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Alterations in neuronal survival and glial reactions after axotomy by ceftriaxone and minocycline in the mouse hypoglossal nucleus

Jun Yamada [a], Shozo Jinno [b,*]

[a] Department of Anatomy and Neurobiology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Department of Developmental Molecular Anatomy, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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ABSTRACT

Some antibiotics are suggested to exert neuroprotective effects via regulation of glial responses. Attenuation of microglial activation by minocycline prevents neuronal death in a variety of experimental models for neurological diseases, such as cerebral ischemia, Parkinson's and Huntington's disease. Ceftriaxone delays loss of neurons in genetic animal models of amyotrophic lateral sclerosis through upregulation of astrocytic glutamate transporter expression (GLT-1). However, it remains largely unknown whether these antibiotics are able to protect neurons in axotomy models for progressive motor neuron diseases. Recent studies have shown that the axotomized motoneurons of the adult rat can survive, whereas those of the adult mouse undergo neuronal degeneration. We thus examined the possible effects of ceftriaxone and minocycline on neuronal loss and glial reactions in the mouse hypoglossal nucleus after axotomy. The survival rate of lesioned motoneurons at 28 days after axotomy (D28) was significantly improved by ceftriaxone and minocycline treatment. There were no significant differences in the cellular densities of astrocytes between ceftriaxone-treated and saline-treated animals. Ceftriaxone administration increased the expression of GLT-1 in the hypoglossal nucleus, while it suppressed the reactive increase of glial fibrillary acidic protein (GFAP) expression to control level. The cellular densities of microglia at D28 were significantly lower in minocycline-treated mice than in saline-treated mice. The time course analysis showed that immediate increase in microglia at D3 and D7 was not suppressed by minocycline. The present observations show that minocycline and ceftriaxone promote survival of lesioned motoneurons in the mouse hypoglossal nucleus, and also suggest that alterations in glial responses might be involved in neuroprotective actions of antibiotics.

In physiological conditions, microglia continuously survey the local microenvironment in the brain [18], while astrocytes are mainly involved in metabolic support of neurons [11]. In addition, glial cells play critical roles in the development and progression of brain pathology [9,22]. Several reports suggest that glial cells might act as a double-edged sword being either detrimental or protective depending on the context [3]. For instance, activated microglia synthesize potentially neurotoxic molecules such as reactive oxygen species, and inflammatory cytokines [9]. Conversely, microglia release some neuroprotective molecules in specific conditions [9]. Activated astrocytes also release a wide variety of neuroprotective and/or neurotoxic mediators [22].

Experimental axotomy allows systematic and detailed study of slow motor neuron death and glial reactions [13]. Interestingly, the axotomized motoneurons of the adult rat can survive, whereas similar axotomy leads to gradual cell death of the injured motoneurons in the adult mouse [25]. Accordingly, the slow neuronal death seen in the adult mouse is thought to be a suitable model for progressive neurodegenerative disorders. After nerve dissection, astrocytes up-regulate within hours the gap junction protein connexin-43, and within one day glial fibrillary acidic protein (GFAP [1]). Concomitantly, microglial cells proliferate and migrate towards the axotomized neuron perikarya [4].

Minocycline, a tetracycline antibiotic, has been shown to inhibit microglial activation and promote neuronal survival in experimental models for neurological diseases [26]. In addition, β-lactam antibiotics, such as ceftriaxone, may protect neurons in animal models of motoneuron diseases and brain ischemia [19,21]. The protective effect of ceftriaxone is considered to be involved in glutamate clearance through increased expression of the astrocytic glutamate transporter, GLT-1. It should also be noted that the therapeutic gain of antibiotics has been contradicted by several studies [7,12]. On the other hand, in spite of the rapidly increasing interest in neuroprotective actions of antibiotics, it remains largely unknown whether these drugs were able to protect neurons in axotomy models.

* Corresponding author. Tel.: +81 92 642 6046; fax: +81 92 642 6202.
E-mail address: sjnno@med.kyushu-u.ac.jp (S. Jinno).

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The aim of this study was to determine whether antibiotics might influence the neuronal survival and glial reactions after hypoglossal axotomy in the adult mouse. Our study is the first to report the improved neuronal survival and altered glial reactions by minocycline and ceftriaxone in the axotomized hypoglossal nucleus of mice.

Adult male C57BL/6 mice (22–25 g body weight, 8–11 weeks old) were used for histological analysis. Every procedure was approved by the Committee of the Ethics on Animal Experiment in the Graduate School of Medical Sciences, Kyushu University, and was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, Rev. 1996). All efforts were made to minimize the number of animals used and their suffering.

Animals were anaesthetized with pentobarbital (45 mg/kg body weight, i.p.), and were positioned supine. At the proximal side of the right hypoglossal nerve bifurcation, the nerve was transected with a pair of scissors, and the nerve stumps were placed not to touch with each other. Samples from sham control animals were taken from animals subjected to identical surgical exposure without axotomy.

Twenty six axotomized mice were divided into three groups, and received daily intraperitoneal injection of drugs or saline from the first day of hypoglossal axotomy (D0): ceftriaxone-treated group (200 mg/kg, Chugai Pharmaceutical Company, Tokyo, Japan; $n = 8$), minocycline-treated group (30 mg/kg, Taiyo Pharmaceutical Industry, Nagoya, Japan; $n = 8$), and saline-treated group (5 ml/kg, $n = 10$). In addition, five mice received daily intraperitoneal injection of minocycline (30 mg/kg, Taiyo Pharmaceutical Industry) from D7. All animals were sacrificed at D28.

Histological and immunocytochemical experiments were conducted by using perfusion fixed sections as described previously [25]. Here we only briefly highlight the primary antibodies; rabbit polyclonal anti-ionized calcium-binding adaptor molecule 1 (Iba1) antibody (1:10,000; Wako Pure Chemical Industries, Osaka, Japan), guinea pig polyclonal anti-GLT-1 antibody (1:5000; Frontier Science, Sapporo, Japan), mouse monoclonal anti-GFAP antibody (1:10,000; Dako, Carpinteria, CA), mouse monoclonal anti-S100β antibody (1:25,000; Sigma–Aldrich, St. Louis, MO), mouse monoclonal anti-synaptophysin antibody (1:25,000; Sigma–Aldrich), and goat polyclonal anti-Olig2 antibody (1:25,000; R&D systems, Minneapolis, MN).

Six sections from three mice were examined from sham-operated animals and axotomized animals, respectively. YOYO-1 (Invitrogen, Carlsbad, CA) was used for morphometric measurements of cell body size. The maximum diameters of individual cell bodies were measured using ImageJ 1.44 (NIMH, Bethesda, MD). Statistical significances of histogram distributions were examined with Kolmogorov–Smirnov test.

Sixteen sections from eight animals were examined from saline-treated group, ceftriaxone-treated group and minocycline-treated group, respectively. Nissl-stained motoneurons in the hypoglossal nucleus were counted by using the Cell Counter plugin of ImageJ 1.44 (NIMH). The survival ratios were calculated by dividing the numbers of motoneurons in the lesioned side by those in the control side.

Eight sections from four mice were selected from each group. Nuclei of S100β-positive astrocytes and Iba1-positive microglia were counted by using DAPI staining. To avoid double counting, the sampling was carried out by using the Cell Counter plugin of ImageJ 1.44 (NIMH).

Eight sections from four animals were selected from each group. The stacks of 20 optical sections (1 μm interval) from hypoglossal region were scanned with a CLSM using an oil immersion objective lens (63×, pixel size = 0.23 μm × 0.23 μm). Single representative optical sections were selected from each stack. The lower threshold value of immunoreactivity was set at 33% of maximum intensity of the gray scale, and the puncta smaller than 0.1 μm^2 were eliminated from the results. Then, the surface area was calculated as the percentage of immunoreactive pixels over the total pixels.

Sections were incubated with mouse monoclonal anti-GFAP antibody (1:10,000; Dako), and then they were incubated with FITC-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in phosphate buffered saline for 3 h at room temperature. GFAP-labeled cells were penetrated with glass microelectrode filled with 2.5% Lucifer yellow (Sigma–Aldrich) in distilled water. The dye was injected into a cell by passing 2 nA negative current in 1 s pulse applied 0.5 Hz for 1 min. The sections containing the labeled cells were re-fixed and processed for immunohistochemistry.

All data sets were analyzed in the statistical environment R version 2.11 (http://www.R-project.org). Comparisons were made using one-way analysis of variance (ANOVA) for paired samples with post hoc Tukey's test. Significant differences were considered to occur when a P value of <0.05 was obtained.

It has been reported that peripheral nerve axotomy might induce neuronal atrophy [17]. We thus aimed to set size criteria for neuronal counting. Considering the reduced expression of neuronal markers in lesioned neurons, we employed three glial markers to discriminate neurons and three major types of glial cells (Fig. 1A). Namely, GFAP, Iba1 and Olig2 were used to label astrocytes, microglia and oligodendrocytes, respectively. The diameter of each cell was estimated by using a cytoplasmic marker, YOYO-1.

There were no significant size differences in glial marker-negative putative neurons between sham-operated and axotomized mice (Fig. 1C). In axotomized animals, the soma size of glial marker-negative putative neurons was significantly larger than that of glial marker-positive cells. Two-sided 95% confidence intervals of soma size of glial marker-negative putative neurons and glial marker-positive glial cells were 11–35.6 μm and 4.5–9 μm, respectively. We thus defined the cell with a soma diameter larger than 10 μm as a neuron.

The possible effects of antibiotics on neuronal cell death were examined at D28 (Fig. 1B and D). The survival ratios of axotomized motoneurons in the hypoglossal nucleus were significantly higher in ceftriaxone-treated ($61.2 \pm 13.9\%$) and minocycline-treated mice ($60.1 \pm 14.1\%$) than in saline-treated animals ($32.2 \pm 11.9\%$). These results indicate the neuroprotective effect of ceftriaxone and minocycline treatment in the mouse hypoglossal axotomy model.

Previous studies have indicated that ceftriaxone regulates the activity of astrocytic glutamate transporters [21]. We therefore examined whether ceftriaxone might affect the reactions of astrocytes following hypoglossal axotomy. In the hypoglossal nucleus, S100β was strongly expressed in astrocytes, while it was also weakly expressed in neurons (Fig. $2A_{1-3}$ [20]). Based on the morphological characteristics and intensity of S100β labeling, we were able to discriminate neurons and astrocytes. At D28, there were no significant differences in the numerical densities of S100β-labeled astrocytes among sham, saline-treated, and ceftriaxone-treated animals (sham = 45.2 ± 8.6 cells/mm^2, saline = 53.5 ± 4.9 cells/mm^2, ceftriaxone = 49.7 ± 7.0 cells/mm^2; Fig. $2A_4$). The density of S100β-labeled astrocytes at D14 showed no significant difference between saline-treated and ceftriaxone-treated mice, neither (saline = 54.9 ± 4.5 cells/mm^2, ceftriaxone = 55.8 ± 5.9 cells/mm^2).

We then examined the possible effect of ceftriaxone on the expression of GLT-1 in the hypoglossal nucleus after axotomy. At D28, the GLT-1-immunoreactive surface area was significantly increased in ceftriaxone-treated mice than in sham-operated and saline-administrated mice (Fig. 2B). Intracellular dye injection into single astrocyte revealed that GLT-1 was expressed in astrocyte processes (Fig. $2C_1$). Immunofluorescent double labeling showed