Expression and Localization of Pituitary Adenylate Cyclase-Activating Polypeptide-Specific Receptor (PAC1R) After Traumatic Brain Injury in Mice

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Abstract: Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic peptide and a well known strong neuroprotectant in various neuronal conditions. PACAP has some receptors and it acts *via* these receptors. PACAP type 1 receptor (PAC1R) is known as the main receptor of PACAP in neuronal damages. On the other hand, the expression and localization of PAC1R have not been elicited well in traumatic brain injury (TBI). In this study, the expression and the cellular localization of PAC1R were investigated immunohistochemically after TBI in mouse controlled cortical impact (CCI) model. The PAC1R positive cells were expressed from 3 h in the peri-contusional area and observed to 7days after the TBI. Using double-immunohistochemistry, the PAC1R immunopositive cells were co-localized with the microglia on 1day and with microglia and astrocyte on 7 days after TBI. These results suggest that PACAP and PAC1R might play an important role in traumatic brain injury as well as other conditions.

Keywords: Pituitary adenylate cyclase-activating polypeptide, PACAP type 1 receptor, traumatic brain injury.

INTRODUCTION

Traumatic brain injury (TBI) is a critical condition in the field of emergency medicine. In the treatment of TBI, the secondary brain damage originated from the complicated factors, such as neuroinflammation, brain edema and secondary brain ischemia, has been considered as main targets in neurocritical care. In spite of the advance in the recent research, the treatment outcome of TBI has not still improved. In the United States, TBI accounts for about 40% of all acute deaths, and the mortality of severe TBI is 51% [1]. In Japan, TBI is one of the major causes of mortality in the younger population.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that was first isolated from ovine hypothalamic extracts in 1989, which belongs to the secretin-glucagon vasoactive intestinal peptide (VIP) family, and it exists in two types of amino acid chains, namely a 38 amino acid chain (PACAP38), and a 27 amino acid chain (PACAP27), which share the same N-terminal amino acids. Both PACAP38 and PACAP27 have similar uniformity to VIP in their amino acid chains. It has been reported that PACAP prevents from neuron strongly in various neuronal injuries [2, 3].

PACAP have 3 types of receptors, PACAP type 1 receptor (PAC1R), VIP type 1 receptor (VPAC1R), and VIP type 2 receptor (VPAC2R). These receptors are G protein-coupled receptors with seven transmembrane domains [2, 3]. It is thought that PAC1R is a main receptor in the neuroprotective effect of PACAP in these receptors [3]. PAC1R binds PACAP38 and PACAP27 with a high affinity, but binds VIP with a 1,000-fold lower affinity [3]. Many PACAP studies which related to cerebral ischemia has been reported. On the other hand, a few studies correlated with TBI have been reported [4, 5], but the PAC1R expression after TBI in mice remains unclear.

In this study, we investigate the PAC1R expressions and the cell identification of the PAC1R expressed cells by immunohistochemical studies.

MATERIALS AND METHODS

Animal

All experimental procedures involving the handling of the animals were approved by the Institutional Animal Care and Use Committee of Showa University (regulation nos. 04093, 04096, 03020, and 02098).

Adult male C57BL mice (20-25g; Saitama Experimental Animal Center, Saitama, Japan) were housed in a temperature- and light-controlled room (lights on at 0600 and off at 1800) and supplied with standard laboratory chow and water ad libitum.

Production of the PAC1R Antibody

The primary antisera were raised in rabbits against a synthetic peptide that corresponded to Cys^{34} to Leu^{47} of the mouse PAC1R, which is located in the amino-terminal ex-

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tracellular domain of all the splice variants [6-8]. The synthetic peptide (CLERIQRANDLMGL) was conjugated to keyhole limpet hemocyanin and then it was injected subcutaneously into the rabbits. The antisera were purified by affinity chromatography using a synthetic peptide conjugated-Affi-Gel 10 column (Pierce Chemical Co., Rockford, IL).

Surgical Procedure

Mice undergoing surgery were maintained in the supine position under anesthesia with 2% sevoflurane in 70% N_2O and 30% O_2 through a nose cone, shaved, mounted in a stereotactic frame and subjected to CCI using a Lighthall stroke-constrained pneumatic compression device adapted for mice [9, 10]. Following a midline incision to expose the skull, a 4-mm craniotomy was made lateral to the sagittal suture, and centered between lambda and bregma. The skull at the craniotomy site was removed with a dural incision. The exposed cortex was injured using a pneumatically controlled impactor device as described previously [11, 12]. The impactor containing a 3-mm diameter flat tip compressed the cortex at a velocity of 5.82 m/s, duration of 47 ms, depth of 1.2 mm, and with a driving pressure of 73 psi. After surgery, the injured area was covered with the dura. Following the suturing of the skin, the animals were placed in an incubator (37°C) until the mice regained consciousness (determined by the return of the righting reflex and increased mobility) in order to prevent hypothermia.

Histology

Mice were anesthetized with sodium pentobarbital (50mg/kg, ip) at 0, 3, 6, 12 h, 1, 2, 4 or 7 days after TBI, and brains were fixed by perfusion with saline followed by 2% paraformaldehyde in 50 mM phosphate buffer (n=4-5). After postfixation, the brain tissues containing injured area were embedded in OCT compound after immersion in 20% sucrose for cytoprotection as a frozen section. The paraffin sections (thickness 4 μm) were used for immunostaining.

Single Immunostaining of PAC1R

For immunoperoxidase labeling, the cryostat sections were incubated in 0.3% hydrogen peroxide (H_2O_2) to block endogenous peroxidase activity and incubated in phosphate-buffered saline (PBS) containing 5% normal horse serum (NHS) to block any nonspecific reaction. The sections were incubated in rabbit anti-PAC1R antibody (1:200) at 4°C overnight. Antibody detection was carried out by incubating the slices with biotinylated goat anti-rabbit IgG (Vector, Burlingame, USA) as a secondary antibody for 120 min, followed by Vectastain ABC (Vector) for 60 min, and then with stable 3,3'-diaminobenzidine complex (DAB; Vector).

Double Immunostaining for Cell Identification

The samples of 1 and 7days after TBI were used to cell identification (n=3). The brain sections were blocked in 5% NHS (for GFAP, CD11b, NeuN, and PAC1R) for 1 h at room temperature. The sections were then immunostained using the following primary antibodies: monoclonal mouse anti-GFAP at a 1:1000 dilution; monoclonal rat anti-CD11b at a 1:500 dilution; monoclonal mouse anti-neuronal nuclei at a 1:1000 dilution; and 2 μg/ml of affinity-purified rabbit polyclonal anti-PAC1R antibody.

The above primary antibodies were diluted in 5% NHS (for GFAP, CD11b, NeuN, and PAC1R) and the sections were incubated with them at 4°C overnight. The sections were then rinsed and incubated with secondary antibodies for 120 min at room temperature. Alexa 546-conjugated goat anti-mouse IgG at a 1:400 dilution (for GFAP and NeuN), biotinylated anti-rat IgG at a 1:200 dilution (for CD11b), and Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes) at a 1:400 dilution (for PAC1R) were used as the secondary antibodies. For CD11b immunostaining, the sections were rinsed and further incubated with a 1:400 dilution of Alexa 546-labeled streptavidin (Molecular Probes) for 30 min at room temperature. For double immunostaining for GFAP and PAC1R, Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) at a 1:400 dilution was used for GFAP. Nuclear staining was performed by 4,6-diamidine-2-phenylindole dihydrochloride (DAPI, 1:10,000; Roche Diagnostics, IN, USA) for 5 min. Both fluorescence and the immunolabeling were detected using a confocal laser microscope (AX-10, Zeiss, Germany).

RESULTS

PAC1R Expressions After Traumatic Brain Injury

In 0 h after TBI (Fig. **1A**, **B**), the PAC1R expression was slightly observed in the cortex.

After 3 h after TBI, the PAC1R positive cells were observed more clearly in the peri-contusional area. The PAC1R immunoreactivity and the numbers of immunopositive cells were gradually increased (the photos of 6, 12h and 2 days were not shown) (Fig. **1**)). Morphologically, small cells like microglia were distributed in the peri-contusional area in 3 h after injury (Fig. **1D**). On day 4 after injury, large cells like astrocyte were also expressed with small cells (Fig. **1G**, **H**). Except for peri-contusional area including the deep white matter and the contra-lateral brain, the strong immunoreactions of PAC1R were not observed.

Identification of the PAC1R-Positive Cells

We observed PAC1R positive cells in peri-contusional area in both samples. On day 1after TBI, the PAC1R positive cells were obviously co-localized with microglia (Fig. **2**). On day 7, PAC1R was not only expressed in microglia but also in the astrocyte (Fig. **3**). Neuron was not co-localized with the PAC1R positive cells in both samples (Figs. **2**, **3**).

DISCUSSION

In our previous study, we have examined the PAC1R expressions and the cellular localization after TBI to estimate the kinetics of PAC1R. The PAC1R positive cells were closely distributed to the peri-contusional area and expressed from the early phase after the insult. Thereafter, PAC1R positive cells were observed in this area up to 7 days after TBI. We also performed cell identification of PAC1R immunopositive cells. The PAC1R expressed in microglia on day 1 after TBI. On day 7^{th} after TBI, PAC1R was also expressed in astrocyte.

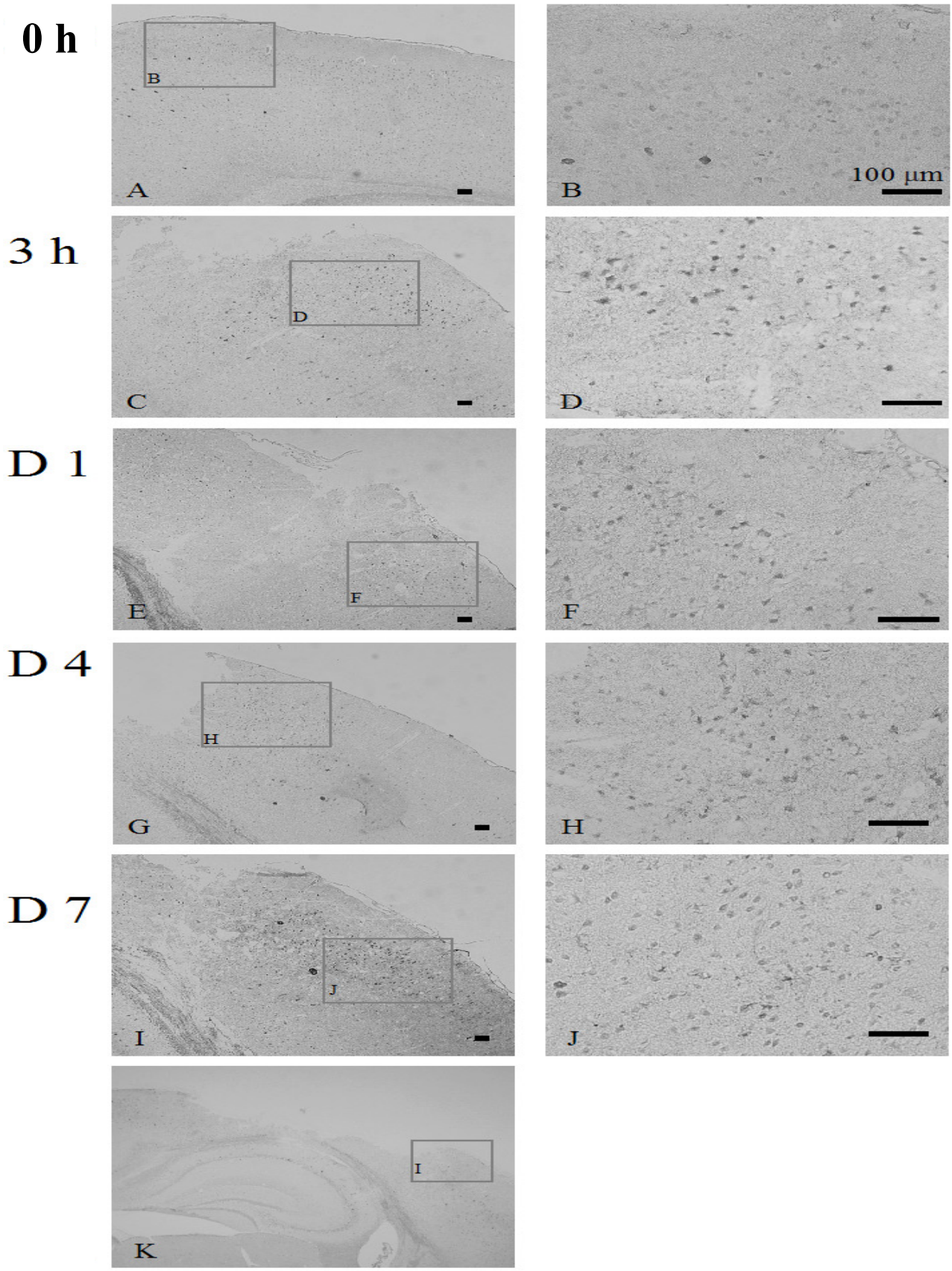

Fig. (1). Light photomicrographs of the time course of the PAC1R positive cells at 0 h (**A**, **B**), 3 h (**C**, **D**), 1 day (**E**, **F**), 4 days (**G**, **H**), and 7 days (**I**, **J**) after traumatic brain injury. PAC1R immunoreactivity was detected in the perifocal area of the trauma lesion at 3 h after brain injury and it was expressed until 7 days post trauma. Boxed areas in **A**, **C**, **E**, **G** and **I** indicated the areas magnified in **B**, **D**, **F** and **H**. Overview of the injured region at 7 days after insult was shown (**K**). Scale bars=100 µm.

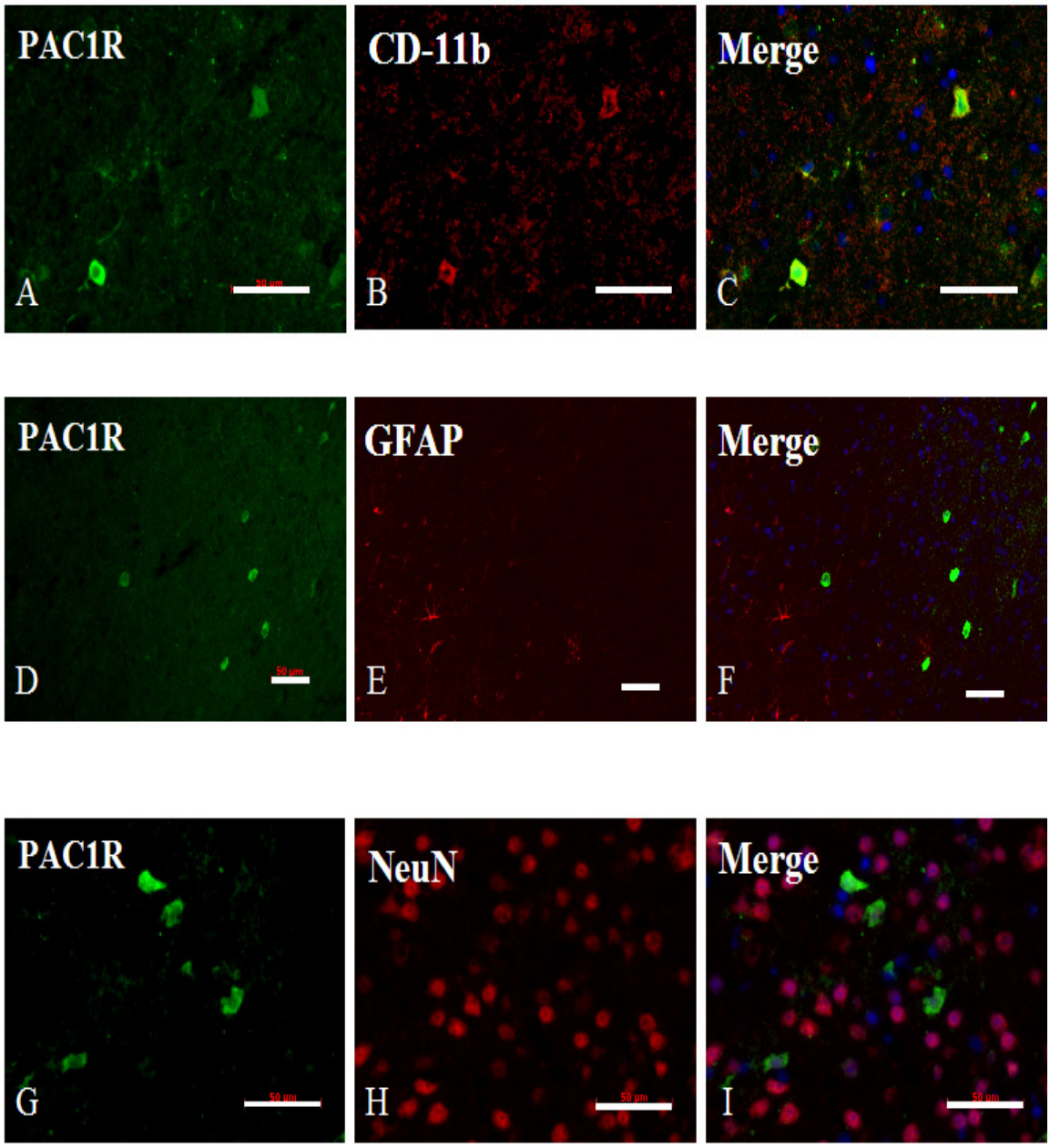

Fig. (2). Double immunostaining of PAC1R with cell markers in the perifocal area of the injured brain at 1 day after the traumatic brain injury. Immunostaining was carried out using antibodies for PAC1R (shown in green) together with CD11-b (**B**), GFAP (**E**), NeuN (**H**) as microglia, astrocytes, and neuron markers (shown in red), respectively. Microglia-like CD11-b immunoreactive cells were co-labeled with PAC1R immunoreactivity (**C**). In the merged images, blue is DAPI staining as counterstaining of nuclei. Scale bars=50 μm.

We also previously reported that PACAP prevents post-ischemic neuronal cell death. PACAP injection into the intracerebroventricular (i.c.v.) space induces Interleukin-6 (IL-6) secretion in cerebrospinal fluid and inhibits c Jun N-terminal kinase (JNK) and p38 phosphorylation in the hippocampus in an after-cardiac-arrest rat model [2, 13]. It was also shown that endogenous PACAP plays a critical role in the prevention of ischemic neuronal cell death *in vivo* and that PACAP suppresses cytochrome *c* release by means of the regulation of bcl-2 in mitochondria. PACAP prevents cerebellar granule neurons from apoptotic cell death through a protein kinase A (PKA)/ protein kinase C (PKC)-dependent inhibition of caspase-3 activity [14]. PACAP regulates phosphorylated extracellular signal-regulated kinase (ERK) directly, and phosphorylated signal transducer and activator of transcription (STAT) 3 indirectly by the generation of IL-6 through PAC1R in neurons [15]. Those signaling pathways leading to apoptosis are inhibited by PACAP i.c.v. administration. PAC1R is actively expressed in different neuroepithelia from the early developmental stages and is expressed in various brain regions during prenatal and postnatal development, and is widely distributed in the embryonic central and peripheral nervous system [16, 17]. It is therefore well-recognized that PACAP have pleiotropic functions in the central nervous system.

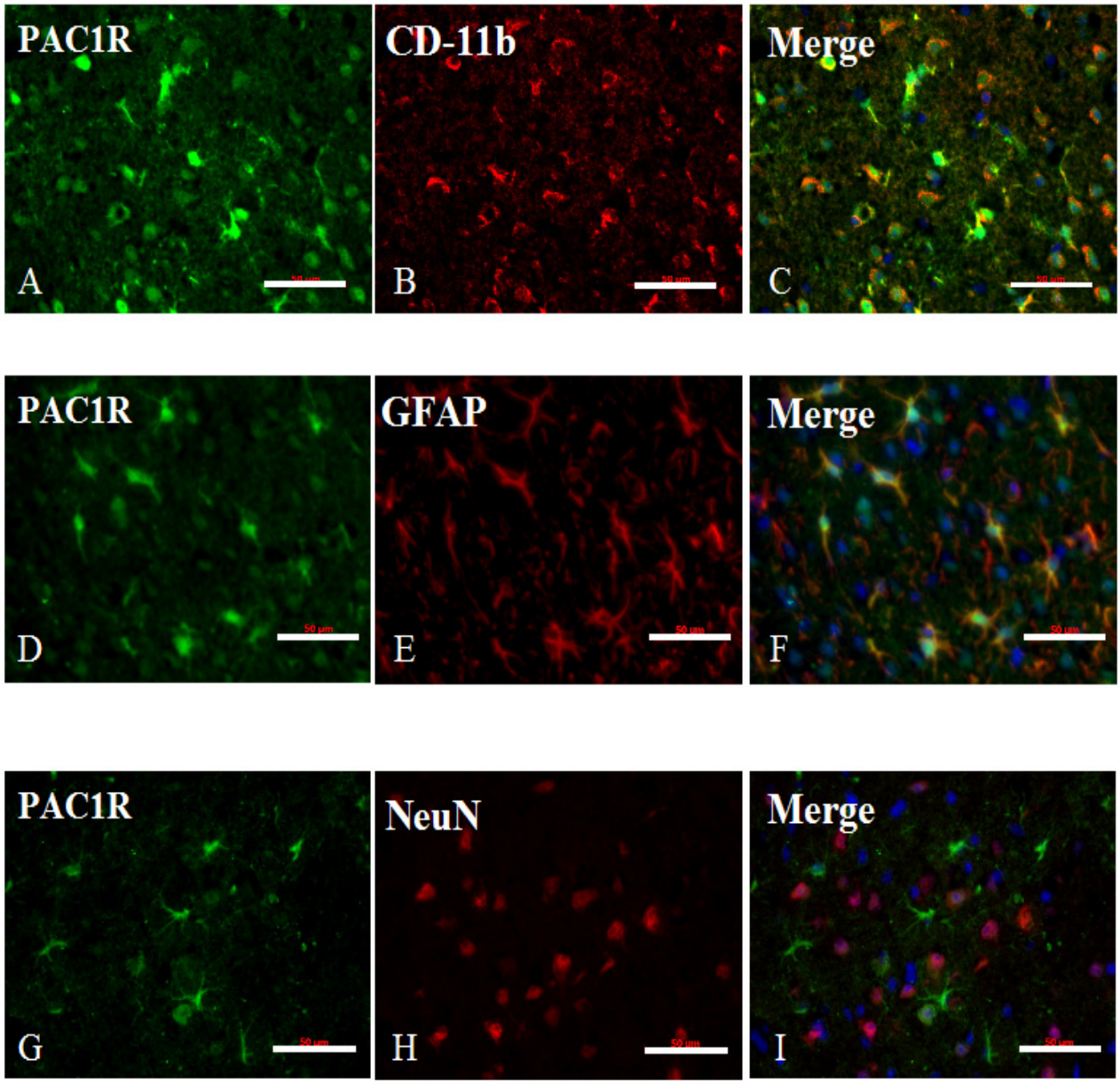

Fig. (3). Double immunostaining of PAC1R with cell markers in the perifocal area of injured brain at 7 days after traumatic brain injury. Immunostaining was carried out using antibodies for PAC1R (shown in green) together with CD11-b (**B**), GFAP (**E**), NeuN (**H**) as microglia, astrocytes, and neuron markers (shown in red), respectively. Microglia-like CD11-b and astrocyte-like GFAP immunoreactive cells were co-labeled with PAC1R immunoreactivity (**C**, **F**). In the merged images, blue is DAPI staining as counterstaining of nuclei. Scale bars=50 μm.

In the current study, PAC1R was expressed not only in the astrocyte, but also in the microglia from the early phase after the TBI. Previous studies reported the amount of PAC1R mRNA to increase after ischemia from day 1 to day 3 [18] and the PAC1R-like immunoreactivity in the reactive astrocytes was intensified until day 7 after the forebrain ischemia [19]. Suzuki *et al.* reported that PAC1R-like immunoreactivity was observed in the reactive astrocytes at day 5after a stab wound, whereas no PAC1R-like immunoreactivity was detected in the reactive astrocyte at 48 h post surgery [5]. In our past study, using ischemic model, PAC1R expression was also observed in neuron [15]. The diversity of PAC1R immunoreactivity might be originated in the differences of animal species and pathophysiolosies. Further experiments will be needed to clarify this point.

CONCLUSION

Traumatic brain injury affected the PAC1R expression in the microglia and astrocyte in the peri-contusional area. These data suggest that PACAP may play an important role involving neuroprotective effect in the complicated pathophysiology of traumatic brain injury.

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REFERENCES

[1] Langlois JA, Rutland-Brown W, Thomas KE. Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations, and Deaths. Atlanta, GA: Centers for Disease Control and Prevention, National Center for Injury Prevention and Control, 2006.

[2] Shioda S, Ozawa H, Dohi K, *et al*. PACAP protects hippocampal neurons against apoptosis: involvement of JNK/SAPK signaling pathway. Ann N Y Acad Sci 1998; 865: 111-7.

[3] Ohtaki H, Nakamachi T, Dohi K, Shioda S. Role of PACAP in ischemic neuronal death. J Mol Neurosci 2008; [Epub ahead of print].

[4] Skoglösa Y, Lewén A, Takei N, Hillered L, Lindholm D. Regulation of pituitary adenylate cyclase activating polypeptide and its receptor type 1 after traumatic brain injury: comparison with brain-derived neurotrophic factor and the induction of neuronal cell death. Neuroscience 1999; 90: 235-47.

[5] Suzuki R, Arata S, Nakajo S, Ikenaka K, Kikuyama S, Shioda S. Expression of the receptor for pituitary adenylate cyclase-activating polypeptide (PAC1-R) in reactive astrocytes. Mol Brain Res 2003; 115: 10-20.

[6] Chatterjee TK, Sharma RV, Fisher RA. Molecular cloning of a novel variant of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor that stimulates calcium influx by activation of L-type calcium channels. J Biol Chem 1996; 271: 32226-32.

[7] Harmar AJ, Arimura A, Gozes I, *et al*. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacol Rev 1998; 50: 265-70.

[8] Dautzenberg FM, Mevenkamp G, Wille S, Hauger RL. Nterminal splice variants of the type I PACAP receptor: isolation, characterization and ligand binding / selectivity determinants. J Neuroendocrinol 1999; 11: 941-9.

[9] Dixon CE, Clifton GL, Lighthall JW, Yaghmai AA, Hayes RL. A controlled cortical impact model of traumatic brain injury in the rat. J Neurosci Methods 1991; 39: 253-62.

[10] Posmantur R, Hayes RL, Dixon CE, Taft WC. Neurofilament 68 and neurofilament 200 protein levels decrease after traumatic brain injury. J Neurotrauma 1994; 11: 533-45.

[11] Sullivan PG, Bruce-Keller AJ, Rabchevsky AG, *et al*. Exacerbation of damage and altered NF-kappaB activation in mice lacking tumor necrosis factor receptors after traumatic brain injury. J Neurosci 1999; 19: 6248-56.

[12] Hall ED, Sullivan PG, Gibson TR, Pavel KM, Thompson BM, Scheff SW. Spatial and temporal characteristics of neurodegeneration after controlled cortical impact in mice: more than a focal brain injury. J Neurotrauma 2005; 22: 252-65.

[13] Dohi K, Mizushima H, Nakajo S, *et al*. Pituitary adenylate cyclase-activating polypeptide (PACAP) prevents hippocampal neurons from apoptosis by inhibiting JNK/SAPK and p38 signal transduction pathways. Regul Pept 2002; 109: 83-8.

[14] Vaudry D, Gonzalez BJ, Basille M, *et al*. The neuroprotective effect of pituitary adenylate cyclase-activating polypeptide on cerebellar granule cells is mediated through inhibition of the CED3-related cysteine protease caspase-3/CPP32. Proc Natl Acad Sci USA 2000; 97: 13390-5.

[15] Ohtaki H, Nakamachi T, Dohi K, *et al*. Pituitary adenylate cyclase-activating polypeptide (PACAP) decreases ischemic neuronal cell death in association with interleukin-6. Proc Natl Acad Sci USA 2006; 103: 7488-93.

[16] Matsuno R, Ohtaki H, Nakamachi T, *et al*. Distribution and localization of pituitary adenylate cyclase activating polypeptide-specific receptor (PAC1R) in the rostral migratory stream of the infant mouse brain. Regul Pept 2008; 145: 80-7.

[17] Watanabe J, Nakamachi T, Matsuno R, *et al*. Localization, characterization and function of pituitary adenylate cyclase-activating polypeptide during brain development. Peptides 2007; 28: 1713-9.

[18] Gillardon F, Hata R, Hossmann KA. Delayed up-regulation of *Zac1* and PACAP type I receptor after transient focal cerebral ischemia in mice. Mol Brain Res 1998; 61: 207-10.

[19] Uchida D, Arimura A, Somogyvari-Vigh A, Shioda S, Banks WA. Prevention of ischemia-induced death of hippocampal neurons by pituitary adenylate cyclase activating polypeptide. Brain Res 1996; 736: 280-6.

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