Nitric Oxide Synthase (NOS) Isoform Expression after Peripheral Nerve Transection in Mice

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Abstract

Localization of the nitric oxide (NO)-producing enzyme, nitric oxide synthase (NOS), and its functions are currently being investigated in several tissues and organs. It has been suggested that NO is involved in nerve cell death and the development of neurodegenerative disease. The purpose of this study was to immunohistochemically investigate expression of NOS to clarify its function in the degeneration and regeneration of transected mouse sciatic nerve. Scattered neuronal NOS (nNOS)-positive Schwann cells observed on the central side of the stump on day 1 after transection showed an increase in number on day 7. None were observed at the stump on day 14, however. Expression of nNOS was observed in axons extending from the stump. The number of nNOS-positive axons increased on day 21. Inducible NOS was expressed in inflammatory cells at the stump on day 1. This positive reaction subsequently weakened by day 7, however. Endothelial NOS was expressed in blood vessels at the stump on day 7, but decreased thereafter. The results of the present study suggest that NO is involved in the proliferation and migration of Schwann cells, as well as in axon regeneration at an early stage following nerve transection.

Key words: Nitric oxide synthase (NOS) — nNOS — Sciatic nerve — Nerve regeneration — Schwann cell

Introduction

Peripheral nerves have the capacity to actively regenerate. The function of these nerves has been reported to recover after damage, but with greater difficulty when the
nerve is completely ruptured. The process of nerve regeneration from peripheral nerve injury has been investigated using morphological methods, genetic modification, and application of iPS cells. When a peripheral nerve is transected, Schwann cells dedifferentiate, proliferate, and migrate to the central side of the stump. These cells then form aggregates, i.e., nerve bridges, and axons extend into these cell aggregates. Cell growth factors, such as nerve growth factor, brain-derived neurotrophic factor, ciliary neurotrophic factor, fibroblast growth factor, transforming growth factor-β, transcription factors, and signaling pathways are involved in this process of nerve regeneration. In addition, several cell adhesion molecules are expressed on the cell membrane of Schwann cells, and these molecules have been reported to play a role in the promotion of nerve regeneration. Schwann cells are closely involved in axon extension by peripheral nerves, and they may also act as a guide, determining the direction of axon regeneration.

The free radical species nitric oxide (NO) is a messenger that targets many types of molecule. Passing freely through the cell membrane without mediation by receptors, it exhibits many functions: in addition to neurotransmission, immune responses, and adjustment to the vascular system, it is also involved in apoptosis and wound healing. There are 3 isoforms of NO-producing nitric oxide synthase (NOS), which have been classified into neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Constantly expressed in cell bodies and axons in the central nervous system and peripheral nerves, nNOS is involved in signal transmission. Expression of iNOS is induced by inflammatory cytokines and lipopolysaccharides, and plays a role in immune responses and host defenses. Expression of eNOS is observed in vascular endothelial cells and platelets, and is involved in blood pressure adjustment through vascular smooth muscle relaxation and inhibition of platelet adhesion and aggregation. However, expression of the 3 types of NOS differs among cells, tissues, and organs, and the type, localization, and function of NOS expressed in each cell, tissue, and organ are still being investigated.

Expression of nNOS has been reported in various types of peripheral nerve, and its expression in nerve cells and Schwann cells has been clarified in vitro. In addition, when a peripheral nerve is subjected to pressure or damaged, nNOS is expressed in Schwann cells surrounding the injured axon. In experiments using transected peripheral nerves, nNOS expression has been detected in the dorsal root ganglion of the axotomy. To our knowledge, NOS expression at the stump and its dynamics in the process of nerve regeneration after transection of the peripheral nerve remain to be investigated.

The purpose of the present study was to investigate immunohistochemically the expression and localization of various types of NOS to elucidate their function in axons and Schwann cells on the central side of the stump after transection of mouse sciatic nerve.

Materials and Methods

1. Experimental animals

Ten-week-old male C57BL/6Jcl mice (Clea Japan, Tokyo, Japan) were used. The mice were allowed free access to pellets for laboratory animals (Oriental Yeast, Tokyo, Japan) and water, and housed at the Ohu University Animal Experimental Study Facility (room temperature, 23°C; humidity, 65%). All the animals were treated in accordance with the Animal Experiment Regulations of Ohu University. Approval of the study protocol was obtained from the Ohu University Animal Experiment Committee (Animal Experiment Approval Number: 2015-24).

2. Surgical method

After peritoneal administration of a mixture of 3 anesthetics (medetomidine, 0.15 mg/kg; midazolam, 2 mg/kg; and butorphanol, 5.0 mg/kg), the lateral side of the
right thigh was shaved, the nerve exposed immediately below the origin of the biceps femoris, and the nerve completely transected in the proximity of the greater trochanter. The 1-mm sciatic nerve distal to the transected region was resected to completely break off continuity; the sciatic nerve stump was transected on the central and peripheral sides. The wound was completely closed with silk thread and surgery completed. After surgery, meloxicam (0.2 mg/kg) was subcutaneously injected as an analgesic every 24 hr for a maximum of 3 days.

3. Sample preparation method

Intraperitoneal pentobarbital sodium (50 mg/kg) was administered after inhalation anesthesia with diethyl ether on days 1, 7, 14, or 21 after transection. A thoracotomy was then performed and physiological saline perfused from the left ventricle, followed by perfusion fixation with 4% paraformaldehyde-phosphate buffer (pH 7.4). The transected sciatic nerve was excised together with the surrounding muscle and tissue. Each specimen was fixed by immersion in the same fixative at 4°C for 24 hr, treated with 30% sucrose solution for anti-freezing, embedded in Tissue Mount (Shiraimatsu, Tokyo, Japan), and rapidly frozen in liquid nitrogen. Ten-micrometer-thick serial sections of the longitudinal sciatic nerve were prepared using a cryostat (MICROM, Germany). Each section was subjected to hematoxylin-eosin and immunohistochemical staining and then observed under a light microscope.

4. Immunohistochemical staining

Each section was treated with 0.3% H$_2$O$_2$-containing methanol solution for 15 min to inactivate endogenous peroxidase, blocked with 10% normal goat or rabbit serum (VECTASTAIN® Elite ABC Kit, VECTOR Lab., CA, USA) for 1 hr, and then reacted with a primary antibody.

A goat anti-human nNOS polyclonal antibody (Life Span BioSciences, WA, USA) (dilution ratio: 1:1,000), rabbit anti-human iNOS polyclonal antibody (Santa Cruz Biotechnology, CA, USA) (dilution ratio: 1:5,000), and rabbit anti-human eNOS polyclonal antibody (Santa Cruz Biotechnology) (dilution ratio: 1:10,000) were used as the primary antibodies. A rabbit anti-S100 protein polyclonal antibody (Nichirei, Tokyo, Japan) (dilution ratio: 1:100) was used to distinguish Schwann cells, while a rabbit anti-human PGP9.5 polyclonal antibody (Ultraclone, UK) (dilution ratio: 1:10,000) was used to label peripheral nerve axons. Each primary antibody was reacted at room temperature for 12 hr.

As secondary antibodies, a biotin-labelled rabbit anti-goat antibody or biotin-labelled goat anti-rabbit antibody (VECTASTAIN® Elite ABC Kit) was reacted at room temperature for 1 hr, followed by a reaction with peroxidase-labelled streptavidin (VECTASTAIN® Elite ABC Kit) at room temperature for 1 hr.

Color was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) (Nichirei). After nuclear staining with 5% methyl green (Mjuto Pure Chemicals, Tokyo, Japan), the sections were observed under a light microscope. In the washing of sections, 0.05 M Tris-hydrochloride buffer (pH 7.6) was used. In the dilution of antisera, 0.1 M phosphate-buffered saline (PBS) (pH 7.4) was used. Normal rabbit or goat serum (DAKO, CA, USA) was used as the negative control in immunostaining instead of the primary antibody.

In double fluorescent immunostaining of nNOS and the S100 protein and that of nNOS and PGP9.5, sections were blocked with 10% normal donkey serum (Santa Cruz Biotechnology) for 1 hr and then reacted with antibody mixture solution at an antibody dilution ratio of 1:100 at room temperature for 12 hr. The sections were then reacted with a mixture solution of an Alexa Fluor 488-labelled donkey anti-goat antibody (Bioss, MA, USA) (dilution ratio: 1:100) and Alexa Fluor 555-labelled donkey anti-rabbit antibody (Bioss) (dilution ratio: 1:100) at room temperature for 2 hr in a dark room and observed under a fluorescence microscope immediately after mounting.
Results

1. Localization of each type of NOS in normal sciatic nerve

The sciatic nerve was surrounded by an epineurium. A large number of nerve fibers were present, forming bundles. Blood vessels were also detected (Fig. 1a).

A weak nNOS-positive reaction was observed in some nerve tissue, and the connective tissue of the epineurium was also partially positive (Fig. 1b). No iNOS-positive reaction was observed in the nerve tissue or surrounding region (Fig. 1c). An eNOS-positive reaction was observed in blood vessels in the nerve tissue, but not in the nerve fibers (Fig. 1d). Schwann cells labelled with the S100 protein were arranged in the direction of the axons in the nerve tissue (Fig. 1e). Staining for PGP9.5 revealed a positive reaction in many axons (Fig. 1f).

Double fluorescent immunostaining with anti-nNOS and anti-S100 protein antibodies showed no co-expression of nNOS and the S100 protein (Fig. 1g–i). In contrast, in double fluorescent immunostaining with anti-nNOS and anti-PGP9.5 antibodies, regions of scattered co-expression of nNOS and PGP9.5 were observed (Fig. 1j–l).

2. Localization of each type of NOS on day 1 after nerve transection

Cells at the nerve stump were swollen, unlike normal cells, and round cell infiltration accompanying an inflammatory reaction was observed (Fig. 2a).

An nNOS-positive reaction was observed in many of the round cells that had infiltrated and densely accumulated at the stump. An nNOS-positive reaction at the stump of the nerve tissue was stronger than that in normal sciatic nerve (Fig. 2b). Staining for iNOS revealed a positive reaction in the round cells that had infiltrated the stump, but not in the nerve tissue (Fig. 2c). Staining for eNOS revealed no strong positive reaction in the nerve stump or surrounding tissue (Fig. 2d). Staining for the S100 protein revealed the accumulation of Schwann cells at the stump, with the pattern of cell arrangement resembling that in normal sciatic nerve, although specific directionality was lost (Fig. 2e). Staining for PGP9.5 revealed a positive reaction in a large number of axons at the stump. The appearance of the axons, however, was more swollen than that observed in normal sciatic nerve (Fig. 2f).

In double immunostaining using anti-nNOS and anti-S100 protein antibodies, nNOS- and S100 protein-positive reactions were noted at the stump, demonstrating co-expression of nNOS and S100 protein, which was not observed in the normal sciatic nerve. Cells expressing nNOS only were also present (Fig. 2g–i). In contrast, in double fluorescent immunostaining using anti-nNOS and anti-PGP9.5 antibodies, no co-expression of nNOS and PGP9.5 was observed in any region (Fig. 2j–l).

3. Localization of each type of NOS on day 7 after nerve transection

On day 7 after transection, the accumulation of a large number of spindle-shaped cells was observed. These formed aggregates extending toward the peripheral side of the stump. At the stump, the swollen cells observed on day 1 were absent. Almost no round cells were observed around the extended nerve bridge. On the other hand, strong proliferation of small-diameter blood vessels was noted (Fig. 3a).

An nNOS-positive reaction was observed in cells scattered around the stump, and positivity was stronger in spindle-shaped cells. These nNOS-positive spindle-shaped cells formed aggregates which extended from the stump toward the peripheral side (Fig. 3b). A weak iNOS-positive reaction was observed in round cells remaining around the stump (Fig. 3c). An eNOS-positive reaction was detected in the blood vessels, which showed an increase around the extended cell aggregates (Fig. 3d). Cells positive for the Schwann cell marker, the S100 protein, accumulated at the stump, forming aggregates, and extended toward the peripheral side (Fig. 3e). In staining for PGP9.5, no PGP9.5-positive axons
**Fig. 1** H-E staining and immunostaining of normal sciatic nerve with anti-NOS, anti-S100 protein, and anti-PGP9.5 antibodies

a) H-E, b) nNOS, c) iNOS, d) eNOS, e) S100, f) PGP9.5, g) nNOS, h) S100, i) nNOS/S100, j) nNOS, k) PGP9.5, l) nNOS/PGP9.5: Sciatic nerve comprised bundles of many nerve fibers with blood vessels (white arrowhead) inside. nNOS-positive reaction was observed in some nerve tissue (black arrow), and eNOS-positive reaction in blood vessels (white arrowhead), but iNOS was negative. Schwann cells labelled with S100 protein (black arrowhead) were arranged in cord pattern in nerve tissue, with many axons labelled with PGP9.5 running through them. No region showed co-expression of nNOS and S100 protein, but nNOS and PGP9.5 were co-expressed in some regions (white arrow).

Scale bar = 100 μm

**Fig. 2** H-E staining and immunostaining with anti-nNOS, anti-S100 protein, and anti-PGP9.5 antibodies at day 1 after nerve transection

a) H-E, b) nNOS, c) iNOS, d) eNOS, e) S100, f) PGP9.5, g) nNOS, h) S100, i) nNOS/S100, j) nNOS, k) PGP9.5, l) nNOS/PGP9.5: Cells at nerve stump (red dotted line) showed swelling and round cells (asterisk) infiltrated the surrounding area. nNOS-positive cells (black arrow) were scattered at stump, and round cells infiltrating around stump (asterisk) were iNOS-positive. No eNOS-positive reaction was observed. Schwann cells labelled with S100 protein (black arrowhead) and axons labelled with PGP9.5 (white arrowhead) were densely present at stump. nNOS, S100 protein, and PGP9.5 were highly expressed at nerve stump, and co-expression of nNOS and S100 protein was observed (white arrow); co-expression of nNOS and PGP9.5 was not observed in any region, however.

Scale bar = 100 μm
Fig. 3  H-E staining and immunostaining with anti-nNOS, anti-S100 protein, and anti-PGP9.5 antibodies at day 7 after nerve transection

a) H-E, b) nNOS, c) iNOS, d) eNOS, e) S100, f) PGP9.5, g) nNOS, h) S100, i) nNOS/S100, j) nNOS, k) PGP9.5, l) nNOS/PGP9.5: Spindle-shaped cells were densely present at nerve stump (red dotted line). These formed cell aggregates (yellow dotted line) which extended outward. nNOS-positive cell aggregates (black arrow) extending from stump were noted, and surrounding round cells were weakly positive for iNOS. eNOS-positive reaction was observed in micro blood vessels (white arrowhead) around cell aggregates. Schwann cells labelled with S100 protein were densely present at stump (black arrowhead), forming cell aggregates, but no axons labelled with PGP9.5 had infiltrated aggregates. nNOS and S100 protein were highly expressed in cell aggregates extending from nerve stump, demonstrating co-expression of nNOS and S100 protein (white arrow); no co-expression of nNOS and PGP9.5 was observed, however.

Scale bar = 100 μm

Fig. 4  H-E staining and immunostaining with anti-nNOS, anti-S100 protein, and anti-PGP9.5 antibodies at day 14 after nerve transection

a) H-E, b) nNOS, c) iNOS, d) eNOS, e) S100, f) PGP9.5, g) nNOS, h) S100, i) nNOS/S100, j) nNOS, k) PGP9.5, l) nNOS/PGP9.5: Cell aggregates extending from nerve stump increased and became dense. nNOS-positive cells (black arrow) were present in nerve tissue extending from stump, but distribution was scarce compared with that on day 7. iNOS was negative, but weak eNOS-positive reaction was observed in blood vessels in extending nerve tissue. There were many S100 protein-labelled Schwann cells (black arrowhead) and PGP9.5-labelled axons (white arrowhead) in extending nerve tissue. nNOS and S100 protein were expressed in nerve tissue extending from nerve stump, but no co-expression was observed. However, a few regions with co-expression of nNOS and PGP9.5 were observed in extending nerve tissue (white arrow).

Scale bar = 100 μm
extended from the stump or infiltrated the
tissue (Fig. 3f).
Double fluorescent immunostaining using
anti-nNOS and anti-S100 protein antibodies
revealed co-expression of nNOS and the S100
protein in cell aggregates extending from the
stump. Co-expression of nNOS and the S100
protein was also frequently detected in scat-
tered cells which had not formed aggregates
(Fig. 3g–i). Double fluorescent immunostain-
ing using anti-nNOS and anti-PGP9.5 anti-
bodies revealed micro axons expressing
PGP9.5, but not nNOS (Fig. 3j–l).

4. Localization of each type of NOS on day
14 after nerve transection
The number of cell aggregates extending
from the stump was higher on day 14 than on
day 7 after transection, resulting in a dense
morphology of nerve tissue (Fig. 4a).
Cells positive for nNOS were observed in
nerve tissue extending from the stump, but in
a smaller number than on day 7 (Fig. 4b).
Cells were negative for iNOS around the
stump (Fig. 4c); a weak eNOS-positive reac-
tion was observed, however, in micro blood
vessels in nerve tissue extending from the
stump (Fig. 4d). Staining for the S100 protein
revealed that the number of Schwann cells
was higher in extending nerve tissue on day
14 than on day 7; and the cell aggregates,
which were long and thin on day 7, were now
wider (Fig. 4e). Staining for PGP9.5 revealed
no PGP9.5-positive axons on day 7, whereas a
large number of axons extending to the
peripheral side were positive on day 14 (Fig.
4f).
Double fluorescent immunostaining using
anti-nNOS and anti-S100 antibodies revealed
many cells expressing only nNOS or the S100
protein; no co-expression of nNOS and the
S100 protein was observed, however (Fig.
4g–i). Double fluorescent immunostaining
using anti-nNOS and anti-PGP9.5 antibodies
revealed many cells expressing only nNOS or
PGP9.5. In addition, co-expression of nNOS
and PGP9.5, which was not observed at day 7,
was detected (Fig. 4j–l).

5. Localization of each type of NOS on day
21 after nerve transection
Axons extending from the stump became
thick and were densely distributed; no epi-
neurium had formed, however. In addition,
blood vessels entering extended nerve tissue
were noted (Fig. 5a).
Cells positive for nNOS were scattered in
extended nerve tissue, with the distribution
density greater than that on day 14 after tran-
section (Fig. 5b). No iNOS-positive reaction
was observed in the extending nerve tissue,
similar to on day 14 (Fig. 5c). Blood vessels
entering densely formed nerve tissue were
positive for eNOS (Fig. 5d). Staining for the
S100 protein revealed that the number of
Schwann cells had further increased in
extended nerve tissue compared with on day
14. On day 21, the Schwann cells were
arranged in a cord-like pattern and had a
similar distribution pattern to that of axons
(Fig. 5e). Staining for PGP9.5 revealed a large
number of long and thin axons extending
toward the peripheral side, and their number
was higher than that on day 14 (Fig. 5f).
Double fluorescent immunostaining using
anti-nNOS and anti-S100 protein antibodies
revealed many cells expressing only nNOS or
the S100 protein, whereas no co-expression
of nNOS and the S100 protein was observed,
similar to on day 14 (Fig. 5g–i). In contrast,
Double fluorescent immunostaining using
anti-nNOS and anti-PGP9.5 antibodies revealed
cexpression of nNOS and PGP9.5, and the
number of these cells was higher than that on
day 14 (Fig. 5j–l).

Discussion
Active regeneration of peripheral nerve tis-
te makes the mouse a popular choice in
such studies\textsuperscript{14}. Therefore, mouse was also
chosen for the present study, in which mor-
phological methods were used to investigate
regeneration after transection. The region
observed comprised regenerated nerve tissue
guided by Schwann cells. To focus on the
regeneration of axons, the central side of the
stump was examined. Nitric oxide synthase is attracting attention as a molecule expressed in both the normal and injured states. Therefore, here we investigated the expression and role of various types of NOS during the process of nerve regeneration.

The results revealed that nNOS was weakly expressed in axons labelled with PGP9.5 in normal sciatic nerve. However, no expression of nNOS was observed in Schwann cells labelled with the S100 protein, demonstrating that nNOS is not expressed in Schwann cells in the normal state. The nNOS-positivity of axons seen here may have been due to neurotransmission, as previously reported. In addition, eNOS was expressed in some vascular endothelial cells in sciatic nerve tissue, indicating that phenomena normally observed in other organs, such as blood flow adjustment, are also present in sciatic nerve tissue. In contrast, no iNOS expression was detected in normal sciatic nerve. One study has noted that iNOS is known to be expressed in inflammatory responses to stimulatory factors such as cytokines and lipopolysaccharides. In the present study, no expression of iNOS was observed because inflammatory reactions do not occur in normal sciatic nerve.

Swelling of the axon stump was noted on the central side on day 1 after transection of the sciatic nerve, which differed from normal conditions, and inflammatory cells infiltrated the stump. Double fluorescent immunostaining for nNOS and S100 protein revealed an nNOS-positive reaction in Schwann cells, which were labelled with the S100 protein in the superficial layer of the stump. This suggests that nerve transection stimulated cells in the superficial layer to express nNOS and produce NO. No nNOS was expressed in PGP9.5-labelled axons at the stump, however, indicating that no NO was produced. In a previous study, Schwann cells cultured after transection of the sciatic nerve expressed nNOS, suggesting the induction of nerve regeneration. These earlier findings suggest that, in the present study, nNOS expression in Schwann cells on day 1 after transection of the nerve was involved in promoting nerve regeneration. Many round cells assumed to
be inflammatory cells were observed in the stump and were iNOS-positive. An iNOS-positive reaction was previously shown to be induced around the ligated region in response to compressive stimulation by sciatic nerve ligation\textsuperscript{12}, suggesting that, in the present study, transection induced inflammatory cytokines, causing an inflammatory reaction. In contrast, no eNOS was expressed in the stump, indicating that it was not involved in the nerve regeneration process. This may have been due to the absence of vascularization on day 1 after transection.

On day 7 after sciatic nerve transection, nNOS-positive cells were observed at the tip of nerve tissue regenerating from the central side of the stump, and these cells formed aggregates which extended toward the peripheral side. These nNOS-positive cells were identified as Schwann cells due to detection of co-expression of nNOS and the S100 protein by double fluorescent immunostaining. Co-expression was detected in many cells scattered around the cell aggregates on day 7, suggesting that the number of nNOS-expressing Schwann cells had increased around the stump. The PGP9.5-labelled axons were short, and no co-expression with nNOS was observed by double fluorescent immunostaining, suggesting that they did not produce NO. In addition, many micro blood vessels expressing eNOS were observed, suggesting that new blood vessels increase around cell aggregates and that eNOS is involved in the repair of transected nerve tissue. When regenerating axons extend, the Schwann sheath is formed followed by axon regeneration toward the Schwann sheath, and Schwann cells influence the direction of axon regeneration\textsuperscript{20}. When a peripheral nerve is transected, Schwann cells that migrate for regeneration may dedifferentiate and return to immature cells. These cells accumulate with fibroblasts on the central side of the stump, forming cell aggregates, termed nerve bridges, and increase Sox2 through EphrinB\textsubscript{2}-EphB2 signal transmission, through which N cadherin is localized on the Schwann cell surface and axons extending from the central side of the stump infiltrate the nerve bridge\textsuperscript{18}. Therefore, Schwann cells may have migrated and formed aggregates, \textit{i.e.}, nerve bridges, to initiate the process of axon extension. Furthermore, signal release in these Schwann cells and the process of aggregation may have been mediated by NO and certain adhesion proteins.

On day 14 after sciatic nerve transection, a further increase was observed in cell aggregates extending from the stump. No co-expression of nNOS and S100 was observed with double fluorescent immunostaining, demonstrating that nNOS expression in the Schwann cells was lost together with subsequent production of NO. In contrast, weak co-expression of nNOS and PGP9.5 was observed in some regions where nerve fibers extended from the stump. This was attributed to axons beginning to express nNOS, initiating production of NO. Furthermore, some axons had penetrated Schwann cell aggregates to a small extent. This suggests that when axons pass through Schwann cell aggregates, the latter stop release of NO.

On day 21 after sciatic nerve transection, the results of double fluorescent immunostaining revealed no expression of nNOS in the Schwann cells, similar to on day 14. Expression of nNOS was observed in axons, however, with distribution and density increasing in comparison to that on day 14. In terms of NO production in regenerating axons, an earlier study also reported expression of nNOS in dorsal root ganglion at 21 days after sciatic nerve transection, indicating that nNOS expression is involved in the promotion of nerve regeneration\textsuperscript{36}. Taken together, this suggests that autocrine NO production plays a role in axon extension. Given the regeneration and extension of axons observed here on day 21, it may be necessary to investigate NO production on the peripheral side of the stump in further investigations.

The results of the present study suggest that NO is produced in axons and Schwann cells through induction of nNOS, and that this takes place in stages corresponding to the nerve regeneration process, whether that be in normal or transected tissue. Strong expres-
sion of nNOS was observed in Schwann cells proliferating soon after transection. On day 14, after the Schwann cell aggregates had been infiltrated by axons, the NO in these cells was lost, and was instead expressed in the axons. This adjustment in expression would not be possible without a specific exchange of information between the regenerated axons and the Schwann cells. Regenerated axons and Schwann cells adhere through cell adhesion molecules. A previous study reported that adhesion molecules, such as the neural cell adhesion molecule, are utilized by Schwann cells as scaffolds for axon extension, and that tight and gap junctions are present between axons and Schwann cells\(^5\). A system has been described in which axons extend through the exchange of specific information, indicating contact between axons and Schwann cells\(^5\). In the present study, axons had infiltrated the Schwann cell aggregates by day 14 after nerve transection. This suggests that the axons and Schwann cells had exchanged information, with NO acting as a transmitter through nNOS. The basement membrane on the surface of the Schwann cell contains proteins considered to play a role in axon elongation, such as laminin, fibronectin, and tenascin-C\(^14\). In the present study, nNOS was expressed in the Schwann cells on days 1 and 7 after nerve transection, suggesting that NO functions in Schwann cells to support the promotion of axon extension. Regeneration of the perineurium and basement membrane has an impact on outcomes after nerve transection, and may be influenced by NO production through nNOS.

**Conflict of Interest**

There is no conflict of interest to be disclosed in this report.

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