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***Chapter 5***

# RESPONSE OF MICROGLIA TO MOTONEURON CELL DEATH IN AXOTOMIZED INFANT RAT FACIAL NUCLEUS

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## ABSTRACT

Young motoneurons are highly vulnerable to insult. Transection of infant rat facial nerve leads to motoneuronal cell death in the ipsilateral nucleus. In this study, we investigated the age at which motoneurons are sensitive or robust to axotomy and determined the stage at which activated microglia emerge as dead-cell scavengers. Following nerve transection, living motoneurons were determined by Nissl staining. At 1 week after transection of the facial nerve in rats aged 2 days (2D), 1 week

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(1W), 2W, 4W, and 8W, the proportions of living motoneurons were approximately 10%, 25%, 75%, 100%, and 95%, respectively. We thus found that younger motoneurons are vulnerable to insult. A time-course experiment revealed that injured motoneurons of 2D rats were almost all alive at 1 day post-insult, but approx. 90% of motoneurons were dead within 3 days post-insult. These results indicated that almost all injured motoneurons die within 3 days post-insult. We next examined the microglial response to the process of motoneuronal cell death by using ionized $Ca^{2+}$ binding adapter molecule-1 (Iba1) and cFms (cFms proto-oncogene) as microglial markers. Immunohistochemical analysis demonstrated that the number of anti-Iba1 antibody-staining cells increased in the axotomized facial nucleus during 3-5 days post-insult. Immunoblotting quantitatively indicated that the levels of Iba1 and cFms in the axotomized facial nucleus were enhanced during 3-5 days post-insult. These results demonstrated that microglia were activated/proliferated from 3 to 5 days post-insult. We further confirmed that the microglia that emerged in the axotomized facial nucleus were anti-CD68 antibody-positive, indicating that the microglia are phagocytic. Taken together, these findings indicate that younger motoneurons more easily die when injured, and that dead motoneurons are cleaned by phagocytic microglia.

**Keywords:** infant rat, axotomy, facial nerve, motoneuron, cell death, microglia

## 1. INTRODUCTION

It is traditionally known that young motoneurons are highly vulnerable to insult. Transection (Aldskogius and Thomander, 1986; Zhang et al., 1995; Newfry and Jones, 1998) or crushing (Torvik and Søreide, 1975; Søreide, 1981) of the facial nerve in infant animals leads to motoneuronal cell death in the ipsilateral nucleus. However, the susceptibility of motoneurons to nerve transection is age-dependent (Olsson and Kristensson, 1979). Infant motoneurons easily die when axotomized, while adult injured motoneurons do not die in the same situation.

The phenomena in which immature motoneurons are vulnerable to nerve injury has been used to assay survival factors for motoneurons. Ciliary neurotrophic factor (CNTF) was the first survival factor known to

rescue injured infant motoneurons that would otherwise undergo cell death (Sendtner et al., 1990). Subsequently, other neurotrophic factors, including neurotrophins (Koliatsos et al., 1993) and transforming growth factor beta (TGFβ superfamily proteins (Zurn et al., 1994), have been continually reported to exert survival effects on injured immature motoneurons.

It has also been known that microglial activation/proliferation is accompanied by motoneuronal cell death in infant axotomized nucleus (Kreutzberg, 1996; Moran and Graeber, 2004). The microglial response has been analyzed mainly by immunohistochemical methods thus far, through which we know that microglia in a lesioned nucleus change their morphology and increase their cell number (Graeber et al., 1998). However, it remains necessary to quantitatively analyze the microglial response. This has not been carried out yet, presumably due to technical difficulties.

In this study, we first analyzed age-dependent motoneuronal cell death in rats aged postnatal 2 days (2D), 1 week (1W), 2 weeks (2W), 4 weeks (4W), and 8 weeks (8W). We next confirmed the expression of microglia-specific proteins in the neonatal stage, and, by using antibodies, carried out a quantitative analysis of microglial response in axotomized facial nucleus.

## 2. MATERIALS AND METHODS

### 2.1. Reagents and Antibodies

Cresyl violet for Nissl staining was obtained from Kanto Chemical (Tokyo, Japan).

Dulbecco's modified essential medium (DMEM) and minimum essential medium (MEM) for culturing neural cells was obtained from Life Technologies Japan (Tokyo, Japan).

Anti-ionized $Ca^{2+}$ binding adapter molecule 1 (Iba1) antibody was supplied by Wako Pure Chemical Industries (Osaka, Japan). Anti-cFms antibody and anti-actin antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-neurofilament (NF-L) antibody

was obtained from Affiniti Research Products (Exeter, UK). Anti-glial fibrillary acidic protein (GFAP) antibody was purchased from Millipore (Temecula, CA, USA). Anti-CD11b antibody and anti-CD68 antibody were from Bio Rad Laboratories (Hercules, CA, USA).

Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology. Alexa Fluor 488-conjugated anti-rabbit IgG, Alexa Fluor 568-conjugated anti-rabbit IgG and Alexa Fluor 568-conjugated anti-mouse IgG were obtained from Life Technologies Japan.

## 2.2. Animals

Wistar rats (8 weeks old, female and male) were purchased from Clea Japan (Tokyo), and the progeny were obtained by home breeding. Rats at ages 2 days (2D), 1 week (1W), 2W, 4W and 8W were subjected to the operation. 2D-, 1W-, and 2W-old rats (male and female) were operated on during nursing, and 4W- and 8W-old rats (male) underwent surgery after independence from their mothers. The rats were kept on a 12-h daylight cycle with food and water. In total, we used 86 rats in this study.

## 2.3. Operation

Animal experiments were carried out in accordance with the guidelines laid down by the NIH regarding the care and use of animals and were approved by the ethics committee of Soka University (approval code: 18002). We also made an effort to minimize the number of animals used in this study.

When 2D-old pups were used, we confirmed in advance that the mother rat did not abandon her pups. Right facial nerves of 2D- and 1W-old rats were axotomized at the stylomastoid foramen under cryoanesthesia. In the cases of 2W-, 4W-, and 8W-old rats, the right facial nerve was cut under diethylether anesthesia, as described previously (Yamamoto et al., 2010). Left facial nerves were left without any

treatment, and the nucleus was used as the control. The rats were reared for 1, 3, 5, 7, or 14 days and then decapitated under anesthesia. The whole brains were removed, frozen on dry ice, and stored at –80°C until used.

## 2.4. Preparation of Microglia, Astrocytes and Neurons

Primary brain cell culture was prepared from 2D-old rats, essentially according to the method described previously (Nakajima et al., 2007). Microglial cells were isolated by shaking the primary culture bottles (Nakajima et al., 2007). The microglia were seeded onto 90-mm dishes (Thermo Fisher Scientific, Waltham, MA, USA) with DMEM containing 10% fetal bovine serum (FBS). The purity was found to be over 99.9% by the assessment of Iba1 staining.

Astrocytes were prepared from the primary brain culture maintained for 3 weeks, as described (Maeda et al., 2009). The astrocytes were subcultured onto 90-mm dishes (Thermo Fisher Scientific) with DMEM containing 10% FBS. The cell purity was estimated to be 98% based on the staining with anti-GFAP antibody.

Neurons were prepared from the cerebral hemispheres of 16-day-old embryonic rat brains essentially according to a previously reported method (Nakajima et al., 2007). The dissociated neurons were seeded onto poly-L lysine-coated 90-mm dishes (Thermo Fisher Scientific) with MEM containing 2% FBS and 1 μM cytosine β-D-arabinofuranoside. After 1 day, the medium was replaced by MEM containing 2% FBS, and the culture was maintained further for 3 days. The purity of the neurons was estimated to be 97% based on staining with anti-NF-L antibody.

## 2.5. Histochemistry (Nissl Staining)

The facial nucleus of each aged brain was cut into 20-μm-thick sections with a cryostat (Leica CM1510; Leica Biosystems, Nussloch, Germany), and the sections were frozen at –80°C until staining.

For Nissl staining, the brainstem sections of 2D-, 1W-, 2W-, 4W-, and 8W-old rats were dehydrated and rehydrated, then stained with 0.5% cresyl violet/1M acetate buffer (pH 3.9) using Nissl staining methods (Konigsmark, 1970). Experiments were usually performed using 3 sections per brainstem.

## 2.6. Immunoblotting

The contralateral and ipsilateral facial nuclei were carefully cut from the frozen brainstem of each aged rat. The cut facial nuclei were solubilized with nonreducing sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, and 5% glycerol] and centrifuged at 100,000 g for 30 min. The supernatant of each facial nucleus was recovered as tissue extract. The protein in the tissue extract was quantified by the method of Lowry et al., (Lowry et al., 1951). The resultant tissue extract was prepared to contain 5% 2-mercaptoethanol, then subjected to immunoblotting for Iba1 (1:1000), cFms (1:200), NF-L (1:400), GFAP (1:1000), and actin (1:2000). The staining methods were described previously (Ichimiya et al., 2013).

## 2.7. Immunocytochemistry

Primary brain cells were prepared from 2D-old rat brain, as described above, and seeded onto the coverslips in wells of a 24-well plate at a density of $10^6$ cells/well. The culture was maintained for 10 days with DMEM containing 10% FBS.

The primary brain cells containing neurons, astrocytes, and microglia on coverslips were fixed with 3.7% paraformaldehyde/phosphate-buffered saline (PBS) for 10 min and blocked with 0.1% bovine serum albumin (BSA)/PBS, as described (Nakajima et al., 2007). For dual staining, the cells were initially incubated with anti-CD11b antibody (1:1000) at 4°C for 16 h and subsequently incubated with anti-Iba1 antibody (1:1000) or anti-cFms antibody (1:200) at 4°C for 16 h. After being rinsed with PBS, the

cells were incubated with a mixture of secondary antibodies (Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 568-conjugated anti-mouse IgG). The cells were sufficiently rinsed and mounted with Shandon PermaFluor (Thermo Fisher Scientific).

## 2.8. Immunohistochemistry

Cryosections prepared from the brainstem of each aged rat were immunohistochemically examined. The sections were fixed in 3.7% paraformaldehyde/PBS and treated sequentially with 50%, 100%, and 50% acetone for 2, 3, and 2 min, respectively. After treatment with 0.1% TritonX100/PBS, the sections were blocked with blocking solution containing 0.1% BSA/PBS (Ichimiya et al., 2013).

The cryosections were stained by the fluorescence method. For single staining, the sections were incubated with anti-Iba1 antibody (1:1000) or anti-cFms antibody (1:200) for 16 h at 4°C, rinsed, and reacted with Alexa Fluor 568-conjugated anti-rabbit IgG (1:1000) or Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000), respectively.

For dual staining, the sections were first incubated with anti-CD11b antibody (1:1000) for 16 h at 4°C and, after being rinsed, they were incubated with anti-Iba1 antibody (1:1000) or anti-cFms antibody (1:200) for 16 h at 4°C. The sections were subsequently incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000) and Alexa Fluor 568-conjugated anti-mouse IgG (1:1000).

These sections were rinsed and mounted with PermaFluor. The specimens were observed by a fluorescence microscope (Eclipse TS100; Nikon, Tokyo, Japan).

## 2.9. Statistical Analysis

The Nissl-stained motoneurons in the facial nucleus were counted, and the densities of protein bands (Iba1 and cFms) in immunoblotting were measured by densitometry using ImageJ software (NIH, Bethesda, MD,

USA). The cell numbers and densities were expressed as the means ± SDs of 3 separate experiments. Differences between the contralateral and ipsilateral nuclei were assessed via Student's *t*-test. In all cases, P values less than 0.05 were considered significant (*P < 0.05, **P < 0.01).

## 3. RESULTS

### 3.1. Age-Dependent Vulnerability of Motoneurons to Nerve Transection

To confirm the age-dependent susceptibility of motoneurons to axotomy, we determined the survivability of motoneurons in axotomized facial nucleus at 7 days post-insult in rats aged 2D, 1W, 2W, 4W, and 8W.

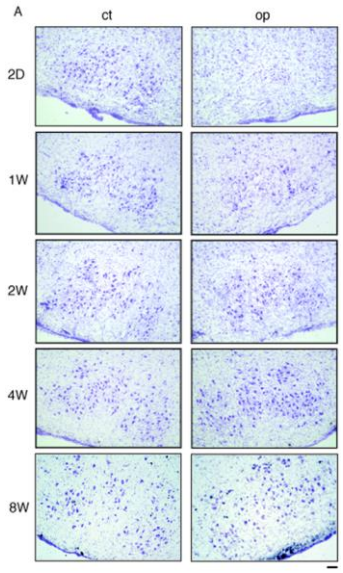


Figure 1A. Determination of living motoneurons in axotomized facial nucleus. A. Nissl staining of axotomized facial nucleus prepared from rats of different ages. Cryosections of brainstem prepared from 2D-, 1W-, 2W-, 4W-, and 8W-old rats whose right facial nerve was cut 7days before were stained by Nissl staining (see Methods). Ipsilateral nucleus (op) and contralateral nucleus (ct) are shown on the right-hand side and left-hand side, respectively. Scale bar represents 100 μm.

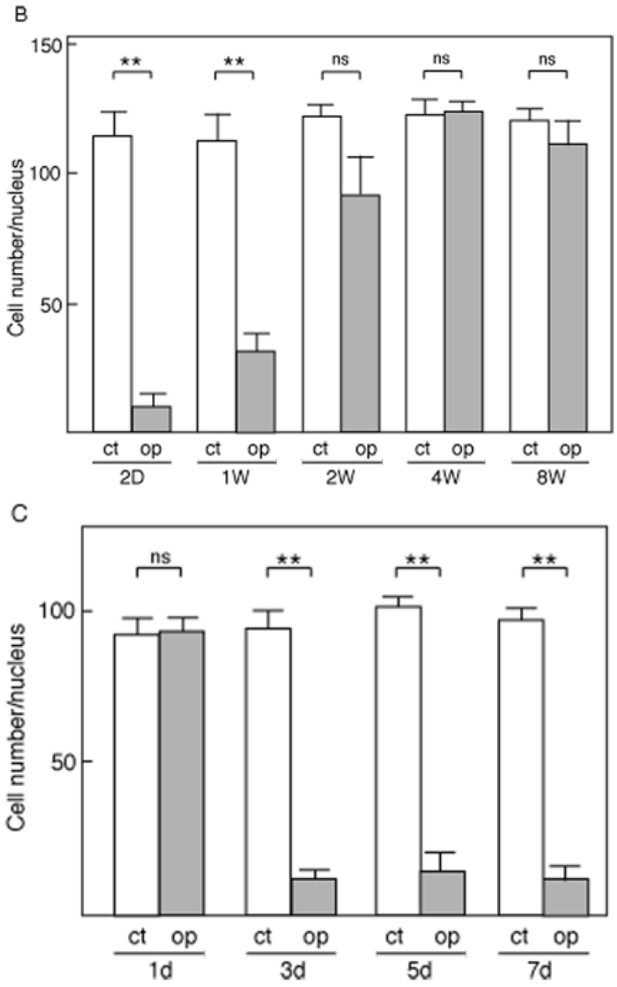


Figure 1B and C. Determination of living motoneurons in axotomized facial nucleus. B. Quantification of Nissl staining. The Nissl-stained motoneurons were counted and statistically compared between the control (ct) and injured facial nuclei (op) taken from 2D-, 1W-, 2W-, 4W-, and 8W-old rats. Data shown are means ± SDs from an experiment using 3 rats (ns: not significant; **P < 0.01). C. Time course of motoneuronal cell death. Right facial nerve in 2D-old rats was transected, and the brains were recovered at 1 day, 3 days, 5 days, and 7days post-insult. The brainstem cryosections prepared at each time point were Nissl-stained as described in Figure 1A and quantified as shown in Figure 1B. The data shown are means ± SDs from three independent experiments (ns: not significant; **P < 0.01).

Nissl staining revealed that 2D-old rats had significant fewer living motoneurons in the axotomized facial nucleus than in that of the control nucleus, whereas in 8W-old rats the number appeared to be the same as that in the control nucleus (Figure 1A). The results in the 1W-, 2W-, and 4W-old rats indicated that less-mature motoneurons tend to die more easily (Figure 1A).

We statistically summarized the proportion of surviving motoneurons in the axotomized facial nucleus of rats of each age. The values of 2D-, 1W-, 2W-, 4W-, and 8W-old rats were 11 ± 5/115 ± 9, 31 ± 7/113 ± 12, 92 ± 15/122 ± 5, 125 ± 4/123 ± 7, and 111 ± 9/120 ± 5, respectively (Figure 1B). We thus confirmed that motoneuronal cell death in axotomized facial nucleus is age-dependent, and that motoneurons die more easily in younger rats than in older ones.

### 3.2. Time Course of Cell Death of Injured Motoneurons

We performed a post-transection time course experiment to clarify the process of motoneuronal cell death in axotomized facial nucleus. After transection of the facial nerve in 2D-old rats, the brains were removed at 1d, 3d, 5d, and 7 days post-insult, and the brainstem sections were stained by Nissl staining. Counting the living motoneurons, we summarized the results in Figure 1C. The values at 1d, 3d, 5d, and 7d post-insult were 93 ± 4/92 ± 5, 11 ± 3/94 ± 16, 14 ± 6/102 ± 3, and 12 ± 4/98 ± 4. The results indicated that at 1 day post-insult, most of the motoneurons were alive, but after 3 days post-insult, many motoneurons died. We found that approximately 90% of motoneurons underwent cell death after 3 days post-insult.

### 3.3. Specific Proteins of Microglia

It is well known that microglia become activated and proliferative in the period of motoneuronal cell death (Graeber et al., 1998). To quantify

the activation/proliferation of microglia in the axotomized facial nucleus of immature rats, we checked in advance the specific proteins in microglia as indicators.

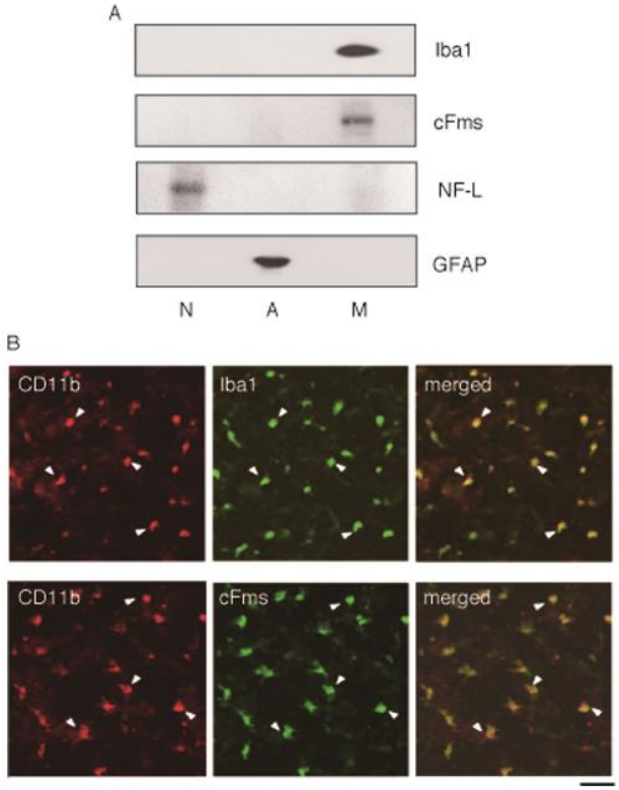


Figure 2. Examination of microglial-specific proteins. A. Expression of Iba1 and cFms proteins in neural cell homogenates. Cellular extract prepared from primary neurons (N), astrocytes (A), and microglia (M) (see Methods) was subjected to immunoblotting for Iba1, cFms, NF-L, and GFAP. B. Immunocytochemistry for CD11b/Iba1 and CD11b/cFms. Primary brain cells on the coverslips in a 24-well plate were dually stained with anti-CD11b antibody and anti-Iba1 (upper panels) or with anti-CD11b antibody and anti-cFms antibody (lower panels). In each case, the merged image is shown on the right-hand side. The scale bar represents 50 μm.

We prepared neurons and glial cells (astrocytes and microglia) from embryonic and neonatal rat brain-derived primary cultures, respectively (see Methods), and examined the reactivity of anti-Iba1 and anti-cFms

antibodies to these cell homogenates in Western blotting. Anti-NF-L antibody and anti-GFAP antibody detected each antigen in neuron homogenate and astrocyte homogenate, respectively (Figure 2A). On the other hand, Iba1 and cFms proteins were detected in only the microglial homogenate (Figure 2A). These results indicated that Iba1 and cFms proteins were expressed only in microglia and not in neurons or astrocytes.

To ascertain the specificity of those antibodies, we carried out immunocytochemistry in neonatal rat brain-derived primary culture. The cells were dually stained with anti-CD11b/Iba1 antibodies or anti-CD11b/cFms antibodies. CD11b has been accepted as a microglia-specific marker, recognizing CR3 complement receptor (Graeber et al., 1988; Choucair-Jaafar et al., 2011). A fluorescence method indicated that anti-Iba1-antibody positive cells are all anti-CD11b-antibody positive (Figure 2B). Furthermore, anti-cFms-antibody positive cells are consistent with anti-CD11b-antibody positive cells (Figure 2B).

These results indicated that anti-Iba1 antibody and anti-cFms antibody can detect Iba1 and cFms molecules specifically, even in immature rat brains. We thus used these antibodies to analyze the microglial response in infant axotomized facial nucleus.

### 3.4. Response of Iba1 to the Transection of Facial Motoneurons

Using anti-Iba1 antibody, we examined immunohistochemically the microglial response in a time course in axotomized facial nucleus in 2D-old rats. The results indicated that at 1 day post-insult, there appears to be no Iba1 staining in axotomized facial nucleus, similar to the case in the control nucleus, but at 3-5 days post-insult the staining intensity in axotomized nucleus became strong or dense compared to that in the control nucleus (Figure 3). At 7 days post-insult, the degree of Iba1 staining became low (Figure 3). Therefore, we found that microglia were highly activated and/or proliferating in axotomized facial nucleus during 3-5 days post-insult, but after that, they became calm.

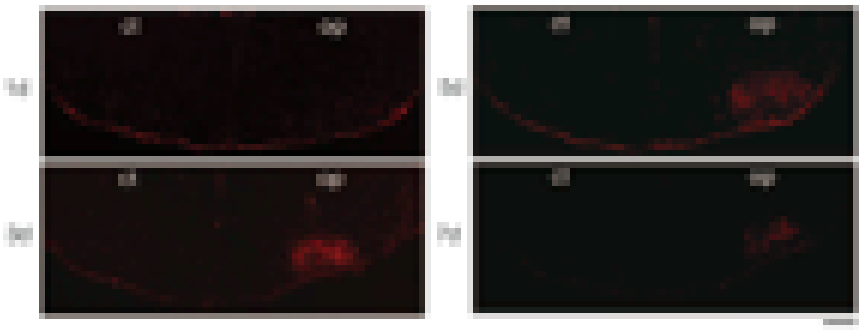

Figure 3. Time course of Iba1 expression in axotomized facial nucleus. Right facial nerve in 2D rats was transected and the brains were recovered at 1 day, 3 days, 5 days, and 7 days post-insult. The brainstem sections of each brain were immunohistochemically stained with anti-Iba1 antibody. Contralateral nucleus (ct) and ipsilateral nucleus (op) are shown on the left-hand side and right-hand side, respectively. The scale bar represents 200 μm.

## 3.5. cFms in Activated/Proliferating Microglia in Axotomized Facial Nucleus

We immunohistochemically investigated whether cFms protein is expressed in microglia in axotomized facial nucleus of 2D-old rats, focusing on 3 days post-insult when Iba1 was strongly expressed. The immunohistochemical image indicated that anti-cFms-antibody significantly stained axotomized facial nucleus, but not control nucleus (Figure 4A). Dual staining with anti-CD11b antibody and anti-cFms antibody indicated that anti-cFms antibody-positive cells were all anti-CD11b-antibody-positive (Figure 4B, 4C), verifying that cFms protein is expressed in microglia. We thus recognized that cFms protein, like Iba1, can be used as a microglial marker in axotomized immature facial nucleus.

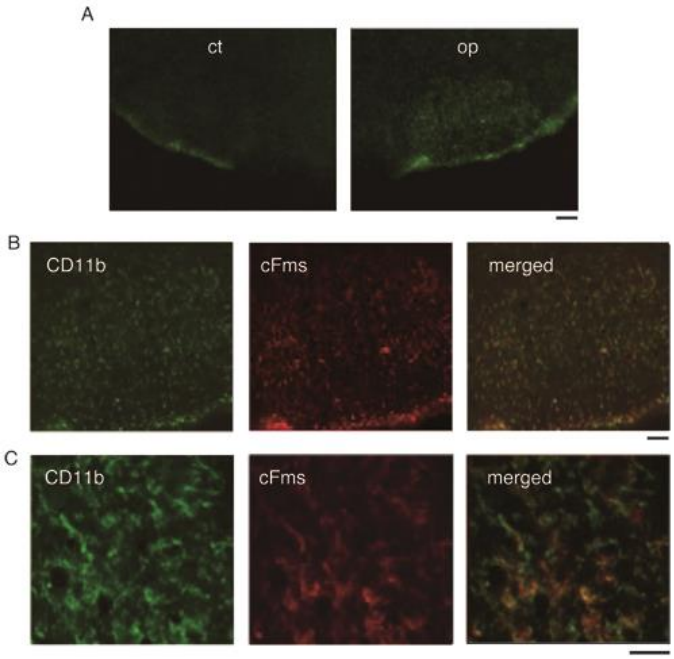


Figure 4. Immunohistochemistry for CD11b/Iba1 and CD11b/cFms. Brainstem sections of 2D-old rats whose right facial nerve was transected 3 days previously were stained with anti-cFms antibody (A) and further dually stained with anti-CD11b and anti-cFms antibody (B and C). Magnified photos are shown in C. In both cases, merged images are shown on the right-hand side. The scale bar in (A) and (B/C) represents 100 μm and 50 μm, respectively.

## 3.6. Quantification of Microglial Reactivity in Axotomized Infant Facial Nucleus

To show quantitatively the microglial response to motoneuronal injury/cell death, we analyzed the levels of Iba1 and cFms in axotomized facial nucleus in 2D-old rats. Western blotting indicated that the levels of Iba1 in operated facial nucleus increased at 3-5 days post-insult and decreased from 7 days to 14 days post-insult (Figure 5A). The values in the ipsilateral nucleus relative to that of the control nucleus (defined as 1.0) are 1.4 ± 0.5, 5.0 ± 1.3, 3.5 ± 0.9, 2.3 ± 1.1, 1.1 ± 0.1 at 1, 3, 5, 7, and 14 days post-insult, respectively (Figure 5A).

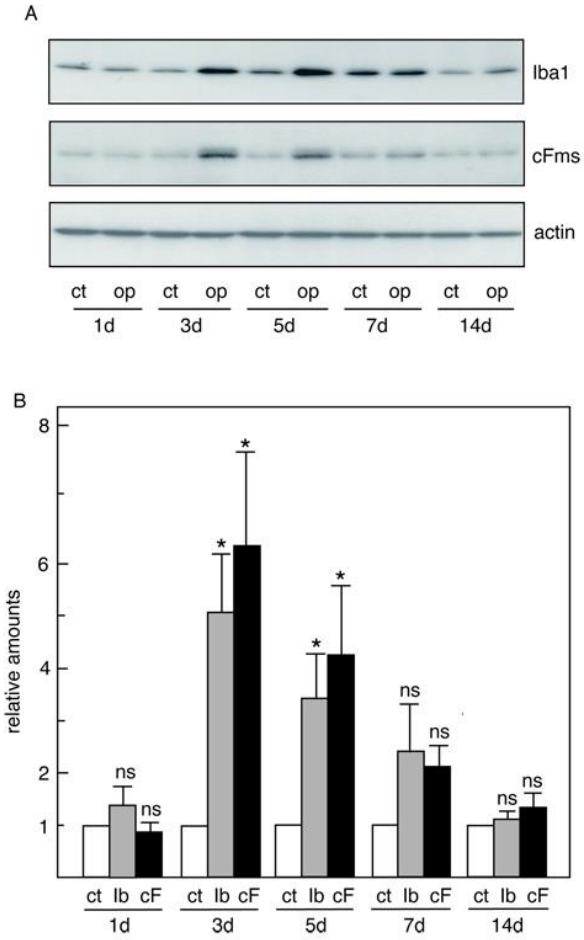


Figure 5. Time-course analysis of Iba1 and cFms in axotomized facial nucleus. A. Immunoblotting for Iba1, cFms, and actin. Right facial nerves of 2D-old rats were transected and the brains were recovered at 1 day, 3 days, 5 days, 7 days, and 14 days post-insult. Contralateral nucleus (ct) and ipsilateral nucleus (op) were cut out from each brain and tissue extracts were prepared (see Methods). These tissue samples were immunoblotted for Iba1, cFms, and actin. B. Statistical analysis of the results of immunoblotting. The intensities of the Iba1 (Ib) and cFms (cF) bands in A were determined by a densitometer, and the values for the operated side were expressed relative to those for the control side (ct) (defined as 1.0). The data shown are means ± SDs from three independent experiments (ns: not significant; *P < 0.05).

Similarly, the levels of cFms in axotomized facial nucleus were examined. The levels were transiently upregulated at 3-5 days post-insult

(Figure 5A). The statistical analysis indicated that the values in injured nucleus relative to control nucleus are 0.9 ± 0.2, 6.3 ± 1.8, 4.3 ± 1.2, 2.1 ± 0.5, 1.3 ± 0.3 at 1, 3, 5, 7, and 14 days post-insult, respectively (Figure 5B).

We found that the profiles of Iba1 and cFms resembled each other. Actin levels were quite equal (constant) between control and axotomized nuclei over 14 days post-insult. These results unambiguously indicated that microglia were significantly activated/proliferated in axotomized facial nucleus at 3-5 days post-insult in 2D-old rats.

## 3.7. Phagocytic Property of Activated/Proliferating Microglia

We confirmed whether the activated/proliferating microglia in axotomized facial nucleus of 2D-old rat are phagocytic or not.

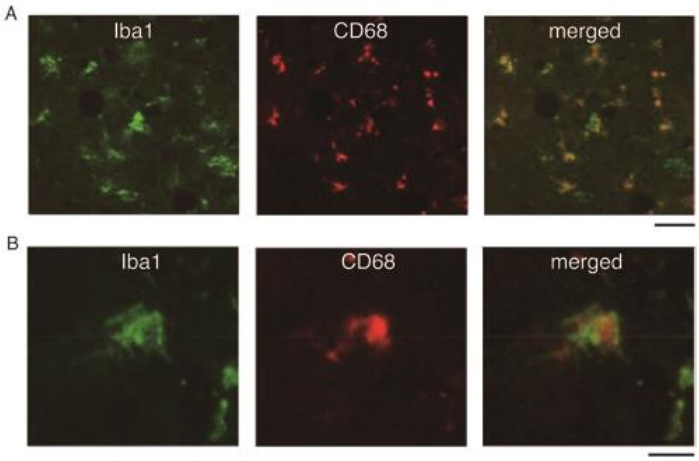


Figure 6. Immunohistochemistry for Iba1/CD68. Brainstem sections prepared from 2D-old rats whose facial nerve was cut 5 days previously were dually stained with anti-Iba1 antibody and anti-CD68 antibody (A). The scale bar represents 50 μm. A magnified view is also shown (B). In both cases, merged images are shown on the right-hand side. The scale bar represents 20 μm.

In this study, CD68 was used as a phagocytic marker because this molecule is a lysosomal membrane protein and highly expressed in phagocytic cells (Holness et al., 1993). In fact, the molecule was found to be effective for evaluating phagocytic microglia (Graeber et al., 1998).

## 4. DISCUSSION

In the present study, we showed that younger facial motoneurons are more vulnerable to axotomy and die more easily. This phenomenon was coincident with previous reports in which the facial nerves of various animals were axotomized, including rats (Aldskogius and Thomander, 1986; Baumgartner and Shine, 1998; Tong and Rich, 1997), mice (Zhang et al., 1995), and hamsters (Newfry and Jones, 1998). This prompted us to wonder if there is a common reason for motoneuronal cell death. The answer may come from the study of neurotrophic factors. Previous studies clarified that injured young motoneurons in axotomized facial nucleus could be rescued by administering neurotrophic factors, including CNTF (Sendtner et al., 1990), BDNF (Sendtner et al., 1992; Koliatsos et al., 1993), insulin-like growth factor (IGF)/leukemia inhibitory factor (LIF) (Hughes et al., 1993), glial cell line-derived neurotrophic factor (GDNF) (Zurn et al., 1994; Yan et al., 1995), and acidic fibroblast growth factor (aFGF) (Cuevas et al., 1995). Such studies allowed us to speculate that infant motoneurons daily obtain the neurotrophic factors they need to survive mainly from their target tissue (expression muscle). Therefore, they cannot survive if their axons are cut off by axotomy, nor can they obtain neurotrophic factors produced in the target tissue. In contrast, adult motoneurons may get neurotrophic factors not only from their target tissue, but also from glial cells distributed around the motoneuron cell bodies. Astrocytes (Zaheer et al., 2001; Lin et al., 2016) and microglia (Nakajima et al., 2001; Nakajima et al., 2007) have been shown to produce/secrete neurotrophic factors such as neurotrophins and GDNF. Thus adult motoneurons, unlike immature motoneurons, might survive by absorbing neurotrophic factors from their environment if the supply from the target

tissue is interrupted. In any event, we can confirm that younger motoneurons in facial nucleus die easily if their axons are transected.

Motoneuronal cell death is a serious matter in the nervous system, and information should be sent to the cells around the injured motoneurons. Microglia are the most plausible cell type in the nervous system for cleaning dead neurons as scavengers. Thus, it is interesting to analyze the response of microglia in axotomized infant facial nuclei in which motoneuronal cell death occurs. We selected Iba1 as a microglial marker (Imai et al., 1996; Ito et al., 1998) and used it to evaluate microglial activation/proliferation. A time-course experiment by immunohistochemical methods indicated that microglia were activated/ proliferating in axotomized facial nucleus at 3-5 days post-insult, but after 5 days post-insult, their reactivity was weakened. These results essentially agreed with our previous report (Graeber et al., 1998). However, the results of immunohistochemistry may not necessarily mean an accurate microglial response because it is difficult to quantify the results of immunohistochemistry carried out by using multiple animals. In the present analysis, we quantified the amounts of Iba1 contained in the extract of facial nucleus taken from the brainstem of multiple rats. Although it has been technically difficult to correctly cut out the facial nucleus from the small brainstem of a 2D-old rat, we overcame this problem by using a conspicuous magnifying glass in a highly accurate freezer box. This is the first time that microglial activation/proliferation in young facial nucleus was quantitatively determined by Iba1.

Previously, in the process of checking microglial-specific proteins, we noticed that cFms protein was expressed in the cell homogenate of primary microglia. cFms was originally identified as a proto oncogene (Müller et al., 1983) and was later identified as a receptor for M-CSF (Yen et al., 1996; Kakiuchi-Kiyota et al., 2014). The receptor protein was strongly suggested to be induced in axotomized facial nucleus in a $^{125}$I-M-CSF binding experiment (Raivich et al., 1991). We analyzed the transition of cFms levels in axotomized adult facial nucleus and showed that cFms protein was expressed in microglia (Yamamoto et al., 2010). However, it remained necessary to examine whether the cFms is expressed in infant

microglia. This point was clarified in this study. Thus, using anti-cFms antibody, we quantified the microglial response in 2D-old rats. The quantified results revealed that the transition profile in axotomized facial nucleus quite resembled that of Iba1. This supported that cFms, expressed in microglia, can be used as an indicator of microglial activation/ proliferation.

To investigate the relationship between motoneuronal cell death and microglial activation/proliferation in axotomized facial nucleus, we compared the profiles of the two. At 1 day post-insult, few motoneurons had died and the levels of Iba1/cFms hde not changed, suggesting a quiescent state. At 3-5 days post-insult, approximately 80-90% of motoneurons had died and the levels of Iba1/cFms had been largely upregulated, suggesting a highly activated state of microglia. After 5 days post-insult, microglial activation/proliferation had markedly subsided, presumably because the dead motoneurons had all been scavenged. It is thus suggested that motoneuronal cell death and microglial activation are intimately associated with each other.

Simultaneously, the phenomenon allowed us to consider an intercellular interaction between injured motoneurons and microglia. What signaling molecule(s) or stimuli from injured motoneurons activate microglia? In the case of infant animals, degenerating neurons would release specific signals known as the “find me” signal (Sokolowski et al., 2014) and the “eat me” signal (Segawa et al., 2015; Bagalkot et al., 2016). Sensing the “find me” and “eat me” signals by means of various receptors, the microglia in the axotomized facial nucleus would transform into phagocytic cells, inducing CD68 protein after 3-5 days post-insult. CD68 is a lysosomal protein and has been used a marker of phagocytic cells like macrophages (Tsang and Chan, 1992; Holness and Simmons, 1993). The phagocytic microglia (macrophages) would actively engulf the dying/dead motoneurons at 3-5 days post-insult. Then, after serving as phagocytic cells, the microglia would gradually return to nonphagocytic cells at 7-14 days post-insult.

Generally, chemokines, including fractalkine (Imai et al., 1997), and nucleotides such as uridine diphosphate (UDP) (Koizumi et al., 2007;

Neher et al., 2014) are classified as “find me” signals, while phosphatidylserine is identified as the major “eat me” signal (Sambrano and Steinberg, 1995; Elward and Gasque, 2003). These molecules might be released from dying motoneurons. It is also speculated that ATP/ADP (Koizumi et al., 2013) is released from injured motoneurons and act as possible stimulators of phagocytic activity of microglia. Thus, these molecules derived from dying motoneurons might be associated with the transformation of microglia to phagocytic cells in the facial nucleus of an infant rat.

As described above, it is suggested that activated/proliferating microglia interact closely with the dying/dead motoneurons in axotomized facial nucleus through the mediation of specific molecules and their receptors. However, little is known about the precise mechanism by which microglia change into macrophage-like cells found in the degenerating facial nuclei of infant animals. This issue will continue to be analyzed in detail in future studies.

## CONCLUSION

We found that younger motoneurons are more susceptible to nerve injury and die more easily. The dead/dying motoneurons were suggested to be cleaned by phagocytic microglia with high levels of Iba1/cFms.

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