Comparison of FGF1 (aFGF) Expression between the Dorsal Motor Nucleus of Vagus and the Hypoglossal Nucleus of Rat

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Neurons in the dorsal motor nucleus of the vagus (DMNV) are more severely affected by axonal injury than most other nerves, such as those of the hypoglossal nucleus. However, the mechanism underlying such a response remains unclear. In this study, we compared the expression of fibroblast growth factor 1 (FGF1), a neurotrophic factor, between the DMNV and the hypoglossal nucleus by RT-PCR and immunohistochemical analyses. RT-PCR showed that the level of FGF1 mRNA expression in the DMNV was lower than that in the hypoglossal nucleus ($P<0.01$). Immunohistochemistry revealed that FGF1 was localized to neurons. FGF1-positive neurons in large numbers were evenly distributed in the hypoglossal nucleus, whereas FGF1-positive neurons were located in the lateral part of the DMNV. Double immunostaining for FGF1 and choline acetyltransferase demonstrated that 22.7% and 78% of cholinergic neurons were positive for FGF1 in the DMNV and hypoglossal nucleus, respectively. A tracing study with cholera toxin B subunit (CTb) demonstrated that cholinergic neurons sending their axons from the DMNV to the superior laryngeal nerve were FGF1-negative. The results suggest that the low expression of FGF1 in the DMNV is due to severe damage of neurons in the DMNV.

Key words: FGF, laryngeal nervous system, dorsal motor nucleus of vagus, hypoglossal nucleus, cholinergic neurons

I.   Introduction

The dorsal motor nucleus of the vagus (DMNV) contains preganglionic parasympathetic neurons sending their axons to the peripheral organs. These preganglionic neurons are affected by axonal injury much more severely than most other nerves [13, 15]. Navaratnam *et al.* reported that only 25% of neurons remain in rat 18 months after the injury of the vagal nerve, whereas 75% of the neurons in the hypo-glossal nucleus survive after the axonal injury [15]. The reason why neurons in the DMNV are more severely damaged by axonal injury remains unclear. However, there is a possibility that some growth factors are involved in the degenerative and regenerative processes that follow the axonal injury. Among growth factors, fibroblast growth factors (FGFs) are of great interest, because of their abundance in the brain and their potent trophic effect on neurons [reviewed in 1 and 17].

FGF1, a member of the FGF family, is a strong mitogen for glial cells and exerts potent trophic effects on neurons [4, 10, 12, 18]. Jacques *et al.* demonstrated that exogenous FGF1 increased the number of axons regenerating the injured vagal nerve and the number of neurons surviving in the

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DMNV at nine weeks after injury [10]. Since FGF1 lacks the signal peptide, FGF1 is thought to be released upon cellular injury and to have a trophic effect on damaged neurons [3, 9]. If this is true, the expression of FGF1 should be at a relatively low level in neurons of the DMNV as compared to neurons of other cranial nuclei, such as the hypoglossal nucleus. To test this hypothesis, we have compared the level of expression of FGF1 in the DMNV and in the hypoglossal nucleus by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical analyses.

II.   Materials and Methods

Animals

This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.). The animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Shiga University of Medical Science. Four Wistar rats weighing 200–250 g were used in this experiment. The animals were housed with food and water available ad libitum under 12:12 hr light-dark schedule.

RNA analysis

Four rats were used for RNA analysis. Under sodium pentobarbital anesthesia (80 mg/kg), the animals were perfused via the ascending aorta with 10 mM phosphate buffered saline (PBS), pH 7.4. The brain was dissected out and quickly frozen. Samples were cut into 40 μm thick sections and mounted on sterilized silane-coated glass slides. The regions of the DMNV and of the hypoglossal nucleus were punched out under a dissection microscope. Total RNA was isolated from both the regions using TRIzol reagent (Life Technology, Rockville, MD, USA). Prior to reverse transcription, the total RNA was incubated for 1 hr with 10 units of RNase-free DNase I (Amersham Biosciences Corp.) and 20 units of recombinant RNase inhibitor (Wako Pure Chemicals, Osaka, Japan) at 37°C, to eliminate any trace of contaminating DNA. Five μg of total RNA was then reversely transcribed for the first strand cDNA synthesis using 80 units of SuperScript II (Gibco BRL, Gaithersburg, MD) and 500 pmol of oligo dT$_{12-18}$ (Amersham Biosciences Corp.) as primers.

The PCR primers used in this study are summarized in Table 1. β-actin mRNA was amplified as an internal control of variable mRNA amounts. The primers for β-actin PCR were designed to encompass different exons, and were expected to yield a 266 bp PCR fragment. The reaction mixture for PCR consisted of 2 ng/μl of the template cDNA, 0.8 μM each of the primers, 0.2 mM of each of four deoxynucleotide triphosphates and 2.0 U *Taq* polymerase (AmpliTaqGold, Perkin Elmer Japan Co., Tokyo, Japan) dissolved in 1×PCR buffer containing 1.5 mM MgCl$_2$. After heat activation for 10 min at 95°C, the sample was amplified using the following profile of thermal cycle: (1) denaturation at 95°C for 30 sec, (2) annealing at 56°C for 30 sec, and (3) extension at 72°C for 60 sec. We performed the PCR for 28–30 cycles. The PCR products obtained were electrophoresed on a 3% agarose gel and stained with ethidium bromide. The staining intensity and area of each fragment were measured by an image analyzer (FMBIO-100, Hitachi Software Engineering Inc., Yokohama, Japan). The relative mRNA level in each band was calculated by comparison with the expression level of the endogenous control β-actin mRNA, which was used as an endogenous control. The normality of the data was first assessed by F-test. F-test showed that P values for the levels of FGF1 and choline acetyltransferase (ChAT) and for the ratio of FGF1 to ChAT were 0.0655, 0.4230, and 0.3915, respectively. The mRNA levels of the DMNV and hypoglossal nucleus were then compared in four rats using Student's t-test. Results were considered significant at $P<0.05$.

Tissue preparations for immunohistochemistry

Five Wistar rats weighing 200–250 g were used in this experiment. Under deep anesthesia with sodium pentobarbital (80 mg/kg body weight), the animals were transcardially perfused with 10 mM PBS followed by ice-cold 4% formaldehyde (FA) in 0.1 M phosphate buffer (PB), pH 7.4. The medulla oblongata was then removed from each animal. The specimens were postfixed for 3 days in the same fixative as used in perfusion, and then immersed for 24 hr in 0.1 M PB containing 15% sucrose and 0.1% sodium azide for cryoprotection. The medulla oblongatae were cut into 20 μm-thick sections using a cryostat. Before staining, these sections were kept for at least 4 days at 4°C in 0.1 M PBS, pH 7.4, containing 0.3% Triton X-100 (PBST).

Table 1.   *PCR primers used in this study*

Gene		Primer Sequence	Corresponding gene sequence	Product size (GenBank file)
FGF1	Upper	5'-ATGGCCGAAGGGGAGATCACAACC-3'	sense to 267–290	468 bp
	Lower	5'-TTAGTCAGAAGATACCGGGAGGGG-3'	antisense to 711–734	(RNHBGF1)
ChAT	Upper	5'-TTCTTTGTCTTGGATGTTGTCAT-3'	sense to 610–632	529 bp
	Lower	5'-AACATTTCAACCTCAACCTTCTGG-3'	antisense to 1115–1138	(from ATG codon*)
β-Actin	Upper	5'-GACCTCTATGCCAACACAGTGCTGT-3'	sense to 2754–2778	259 bp**
	Lower	5'-CTAGAAGCATTTGCGGTGCACGATG-3'	antisense to 3108–3132	(RATACCYB)

* refers to references 2 and 7, ** excluding intron E.

Primary antibody

The specificity and characterization of the antibodies were done using Western blot analysis and an immuno-absorption test.

Western blot analysis and immunoabsorption test

For Western blots, a male Wistar rat weighing 250 g was perfused with 10 mM PBS, pH 7.4, under deep anesthesia with sodium pentobarbital (80 mg/kg). The medulla oblongata was dissected out and homogenized in 5 volumes of ice-cold 50 mM Tris-HCl (pH 7.4) containing 0.5% Triton X-100 and protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany; one tablet/10 ml). The homogenates were centrifuged at 12,000 g for 20 min at 4°C. The supernatants were collected as a crude protein fraction. Protein concentration was assayed using Lowry's method [12]. Fifty µg of the crude extracted protein, 50 ng of human recombinant FGF1 (140 amino acid form, M.W. 15.8 kDa; Wako Pure Chemicals, Osaka, Japan) and prestained Precision protein standards (Bio-Rad, Hercules, CA) were electrophoresed on a 15% sodium dodecyl sulfate-polyacrylamide gel under a reducing condition, and then transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Tokyo, Japan). The membrane was blocked for 1 hr with 5% skim milk in 25 mM Tris-buffered saline (TBS, pH 7.4) at room temperature, and further incubated overnight with the monoclonal antibody against FGF1 (1 µg/ml) in 25 mM TBS containing 1% skim milk at room temperature. After washing with 25 mM TBS containing 0.1% Tween-20 (Bio-Rad, Hercules, CA), the membrane was reacted for 2 hr with a peroxidase-coupled F (ab')$_2$ anti-mouse IgG (Histofine, Nichirei Corp., Tokyo, Japan; diluted 1:50). Peroxidase labeling was detected by incubating with 0.02% 3,3'-diaminobenzidine in 50 mM Tris-HCl buffer (pH 7.6) containing 0.3% nickel ammonium sulfate.

For immunoabsorption test, the FGF1 antibody at the same dilution as used for immunohistochemistry was pre-incubated overnight at 4°C in 0.5 ml of PBST with or without 10 µg/ml of the human recombinant FGF1. The FGF1/IgG complex was removed by incubation for 1 hr at room temperature with 0.5 ml of heparin-Sepharose beads (Amersham Pharmacia Biosciences Corp., Piscataway, NJ) and centrifugation at 15,000 rpm for 20 min at 4°C. The supernatant was used as the immunostain in the immunohistochemical analysis described below.

FGF1 immunohistochemistry

Before staining, the sections were incubated for 30 min in PBST containing 0.5% hydrogen peroxide at room temperature in order to quench endogenous peroxidase. After several washes with PBST, the sections were incubated with the mouse monoclonal antibody against FGF1 (1 µg/ml) at 4°C for 2 days. The sections were washed as above and incubated for 1 hr with biotinylated anti-mouse IgG (diluted 1:1000 in PBST; Vector Laboratories, Burlingame, CA, USA) at room temperature. The sections were washed as above and incubated for 1 hr with avidin-biotinylated perox-

idase complex (diluted 1:4000 in PBST; Vector Laboratories) at room temperature. After washing above, a purple color was developed with 0.02% 3,3'-diaminobenzidine and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.6). The free-floating sections were mounted on gelatin/chrome-coated glass slides and air-dried.

Double immunofluorescence for FGF1 and ChAT or pChAT

We used double immunofluorescence staining to visualize FGF1 and ChAT simultaneously. The sections were incubated for 2 days at 4°C with a mixture of mouse anti-FGF1 monoclonal antibody (1 µg/ml) and goat anti-ChAT antibody (AB-144p, diluted 1:1000; Chemicon International, Temecula, CA, USA). The sections were incubated for 4 hr at room temperature with a mixture of Alexa 488-conjugated anti-mouse IgG (1:500; Molecular Probes, Eugene, OR, USA) and Alexa 594-conjugated anti-goat IgG (1:500; Molecular Probes). PBST was used to dilute the antibodies and wash the sections between each step. The free-floating sections were mounted on gelatin-coated glass slides and examined under a confocal laser-scanning microscope (Bio-Rad, Hercules, CA, USA) as described previously [7, 16].

Image analyses

The image data were obtained from ten sections of two rats. We measured areas of all nucleated neurons positive for FGF1 (87 cells in the DMNV and 261 cells in the hypoglossal nucleus) or ChAT (385 cells in the DMNV and 302 cells in the hypoglossal nucleus) using the National Institutes of Health Image software on a Windows computer. After assessing the normality of the data, the statistical differences were determined by Student's t-test. Results were considered significant at $P<0.05$.

Cholera toxin B subunit (CTb) tracing

Under anesthesia with sodium pentobarbital (40 mg/kg body weight), 1% cholera toxin B subunit (CTb; List Biological Lab., Campbell, CA, U.S.A.) was injected with a Hamilton syringe into the right superior laryngeal nerve of each animal as a neuronal tracer. After 5 days, the animals were deeply anesthetized and perfused with 10 mM PBS followed by ice-cold 4% formaldehyde in 0.1 M PB. The medulla oblongata was removed and processed for immunohistochemistry as above.

Double immunofluorescence for FGF1 and CTb

For simultaneous visualization of FGF1 and CTb, we employed a double immunofluorescence method using the mouse anti-FGF1 antibody and goat anti-choleragenoid antibody. The sections were incubated for 3 days at 4°C with a mixture of the mouse anti-FGF1 antibody (1 µg/ml) and goat anti-choleragenoid antibody (diluted 1:100,000; List Biological Lab.). After washing with PBST several times, the sections were incubated for 4 hr at room temperature with mixture of Alexa 488 conjugated anti-mouse IgG (1:500; Molecular Probes) and Alexa 594 conjugated anti-goat IgG (1:500; Molecular Probes). After washing with PBST, the

free-floating sections were mounted on gelatin-chrome-coated glass slides and then examined under the confocal laser-scanning microscope (Bio-Rad).

III. Results

RT-PCR analysis

Figure 1 shows a typical example of RT-PCR experiments with primer sets for FGF1 (Fig. 1A), ChAT (Fig. 1B) and β-actin (Fig. 1C). The expression of ChAT and β-actin mRNAs appears to be almost the same in the DMNV and the hypoglossal nucleus, whereas the expression of FGF1 mRNA appears lower in the DMNV than in the hypoglossal nucleus. Semi-quantitation of the data showed that the expression of FGF1 and ChAT mRNAs was lower in the DMNV than the hypoglossal nucleus ($P<0.01$, Table 2). The ratio of FGF1 mRNA to ChAT mRNA was significantly

Fig. 1.   RT-PCR analysis using primer sets for FGF1 (**A**), ChAT (**B**) and β-actin (**C**) amplification. H and D indicate samples from the hypoglossal nucleus and the DMNV, respectively. M indicates molecular marker.

Table 2.   *Relative levels of FGF-1 and ChAT mRNAs in the DMNV and hypoglossal nucleus*

FGF-1	
Hypoglossal nucleus	69912.5±1598.1
DMNV	34174.0±4390.0*
ChAT	
Hypoglossal nucleus	64016.5±13852.8
DMNV	45524.5±12265.6*
FGF-1/ChAT	
Hypoglossal nucleus	110.5±21.2
DMNV	79.9±25.2*

* $P<0.01$ (n=4).

lower in the DMNV than the hypoglossal nucleus ($P<0.01$, Table 2).

Specificity of the FGF1 antibody

On Western blot analysis, the mouse monoclonal anti-FGF1 antibody stained the 15.8 kDa recombinant FGF1 (140 amino acid form) and a single band with a molecular weight of about 16.5 kDa in rat medulla oblongata (Fig. 2A). As reported previously [18], the antibody clearly stained the cytoplasm of some neurons (Fig. 2B), and the staining was abolished when the antibody was preabsorbed with 10 μg/ml of FGF1 (Fig. 2C).

Distribution of FGF1 in rat DMNV and hypoglossal nucleus

Figure 3 shows the distribution of FGF1-positive neurons in rat DMNV and hypoglossal nucleus. In agreement with the RT-PCR data, there were fewer FGF1-positive neurons in the DMNV than in the hypoglossal nucleus. Positive neurons were seen mainly in the lateral part of the DMNV (arrows in Fig. 3A and B). In the hypoglossal nucleus, FGF1-positive neurons were evenly distributed rostro-caudally (Fig. 3A and B).

Figure 4 shows the typical examples of double immunostaining for FGF1 (green) and ChAT (red). Most of the ChAT-positive neurons in the hypoglossal nucleus contained FGF1. In the DMNV, a small number of ChAT neurons in the lateral part were positive for ChAT and a large number of ChAT-positive neurons in the medial part were negative for FGF1 (Fig. 4). Quantitatively, 22.7% and 78% of ChAT-positive neurons were positive for FGF1 in the DMNV and hypoglossal nucleus, respectively (Table 3). In the hypoglossal nucleus, areas with FGF1-positive neurons and ChAT-positive neurons were of approximately the same size, whereas in the DMNV, areas of FGF1-positive neurons were significantly smaller than areas of ChAT-positive neurons (Table 4).

We used CTb tracing to determine if in the DMNV, cholinergic neurons sending their axon to the larynx contain FGF1. As seen in Figure 5, FGF1-positive neurons were a different subpopulation from CTb-positive neurons (Fig. 5).

IV. Discussion

RNA analysis

RT-PCR analysis showed that the level of FGF1 mRNA expression was lower in the DMNV than that in the hypoglossal nucleus, while the expression of β-actin mRNA did not differ between the two nuclei. Unexpectedly, ChAT mRNA was expressed at a lower level in the DMNV than in the hypoglossal nucleus. The reasons behind this observation are unclear. However, because the area of the DMNV is smaller than that of the hypoglossal nucleus, it is possible that surrounding areas were included when we punched out the DMNV. Thus, we calculated the ratio of FGF1 mRNA to ChAT mRNA. The ratio was also significantly lower in the DMNV than in the hypoglossal nucleus.
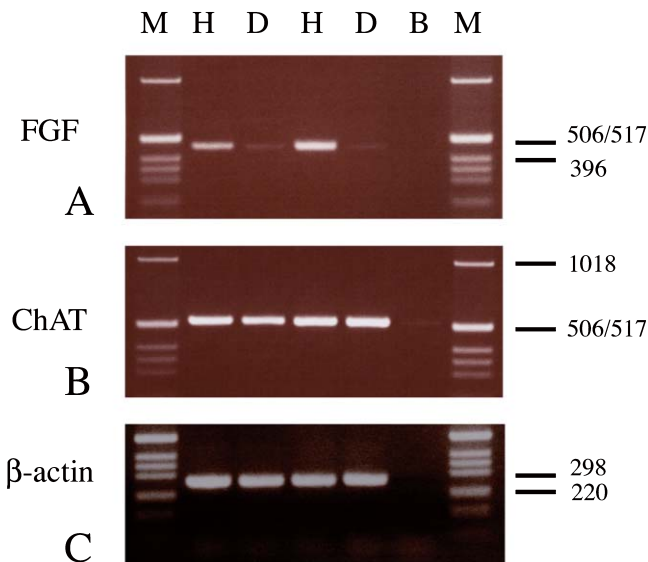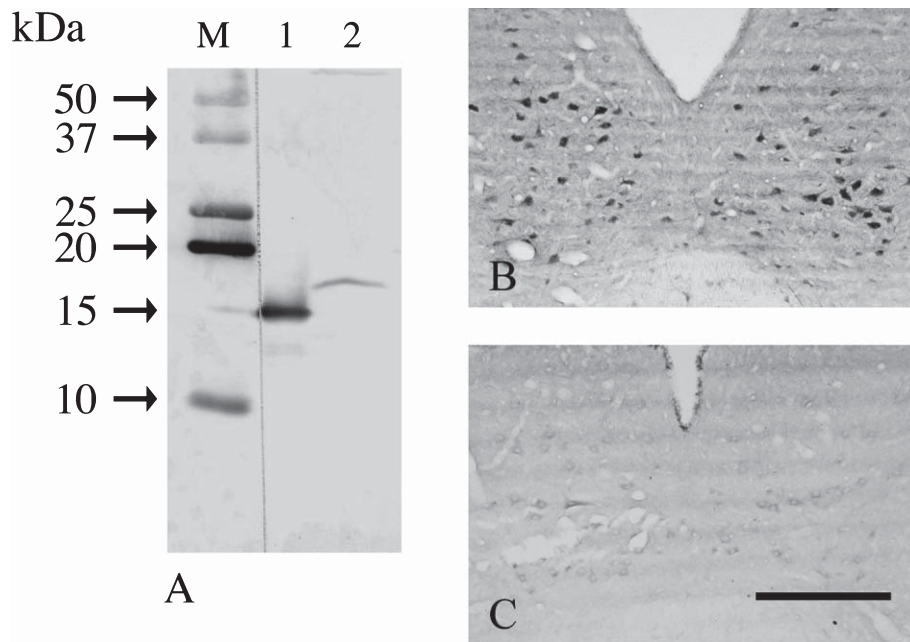
Fig. 2. Detection of FGF1. (**A**) Western blot analysis of recombinant FGF1 (140 amino acid form, 15.8 kDa; lane 1) and crude protein extract from rat medulla oblongata (50 μg protein loaded; lane 2) with mouse anti-FGF1 antibody (diluted to 1 μg/ml). M indicates molecular marker. (**B**) Immunostaining for FGF1 in rat medulla oblongata with mouse anti-FGF1 antibody (diluted to 1 μg/ml). (**C**) Preabsorption of the mouse anti-FGF1 antibody with 10 μg/ml of recombinant FGF1 abolishes staining of FGF1 in rat medulla oblongata.
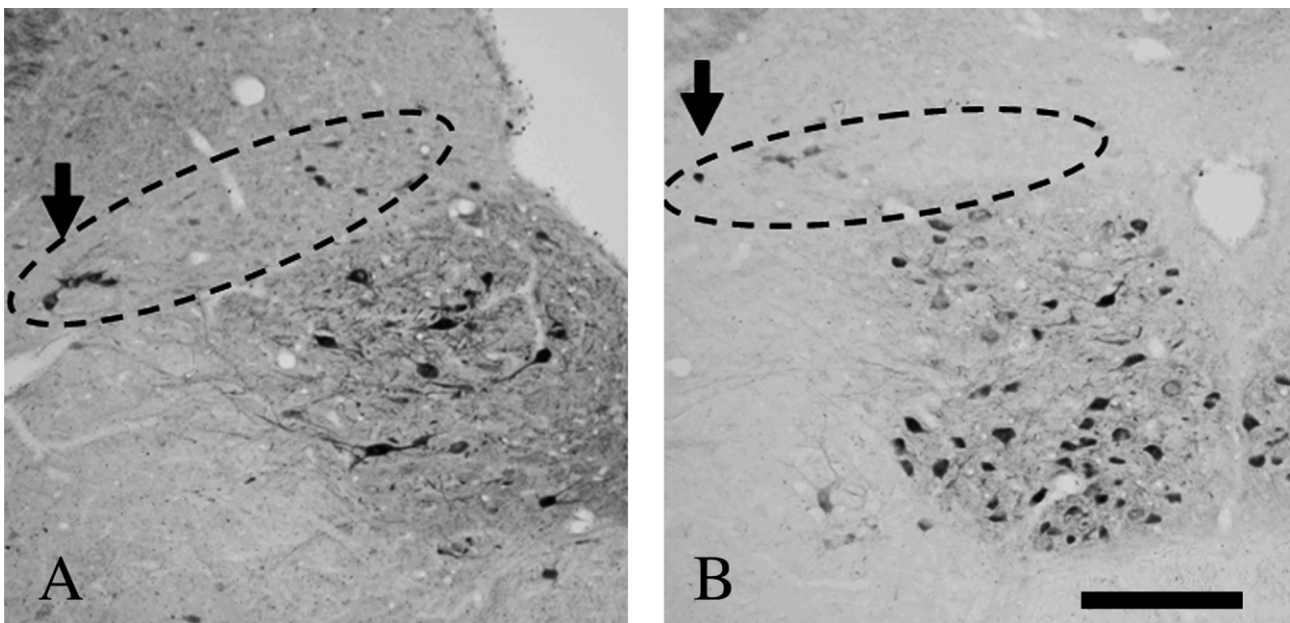

Fig. 3. FGF1 immunoreactivity in the medulla oblongata at bregma levels of −13.30 mm (**A**) and −13.80 mm (**B**). Bar=200 μm.

Characterization of FGF1 antibody

On Western blot analysis, the FGF1 monoclonal antibody was recognized with the 15.8 kDa of recombinant human FGF1 (140 amino acid form) [5]. In rat medulla oblongata homogenate, both antibodies detected a single band with a molecular weight of 16.5 kDa, which corresponds to the molecular weight of a native form of FGF1 reported in previous studies [20, 21, 22]. Although the 140 amino acid form of FGF1 was first purified from bovine brain [5], the native form of FGF1 is thought to be extended at the N-terminal end [11].

To prepare the reagent for the immunoabsorption test, we used heparin-coated sepharose beads to remove the FGF1/FGF1 antibody complex, because FGF1 often reacts
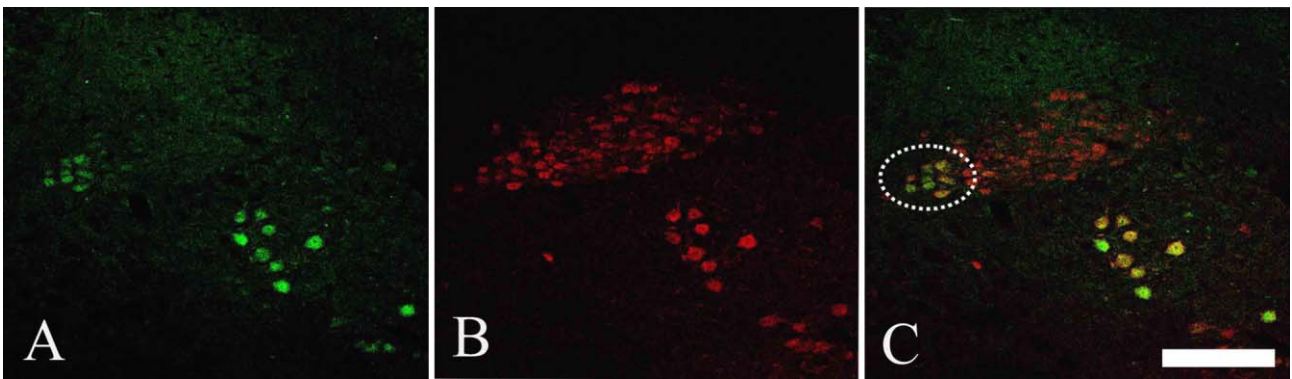
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Fig. 4. Double immunostaining for FGF1 and ChAT in the medulla oblongata. (**A**) FGF1-immunoreactive neurons (green) are seen in the DMNV and hypoglossal nucleus. (**B**) ChAT-immunoreactive neurons (red) are observed in the DMNV and hypoglossal nucleus. (**C**) Merged image of (**A**) and (**B**). Bar=200 μm.
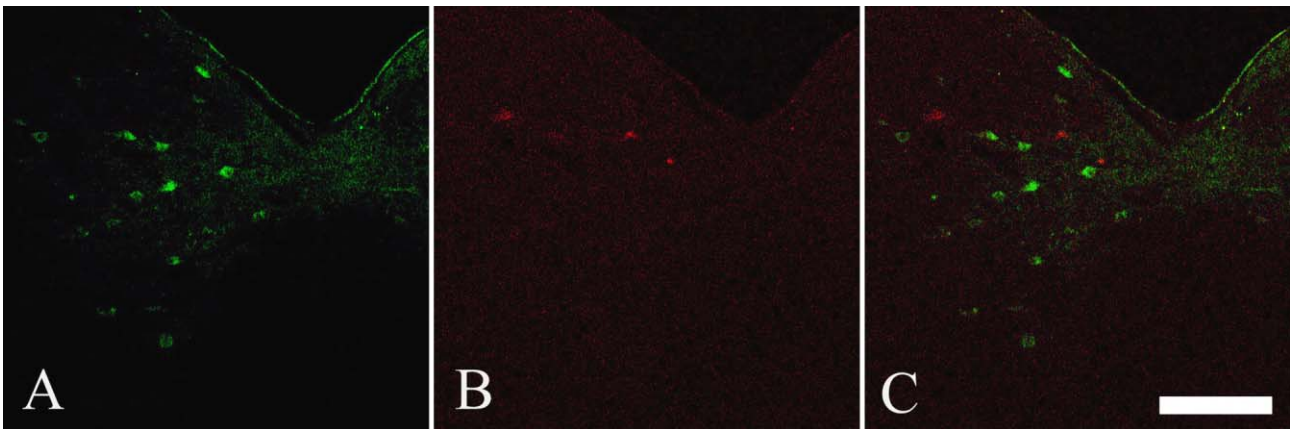

Fig. 5. Double immunostaining for FGF1 and CTb in the DMNV. (**A**) FGF1-immunoreactive neurons (green) and (**B**) CTb-immunoreactive neurons (red) are seen in the DMNV. (**C**) Merged image of (**A**) and (**B**). Bar=200 μm.

Table 3. *Quantitation of FGF1 and ChAT in the DMNV and hypoglossal nucleus*

DMNV		Hypoglossal nucleus	
FGF1/ChAT	ChAT/FGF1	FGF1/ChAT	ChAT/FGF1
22.7%	93.1%	78.0%	81.8%

Table 4. *Area-frequency distribution of FGF1-positive and ChAT-positive neurons in the DMNV and hypoglossal nucleus*

	DMNV (μm^2)	Hypoglossal nucleus (μm^2)
FGF1	976.6±373.2*	1843.4±502.3
ChAT	1233.9±388.5	1946.7±582.2

* *P*<0.01 (between FGF1 and ChAT-positive neurons).

with heparan sulphate in tissues. Staining of medulla oblongata sections for FGF1 was abolished using the antibody preabsorbed with 10 μg/ml of FGF1. These results indicate that the anti-FGF1 antibody stains FGF1 in rat tissues.

Comparison of FGF1 expression between the DMNV and the hypoglossal nucleus

The RT-PCR results were corroborated by the results of the immunohistochemical examination, which showed a lower expression of FGF1 in the DMNV than the hypoglossal nucleus. In the hypoglossal nucleus, 78% of cholinergic neurons were positive for FGF1, while only 22% of cholinergic neurons in the DMNV contained FGF1. In the DMNV, FGF1-positive neurons were distributed mainly in the lateral part of the nucleus. Such areas in the DMNV contain many preganglionic cells sending their fibers to the ileum and colon [19]. Previous reports indicated that neurons projecting to the larynx are distributed to the rostral part of the DMNV [6, 23], but details of the studies were not shown. The present study, which uses CTb tracing, demonstrated that FGF1-positive neurons were a different subpopulation from neurons projecting their axons to the larynx.

Preganglionic neurons in the DMNV are much more severely affected by axonal injury than most other nerves [13, 15]. For example, eighteen months after injury of the vagal nerve, only 25% of the neurons could be found in rat

DMNV, whereas 75% of the hypoglossal nucleus neurons had survived [15]. The reason why neurons in the DMNV are severely damaged by axonal injury remains unclear. However, our results suggest that the low expression of FGF1 in neurons in the DMNV may be responsible for their susceptibility to axonal injury. The study by Jacques *et al.* who reported that a single administration of FGF1 into the injured axon of the vagus enhanced the survival of neurons in the DMNV [10], supports this possibility. Thus, FGF1 supplementation may be useful in the treatment of vagal nerve injury.

V.   Conclusion

RT-PCR analysis and immunohistochemistry demonstrated a lower expression of FGF1 in the DMNV than the hypoglossal nucleus. CTb tracing confirmed that FGF1 was not localized to cholinergic neurons sending their axon to the larynx. Since FGF1 is released from damaged neurons and acts as a trophic factor, the low expression of FGF1 in the DMNV may account for the susceptibility of preganglionic parasympathetic neurons to axonal injury.

VI.   References

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