－原著－

歯根膜ルフィニ神経終末の再生過程における ASIC3 発現様式の変化

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Alterations in the Expression Pattern of the Acid-Sensing Ion Channel 3（ASIC3）during the Regeneration of Periodontal Ruffini Endings

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Abstract

The acid-sensing ion channel 3 (ASIC3), belonging to the epithelial sodium-channel/degenerin (ENaC/DEG) superfamily, is regarded as an essential ion channel for mechanosensation. Our recent study has confirmed the expression of ASIC3 in the mechanoreceptive Ruffini endings in the periodontal ligament of mice incisors, indicating that this molecule is a new marker for mechanoreceptors. Although the periodontal Ruffini endings have been reported to have a potential for high neuroplasticity, no information is available regarding the role of ASIC3 during the regeneration process after nerve injury. This study was therefore undertaken to examine changes in the expression pattern of ASIC3 in the periodontal ligament and trigeminal ganglion of the rats. After transection of the inferior alveolar nerve (IAN), cryostat sections – including the lingual ligament of the lower incisors and trigeminal ganglia – were processed for immunohistochemical procedures. As previously reported, the periodontal Ruffini endings disappeared on postoperative (PO) Day 3, and began regeneration from PO Day 7 to return to their normal morphology by PO Day 28, as shown by immunohistochemistry for protein gene product 9.5, a neuronal marker. However, no apparent immunoreaction for ASIC3 was ever recognizable in the periodontal ligament throughout this observation period. On the other hand, a strong immunoreaction for ASIC3 occurred in the satellite cells encircling the trigeminal ganglion neurons on the ipsilateral side. These findings indicate that ASIC3 is not directly involved in the axonal regeneration of the periodontal Ruffini endings but rather in neuron-glial interactions such as control of the neuronal activity in the trigeminal ganglion.

抄録

Acid-sensing ion channel 3 (ASIC3) は，上皮性ナトリウムチャネル/ degenerin (ENaC/DEG) 遺伝子スーパーファミリーに属する非電位依存性陽イオンチャネルで，機械刺激受容に重要な役割を担う。近年，我々は ASIC3 が歯根膜の機械受容器である歯根膜ルフィニ神経終末に発現することを報告し，機械受容器の新たなマーカーとなり得ることを示した。歯根膜ルフィニ神経終末は，高い神経可塑性を有することが報告されているが，神経傷害後の再生過程における ASIC3 の役割については全く不明である。従って本研究では，ラットの歯根膜ルフィニ神経終末および三叉神経節における下歯槽神経傷害後の ASIC3 の発現様式の変化を検討した。下歯槽神経切断後，切歯舌側歯根膜を

含む下顎骨と三叉神経節の凍結切片を作製し，神経線維のマーカーである protein gene product 9.5 (PGP 9.5) と ASIC3 の局在を免疫組織化学的手法を用いて検索した。過去の報告と同様に，切歯歯根膜ルフィニ神経終末は，下歯槽神経切断 3 日後に完全に消失したが，7 日後には再生を開始し，切断後 28 日目には正常なルフィニ神経終末の形態に回復した。しかしながら，観察期間を通して ASIC3 の免疫陽性反応は歯根膜ルフィニ神経終末には認められなかった。一方，切断側の三叉神経節では神経細胞周囲の衛星細胞が強い ASIC3 免疫陽性反応を示した。これらのことから，ASIC3 は歯根膜ルフィニ神経終末の再生過程において，軸索終末の形態回復に直接的には関与しないが，三叉神経節における神経活性の調節といった neuron-glia interaction に関与する可能性が示唆された。

Introduction

Acid-sensing ion channels (ASICs) belong to the epithelial sodium-channel/degenerin (ENaC/DEG) superfamily[1-3]. ASICs consisting of 7 isoforms[2,4] have been reported to be distributed throughout the central and peripheral nervous systems in mammals[5-7]. Previous studies have suggested the involvement of the neuronal ASICs in acid sensing as well as nociception and mechanoception[5,8-11]. In fact, ASIC1, 2, and 3 have been confirmed in the mechanoreceptive neurons of the dorsal root ganglia[12] as have ASIC2a and ASIC3 in cutaneous mechanoreceptors including Meissner corpuscles, Merkel nerve endings, and palisades of lanceolate nerve endings[5,8,13]. Predominant expressions of sensory neurons have suggested that ASIC3 is an essential channel for mechanosensation[5,8,9,13,14], as confirmed by ASIC3 expression in the trigeminal neurons, which participate in the sensation and modulation of nociceptive and mechanoreceptive signals[15-17].

The periodontal ligament is a dense connective tissue which receives a rich supply of sensory nerves. The periodontal sensory endings are divided into nociceptive free endings and mechanoreceptive specialized nerve terminals[18]. In spite of variance among species or kinds of teeth, a series of morphological studies have suggested that the Ruffini ending is a primary mechanoreceptor in the periodontal ligament[19,20]. Although the periodontal Ruffini ending lacks a distinctive fibrous capsule different from the Ruffini endings originally reported by Ruffini, this mechanoreceptive nerve terminal is morphologically characterized by dendritic ramifications of expanded axon terminals filled with abundant mitochondria and by an association with the terminal Schwann cells, analogues to lamellar cells of the Pacinian corpuscle or laminar cells of the Meissner corpuscle[18,19,21-23].

Active tissue remodeling takes place in the periodontal ligament with the constant exposure of occlusal forces[24]. Indeed, it has been shown that the turnover rate of the collagen fibers in the periodontal ligaments is about five times faster than that in other tissues[25-27]. This indicates that the periodontal nerve fibers have to adapt to the active tissue remodeling of the periodontal ligament. Previous experimental studies on tooth movement[28,29] and traumatic occlusion[30,31] have shown the rearrangement of the periodontal nerves and changes in the shape of the nerve endings. Furthermore, when the inferior alveolar nerve (IAN) is cut at the level of the mandibular foramen, the morphology and density of the periodontal Ruffini ending almost recover by postoperative 1 month[32,33]. Therefore, many researchers agree with the notion that the periodontal Ruffini ending has a high potential for neuroplasticity. Our regeneration studies using neurotrophin gene knockout mice have indicated the possibility that various kinds of molecules control the regeneration of the periodontal Ruffini endings in a stage-specific manner[34-36]. We have recently demonstrated ASIC3 immunoreaction in the axoplasm of the periodontal Ruffini endings as well as in the medium-sized trigeminal neurons which mediate mechanotransduction[17] in the mouse, suggesting a possible role for ASIC3 during the regeneration process of the periodontal Ruffini endings. To date, however, no information is available regarding changes in the ASIC3 expression in the periodontal ligament and trigeminal ganglion during nerve regeneration. Therefore, this study investigated changes in the localization of ASIC3 in the periodontal ligament and trigeminal ganglion using a rat IAN injury model to determine the role of ASIC3 in the regenerating Ruffini endings.

Materials and Methods

Animal preparation

All animal experiments were approved and performed according to the guidelines of the Niigata University Intramural Animal Use and Care Committee

(approval number # 47-4).

Thirty male Wistar rats, 8 weeks of age and weighing 250-300 g at surgery, were used in this experimental study. Transection of the IAN on one side which was randomly selected was performed according to the methods described by Atsumi et al. [37,38] and Harada et al.[34]. Briefly, under anesthesia by an intraperitoneal injection of 4% chloral hydrate (400 mg/kg), the masseter muscle at the side was torn to expose the buccal surface of the mandibular bone, and a small amount of the bone covering the mandibular canal was removed. After transection of the exposed IAN with fine scissors on the side, the cut ends of the nerve were returned into the mandibular canal (experimental group, n = 25). The contralateral side of the trigeminal ganglion was observed as the sham side, which was without any nerve transection. Five rats without any surgical treatment served as a control group. No postoperative treatment such as the administration of antibiotics was given to the operated rats. The animals of the experimental group were allowed to survive for 3, 7, 14, 21, and 28 days after surgery (n = 5 at each stage).

Tissue preparation

At the appropriate survival period, animals were deeply anesthetized with 8% chloral hydrate (400 mg/kg) and perfused transcardially with a fixative containing 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.4). The trigeminal ganglia and the mandibles were removed en bloc and immersed in the same fixative at 4℃ overnight. The mandibles were decalcified with a 10% ethylene diamine tetraacetic acid disodium (EDTA-2Na; Dojindo Laboratories, Kumamoto, Japan) solution for 4 weeks at 4℃ with slight agitation. After demineralization, frozen sections of mandibles including the incisors were serially prepared at a thickness of 25 μm in a cryostat, collected in phosphate-buffered saline, pH 7.4, and treated as free-floating sections. Additionally, cryostat sections of the trigeminal ganglion were cut at a thickness of 8 μm.

Immunohistochemistry

Cryostat and free-floating sections were processed for immunohistochemistry according to our protocol as previously reported[17,35,36]. The sections were incubated with a primary antiserum overnight at 4℃. The primary antisera used in this study are shown in Table 1. The incubated sections were then reacted with a biotinylated goat anti-rabbit IgG (1:1000, Vector Lab., Burlingame, CA) and subsequently with a peroxidase-conjugated avidin (ABC Kit, Vector Lab.) for 90 min each at room temperature. The immunoreaction sites were visualized by incubation in a 0.05 M Tris buffer (pH 7.6) containing 0.025% 3,3'-diaminobenzidine (DAB) and 0.03% hydrogen peroxide. The immunostained sections were counterstained with 0.03% methylene blue.

Furthermore, we employed a double immunostaining with ASIC3 and PGP 9.5, S-100 protein, or Calcitonin gene related peptide (CGRP) in the trigeminal ganglion

Table 1　Antibodies used for DAB staining

	Primary antibodies	Dilution
ASIC3	Rabbit polyclonal anti-rat ASIC3 (Osenses Pty., Keswick, Australia)	1:500
PGP9.5	Rabbit polyclonal anti-human PGP 9.5 (Ultraclone, Co. Ltd., Cambridge, U.K.)	1:10,000

Table 2　Antibodies used for double staining

	Primary antibodies	Fluorescent dye
ASIC3	Rabbit polyclonal anti-rat ASIC3 (1:500, Osenses Pty., Keswick, Australia)	FITC (1:100, Vector Lab.)
PGP9.5	Mouse monoclonal anti-human PGP 9.5 (1:5,000, Ultraclone, Co. Ltd., Cambridge, U.K.)	Texas red (1:100, Vector Lab.)
S100 protein	Mouse monoclonal anti-human S-100 protein (1:1,000, Sigma-Aldrich, Inc., St. Louis, MO)	Texas red (1:100, Vector Lab.)
CGRP	Mouse monoclonal anti-rat CGRP (1:3,300, Sigma-Aldrich, Inc., St. Louis, MO)	Texas red (1:100, Vector Lab.)

and the periodontal ligament. Information on the double immunostaining is shown in Table 2.

Results

ASIC3 expression in the periodontal ligament in the control group

Immunohistochemistry for ASIC3 depicted structures with dendritic profiles in the periodontal ligament of rat incisors (Fig. 1a, c). The positive structures were restricted to the vascular zone but never in the non-vascular zone of the incisor ligament; these were referred to as the alveolus- and tooth-related parts, respectively. In a combination of immunohistochemistry with ASIC3 and PGP 9.5, the immunoreactions appeared co-localized in the ramifications of the thick axonal profiles (Fig. 1b). Judging from their morphology and location, these ramified structures with ASIC3 were regarded as periodontal Ruffini endings, as we have reported[20,34]. In addition, ASIC3 positive cells with round profiles were associated with these axonal branches of the periodontal Ruffini endings with the co-localization ASIC3 and PGP 9.5 (Fig. 1a, b). The ASIC3 immunoreactions were found in their cytoplasm but not in the indented nucleus (Fig. 1a). These cells were devoid of any PGP 9.5 immunoreaction, however (Fig. 1b). On the other hand, a double staining with ASIC3 and S-100 protein demonstrated the co-localization of these immunoreactions in the axon terminals of the periodontal Ruffini endings as well as in the cytoplasm of the rounded cells (Fig. 1c, d). Since the periodontal Ruffini endings have been reported to have an association with rounded telo-glia cells[19], these rounded cells with S-100 immunoreaction could be identified as terminal Schwann cells. However, a part of these terminal Schwann cells lacked ASIC3 immunoreaction (Fig. 1d).

Changes in PGP9.5 and ASIC3 expression after transection of the IAN

On postoperative (PO) Day 3, nerve injury to the IAN induced a complete disappearance of PGP 9.5-positive neural elements including the periodontal Ruffini endings in the lower incisor ligament (Fig. 2a). A few PGP 9.5-positive – but very weak – nerve fibers beaded in appearance were found near the blood vessels in the periodontal ligament on PO Day 7 (Fig. 2b), and the

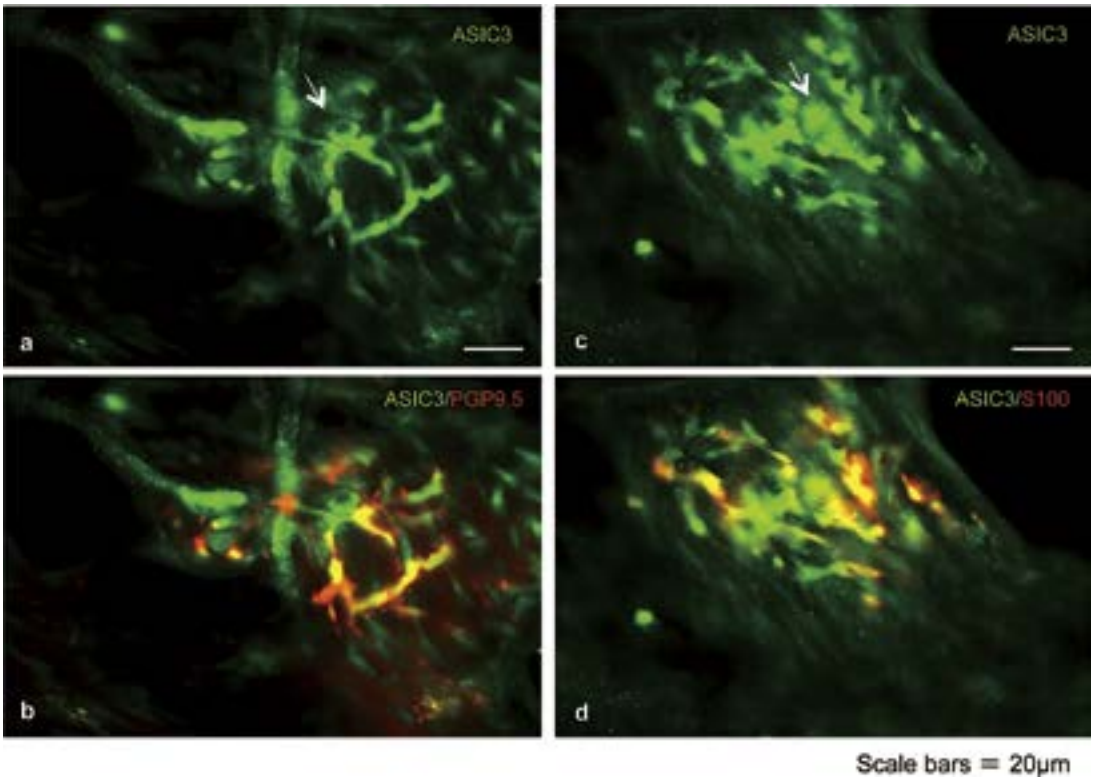

Fig. 1 Fluorescent micrographs showing the periodontal Ruffini endings in the rat lower incisors in the control group, as demonstrated with a double staining with ASIC3 colored green and either protein gene product 9.5 (PGP 9.5) (a, b) or S-100 protein colored red (c, d). Arrows indicate the terminal Schwann cells associated with periodontal Ruffini endings. Scale bars = 20 μ m.

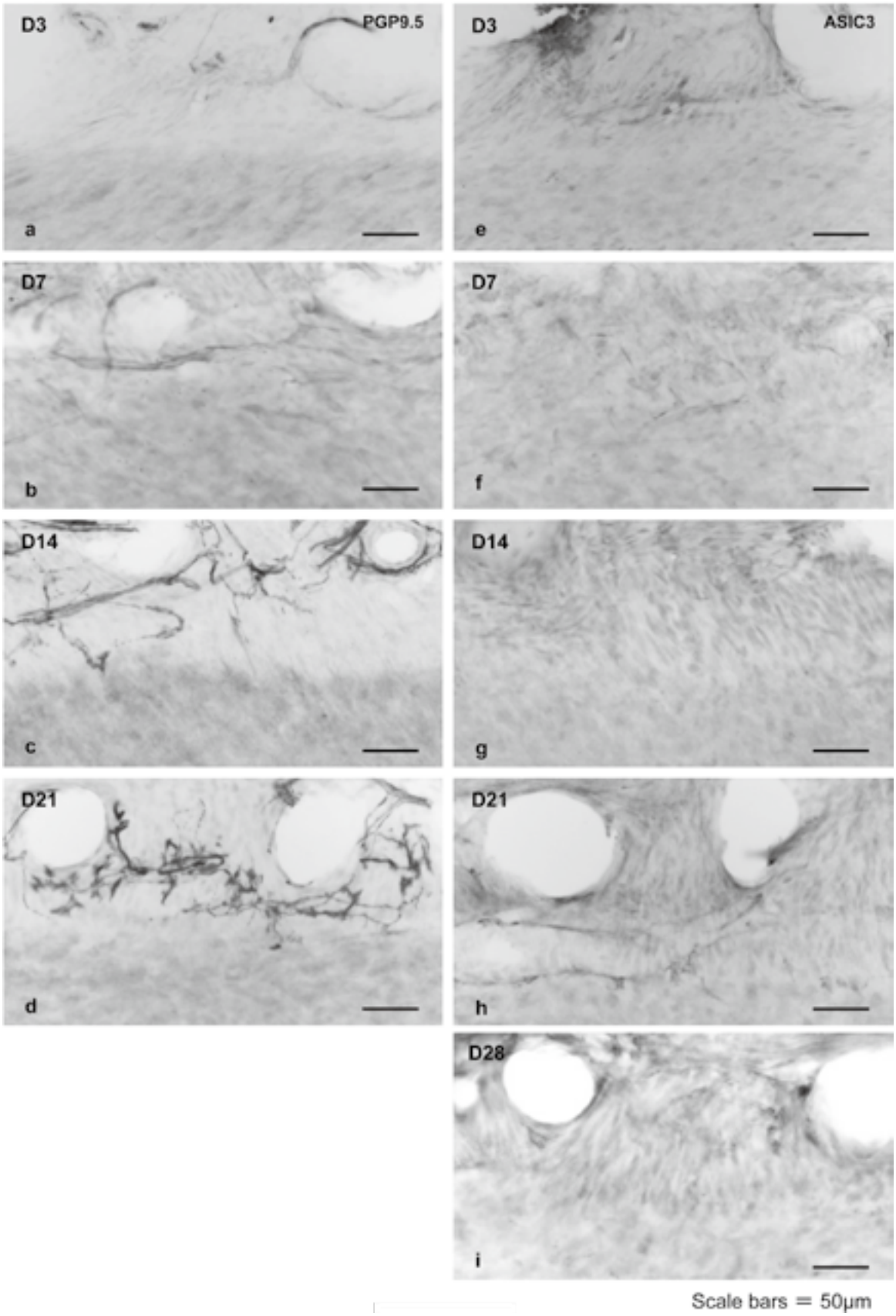

Fig. 2　Micrographs showing changes in immunoreactions for protein gene product 9.5 (PGP 9.5) (a-d) and for ASIC3 (e-i) on postoperative Days 3 (a, e), 7 (b, f), 14 (c, g), 21 (d, h) and 28 (i). DAB development, counter-stained with methylene blue. Scale bars＝50 μ m.

nerve fibers increased in number day by day. The dendritic ramifications composed of thin nerve fibers appeared on PO Day 14 (Fig. 2c), and typical Ruffini endings were first recognizable in the periodontal ligament of the rat incisors on PO Day 21 (Fig. 2d). Thereafter, the periodontal Ruffini endings with extensive ramifications of the thick axon terminals continued to increase by PO Day 28 (data not shown). In ASIC3 immunohistochemistry, however, transection of the IAN reduced any immunoreaction in the periodontal ligament throughout the observation period (Fig. 2e-i), except for some cellular elements with a weak reaction along the blood vessels found on PO Day 21 when the typical Ruffini endings had appeared (Fig. 2h).

ASIC3 expression in the trigeminal ganglion in the control group

The trigeminal ganglia in the control group contained many ASIC3 positive cells of various sizes (Fig. 3a). These ASIC3 positivities were localized in the PGP 9.5 immunoreactive cells in the trigeminal ganglion, indicating that ASIC3 immunoreaction was localized in the trigeminal ganglion neurons. Furthermore, some satellite cells also showed a relatively weak ASIC3 immunoreaction (Fig. 3b). This weak immunoreaction often took on a dot-like appearance in the cytoplasm of the satellite cells, whose outlines were unclear. A double immunostaining with ASIC3 and CGRP demonstrated that one marker for nociceptive neurons – some ASIC3 positive neurons, lacked CGRP neurons (arrowheads in Fig. 3c, d). However, a few neu-

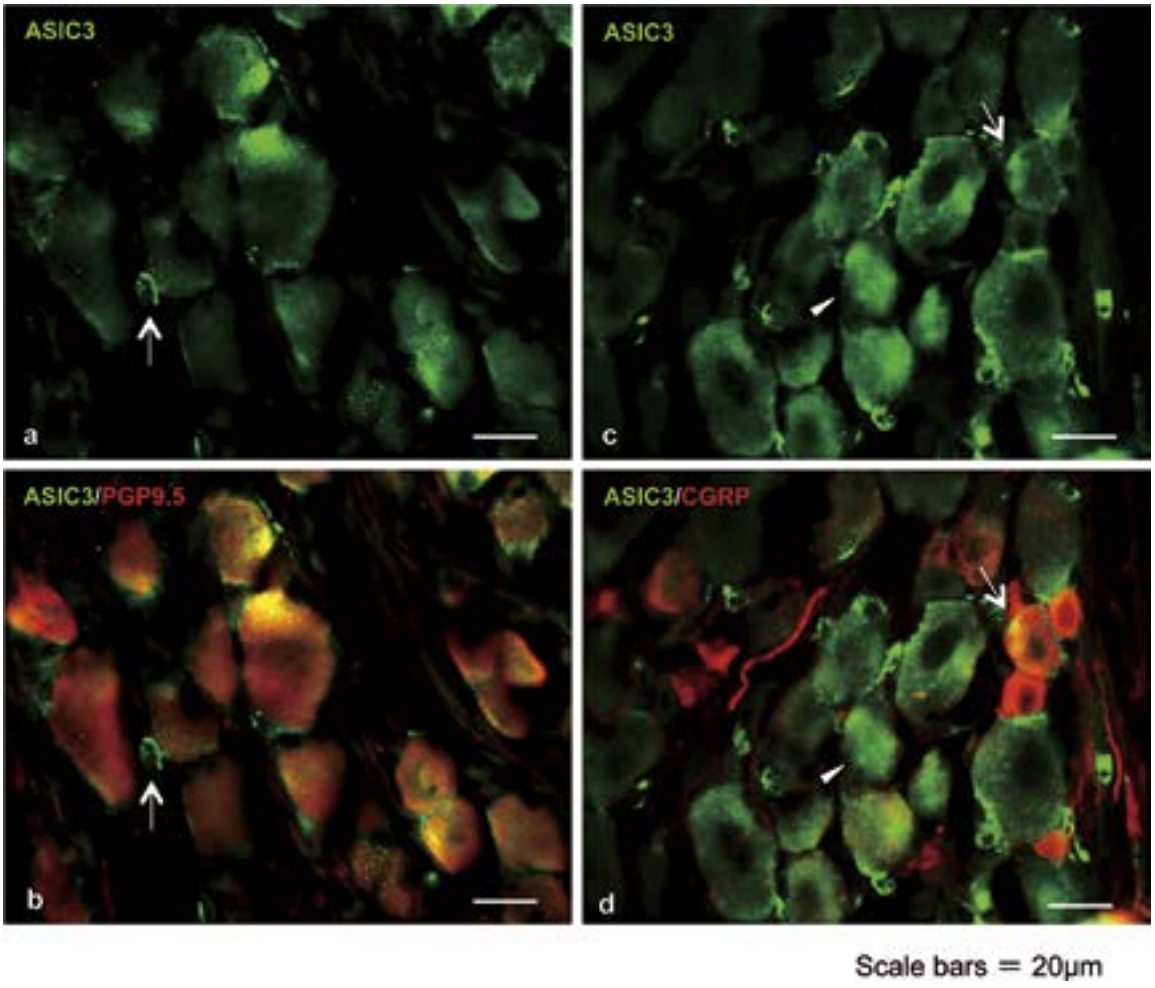

Fig. 3 Fluorescent images of the rat trigeminal ganglion in the control group, as demonstrated with a double staining with ASIC3 (green) and either protein gene product 9.5 (PGP 9.5) (red in b) or calcitonin gene related peptide (CGRP) (red in d). Figures b and d are merged images of ASIC3/PGP 9.5 and ASIC3/CGRP, respectively. Arrows in a and b indicate ASIC3 positive satellite cells. Arrows and Arrowheads in c and d indicate ASIC3 positive neurons with CGRP and without CGRP, respectively. Scale bars＝20 μ m.

rons with CGRP immunoreaction were also positive for the ASIC3 immunoreaction (arrow in Fig. 3c, d).

Changes in ASIC3 expression in the trigeminal ganglion during regeneration after transection of the IAN

Throughout the observation of the contralateral side, the expression pattern of ASIC3 immunoreaction in

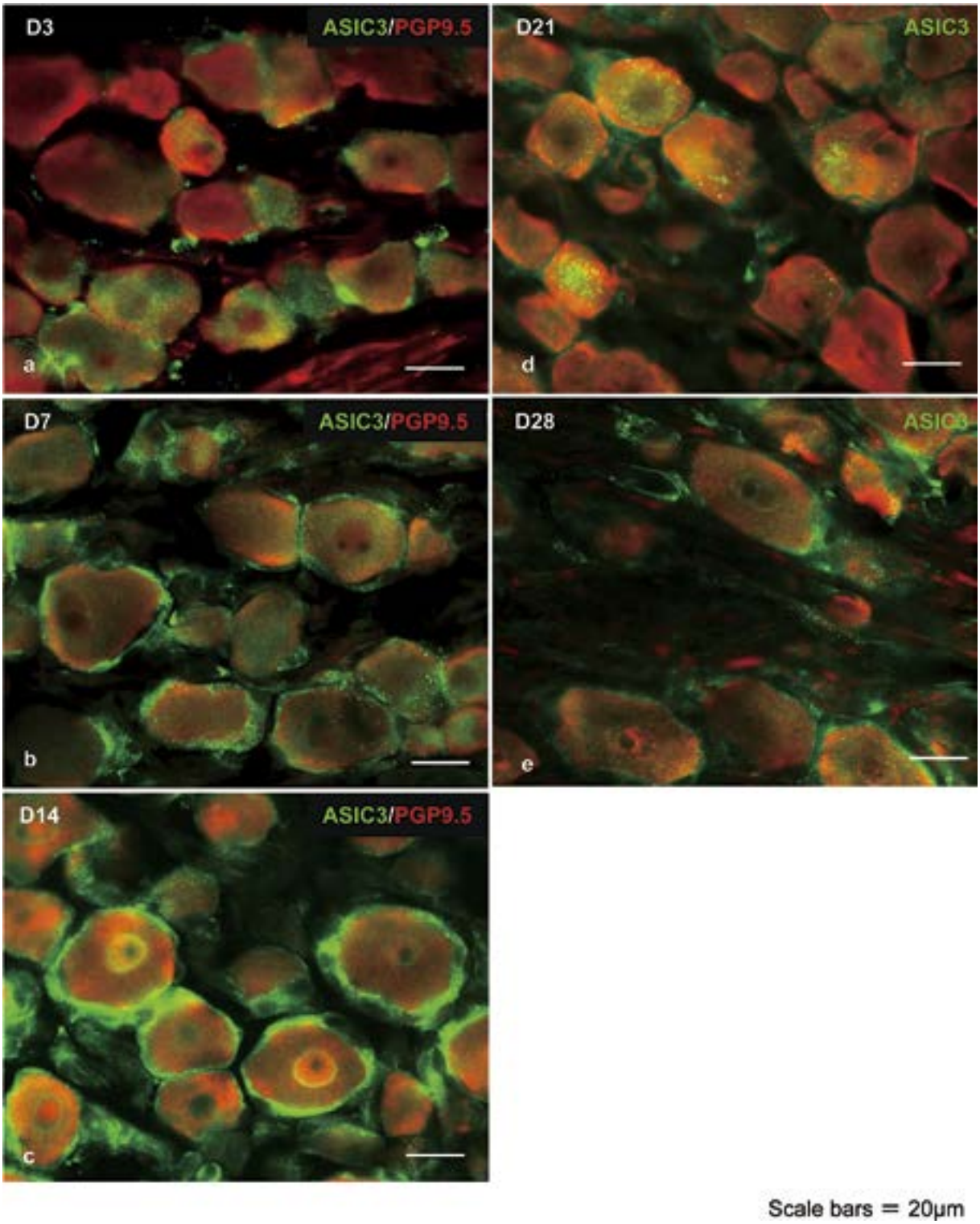

Fig. 4　Merged images of ASIC3 and protein gene product 9.5 (PGP 9.5) immunoreactions (a-c) and fluorescent images of ASIC3 immunoreaction (d, e) on postoperative Days 3 (a), 7 (b), 14 (c), 21 (d) and 28 (e) in the trigeminal ganglion of the ipsilateral side in the experimental group. Scale bars = 20 μ m.

the satellite cells remained unchanged; some satellite cells had immunoreactions with a granular like appearance in their cytoplasm (data not shown).

On PO Day 3 when the periodontal nerve fibers had disappeared from the lingual ligament, no remarkable changes in ASIC3 existed between the ipsilateral and contralateral sides in the experimental group; ASIC3 positivity was found in the trigeminal neurons and satellite cells (Fig. 4a). However, the IAN transection induced a clear ASIC3 expression in the satellite cells on the ipsilateral side on PO Day 7 (Fig. 4b). These ASIC3 reactive satellite cells were often observed surrounding ASIC3 immuno-positive or negative neurons (Fig. 4b). On PO Day 14, ASIC3 immunoreaction in the satellite cells became clearer and stronger than at the previous stages (Fig. 4c). Compared to the previous stages, the satellite cells exhibited thicker profiles with an intense ASIC3 immunoreaction. In accordance with the progress in the regeneration of the periodontal nerves, the ASIC3 immunoreaction had gradually weakened to return to a normal level (Fig. 4d, e). No apparent change in ASIC3 immuno-expression was recognizable in the trigeminal neurons in the ipsilateral or contralateral sides of the experimental group.

Discussion

This immunohistochemical study demonstrated chronological changes in ASIC3 expression in the trigeminal ganglion as well as the regeneration process of the periodontal nerves in the rat periodontal ligament after transection of the IAN. The time course of the regeneration of the rat periodontal nerve was in accordance with our previous reports [34, 35, 39]; the IAN transection induced a complete disappearance of the periodontal nerves in the lower lingual ligament by PO Day 3, and a commencement of the regeneration from PO Day 7 to finish the morphological recovery of the periodontal Ruffini endings by PO Day 28. Although our recent study reported the localization of ASIC3 in the intact Ruffini endings of the periodontal ligament[17], no information is available regarding the changes in ASIC3 expression during the regeneration of the periodontal nerve fibers after nerve injury. This study confirmed the expression of ASIC3 in the periodontal Ruffini endings as Rahman et al.[17] have previously reported. Furthermore, this is the first report to demonstrate an alteration in the expression of ASIC3 in the periodontal Ruffini endings as well as in the trigeminal ganglion during the regeneration process.

Our findings on the localization of ASIC3 in the trigeminal ganglion and the periodontal Ruffini endings differ from the previous report[17]; we found positive reactions in some glial cells including the terminal Schwann cells and trigeminal satellite cells. We are at a loss to explain the difference in ASIC3 immuno-expression pattern between the previous and present studies. Since we used the same kind of antibody to ASIC3 in both studies, a possible explanation might be the different species: mice versus rats (this study). Although ASIC3 immunoreactions have been shown in the non-neural tissues including the testis, pituitary gland, lung, epithelial cells, and bone[40, 41], the role of ASIC3 in non-neural tissues remains unclear. Current observation of the glial expression may indicate a new role for ASIC3.

It is noteworthy that the periodontal Ruffini endings – including the terminal Schwann cells – lost their ASIC3 immunoreaction during the regeneration process after transection of the IAN. As shown in this and the previous study, the morphology and distribution pattern returned to normal by PO Day 28, indicating a possibility that ASIC3 is not directly related to the regeneration process of the periodontal Ruffini endings. However, a time lag between physiological and morphological recoveries is known to exist during the regeneration process in a tooth re-plantation model; the neural activity in the replanted tooth did not seem to recover to that of the normal tooth[42], and the jaw-opening reflex had a raised threshold and increased latency in the replanted teeth[43]. These findings may explain the reason for the complete disappearance of ASIC3 by PO Day 28 if we consider that neuronal ASIC3, an ion channel for mechanosensation[5, 8, 9, 13], is not involved in neural regeneration. Since our observation period concludes by PO Day 28, a longer observation is needed for verifying this hypothesis.

Electrophysiological studies have shown that injury to the trigeminal nerves can result in trigeminal ganglion neurons with unusual peripheral receptive fields[44]. Previous morphological studies have additionally demonstrated that injury to the trigeminal nerve also induced neuroanatomical changes in the trigeminal ganglion, including alterations in neuropeptide levels[45] and neuronal cell death[46]. This immunohistochemical study found an up-regulation of ASIC3

immunoreaction in the satellite cells in the trigeminal neurons on the ipsilateral side of the experimental group; the satellite cells with ASIC3 immunoreaction showed the strongest immuno-intensities and thicker profiles encircling the trigeminal neurons at PO Day 14. Stephenson and Byers[47] demonstrated the temporal up-regulation of glial fibrillary acidic protein (GFAP) in the rat satellite cells in response to dental injury; the satellite cell reaction was confined to the somatotopic region of the ganglion that corresponded to the zone of damage in the periphery. A similar expression of GFAP immunoreaction was found in the satellite cells of the dorsal root ganglion after transection of the sciatic nerve[48]. These studies on temporal GFAP expression have suggested the presence of neuron-glial cell interactions in the sensory ganglion. Although there still has been no information provided on the role of ASIC3 in the glial cells, we can easily postulate the involvement of ASIC3 in the neuron-glial cell interactions in the trigeminal ganglion. This idea may be supported by the putative role of alterations in extracellular ionic concentrations between neurons and glial cells by changing neuronal activity[47]. It would seem appropriate to consider that the alteration in neuronal activity is mediated by ASIC3, which is up-regulated in the satellite cells after transection of the IAN.

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