Up-regulation of β_2-adrenergic receptor immunoreactivity in astrocytes in the spinal cord after dorsal rhizotomy

Teruyoshi Kondo*, Kei-ichiro Nakamura**, Norito Takamura***, Jin Tokunaga***

Abstract

Expression pattern of β_2-adrenergic receptor (β_2-AR) in the dorsal entry zone and dorsal funiculus was examined from 2 to 7 days after unilateral transection (rhizotomy) of the C4 and C5 dorsal roots. Rhizotomy induced a marked increase of β_2-AR immunoreactivity (IR) in the regions of the dorsal root entry zone and dorsal funiculus containing the central processes of injured primary sensory neurons. The expression of β_2-AR-IR in regions of the dorsal root entry zone and dorsal funiculus continued to increase with time. β_2-AR-immunoreactive cells co-expressed GFAP-IR and were positive for nestin, which is characteristic of reactive astrocytes. A population of β_2-AR-immunoreactive cells was labeled with Ki-67, a marker of cell proliferation, indicating that some of them went into cell mitotic state. A subpopulation of β_2-AR-immunoreactive cells also exhibited FGF-2-IR. These findings suggest that β_2-AR may play important roles in astrocytic activation which is associated with the induction of synthesis of growth factor such as FGF-2.

Key words: β_2-adrenergic receptor, astrocyte, dorsal rhizotomy, spinal cord
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INTRODUCTION

Centrally projecting axons from the dorsal root ganglia enter the spinal cord by means of the dorsal root entry zone. Dorsal root rupture injury to the brachial plexus, which results in severe impairment of hand function, is caused by rupture of the root resulting in an interruption of the primary afferent pathway between the dorsal root ganglion and the dorsal root entry zone[1]. After dorsal root injury, centrally directed axons regenerate as far as the dorsal root entry zone, but axonal regeneration through the dorsal root entry zone is arrested by activation of astrocytes[2,3].

The cervical dorsal rhizotomy is a useful model to study the underlying mechanisms involved in the astrocytic barrier to axonal regeneration[4]. Dorsal rhizotomy leads to nerve fiber degeneration, such as Wallerian degeneration of primary afferents in the spinal cord[5]. Concomitantly, this degeneration is accompanied by the activation of astrocytes, which is characterized by the proliferation and hypertrophy of astrocytes and increased production of glial fibrillary acidic protein (GFAP)[6]. In addition, reactive astrocytes start to re-express nestin, an intermediate filament protein characteristic of undifferentiated neuroepithelial cells[7].

After brain injury, a pronounced increase in β_2-adrenergic receptor (β_2-AR) expression has been detected predominantly in activated glia cells[8,9]. Pharmacological studies have demonstrated that β_2-AR stimulation induces astroglial proliferation and activation in brain tissues[10,11]. Furthermore, activation of astrocytes by β_2-AR agonists has been shown to increase the

*Department of Clinical Engineering, School of Health Science, Kyushu University of Health and Welfare, 1714-1 Yoshino-cho, Nobeoka, 882-8508, JAPAN
**Department of Anatomy, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, JAPAN
***Second Department of Clinical Pharmacy, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, 1714-1 Yoshino-cho, Nobeoka 882-8508, JAPAN

synthesis of fibroblast growth factor-2 (FGF-2) in ischemic brain tissue[12]; however, no information is available concerning the relationship between astrocyte activation and the expression of β_2-AR-IR and FGF-2-IR in astrocytes following dorsal rhizotomy. Therefore, we have examined changes in the expression of β_2-AR and FGF-2-IR in the regions of the dorsal root entry zone and dorsal funiculus containing degenerating primary afferents after rhizotomy.

MATERIALS AND METHODS

Animals and surgery

Animal procedures were approved by the Institutional Animal Care and Use Committee at Kurume University School of Medicine and Kyushu University of Health and Welfare. Twelve male Wistar rats weighing 300-400 g were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The left cervical dorsal roots were exposed at the cervical level by hemilaminectomy under an operating microscope and the dorsal roots were sectioned from C5 to C7, using an extradural approach (n=3 for each postoperative survival time). Wounds were closed with silk sutures, and buprenorphine (0.01 mg/kg, subcutaneously) was administered every 12h for a total of four doses. Four animals were subjected to sham operations: hemilaminectomy was performed but the roots were left intact. After postoperative survival times of 1, 2, 4, 7 days, the deeply anesthetized animals were perfused through the ascending aorta with heparinized saline (1,000 U/ml) followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB). The C5 to C7 spinal cord segments were removed, postfixed, and cryoprotected overnight in 20% sucrose in 0.1M PB at 4 ℃. Ten μm-thick transverse spinal cord sections were cut on a cryostat and stored at -80℃ .

Immunohistochemistry

Sections were treated with 3% H_2O_2 in 0.1 M phosphate buffered-saline (PBS, pH 7.4) to eliminate endogenous peroxidase activity and incubated in a blocking solution of 10% normal donkey serum and 1% bovine serum albumin (BSA) in PBS with 0.2% Triton-X 100. They were then incubated for 24 hrs at 4 ℃ with rabbit polyclonal anti-β_2-AR antibody raised against the C-terminal 20 amino acids of the human β_2-AR (1: 400; Santa Cruz). After several washes in PBS, sections were incubated for 1 hr at room temperature in biotinylated donkey anti-rabbit IgG, (1: 400, Jackson ImmunoRresearch), rinsed in PBS, and then incubated for 1 hr at room temperature with ABC complex (Streptavidin Biotin Complex Peroxidase Kit, Nacalai Tesque) according to the manufacture's instructions. All antibodies were diluted with blocking solution. After washing in PBS, the immunoperoxidase reaction product was visualized using Vector VIP® as a chromogen. Omission of the primary antibodies in the incubation medium resulted in a complete absence of immunostaining. The specificity of anti-β_2-AR antibody was tested by preincubation of the primary antibody with 50 μg/ml antigen peptide (Santa Cruz), which abolished the specific immunoreaction, although weak and diffuse staining in the nuclei was occasionally seen.

Double immunofluorescence

Sections were preincubated in blocking solution as described above and incubated for 24 hrs at 4℃ with rabbit polyclonal anti-β_2-AR antibody (1: 200) mixed with one of the following antibodies: mouse monoclonal anti-GFAP antibody (marker for astrocytes, 1: 10,000, Sigma), mouse monoclonal anti-nestin antibody (marker for reactive astrocytes, 1: 600; BD Biosciences Pharmingen), mouse monoclonal anti-ki-67 antibody (nuclear antigen of proliferating cells, 1: 200, BD Biosciences Pharmingen). After several rinses in PBS, sections were incubated for 1 hr at room temperature with biotinylated donkey anti-mouse IgG (1: 400), rinsed with PBS, and then incubated for 1 hr at room temperature with a mixture of Alexa 488 conjugated donkey anti-rabbit IgG (1: 500, Molecular Probes) and Alexa 568 conjugated donkey anti-mouse IgG (1: 500, Molecular Probes).

For double labeling for β_2-AR and FGF-2, tyramide signal amplification fluorescence procedures[13] were used. Spinal sections were incubated with β_2-AR antibody (1: 5,000). After using the ABC method and tyramide signal amplification (TSA™ biotin system; PerkinElmer) for 6 min at room temperature, sections were incubated with Alexa 488 conjugated streptavidin (1: 1,000; Molecular Probes). Next, sections were incubated with rabbit polyclonal FGF-2 antibody (1: 500;

Santa Cruz), and then with Alexa 568 conjugated donkey anti-rabbit IgG (1: 500, Molecular Probes). Sections were rinsed with PBS and coverslipped with a PermaFluor aqueous mount (Thermo).

RESULTS

In sham-operated animals and on the unoperated side of experimental animals, β_2-AR immunoreactivity (β_2-AR-IR) showing the appearance of fibers was concentrated in the superficial layers of the dorsal horn (Fig. 1B). In addition to neuropil staining, weakly immunoreactive glial structures, as judged by their morphological configuration, were found in both the white and gray matter as well as the dorsal root entry zone (Fig. 1B). At 1 day after rhizotomy, no change in β_2-AR-IR in these areas was observed. Two days after injury, β_2-AR-IR began to increase on the rhizotomy side (Fig. 1A) compared to the contralateral side (Fig. 1B) in the dorsal root entry zone and dorsal funiculus. Large immunoreactive cells were prominent, especially at the boundary of the dorsal root entry zone, where they formed a distinct layer separating central nervous system and peripheral nervous system tissue (Fig. 1E). This increase in β_2-AR label on the ipsilateral side (Fig. 1C, F) compared to the contralateral side (Fig. 1D) was more pronounced at 7 days.

GFAP-IR in the ipsilateral (Fig. 2B) dorsal root entry zone and dorsal funiculus compared to the contralateral side (Fig. 2F) was up-regulated concomitantly with β_2-AR-IR at 2 days after rhizotomy and showed a gradually increasing expression with longer survival times (Fig. 2D). Double labeling revealed that most β_2-AR-immunoreactive profiles (Fig. 2A, C, E) coexist with GFAP- IR (Fig. 2B, D, F).

Nestin expression was almost undetectable in sham-operated animals and on the unoperated side of experimental animals. Nestin-IR first appeared at 2 days after rhizotomy in the ipsilateral dorsal root entry zone and dorsal funiculus (Fig. 3B); thereafter, the number of nestin-immunoreactive profiles increased in these areas until 7 days (Fig. 3D), which was the longest observation time. Double labeling showed significant colocalization between β 2-AR-IR (Fig. 3A, C) and nestin-IR (Fig. 3B, D).

Only a few Ki-67-positive cells were observed in the dorsal root entry zone and dorsal funiculus of sham-operated animals and the unoperated side of experimental animals (Fig. 4D). Numerous Ki-67-immunoreactive cells were found from 2(Fig. 4B) to 4 days in the dorsal root entry zone and dorsal funiculus ipsilateral to rhizotomy, but by 7 days after injury, their number had declined markedly (data not shown). Colocalization between β_2-AR-IR (Fig. 4A) and Ki-67-IR (Fig. 4B) was found only on the rhizotomy side.

A few FGF-2-immunoreactive cells were observed in the contralateral dorsal root entry zone and dorsal funiculus. There was a significant increase in FGF-2-IR in the dorsal root entry zone and dorsal funiculus ipsilateral to rhizotomy. Some β_2-AR-immunoreactive cells were also stained for FGF-2 (Fig. 5A, B).

DISCUSSION

The results indicate that dorsal rhizotomy increases β_2-AR-IR in activated astrocytes of the dorsal root entry zone and dorsal funiculus occupied by the injured primary sensory axons after dorsal rhizotomy. In addition, dorsal rhizotomy leads to increased FGF-2-IR in activated β_2-AR-immunoreactive astrocytes. Although a causal relationship has not been established, these data provide suggestive evidence that β_2-AR and FGF-2 play a role in the activation of astrocytes following dorsal rhizotomy.

Our finding of the increased expression of β_2-AR-IR in activated astrocytes is in accordance with previous observations of damaged optic nerves[8] and the brain subjected to focal ischemia[9]. The increase in astrocyte β_2-AR after injury appears to be a general property of astrocytes that is distributed throughout the mammalian central nervous system. β_2-AR activation has been involved in the hypertrophy of astrocytes by up-regulation of the synthesis of GFAP[14] and the regulation of GFAP assembly[15]. Furthermore, β_2-AR stimulation has been shown to increase FGF-2 synthesis in cortical astrocytes[12]. Taken together with these data, the up-regulation of FGF-2 in β_2-AR-immunoreactive activated astrocytes suggests that released FGF-2 may act in an autocrine and/or paracrine manner to further augment astrocyte activation. FGF-2 has indeed been reported to induce both GFAP mRNA[16].

Activation of astrocytes became prominent, especially at the boundary of the dorsal root entry zone, 2 days after rhizotomy. The functional significance of this response is as yet unclear. It has been demonstrated that proteoglycans responsible for inhibiting axonal regeneration through the dorsal root entry zone are abundant in the dorsal root entry zone following dorsal root injury[17, 18]. β_2-AR on astrocytes may be implicated in regulation of the synthesis of such inhibitory molecules.

REFERENCES

1 Nomura,N., Furuta,A., Iwaki,T.: Dorsal root rupture injury induces extension of astrocytic processes into the peripheral nervous system and expression of GDNF in astrocytes. Brain Res. 950: 21-30, 2002.

2 Carlstedt,T.: Nerve fibre regeneration across the peripheral-central transitional zone. J.Anat.190: 51-56, 1997.

3 McPhail,L.T., Plunet,W.T., Das,P., et al.,: The astrocytic barrier to axonal regeneration at the dorsal root entry zone is induced by rhizotomy. Eur.J.Neurosci. 21: 267-270, 2005.

4 Livesey,F.,J. and Fraher.,J.P.: Experimental traction injuries of cervical spinal nerve roots: a scanning EM study of rupture patterns in fresh tissue. Neuropathol. Appl. Neurobiol. 18:376-386, 1992.

5 George,R. and Griffin,J.W.: Delayed macrophage responses and myelin clearance during Wallerian degeneration in the central nervous system: The dorsal radiculotomy model. Exp. Neurol.129: 225-236, 1994.

6 Liu,L., Persson J.K.E., Svensson,M., et al.: Glial cell responses, complement, and clusterin in the central nervous system following dorsal root transection. Glia 23:221-238, 1998.

7 Kozlova,E.N.: Differentiation and migration of astrocytes in the spinal cord following dorsal root injury in the adult rat. Eur. J. Neurosci. 17: 782-790, 2003.

8 Mantyh, P.W., Rogers, S.D., Allen, C.J., et al.: β_2-adrenergic receptors are expressed by glia in vivo in the normal and injured central nervous system in the rat, rabbit, and human. J. Neurosci. 15: 152-164, 1995.

9 Imura,T., Shimohama,S., Sato,M., et al.: Differential expression of small heat shock proteins in reactive astrocytes after focal ischemia: possible role of β_2-adrenergic receptor. J. Neurosci. 19: 9768-9779, 1999.

10 Hodges-Savola, C., Rogers,S.D., Ghilardi,J.R., et al.: Beta-adrenergic receptors regulate astrogliosis and cell proliferation in the central nervous system in vivo. Glia 17: 52-62, 1996.

11 Junker,V., Becker,A., Hüne,R., et al.: Stimulation of β_2-adrenoceptors activates astrocytes and provides neuroprotection. Eur. J. Pharmacol. 446: 25-36, 2002.

12 Culmsee,C., Stumm,R.K., Schäfer,M.K., et al.: Clenbuterol induces growth factor mRNA, activates astrocytes, and protects rat brain tissue against ischemic damage. Eur. J. Pharmacol. 379: 33-45, 1999.

13 Hunyady,B., Krempels,K., Harta,G., et al.: Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining. J. Histochem. Cytochem. 44: 1353-1362,1996.

14 Segovia,j., Lawless,G.M., Tillakaratne,N.J., et al.: Cyclic AMP decreases the expression of a neuronal marker (GAD67) and increases the expression of an astroglial marker (GFAP) in C6 cells. J.Neurochem. 63: 1218-1225, 1994.

15 McCarthy,K.D., Prime,J., Harmon,T., et al.: Receptor-mediated phosphorylation of astroglial intermediate filament proteins in cultured astroglia. J.Neurochem. 44: 723-730, 1985.

16 Gomez-Pinilla,F., Miller,S., Choi,J., et al.: Heparan sulfate potentiates the autocrine action of basic fibroblast growth factor in astrocytes: an in vivo and in vitro study. Neuroscience 76: 137-145, 1997

17 Zhang,Y., Tohyama,K., Winterbottom,J.K., et al.: Correlation between putative inhibitory molecules at the dorsal root entry zone and failure of dorsal root axonal regeneration. Mol.Cell.Neurosci. 17: 444-459, 2001.

18 Beggah,A.T., Dours-Zimmermann,M.T., Barras,F.M., et al.: Lesion-induced differential expression and cell association of Neurocan, Brevican, Versican V1 and V2 in the mouse dorsal root entry zone. Neuroscience 133: 749-762, 2005.

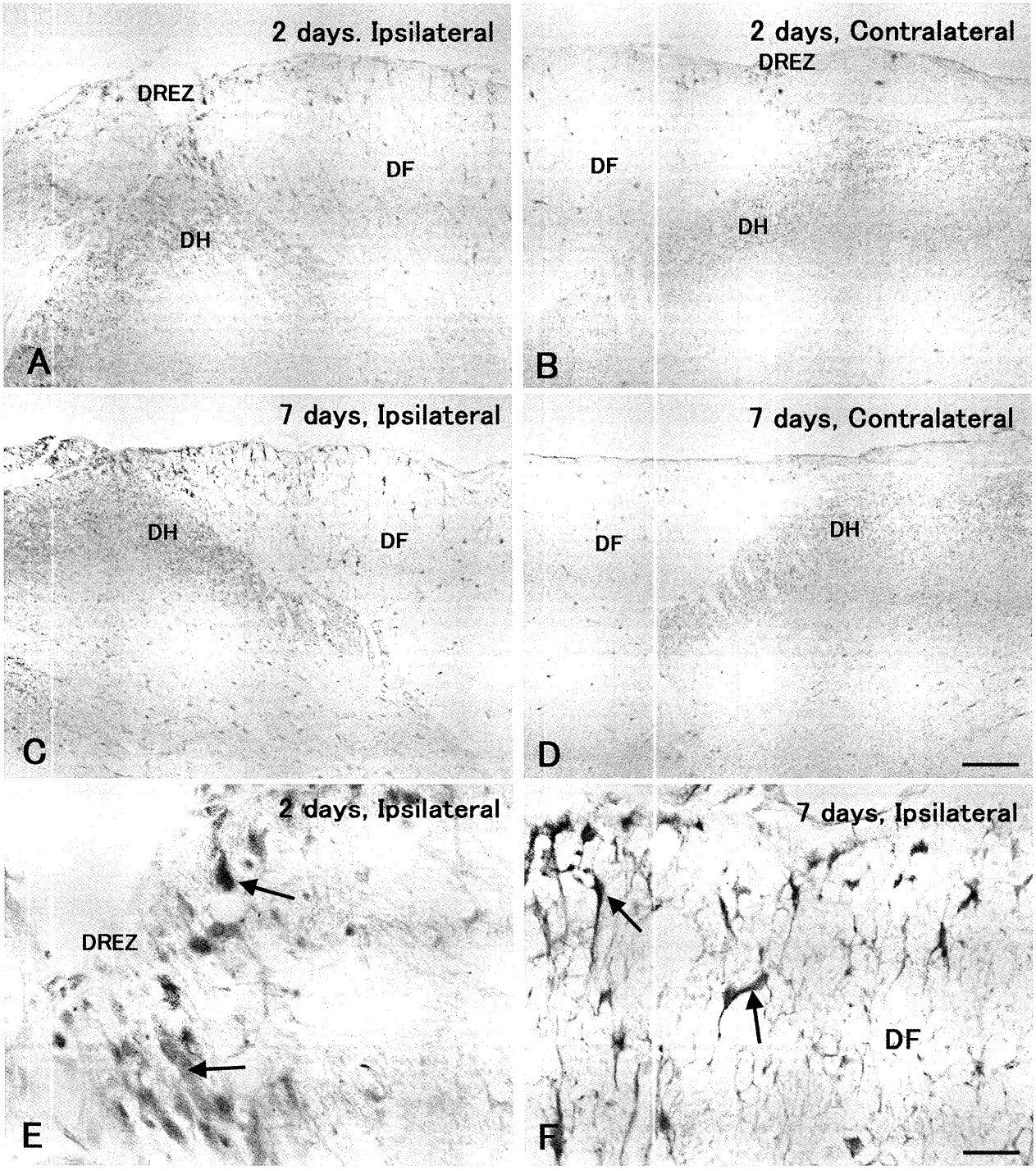

Fig. 1. β_2-AR-IR in the dorsal root entry zone (DREZ) and dorsal funiculus (DF) 2 days (A, B,) and 7 days (C, D,) after dorsal rhizotomy. E: Higher magnification of the dorsal entry zone 2 days after dorsal rhizotomy, showing large immunoreactive cells (arrows) concentrated at the boundary of the dorsal entry zone. F: Higher magnification of the dorsal entry zone 7 days after dorsal rhizotomy, showing intensely labeled cell bodies and their hypertrophic processes (arrows). Scale bars=200 μm (A-D) and 50 μm (E, F).

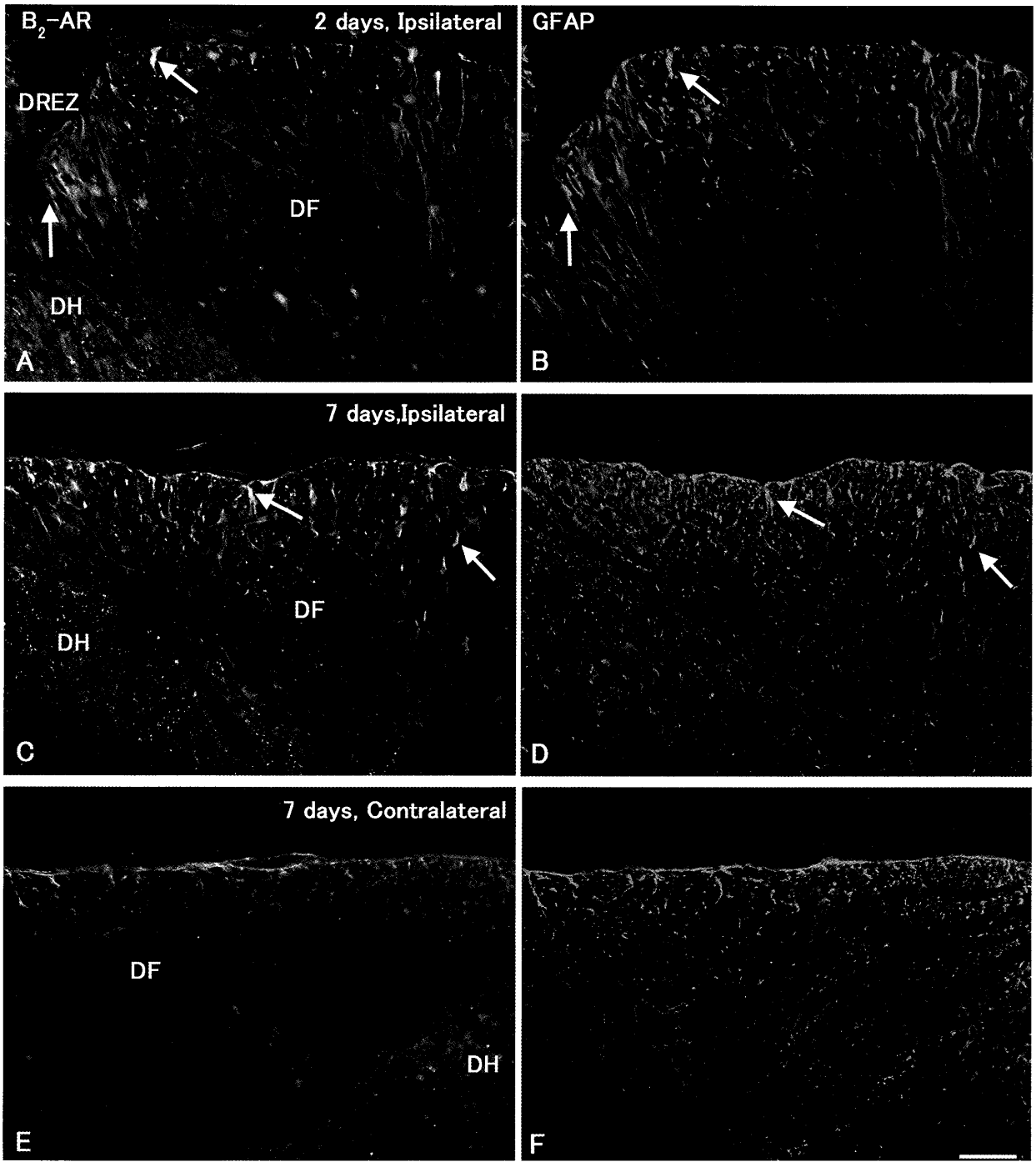

Fig. 2. Double immunostaining with antibodies to β_2-AR (A, C, E) and GFAP (B, D, F) in the dorsal root entry zone (DREZ) and dorsal funiculus (DF) 2 days (A, B) and 7 days (C-F) after dorsal rhizotomy. Note that most β_2-AR-immunoreactive (A, C, arrows) cells are co-localized with GFAP- IR (B, D, arrows). Scale bar = 100 μm.

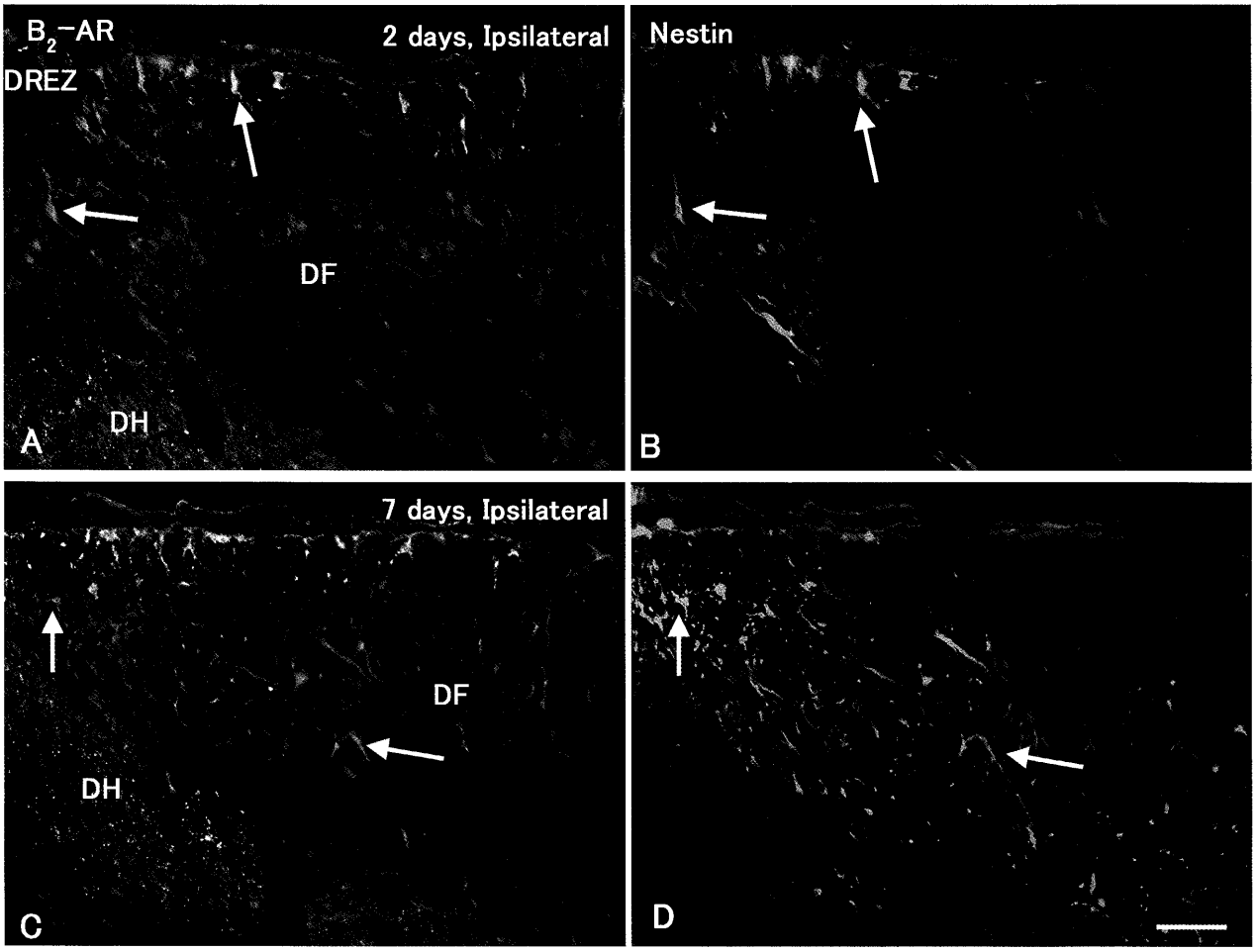

Fig. 3. Double immunostaining with antibodies to β_2-AR (A, C) and nestin (B, D) in the dorsal root entry zone (DREZ) and dorsal funiculus (DF) 2 days (A, B) and 7 days (C, D) after dorsal rhizotomy. Note that many β_2-AR-immunoreactive cells (A, C, arrows) also express nestin-IR (B, D, arrows). Scale bar = 100 μm.

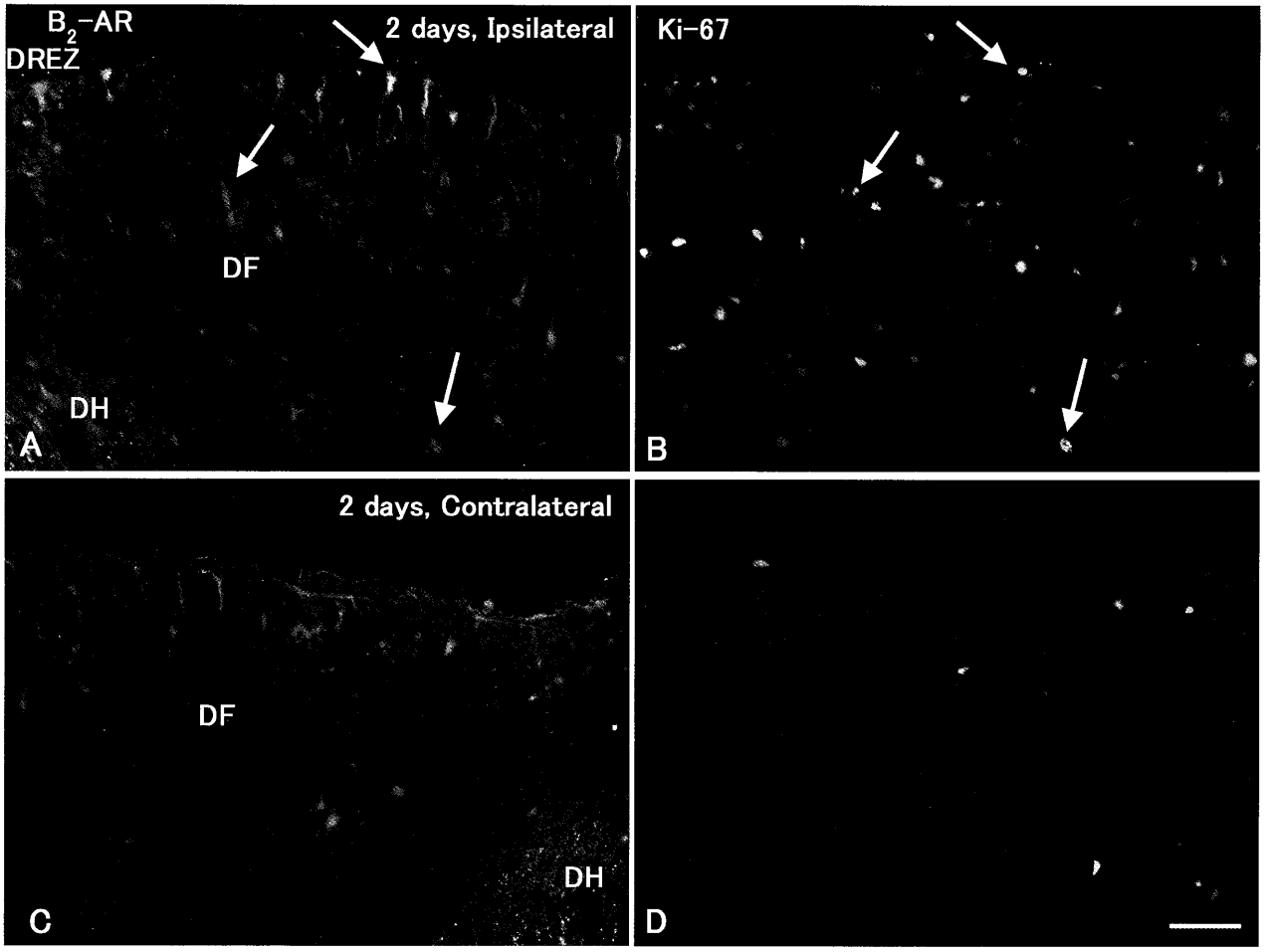

Fig. 4. Double immunostaining with antibodies to β_2-AR (A, C) and Ki-67 (B, D) in the dorsal root entry zone (DREZ) and dorsal funiculus (DF) 2 days after dorsal rhizotomy. Note that some Ki-67-immunoreactive nuclei (B, arrows) are localized to β_2-AR-immunoreactive cells (A, arrows) on the rhizotomy side but not to the contralateral side (C, D). Scale bar = 100 μm.

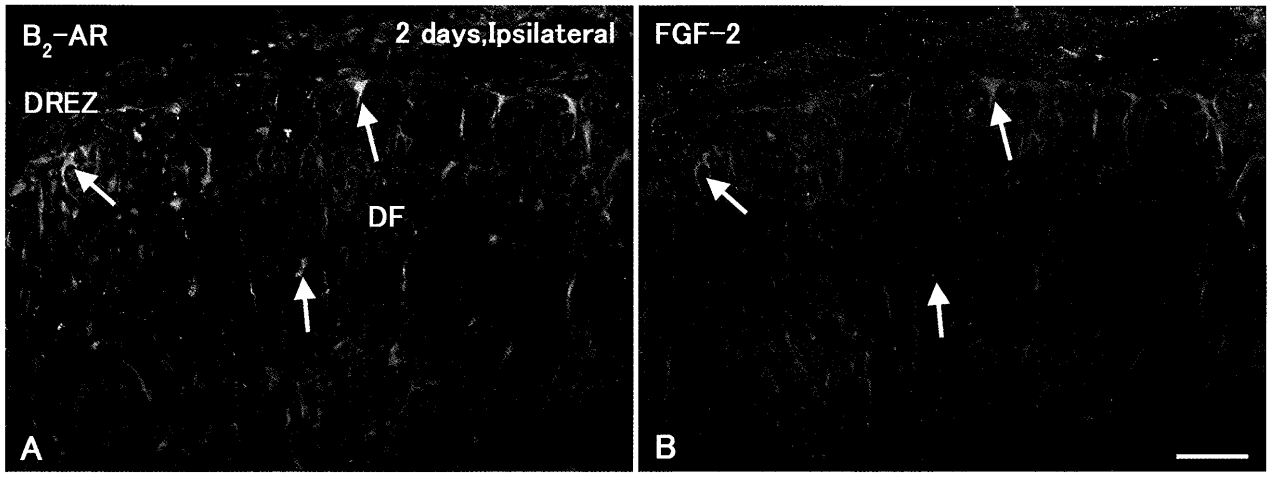

Fig. 5. Double immunostaining with antibodies to β_2-AR (A) and FGF-2 (B) in the dorsal root entry zone (DREZ) and dorsal funiculus (DF) 2 days after dorsal rhizotomy. Arrows indicate some of dually stained cells. Scale bar = 100 μm.

後根切除による脊髄アストロサイトにおけるβ_2アドレナリン受容体免疫活性の増加

近藤　照義*、中村桂一郎**、高村　徳人***、徳永　　仁***

九州保健福祉大学保健科学部臨床工学科　〒882-8508　宮崎県延岡市吉野町1714－1
**久留米大学医学部解剖学講座顕微解剖・生体形成部門　〒830-0011　福岡県久留米市旭町67
***九州保健福祉大学薬学部臨床薬学第二講座　〒882-8508　宮崎県延岡市吉野町1714－1

要　旨

片側後根切除後１日から７日において、脊髄の後根侵入域 及び後索におけるβ_2アドレナリン受容体（β_2AR）の分布様式の変化を免疫組織化学的に検索した。後根切除後２日において、術側のβ_2AR免疫活性は対側に比べて増加し、その増加は切除後７日において顕著であった。β_2AR免疫陽性細胞のほとんどはアストロサイトのマーカーであるGFAPや活性型アストロサイトのマーカーであるネスチンの免疫活性を示した。β_2AR免疫陽性細胞には、時々、増殖マーカーであるKi-67の免疫活性が認められた。β_2AR免疫陽性細胞には、しばしば、FGF-2の免疫活性の増加が認められた。後根切除によるβ_2AR免疫活性の増加及びβ_2AR免疫陽性細胞におけるGFAP、Ki-67、FGF-2の共存関係を考慮すると、脊髄損傷においてβ_2-ARはFGF-2合成を介してアストロサイトの増殖や活性化に関与している可能性が示唆された。

キーワード：β_2アドレナリン受容体、アストロサイト、後根切除、脊髄
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