Histol Histopathol (2007) 22: 1327-1335
DOI: 10.14670/HH-22.1327

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Low expression of FGF1 (Fibroblast growth factor-1) in rat parasympathetic preganglionic neurons

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Summary. Fibroblast growth factor-1 (FGF1), a member of the FGF family of growth factors, is localized in cholinergic neurons where it has trophic activity. We recently reported that cholinergic neurons in the dorsal motor nucleus of the vagus (DMNV) contain little FGF1, raising the possibility that FGF1 is not localized to parasympathetic preganglionic cholinergic neurons. To clarify this issue, we investigated the co-localization of FGF1 with cholinergic neuron markers in the Edinger-Westphal nucleus (EWN), salivatory nucleus, DMNV, and sacral parasympathetic nucleus by double immunofluorescence using antibodies to FGF1 and choline acetyltransferase (ChAT). The neurons in the EWN were devoid of FGF1. In the salivatory nucleus, 13% of ChAT-positive neurons were also positive for FGF1. In the DMNV, only 8% of ChAT-positive neurons contained FGF1, and in the sacral parasympathetic nucleus, 18% of ChAT-positive neurons were FGF1-positive. We also confirmed that a large number of ChAT-positive motor neurons in the oculomotor nucleus, facial nucleus, hypoglossal nucleus, and spinal motor neurons contained FGF1. The results confirmed that parasympathetic preganglionic neurons are largely devoid of FGF1, which is a unique feature among cholinergic neurons.

Key words: FGF, Parasympathetic preganglionic neurons, Cholinergic neurons, Amyotrophic lateral sclerosis

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Introduction

Fibroblast growth factor 1 (FGF1), one of the most extensively studied members of the fibroblast growth factor family, is a trophic factor for many different neurons in the central and peripheral nervous systems. In the central nervous system, FGF1 is localized to specific neuronal subpopulations in the subcortical areas, particularly cholinergic neurons (Elde et al., 1991; Stock et al., 1992; Eckenstein et al., 1994). These include cholinergic neurons in the striatum and basal forebrain (Stock et al., 1992; Bizon et al., 1996) as well as motor neurons in the brainstem and spinal cord (Elde et al., 1991; Kage et al., 2001).

Previous studies using cell culture demonstrated the potent trophic effects of FGF1 on cholinergic neurons *in vitro* (Deloulme et al., 1991; Sendtner et al., 1991; Sweetnam et al., 1991) and *in vivo*. Administration of FGF1 reduced degeneration of nucleus basalis magnocellularis cholinergic neurons after cortical ablation (Figueiredo et al., 1993) and degeneration of septal cholinergic neurons in aging (Oomura et al., 1996; Tooyama et al., 1997; Sasaki et al., 1999), as well as enhancing the survival of motor neurons after spinal injury (Teng et al., 1998; Lee et al., 2006). FGF1 also prevents cell death in motor neurons after axonal injury (Cuevas et al., 1995; Laird et al., 1995; Jacques et al., 1999). These data implicated FGF1 as one of the most important trophic factors for cholinergic neurons in the central nervous system.

The parasympathetic preganglionic cholinergic neurons in the dorsal motor nucleus of the vagus (DMNV) are more severely affected by axonal injury than most other cholinergic neurons in the cranial nuclei (Lewis et al., 1972; Aldskogius et al., 1980; Navaratnam et al., 1998). However, the underlying mechanism for

this increased susceptibility remains unclear. We recently reported a distinct lack of FGF1 in parasympathetic preganglionic cholinergic neurons of the DMNV (Okano et al., 2006), questioning the presence of FGF1 in all cholinergic neurons in the central nervous system including the parasympathetic preganglionic neurons. To clarify this issue, we investigated a possible co-localization of FGF1 with cholinergic neuron markers in parasympathetic preganglionic nuclei such as the Edinger-Westphal nucleus (EWN), salivatory nucleus, DMNV, and sacral parasympathetic nucleus using double immunofluorescence techniques.

Materials and methods

Tissue preparation

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 the Shiga University of Medical Science. Four male Wistar rats weighing 200 to 300 g were used for the immunohistochemistry. They were deeply anesthesized with sodium pentobarbital (80 mg/kg), and transcardially perfused with 10 mM phosphate-buffered saline (PBS) followed by 300 ml of cold fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains and lumbosacral spinal cord were then removed from each animal and postfixed for 24 hours in the same fixative used for the perfusion. Samples were then cryoprotected by immersion for 24 hours in 0.1 M PB containing 15% sucrose and 0.1% sodium azide. Each sample was mounted on a cryostat holder using Tissue Tek compound (Sakura Finetek, CA, USA), and frozen on dry ice. A cryostat was used to cut 20-mm sections, which were placed for at least 4 days at 4°C in 0.1 M PBS containing 0.3% Triton X-100 (PBST, pH 7.4) to increase the penetration of antibodies into the tissues.

Western blot analysis

For Western blot, male Wistar rats weighing 300 g were perfused with 10 mM PBS, pH 7.4, under deep anesthesia with sodium pentobarbital (80 mg/kg). The brainstem 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; 1 tablet/10 ml). The homogenates were centrifuged at 12,000g for 20 minutes at 4°C. The supernatants were collected as a crude protein fraction. Protein concentration was assayed using Lowry's method (Lowry et al., 1951). Fifty micrograms of the crude extracted protein and prestained precision protein standards (BioRad; Hercules, CA) were electrophoresed on a 15% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions, and then transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore; Tokyo, Japan). The membrane was incubated for 1 hour with 5% skimmed milk powder in 25 mM Tris-buffered saline (TBS; pH 7.4) at room temperature, and further incubated overnight with the monoclonal antibody against FGF1 (Imamura et al., 1994, Okano et al., 2006) at a dilution of 1:4000 in 25 mM TBS containing 0.5% skimmed milk powder at 4°C. After washing with 25 mM TBS containing 0.1% Tween-20 (BioRad), the membrane was incubated for 1 hour with a peroxidase-labeled anti-mouse IgG (ImmunoPure, Pierce, Rockford, IL, USA; diluted 1:10000). The peroxidase labeling was detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Immunohistochemistry

Free-floating sections were incubated for 3 days at 4°C with the goat polyclonal antibody to choline acetyltransferase (ChAT, AB-144p, diluted 1:500; Chemicon; Temecula, CA) or the mouse monoclonal antibody to FGF1 (diluted 1:4,000). After washing with PBST, the sections were incubated with biotinylated anti-goat IgG or anti-mouse IgG (diluted 1:1000; Vector Laboratories; Burlingame, CA) for 1 hour at room temperature. The sections were then washed with PBST and incubated with avidin-biotin peroxidase complex (diluted 1:4000; Vector Laboratories) for 1 hour at room temperature. After washing, a purple color was developed with 0.02% 3,3'-diaminobenzidine, 0.3% nickel ammonium sulfate and 0.0045% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.6). The free-floating sections were mounted on gelatin-chrome-coated glass slides, washed in running water, dried through an ascending series of alcohol, cleared in xylene, and mounted using Entellan (Merck, Darmstadt, Germany).

As reported previously (Okano et al., 2006), the staining was abolished using the antibody preabsorbed with 10 mg/ml of FGF1 (data not shown).

Double immunofluorescence histochemistry for FGF1 and ChAT

For simultaneous visualization of FGF1 and ChAT, a double immunofluorescence method was used. The sections were incubated in primary antibodies for 3 days at 4°C with a mixture of mouse anti-FGF1 antibody (diluted 1:4000) and goat anti-ChAT antibody (AB-144p, diluted 1:1000; Chemicon). The sections were then incubated for 4 hours at room temperature in a mixture of Alexa 488-conjugated anti-mouse IgG (1:500; Molecular Probes; OR) and Alexa 546-conjugated anti-goat IgG (1:500; Molecular Probes). PBST was used to dilute the antibodies and wash the sections between steps. The free-floating sections were mounted on gelatin-coated glass slides, cover-slipped using 50% glycerol, and examined with a confocal laser-

scanning microscope (BioRad).

Quantification of labeled neurons

Four male Wistar rats were used for quantitative analysis of FGF1-positive and/or ChAT-positive neurons. The brainstem and spinal cord from each rat were dissected out and cut into serial 20-μm sections using a cryostat. After double immunostaining, these sections were mounted on gelatin-coated glass slides and examined under the confocal laser-scanning microscope. To avoid counting the same cells twice, we selected sections for counting according to the atlas of Paxinos and Watson (1986). For the EWN, we used a total of 8 sections from 4 rats at levels -5.30 mm and -6.70 mm from the Bregma. For the salivatory nucleus, two sections from each rat (total 8 sections) were used at levels -10.30 mm and -11.00 mm from the Bregma. For the DMNV, four sections from each rat (total 16 sections) were selected at levels -12.80 mm, -13.30 mm, -13.80 mm and -14.30 mm from the Bregma. For the sacral parasympathetic nucleus, we analyzed 8 sections at L6 and S1 spinal cord levels obtained from 4 rats. The EWN and DMNV were divided into squares of 0.85 mm^2, and the salivatory and sacral parasympathetic nucleus into squares of 0.21 mm^2. The image in each square was digitized and saved to disk. Numbers of FGF1-positive and/or ChAT-positive cells were counted by eye from saved images.

To compare the colocalization pattern of FGF1 and ChAT in parasympathetic preganglionic neurons with that in motor neurons, we counted the number of FGF1-positive and/or ChAT-positive cells in the oculomotor, facial, and hypoglossal nucleus as well as spinal motor neurons in the ventral horn. In all regions, only neurons with visible nuclei were counted.

Acetylcholinestrase histochemistry

Acetylcholinestrase (AChE) histochemistry was performed according to the method of Tago et al. (1986). Before staining, cryostat sections of midbrain were treated for 20 minutes with 0.3% hydrogen peroxide in 0.1 M maleate buffer (pH 6.0) at room temperature to eliminate endogenous peroxidase activity. After washing in 0.1 M maleate buffer, the sections were first incubated for 2 hours in a solution containing 18 mM acetylthiocholine iodide, 5 mM $K_3Fe(CN)_6$, 30 mM CuSO4, and 50 mm sodium citrate in 0.1 M maleate buffer at room temperature. After washing with 50 mM Tris-HCl buffer (pH 7.6), the specimens were reacted with 0.02% 3,3'-diaminobenzidine, 0.3% nickel ammonium sulfate and 0.0045% hydrogen peroxide in 50 mM Tris-HCl buffer.

Results

Western blot analysis of the rat brainstem homogenate showed a single FGF1-immunopositive band corresponding to a molecular weight of approximately 16.5 kDa (Fig. 1), in agreement with previous reports (Okano et al., 2006).

In the EWN at level -6.70 mm, neither ChAT-positive (red) nor FGF1-positive (green) neurons were observed at the level of the oculomotor nucleus (Fig. 2A,B). In the oculomotor nucleus, 81% of ChAT-positive neurons contained FGF1 (Table 1). At level -5.30 mm from the Bregma, a small number of ChAT-positive neurons were observed in the ventral part of the EWN (Fig. 2D). In contrast, most of the neurons in the EWN were positive for AChE (Fig. 2E). No neurons in the EWN were positive for FGF1 at the -5.30 mm level (Fig. 2F).

Double immunostaining for FGF1 and ChAT in the salivatory nucleus and facial nucleus (Fig. 3A-C) at the -10.30 mm level revealed some ChAT-positive neurons in the salivatory nucleus (circle in Fig. 3A), and they lacked FGF1 (Fig. 3B,C). In the facial nucleus, the majority of ChAT-positive neurons also stained for FGF1 (Fig. 3A-C). Quantitatively, 13% and 97% of ChAT-positive neurons were positive for FGF1 in the salivatory

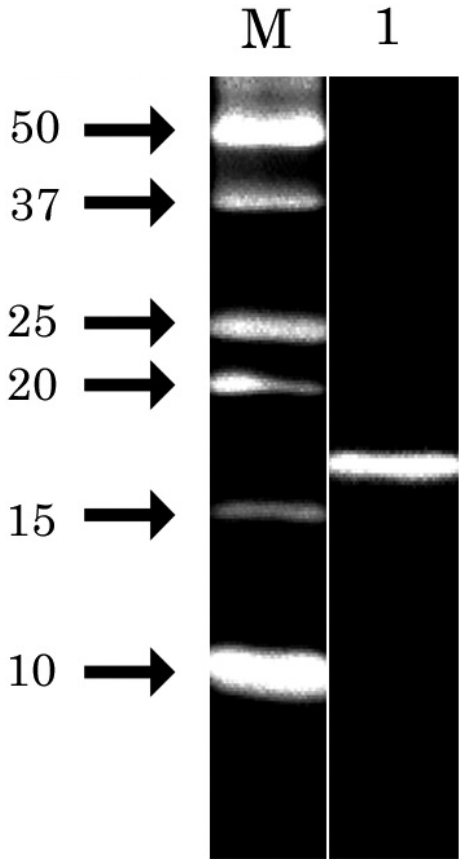

Fig. 1. Western blot analysis using the mouse anti-FGF1 antibody. Fifty μg of the crude rat brainstem extract was electrophoresed (lane 1) and immunoblotted for FGF1 to reveal a 16.5-kDa band. M indicates a molecular marker.

nucleus and facial nucleus, respectively (Table 1).

Figure 4 shows a typical example of double immunostaining for FGF1 and ChAT in the DMNV and hypoglossal nucleus at -13.30 mm from the Bregma. In the DMNV and hypoglossal nucleus, almost every neuron was labeled by the ChAT antibody (Fig. 4A), but only a small number of these in the lateral part of the DMNV were also positive for FGF1 (Fig. 4B,C). In fact, a large number of ChAT-positive neurons were completely negative for FGF1 (Fig. 4). In the hypoglossal nucleus, most of the ChAT-positive neurons contained FGF1. Quantitatively, 8% and 93% of ChAT-

Table 1. Numbers of neurons doubly stained for ChAT and FGF1 as percentages of total ChAT-positive neurons.

Parasympatheitc pregangionic nuclei	Edinger-Westphal nucleus	Salivatory nucleus	DMNV	Sacral parasympathetic nucleus
	0%	13%	8%	18%
Motor nuclei	Oculomotor nucleus	Facial nucleus	Hypoglossal nucleus	Sacral spinal motor nucleus
	81%	97%	93%	96%

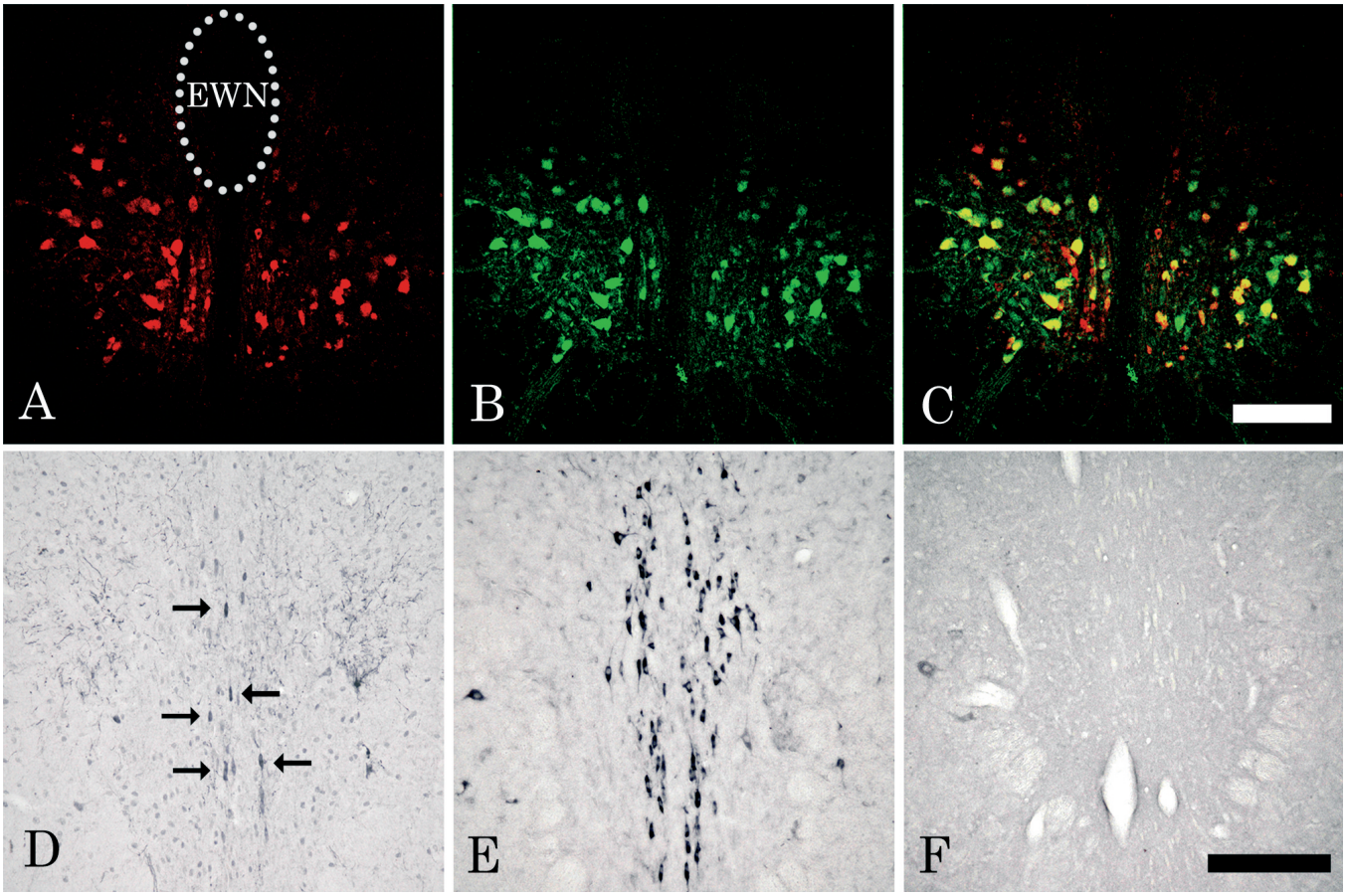

Fig. 2. Photomicrographs of the EWN and oculomotor nucleus. **A-C.** Double immunostaining for ChAT (red) and FGF1 (green) at levels -6.70 mm from the Bregma. **A.** ChAT-immunoreactive neurons. **B.** FGF1-immunoreactive neurons. **C.** Merged image of **(A)** and **(B)**. In the oculomotor nucleus, many of the ChAT-positive neurons contained FGF1 **(A-C)**. In the EWN, neither ChAT-positive nor FGF1-positive neurons were observed. **D-F.** Immunohistochemical staining for ChAT **(D)** and FGF1 **(F)**, and histochemical staining for AChE **(E)** at levels -5.30 mm from the Bregma. A small number of neurons in the EWN are positive for ChAT (arrows in **D**), but most are positive for AChE **(E)**. No neurons were positive for FGF1 **(F)**. Scale bar: 200 μm.

positive neurons were positive for FGF1 in the DMNV and hypoglossal nucleus, respectively (Table 1).

Staining for FGF1- and ChAT-positive neurons was also carried out on the sacral parasympathetic nucleus (Fig. 5A-C) and spinal motor neurons in the ventral horn (Fig. 5D-F) at the S1 spinal cord level. There were many ChAT-immunopositive neurons in the sacral parasympathetic nucleus (Fig. 5A), but only a few showed positive for FGF1 (Fig. 5B, C). Quantitatively, 18% of ChAT-positive neurons contained FGF1 in the sacral parasympathetic nucleus (Table 1), while in the spinal motor neurons, 96% of the ChAT-positive neurons contained FGF1 (Fig. 5D-F, Table 1).

Discussion

The present study demonstrated that parasympathetic preganglionic neurons such as the EWN, salivatory nucleus, DMNV, and sacral parasympathetic nucleus contain little FGF1. In contrast, the majority of cholinergic neurons in motor nuclei, including the oculomotor nucleus, facial nucleus, hypoglossal nucleus, and sacral spinal motor neurons, were positive for FGF1. This agrees with previous findings of high levels of FGF1 expression in motor neurons (Elde et al., 1991; Stock et al., 1992; Koshinaga et al., 1993; Kage et al., 2001). FGF1 is also present in cholinergic neurons of the

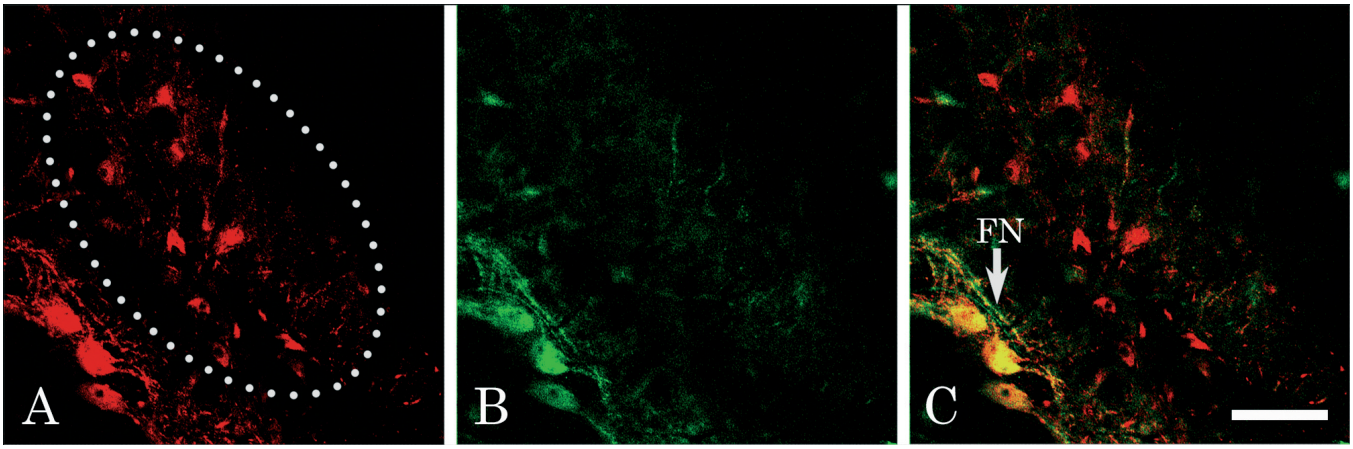

Fig. 3. Double immunostaining for ChAT (red) and FGF1 (green) in the salivatory nucleus and facial nucleus. **A.** ChAT-immunoreactive neurons. **B.** FGF1-immunoreactive neurons. **C.** Merged image of **(A)** and **(B).** In the salivatory nucleus, ChAT-positive neurons (circle in **A**) contained little FGF1. In the facial nucleus (FN), ChAT-positive neurons also contained FGF1. Bar: 100 μm.

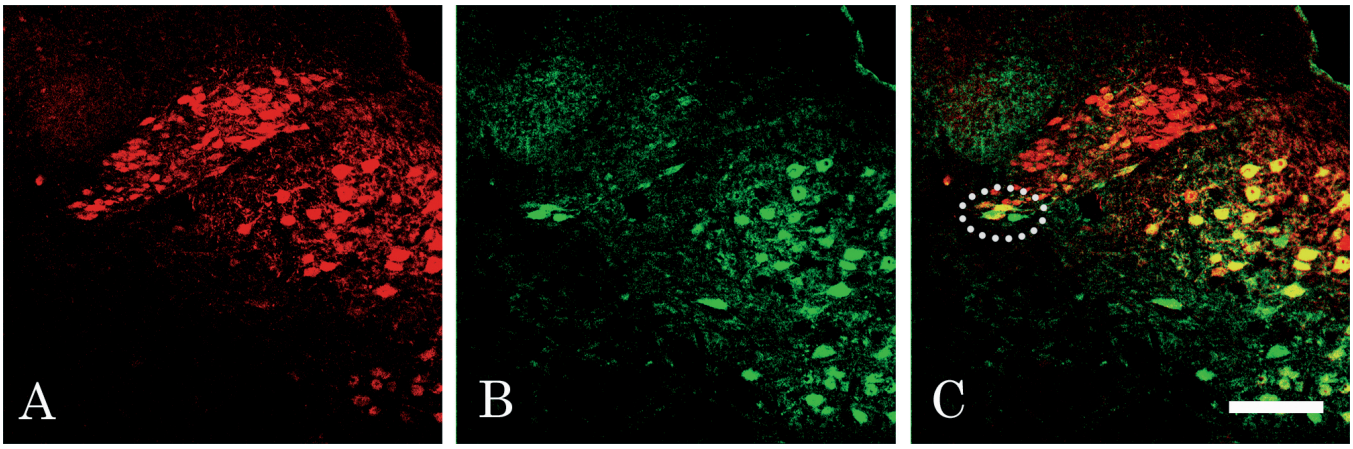

Fig. 4. Double immunostaining for ChAT (red) and FGF1 (green) in the DMNV and hypoglossal nucleus at level -13.30 mm from the Bregma. **A.** ChAT-immunoreactive neurons. **B.** FGF1-immunoreactive neurons. **C.** Merged image of (**A**) and **(B)**. In the DMNV, a few ChAT neurons in the lateral part were also positive for FGF1 (circle in **C)**, but most ChAT-positive neurons were negative for FGF1. In the hypoglossal nucleus, most ChAT neurons also contained FGF1. Scale bar: 200 μm

striatum and basal forebrain (Stock et al., 1992; Bizon et al., 1996), and postganglionic parasympathetic ganglia (Okano et al., 2006). It seems then that parasympathetic preganglionic cholinergic neurons comprise a unique subpopulation.

The neurons in the EWN innervate the ciliary ganglion via the third cranial nerve, controlling lens accommodation and pupillary constriction, according to the clinicopathological studies in human (Warwick, 1954). The rat EWN is located dorsomedially to the oculomotor nucleus at the level of and rostral to the oculomotor nucleus, according to the rat brain atlas (Paxinos and Watson, 1986). Neurons in the EWN have been visualized previously by injection of an anterograde tracer to show axon projection to the ciliary ganglion (Klooster et al., 1993) and by labeling transsynaptic transport in the eye (Smeraski et al., 2004). An in situ hybridization and immunohistochemical study demonstrated ChAT-positive neurons in the EWN (Armstrong et al., 1983; Ruggiero et al., 1990; Lauterborn et al., 1993), and showed that the parasympathetic preganglionic neurons innervating the lens and pupils are located in the EWN in rat.

In our study, however, a small number of ChAT-positive neurons were observed in the EWN rostral to the oculomotor nucleus and no ChAT-positive neurons were observed at the level of the oculomotor nucleus, whereas most of the neurons in the EWN contained AChE. ChAT is considered the most reliable marker for cholinergic neurons (Storm-Mathisen, 1977). AChE is not specific for cholinergic structures, and can also be localized in sensory fibers and catecholaminergic neurons (Koelle, 1955, Jacobowitz and Palkovits, 1974). The results suggest that parasympathetic preganglionic neurons constitute only a small population in the EWN. The hypothesis is supported by previous studies. Urocortin, a corticotrophin-releasing factor, is another marker of neurons in the EWN (Yamamoto et al., 1998; Kozicz, 2003; Laursen and Rekling, 2006). Some previous studies using urocortin demonstrated that neurons in the EWN did not contain ChAT (Ryabinin et al., 2005; Weitemier et al., 2005). In addition, in situ

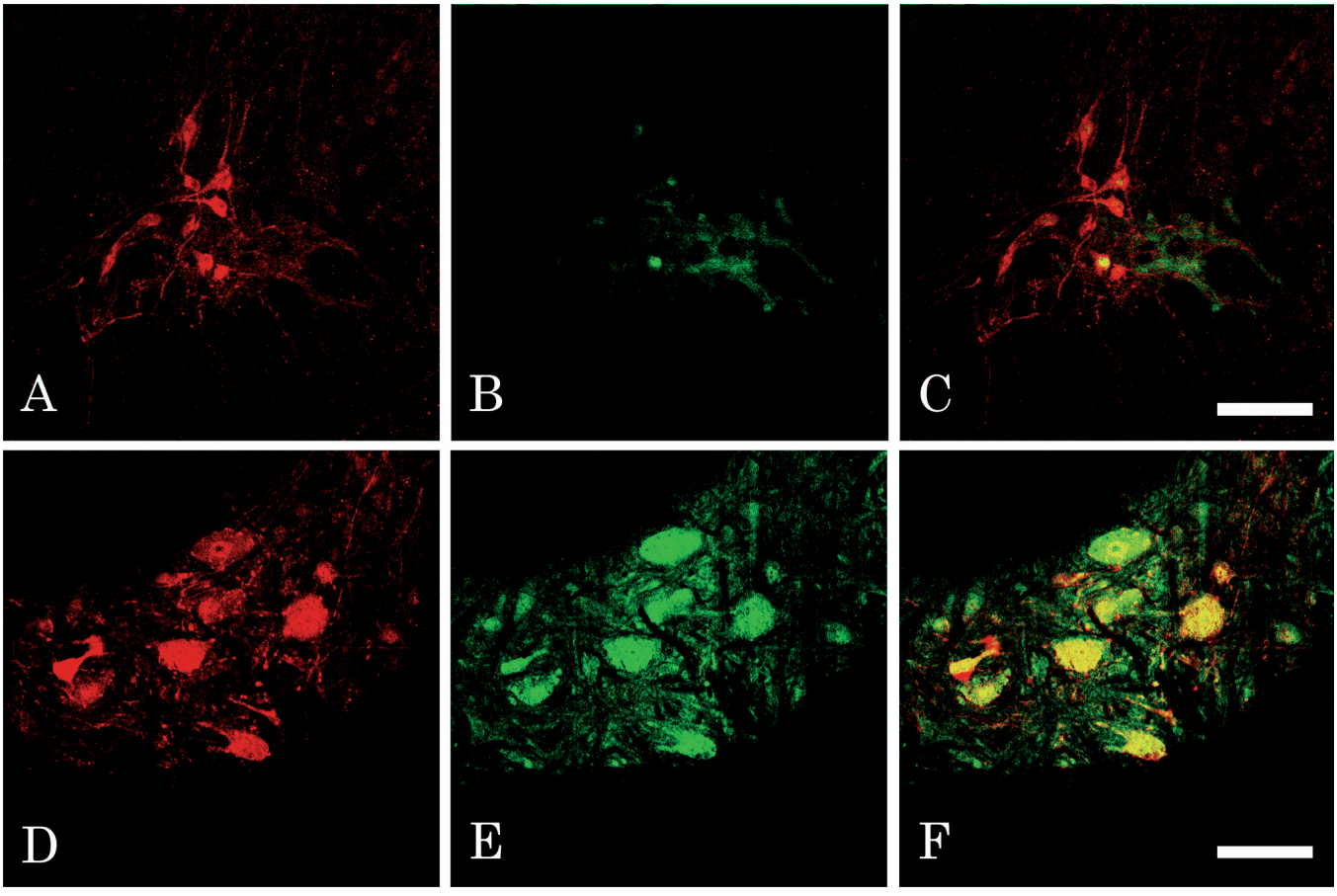

Fig. 5. Double immunostaining for ChAT (red) and FGF1 (green) in the sacral parasympathetic nucleus and spinal motor neurons in the ventral horn at the S1 spinal cord level. **A, D.** ChAT-immunoreactive neurons. **B, E.** FGF1-immunoreactive neurons. **C.** Merged image of (**A**) and (**B**). **F.** Merged image of (**D**) and (**E**). **A-C.** In the sacral parasympathetic nucleus, a few ChAT neurons were positive for FGF1. **D-F.** In the spinal motor neurons, most ChAT neurons contain FGF1. Scale bar: 100 μm.

hybridization demonstrated less ChAT-expressing cells in the EWN than in the oculomotor nucleus (Lauterborn et al., 1993). Armstrong et al. (1983) also demonstrated immunohistochemically that neurons in the EWN expressed less ChAT than the other groups, in some cases barely above background levels. Moreover, a tracer study demonstrated EWN neurons also projecting to the facial nucleus, inferior olive, lateral parabrachial nucleus, lateral reticular nucleus, spinal trigeminal nucleus (Klooster et al., 1993), cerebellum (Sekiya et al., 1984, Roste and Dietrichs, 1988), and spinal cord (Loewy et al., 1978; Maciewicz et al., 1983). Taken together with our results, it seems that some neurons in the EWN send their axons to the ciliary ganglion to control lens accommodation and pupiliary constriction, but many of the neurons in the EWN have no preganglionic oculomotor function. Nevertheless, no neurons in the EWN in this study contained FGF1.

In contrast to the EWN, the salivatory nucleus contains many ChAT-positive neurons (Senba et al., 1987; LeDoux et al., 2001; Cuthbertson et al., 2003). The salivatory nucleus is divided into the superior and inferior salivatory nucleus based on the trajectory of the efferent projection pattern. Parasympathetic neurons in the superior salivatory nucleus send their axons through the facial nerve to control various targets such as the submandibular and sublingual salivary glands, anterior part of the tongue, lacrimal gland, and nasal and palatine mucosa. Parasympathetic neurons in the inferior salivatory nucleus send their axons through the glossopharyngeal nerve to regulate the parotid and von Ebner salivary gland. However, it is difficult to divide the superior and inferior salivatory nucleus clearly due to the degree of overlap (Nicholson and Severin, 1981). Thus, we counted FGF1-positive neurons in the salivary nucleus including both superior and inferior salivatory nucleus. Only 13% of ChAT-labeled neurons in the salivatory nucleus showed FGF1-immunoreactivity. These neurons were located in the dorsal part of the salivatory nucleus that innervates submandibular and parotid glands (Nicholson and Severin, 1981). ChAT-positive neurons in the lateral part of the salivatory nucleus contained no FGF1; these innervate the lacrimal gland as well as the nasal and palatine mucosa (LeDoux et al., 2001).

In the DMNV, 8% of ChAT-positive neurons also contained FGF1. These doubly labeled neurons were located mainly in the lateral part of the DMNV, which contain many preganglionic cells extending to the ileum and colon (Satomi et al. 1978). These results are in accord with our previous reports (Okano et al., 2006).

The sacral parasympathetic nucleus is localized in the lateral horn of the L6-S1 spinal cord segments in rat (Hancock and Peveto, 1979; Nadelhaft and Booth, 1984). In agreement with previous reports (Senba et al., 1987; Burnett et al., 1995; Papka et al., 1995; Hoang et al., 2003), ChAT-positive neurons were observed in the sacral parasympathetic nucleus, with only 18% of these showing FGF1-immunoreactivity.

The functional meaning of low expression of FGF1 in parasympathetic preganglionic neurons remains unclear in this study. However, previous studies showed severe damage to these neurons following axonal injury. Aldskogius et al. (1980) reported that parasympathetic preganglionic neurons in the DMNV were more severely damaged by axonal injury than the motor neurons in the hypoglossal nucleus. In their report, only 30% of the neurons could be found in rat DMNV 164 days after injury of the vagal nerve, whereas 75% of the hypoglossal nucleus neurons had survived. Sacral parasympathetic nucleus neurons also exhibited a more prominent decline in ChAT expression than pelvic motor neurons in L5-S2 ventral root avulsion (Hoang et al., 2003). Since FGF1 lacks a signal peptide, it is thought to be released upon cellular injury and have a trophic effect on damaged neurons (Eckenstein et al., 1991; Ishikawa et al., 1992). Our study shows, however, that these parasympathetic preganglionic neurons contain little FGF1. We propose that the low expression of FGF1 in parasympathetic preganglionic neurons is related to their susceptibility to axonal injury.

In contrast to axonal injury, neurons in the sacral parasympathetic nucleus are selectively preserved in amyotrophic lateral sclerosis (ALS), a progressive disease of motor neurons (Konno et al., 1986; Bergmann et al., 1995). Cassina et al. (2005) reported that FGF1 could activate spinal cord astrocytes in a manner that decreased motor neuron survival in cocultures in an ALS animal model. They also suggested that FGF1 released by oxidative stress from motor neurons might have a role in activating astrocytes, which could in turn initiate motor neuron apoptosis in ALS through a p75 neurotrophin receptor-dependent mechanism. If their hypothesis is true, motor neurons that strongly express FGF1 would be severely damaged in ALS, while parasympathetic preganglionic neurons, containing small amounts of FGF1, would only undergo slight injury. Further study is needed to clarify this issue.

Conclusion

The present study demonstrated that parasympathetic preganglionic neurons such as in the EWN, salivatory nucleus, DMNV, and sacral parasympathetic nucleus contain little FGF1. FGF1 is thought to be released upon cellular injury and act as a trophic factor on damaged neurons. These results suggest that the low expression of FGF1 in parasympathetic preganglionic neurons is associated with their susceptibility to axonal injury.

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Accepted May 25, 2007