8 mM $CaCl_2$, 1.3 mM KCl, 50 mM Tris, pH 7.4) with the addition of protease inhibitor (Protease Inhibitors Set, BMS, USA) at 4°C using a glass homogenizer. The homogenized solution was then centrifuged at 15,000 rpm at 4°C to obtain the supernatant, excluding the tissue fragments and lipid layer. A portion of the obtained supernatant was collected and the protein content was quantified using the Bradford assay. The total extracted proteins were subjected to electrophoresis using 12% Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride transfer membrane (Pall, Ann Arbor, MI, USA) at 100 V for 2 hours. The transferred membrane was equilibrated in a pH 7.4 TBS solution containing 0.1% Tween-20 for 10 minutes and then blocked with 5% non-fat dry milk at 4°C for 1 hour to prevent non-specific immune reactions. The membrane was then incubated with primary antibody solution diluted 1:1000 at 4°C for 12 hours, washed three times with TBST for 10 minutes each and then incubated with the corresponding secondary antibody for 2 hours at room temperature. The primary antibodies used were rabbit monoclonal anti-TH antibody (Chemicon), mouse monoclonal anti-beta-actin antibody (Santa Cruz), mouse monoclonal anti-OX42 antibody (Serotec), mouse monoclonal anti-OX6 antibody (Serotec) and mouse monoclonal anti-ED1 antibody (Serotec). The membrane was then washed three times with TBST for 10 minutes each and incubated with the corresponding secondary antibody at room temperature for 2 hours. After washing three times with TBST for 10 minutes each, the membrane was incubated with ECL reagent (Pierce, Rockford, IL, USA) for 1 minute and then exposed to X-ray film for 30 seconds and developed.

## RESULTS AND DISCUSSION

### Dopaminergic neuronal loss following nigrostriatal pathway lesion

To assess the viability of dopaminergic neurons following MFB axotomy, TH immunohistochemical staining was performed. The results demonstrated a gradual reduction in the number of dopaminergic neurons exhibiting immunopositive reactions to the TH antibody in the substantia nigra as time elapsed after the procedure (Fig 1A).

To determine the extent of changes in tyrosine hydroxylase (TH) enzyme expression in response to the reduction in the number of dopaminergic neurons, a western blot assay was performed. The results showed that, at 7 days post-surgery, there was minimal change in TH enzyme expression compared to the contralateral side. However, after 14 days, there was a reduction of approximately 57% and after 28 days, a reduction of approximately 64% in TH enzyme expression was observed (Fig 1B, C).

### OX42 immunoreactivity

In the contralateral substantia nigra, numerous resting microglial cells exhibiting immunopositive reactions to OX42 were observed. These cells had small cell bodies and exhibited a ramified microglial morphology, characterized by several thin, elongated cellular processes. They were distributed in higher density in the nigral region compared to the surrounding areas, although within the substantia nigra itself, they showed a relatively uniform distribution density. In the ipsilateral side, there was a significant increase in the number of microglial cells exhibiting strong immunopositive reactions to OX42 at 7 days post-surgery. These cells were densely clustered. Although there was a slight decrease in this phenomenon at 14 and 28 days, they still displayed a high level of OX42 immunoreactivity compared to the contralateral side (Fig 2A, B). The OX42 immunopositive microglial cells in the ipsilateral side exhibited enlarged cell bodies and shorter, thicker cellular processes while maintaining a ramified microglial cell morphology. No amoeboid microglial cells were observed (Fig 2B).

The protein expression level of CD11b in the substantia nigra was compared using Western blot analysis. The results showed that at 7 days post-surgery, the expression level of CD11b protein was increased by more than 8-fold compared to the contralateral side. After 14 and 28 days, the expression level was still approximately 4-fold higher than that of the contralateral side (Fig 2C, D).

### ED1 and OX6 immunoreactivity

In the contralateral side, very few microglial cells exhibiting immunopositive reactions to ED1, a lysosomal protein, were detected. However, a small number of cells showing immunopositive reactions to OX6, a marker for MHC class II, were observed and they exhibited a ramified microglial cell morphology.

In the ipsilateral side, numerous cells exhibited strong immunopositive reactions to ED1 and OX6 at 7 days post-surgery and they were densely clustered. Although there was a slight decrease at 14 and 28 days, they still showed a higher level of immunopositive reactions compared to the contralateral side (Fig 3, 4). The OX6 immunopositive microglial cells in the ipsilateral side displayed enlarged cell bodies and shorter, thicker cellular processes, resembling ramified microglial cells.

Western blot analysis showed that the immune activity of ED1 in the black substance was almost undetectable in the control group, but significantly increased in the treatment group at 7 days after the procedure. At 14 and 28 days after the procedure, the immune activity decreased by about half compared to 7 days, but still remained high (Fig 3C, D). On the other hand, the immune activity of OX6 was significantly high in the control group and the OX6 immune activity in the treatment group showed a similar trend to ED1 (Fig 4C, D).

**Double immunohistochemical staining with OX6/TH**

To investigate the relationship between activated microglia and dopaminergic neurons in the substantia nigra following MFB lesion, double immunostaining was performed using

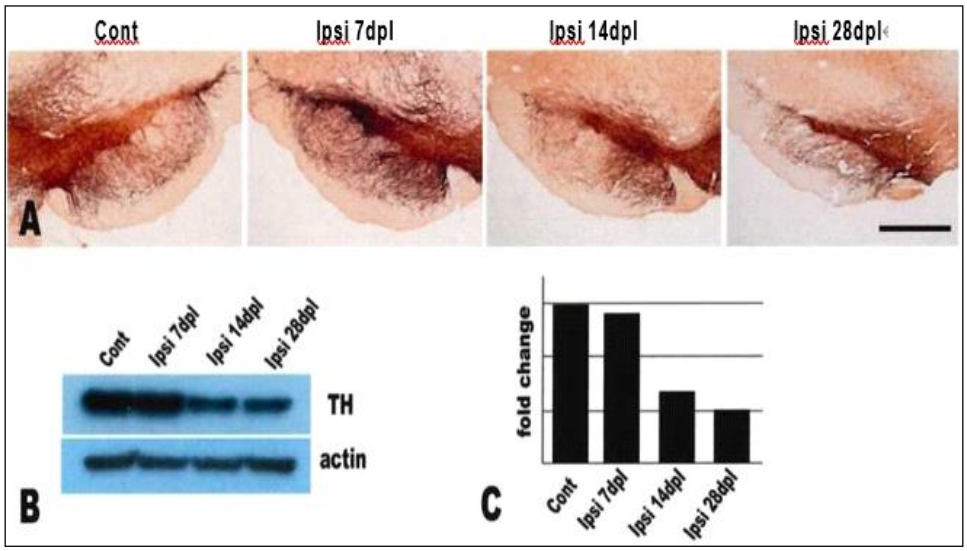


**Fig 1:** (A) TH immunohistochemistry in the contralateral (Cont) and ipsilateral SN at 7, 14 and 28 days after MFB axotomy. (B) Changes in expression level of TH by western blot assay. (C) Quantification of results from western blot assay in (B). The density of each TH band was normalized against that of beta-actin. Scale bar in (A) represent 700 μm.

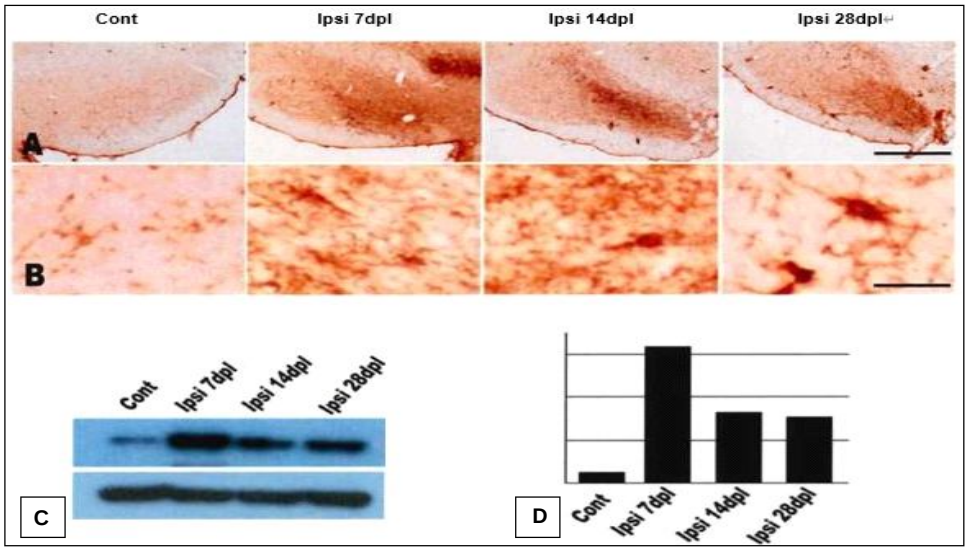


**Fig 2:** (A) OX42 immunohistochemistry in the contralateral (Cont) and ipsilateral SN at 7, 14 and 28 days after MFB axotomy. (B) High magnified images of (A). (C) Changes in expression level of OX42 by western blot assay. (D) Quantification of results from western blot assay in (C). The density of each OX42 band was normalized against that of beta-actin. Scale bars in (A) and (B) represent 600 μm and 20 μm, respectively.

OX6 and TH antibodies. The results showed that activated microglia, exhibiting immunopositivity for OX6, were closely associated with the dendrites and cell bodies of dopaminergic neurons, displaying immunopositivity for TH (Fig 5).

**MFB axotomy model**

Apart from relatively recent animal models utilizing genetic modifications in certain insects or non-human primates, there are currently two main approaches widely used to replicate Parkinson's disease in animal models. One approach involves the use of specific drugs, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Ballard *et al.*, 1985) or 6-OHDA (6-hydroxydopamine) (Ungerstedt, 1971), to selectively induce the degeneration of dopaminergic neurons in the midbrain. The other approach involves the retrograde degeneration of dopaminergic neurons by axonal transection, where the axons of dopaminergic neurons are severed to induce their degeneration (Reis *et al.*, 1978). As more than 90% of midbrain dopaminergic neurons send their axons to the ipsilateral striatum through the nigrostriatal pathway (Fass and Butcher, 1981), selective MFB axotomy using a retractable wire knife, as developed by Scouten and

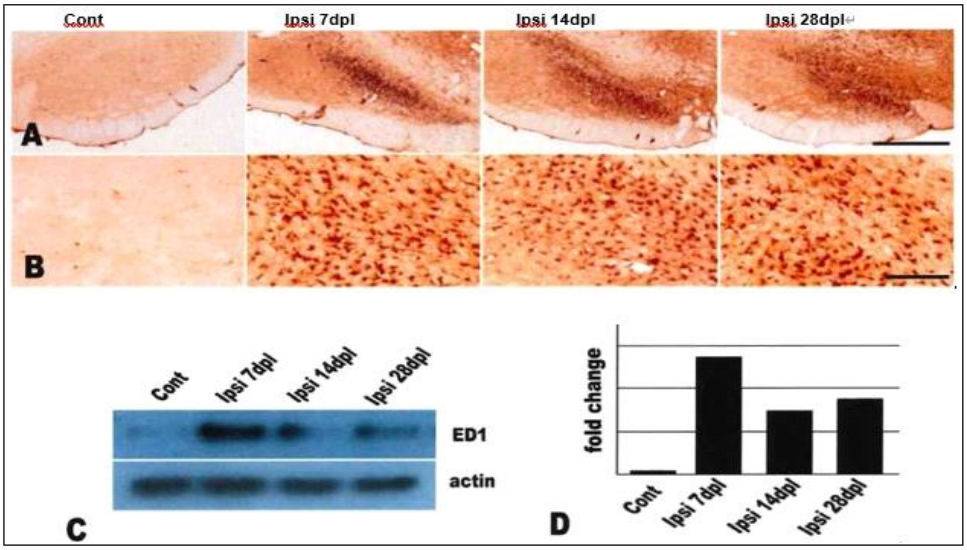


**Fig 3:** (A) ED1 immunohistochemistry in the contralateral (Cont) and ipsilateral SN at 7, 14 and 28 days after MFB axotomy. (B) Magnified images of (A). (C) Changes in expression level of ED1 by western blot assay. (D) Quantification of results from western blot assay in (C). The density of each ED1 band was normalized against that of beta-actin. Scale bars in (A) and (B) represent 600 ìm and 100 μm, respectively.

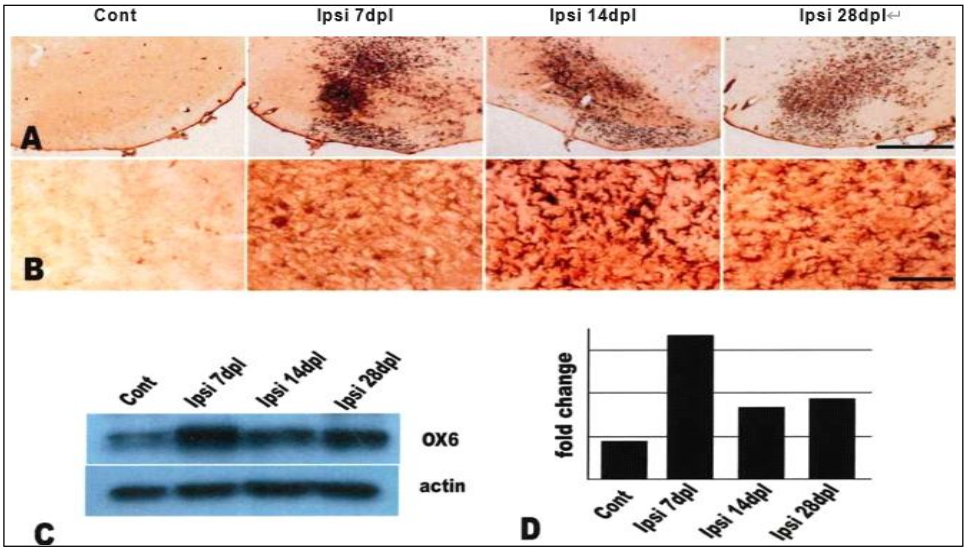


**Fig 4:** (A) OX6 immunohistochemistry in the contralateral (Cont) and ipsilateral SN at 7, 14 and 28 days after MFB axotomy. (B) Magnified images of (A). (C) Changes in expression level of OX6 by western blot assay. (D) Quantification of results from western blot assay in (C). The density of each OX6 band was normalized against that of beta-actin. Scale bars in (A) and (B) represent 600 μm and 100 μm, respectively.

colleagues (Scouten *et al.*, 1981), leads to the degeneration of a significant number of dopaminergic neurons in the substantia nigra pars compacta within the midbrain (Sugama *et al.*, 2003).

The selective MFB axotomy offers the advantage of inducing chronic and long-term degeneration of dopaminergic neurons within the adult central nervous system. For example, when 6-OHDA is administered to the MFB, it is known to cause the death of approximately 95% of midbrain dopamine neurons within 3 days (Schmidt *et al.*, 1983). On the other hand, in the case of MFB axotomy, after the procedure, around 20-30% of dopamine neurons are reported to survive even after 70 days post-surgery (Cho *et al.*, 2006). From this perspective, MFB axotomy is a valuable method for comparing and investigating the relationship between neuronal cell death and the surrounding environment, particularly the interactions with glial cells. Another advantage of MFB axotomy is that the non-lesioned hemisphere of the brain can serve as a control group, allowing for direct comparisons.

According to previous studies, approximately 26% of dopaminergic neurons were reported to degenerate after 7 days post-surgery, approximately 39% after 14 days and approximately 46% after 28 days (Cho *et al.*, 2006). However, the results of Western blot assay in this study showed minimal changes in tyrosine hydroxylase expression levels at 7 days post-surgery. After 14 days, there was a decrease of approximately 57% in tyrosine hydroxylase enzyme levels and after 28 days, there was a decrease of approximately 64%. These findings differ from previous studies. This discrepancy is believed to be attributed to the cumulative amount of residual enzyme present in certain regions of the MFB and the synthesis of tyrosine hydroxylase enzyme in dopaminergic neurons within the ventral tegmental area. However, overall, there was a decrease in the total amount of synthesized tyrosine hydroxylase enzyme over time, indicating a progressive degeneration of dopaminergic neurons.

**The activation of microglia in response to dopaminergic neuron degeneration**

Microglia, which serve as key immune cells in the central nervous system, share similarities in function with monocytes and macrophages, which perform similar functions in the periphery. However, microglia also possess distinct morphological and molecular characteristics that set them apart (Sedgwick *et al.*, 1991).

Previous researchers have proposed that when resting ramified microglia are activated by environmental changes, their cell bodies enlarge and their cellular processes become shorter and thicker, transitioning into activated ramified microglia. It has been suggested that once these activated microglia engage in phagocytic activity, they transform into an ameboid microglial form resembling peripheral macrophages (Mor *et al.*, 1999). Indeed, Stence *et al.*, 2001) have reported research findings that activated ramified microglia, based on hippocampal tissue culture experiments, do not engage in phagocytic activity towards surrounding tissues. However, in these research findings, the researchers were able to confirm that activated ramified microglia, induced by the demise of dopamine neurons, also exhibited an immune-positive response to the ED1 antibody. This demonstrates that activated ramified microglia are indeed engaged in active phagocytic activity.

**Activated microglia**

Microglia, present in major parts of the central nervous system, are not uniformly distributed in terms of their morphology and density. Specifically, in the brains of many mammals, including humans, microglia are known to have the highest density in the substantia nigra (Lawson *et al.*, 1990). In other words, dopaminergic neurons in the substantia nigra are more susceptible to specific factors derived from microglia compared to neurons in other regions (Kim *et al.*, 2000).

One of the major pathological features in the brains of Parkinson's disease patients is the localized accumulation of activated microglia within the substantia nigra (Grünblatt *et al.*, 2000; Knott *et al.*, 2000). These previous studies suggest that the activation of microglia due to brain inflammation can act as a risk factor for the progressive demise of dopaminergic neurons. In other words, the implication is that activated microglia play a significant role in the onset and progression of Parkinson's disease.

We confirmed through TH/OX6 dual immunostaining that these microglia were spatially closely linked to the surrounding dopaminergic neurons. This indicates that activated microglia have the potential to negatively impact neighboring healthy neurons.

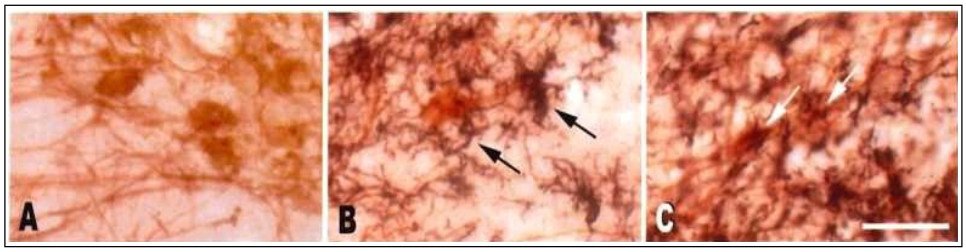


**Fig 5:** High magnifications of TH and OX6 double labeling in the contralateral (A) and ipsilateral (B and C) SN at 14 dpi. OX6 positive (black) activated microglia specifically stick to TH immunoreactive nigrostriatal fibers (arrows in B), and soma (arrows in C). Scale bar represents 30 µm.

## CONCLUSION

The researchers confirmed that the demise of dopaminergic neurons induced by MFB axotomy, triggers the activation of microglia. It suggests that controlling the excessive activation of microglia could be a viable strategy to delay the demise of damaged neurons and promote the survival of surrounding healthy neurons. By modulating the activity of microglia and mitigating their detrimental effects, it may be possible to protect neuronal cells from further damage. This research study represents a paradigm shift from the previous research direction, which aimed to understand the mechanisms of neuronal cell death and manipulate specific signaling pathways to inhibit or delay cell death.

**Conflict of interest:** None.

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