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A simple immunohistochemical method for detecting microglia in rat brain

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Abstract

We attempted to stain ramified and activated microglia in vivo by a direct fluorescent antibody technique (DFAT) using SPICA Dye™ 568-conjugated anti-Iba1 antibody (SPICA-Iba1 antibody). This simple method specifically stained ramified microglia in rat cerebral cortex, and the quality was quite comparable to that obtained by an indirect fluorescent antibody technique (IFAT) and an avidin biotin-peroxidase complex method (ABCM). The DFAT also clearly stained activated microglia in axotomized rat facial nucleus, and the quality was equivalent to that obtained by the ABCM. A time-course experiment revealed that 3-hour incubation is enough to observe microglial morphology. We also observed that the DFAT can detect microglia in primary cell cultures derived from newborn rat brain. Our results demonstrated that this simple DFAT method using SPICA-Iba1 antibody is significantly efficient for observing microglia in vivo as well as in vitro.

Keywords: Microglia; Immunohistochemistry; SPICA Dye™ 568, Ramified microglia; Activated microglia

Introduction

Microglia have been a focus of researchers' attention from the physiological/pathological point of view, and the morphology, the mobility, surface antigens, biological activity, cellular characteristics, and intercellular interaction, in the mammalian brain have been investigated by multifaceted studies in the field of neuroscience [1][2]. One of the most fundamental features of microglia in the brain in vivo is that the cell shape is easily changed, as is the cell number. Historically, microglia have been described to be present at the early developmental stage mainly as amoeboid microglia and to change into process-bearing ramified microglia with the brain's development [3][4]. Microglia in the normal adult brain exhibit long process-bearing morphology; i.e., the so-called 'resting' microglia (ramified microglia), but they transform to short- or non-process-bearing activated microglia in pathological conditions [5]. Researchers often encounter situations in which information related to the cell morphology, cell density, or localization of microglia in vivo is desired.

If such information could be obtained in a quick experiment, the research can proceed sooner; however, a relatively long time has been necessary to obtain information about microglia in vivo with the use of general immunohistochemical methods as the avidin biotin complex method (ABCM) [6] and the indirect fluorescent antibody technique (IFAT) [7]. These methods are time-consuming. Here, we introduce a simple direct fluorescent antibody technique (DFAT) using SPICA Dye™ 568-conjugated anti-Iba1 antibody (SPICA-Iba1 antibody) with which ramified microglia or activated microglia in vivo can be easily stained in a short time, providing valuable information about microglial morphology, cell density, and localization.

Materials and methods

Reagents

Paraformaldehyde (PFA) was obtained from Kanto Chemical Corp (Tokyo), and 3,3' diaminobenzidine tetrahydrochloride (DAB) was purchased from Dojindo Laboratories (Mashiki, Japan). VectaMount and PermaFluor were purchased from Vector Laboratories (Burlingame, CA, USA) and Thermo Fisher Scientific (Waltham, MA), respectively.

Antibodies

For the detection of microglia, we used anti-ionized $Ca2^{+}$ binding adapter molecule 1 (Iba1) antibody [8]. Rabbit polyclonal antibody against Iba1 and SPICA Dye™ 568-conjugated anti- Iba1 antibody were purchased from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). The Vectastain ABC kit (including biotinylated anti-rabbit IgG) was obtained from Vector Laboratories. Anti-CD11b antibody (OX42) was purchased from Cosmo Bio Co (Tokyo). Alexa Fluor 488-conjugated anti-mouse IgG was supplied by Jackson Immunoresearch Laboratories (West Grove, PA).

Animals

Eight-week-old male Wistar rats were used. They were kept on a 12-h daylight cycle with food and water ad libitum. The animal experiments were carried out in accordance with the guidelines laid down by the U.S. NIH regarding the care and use of animals and were approved by the ethics committee of Soka University (approval code: 2012).

Preparation of adult rat brain

Rats were deeply anesthetized with chloral hydrate and perfused transcardially with 100 mL of phosphate-buffered saline (PBS), followed by 100 mL 4% paraformaldehyde (PFA)/PBS. The whole brains were removed and soaked in 4% PFA/PBS. After post-fixation, the brains were further immersed in 10%, 20% and 30% sucrose/PBS step by step, and stored at -80° until use.

Preparation of adult rat brain after axotomy of a facial nerve

The right facial nerve of adult rats was transected at the stylomastoid foramen under diethylether anesthesia [9]. The left facial nerve was left without operation. At 5 days post- insult, the rats were continuously perfused with 100 mL PBS and 100 mL 4% PFA/PBS in a transcardial manner, as described above. The brains were post-fixed with 4% PFA/PBS, dipped in 10%, 20% and 30% sucrose/PBS, and stored at -80°C until use.

Preparation of cryosections

The brain tissue was cut into 20-μm-thick coronal sections with a cryostat (Leica CM1860; Leica Biosystems, Nussloch, Germany), and the sections were frozen at -80°C until use.

Immunohistochemistry

The DFAT protocol using SPICA-Iba1 antibody

The DFAT was first used in the 1970s [10][11][12]. In this study, we used the DFAT along with SPICA Dye™ 568-conjugated anti-Iba1 antibody for the detection of microglia in vivo. Cryosections were rinsed with 0.3% Triton X-100/10 mM PBS (0.3% TXPBS) two times and then blocked with 1% bovine serum albumin (BSA)/0.3% TXPBS for 1 h. After that, SPICA-Iba1 antibody (1:200) diluted with 1% BSA/0.3% TXPBS was applied to the cryosections, which were then incubated for 3, 6, 9, or 16 h at room temperature. The sections were rinsed with 0.3% TXPBS three times and mounted with PermaFluor.

The IFAT protocol

We have already examined microglia in vivo by the IFAT using rabbit anti-Iba1 antibody and a fluorescent secondary antibody [13]. In the present study, microglia in rat cerebral cortex were stained according to the routine method. Cryosections were rinsed with 0.3% TXPBS two times and blocked with 1% BSA/0.3% TXPBS for 1 h at room temperature. The sections were then incubated with rabbit anti-Iba1 antibody (1:1000) diluted with 1% BSA/0.3% TXPBS at 4°C for 16 h. After being rinsed with 0.3% TXPBS, the sections were incubated with Alexa Fluor 488-anti-rabbit IgG (1:1000) at room temperature for 3 h. The sections were then rinsed with 0.3% TXPBS and mounted with PermaFluor.

The ABCM protocol

Cryosections were rinsed with 0.3% TXPBS twice and incubated with 3% H2O2/80% methanol at -20°C for 20 min to quench endogenous peroxidase. The sections were then rinsed with 0.3%TXPBS twice and blocked with 1% BSA/0.3% TXPBS for 1 h at room temperature. Anti- Iba1 antibody (1:1000) diluted with 1% BSA/0.3% TXPBS was applied to the blocked sections for 16 h at 4°C. After being rinsed with PBS two times, the sections were incubated with biotinylated anti-rabbit IgG (1:200) for 1 h. The sections were rinsed with PBS two times and incubated with avidin/biotin complex solution for 1 h at room temperature. After the sections were rinsed with PBS three times, the antigen/antibody/avidin biotin complex on the sections was visualized by adding 0.006% H2O2/PBS and 0.5 mM DAB/PBS. The sections were dehydrated and mounted with VectaMount.

Immunocytochemical detection of microglia in primary brain cell cultures

Primary cell cultures were prepared from newborn rat brain as described [14]. Brain cells (5× 105) were seeded on each well of a 24-well plate, and at 10 days after the

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seeding the coverslips on which brain cells had adhered were fixed with 4% PFA/PBS and treated with 0.3% TXPBS. The cells on the coverslip were blocked with 1% BSA/0.3% TXPBS for 1 h at room temperature. SPICA-Iba1 antibody (1:200) diluted with 1% BSA/0.3% TXPBS was added on the coverslips and incubated for 3 h at room temperature. The coverslips were rinsed with 0.3% TXPBS three times and mounted with PermaFluor.

In the case of dual staining, brain cells on the coverslips were fixed and blocked as described above and incubated with anti-CD11b antibody (1:100) for 16 h, followed by Alexa Fluor 488- anti mouse IgG (1:1000) for 3 h. After being rinsed with 0.3% TXPBS, the coverslips were incubated with SPICA-Iba1 antibody (1:200) for 3 h, rinsed with 0.3% TXPBS and mounted.

Results

Detection of ramified microglia in adult rat cerebral cortex

We first attempted to stain ramified microglia in the brain by using the direct fluorescent antibody technique (DFAT). Cryosections of adult rat cerebral cortex were incubated for 3 h with SPICA Dye™ 568-conjugated anti-Iba1 antibody (SPICA-Iba1 antibody) as described in Materials and Methods. As shown in Figure 1A, the SPICA-Iba1 antibody reliably recognized many ramified microglia.

The microglial images in the DFAT were compared with those obtained by the indirect fluorescent antibody technique (IFAT) and avidin biotin-peroxidase complex method (ABCM). With the IFAT, we could see clearly microglial cell bodies as well as the processes (Fig. 1B). The ABCM also revealed a clear microglial appearance as ramified microglia (Fig. 1C). We thus observed that the microglial images obtained by the DFAT were quite comparable to those obtained by the IFAT and the ABCM.

Focusing on the appearance of the microglia, we confirmed the staining of ramified microglia by SPICA-Iba1 antibody. We could easily see many ramified microglia in a wide view (Fig. 2A). In the enlarged pictures, we observed that the microglia extended long processes or branched processes around the cell body, indicating the typical morphology of ramified microglia (Fig. 2B).

Detection of activated microglia in axotomized facial nucleus

Activated microglia can be observed in an axotomized rat facial nucleus [15]. These activated microglia were recognized by anti-Iba1 antibody, but hardly by anti-MHC class II antibody (OX-6) and anti-CD68 antibody, suggesting that they are not antigen-presenting cells and phagocytic cells. Using this injury model, we attempted to stain the activated microglia by the DFAT using SPICA-Iba1 antibody. In the control nuclei, ramified microglia were weakly stained (Fig. 3A). On the other hand, many activated microglia were seen in the axotomized facial nuclei (Fig. 3A). In the magnified images (Fig. 3A), we noted a ring-like staining indicating that activated microglia surrounded the motoneuron cell body [16].

We compared the images of activated microglia obtained by the DFAT using SPICA-Iba1 antibody to those obtained with the ABCM. In low-powered views, we could see activated microglia as brown staining in axotomized facial nuclei (Fig. 3B). In enlarged images, we observed ramified microglia alone in control nuclei (Fig. 3B), whereas we observed activated microglia with a ring-structure that was also seen in the images revealed by the DFAT (Fig. 3A). We thus concluded that the quality of the images obtained with the use of the SPICA- Iba1 antibody is sufficient to observe the morphology of activated microglia in vivo.

Time-course experiment

The reaction of SPICA Dye™ 568-conjugated anti-Iba1 antibody

We further examined how long it takes to obtain microglial images with sufficient quality by the DFAT using the SPICA-Iba1 antibody. Tissue sections are often incubated with the primary antibody for 16 h (overnight) as a matter of convenience. Herein, 16-h incubation resulted in good-quality microglial images (data not shown). We tested the utility of short-term incubation, i.e., 3, 6, and 9 h. The results of this time-course experiment revealed that the intensity of the staining tended to increase with longer incubation times (Fig. 4A–C). We observed that even the 3-h incubation resulted in an unambiguous image of activated microglia with sufficient quality. We thus recommend the use of the DFAT with the SPICA- Iba1 antibody when quick evaluations of microglia are needed.

Detection of microglia in rat brain primary cultures

We attempted to detect microglia in primary cell cultures derived from newborn rat brains by using the DFAT with SPICA-Iba1 antibody. Coverslips on which primary brain cells had adhered were incubated with SPICA-Iba1 antibody for 3 h and then fixed. As shown in Figure 5A, many microglia were detected under the fluorescence microscope.

We next examined the localization of SPICA-Iba1 antibody-recognizing cells in the primary cell cultures by phase-contrast view. Some of the cells among the many adherent cells on the coverslips were stained as microglia (Fig. 5B, phase-contrast and fluorescent views). The arrows in the figure indicated the microglia stained with SPICA-Iba1 antibody. With this method, it was possible to observe microglia in an in vitro system.

The SPICA-Iba1 antibody-binding cells were confirmed to be microglia by dual staining with anti-CD11b

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antibody (OX42). As seen in Figure 5C,D, the SPICA-Iba1 antibody-binding cells were all anti-CD11b antibody-positive cells, demonstrating that the SPICA-Iba1 antibody- positive cells are microglia (Fig. 4C,D). These microglia were also stained by anti-CD68 antibody (ED1), suggesting that they are phagocytic. We thus contend that the DFAT using SPICA-Iba1 antibody is applicable to immunocytochemistry for microglia.

Discussion

Microglia have been regarded as 'a sensor cell in the central nervous system' because they change their morphology and/or cell density in response to even a small abnormality of the brain [5]. Thus, if a transformation of microglia or a slight change of cell shape/processes is observed in microglia, it indicates that an adverse event (e.g., injury, disease, abnormal transmission) has occurred in the brain. Morphological changes and/or changes in the cell density of microglia have been detected in many pathological states [17][18][19] and insult conditions [20][21] of the brain. There are circumstances in when the information about microglia in a pathological brain or experimentally treated brain is needed as quickly as possible, and our present findings indicate that the time needed to obtain such information can be reduced.

Our present findings demonstrated that a simple DFAT using SPICA-Iba1 antibody could clearly stain ramified microglia in normal cerebral cortex (Fig. 1A) and activated microglia in axotomized facial nuclei (Fig. 3A). Ramified microglia were clearly revealed by 3-h incubation with the SPICA-Iba1 antibody (Fig. 1A, Fig. 2), and the quality of the images is comparable to those obtained with the IFAT (Fig. 1B) and the ABCM (Fig. 1C). Activated microglia in axotomized facial nuclei were also observed by 3-h incubation with SPICA-Iba1 antibody (Fig. 3A). The imaging profile was almost the same as that provided by the ABCM (Fig. 3B).

We further investigated the incubation time for staining activated microglia with SPICA-Iba1 antibody, and showed that even the shortest (3-h) incubation-stained activated microglia clearly (Fig. 4A). Thus, the short-time DFAT using SPICA-Iba1 antibody was very useful for a quick evaluation of the morphology and localization of microglia.

In addition to immunohistochemical detection, we immunocytochemically detected microglia in brain primary cell cultures (Fig. 5A). The short-time incubation with SPICA-Iba1 antibody led to a specific staining of microglia in cultures in which presumably astrocytes, oligodendrocytes, and neurons were present (Fig. 5 C, D). This method allowed us to obtain precise information about the microglial shape/number in vitro.

To further delineate the efficacy of the DFAT using SPICA-Iba1 antibody, we provide the outlines of the general protocols of the DFAT, IFAT, and ABCM used in vivo and the DFAT used in vitro in Table 1. Of course, several methods are available to stain microglia when enough time is available. However, the DFAT defined herein with 3-h incubation is quite advantageous for staining microglia both in vivo and in vitro when researchers are in a hurry.

Conclusion

Taken together, our findings demonstrated that a simple DFAT using SPICA Dye™ 568 conjugated anti-Iba1 antibody is useful for staining ramified and activated microglia within a short time in immunohistochemical studies as well as for detecting microglia in immunocytochemical studies.

Conflicts of Interest

The authors have no conflict of interest to declare.

Acknowledgements

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Table 1. Immunohistochemical methods and the lengths of the protocols

Methods	Time
Immunohistochemistry	
1. Direct fluorescent antibody technique (DFAT)	
SPICA Dye™ 568-conjugated anti-Iba1 antibody	3–16 h
2. Indirect fluorescent antibody technique (IFAT)	
Anti-Iba1 antibody (primary antibody) Alexa Fluor 488-conjugated anti-rabbit IgG (secondary antibody)	16 h 3 h
3. Avidin biotin-peroxidase complex method (ABCM)	
Anti-Iba1 antibody (primaryantibody) Biotinylated anti-rabbit IgG (secondaryantibody) Incubation with avidin-biotincomplex Color development	16 h 1 h 1 h 15min
Immunocytochemistry	
1. Direct fluorescent antibody technique (DFAT)	
SPICA Dye™ 568-conjugated anti-Iba1 antibody	3-16 h

The immunohistochemical protocol of the DFAT using SPICA-Iba1antibody, the IFAT, and the ABCM, and the immunocytochemical DFAT using SPICA-Iba1antibody are listed along with the time necessary for each method.

Figures

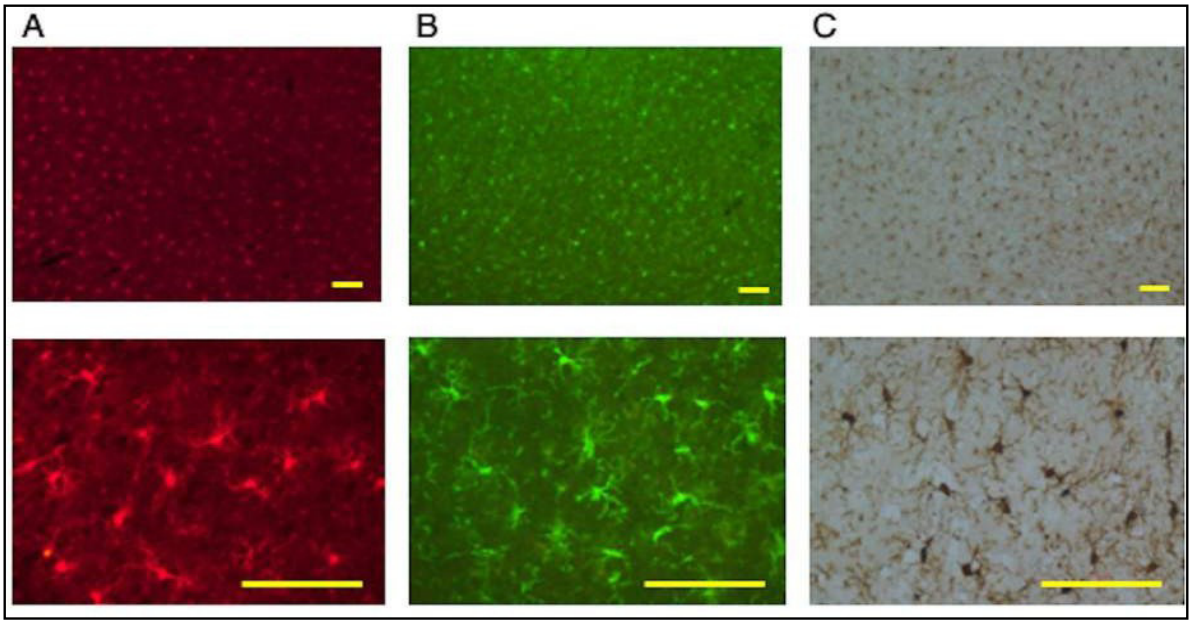

Fig. 1: Detection of ramified microglia; comparison of the three methods. A: SPICA-Iba1 antibody was incubated for 3 h on cryosections of rat cerebral cortex. With the IFAT (B) and the ABCM (C), anti-Iba1 antibody was incubated for 16 h. Scale bar: 100 mm.

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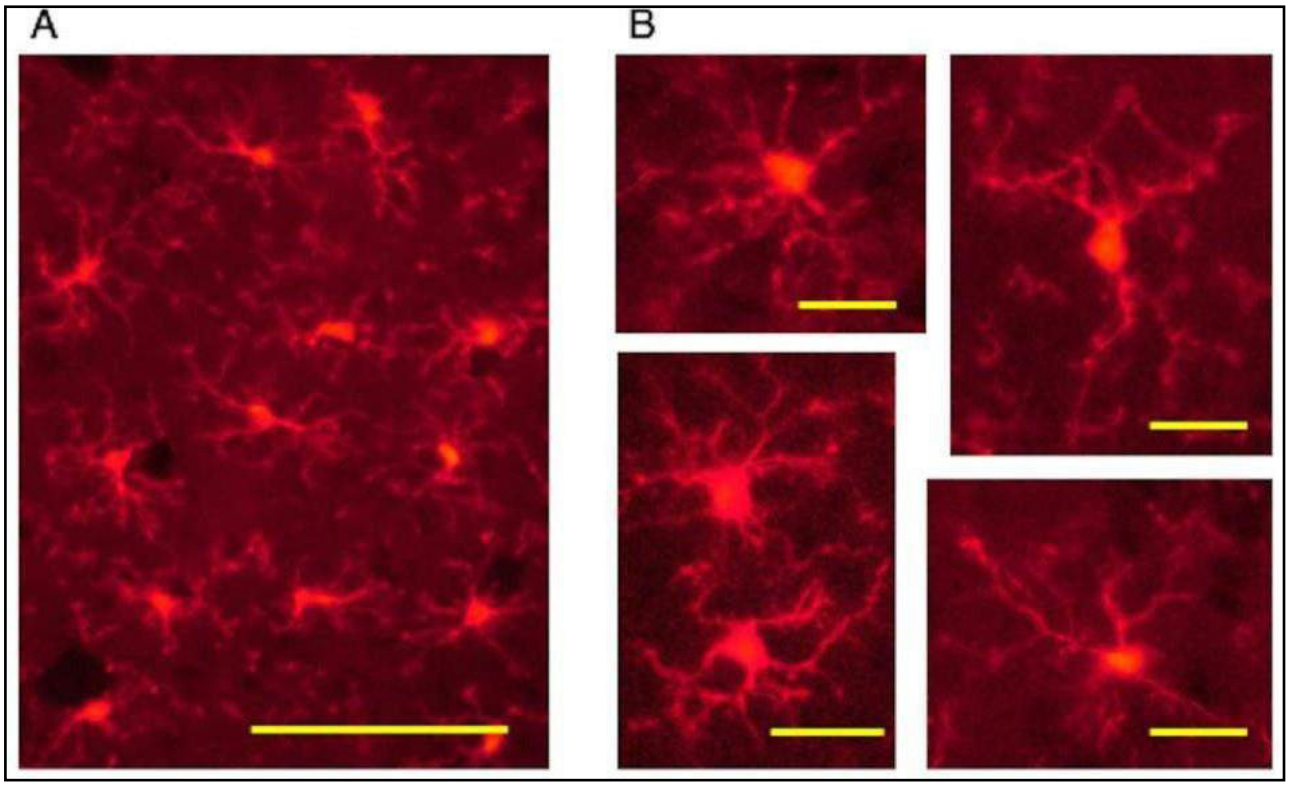

Fig.2: Appearanceoframifiedmicroglia.Sectionsofadultratcerebralcortexwereincubatedwiththe DFAT using SPICA-Iba1 antibody for 3 h, and the image in wide view is shown in panel A. Scale bar: 100 mm. Typical appearance of ramified microglia is presented in panel B. Scale bar: 20mm.

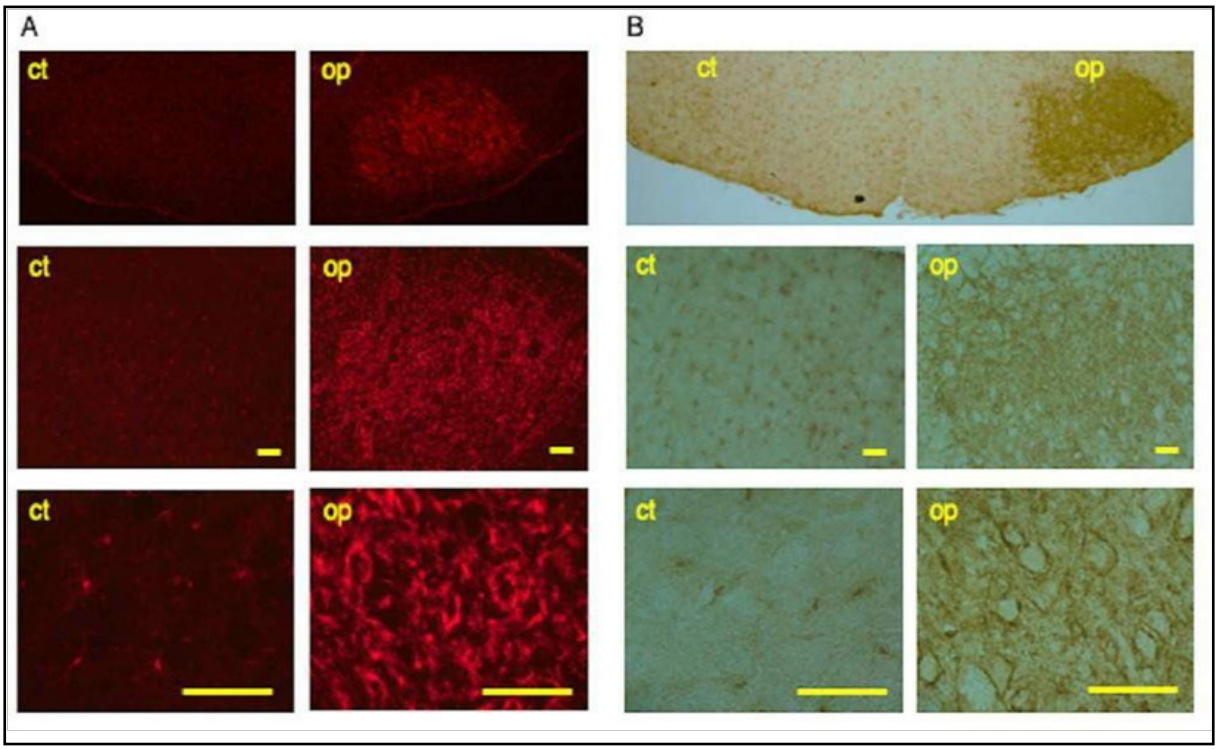

Fig. 3: Detection of activated microglia in axotomized facial nucleus. Cryosections of axotomized rat facial nuclei were stained for microglia by the DFAT using SPICA-Iba1 antibody (A) and the ABCM (B). In panel A, SPICA-Iba1 antibody was incubated for 3 h. A control nucleus (ct) and an axotomized nucleus (op) are shown at the *left side* and *right side*, respectively. In panel B, a control nucleus (ct) and an axotomized nucleus (op) are shown at the *left side* and *right side*, respectively. Scale bar: 100 mm.

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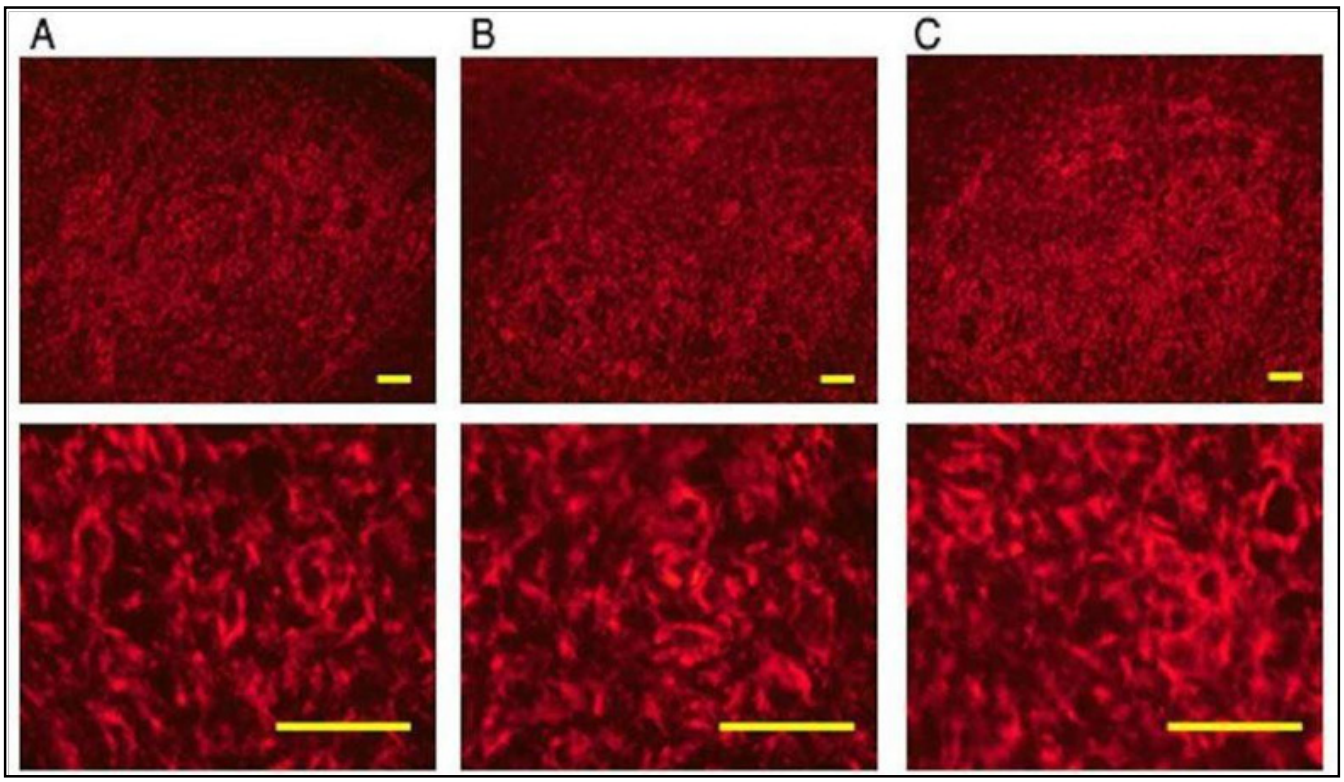

Fig. 4: Time-course experiment in SPICA-Iba1 antibody binding. Cryosections of axotomized facial nucleiwereincubatedwithSPICA-Iba1antibody for3h(A),6 h(B)or9h(C)andmounted.Scalebar: 100mm.

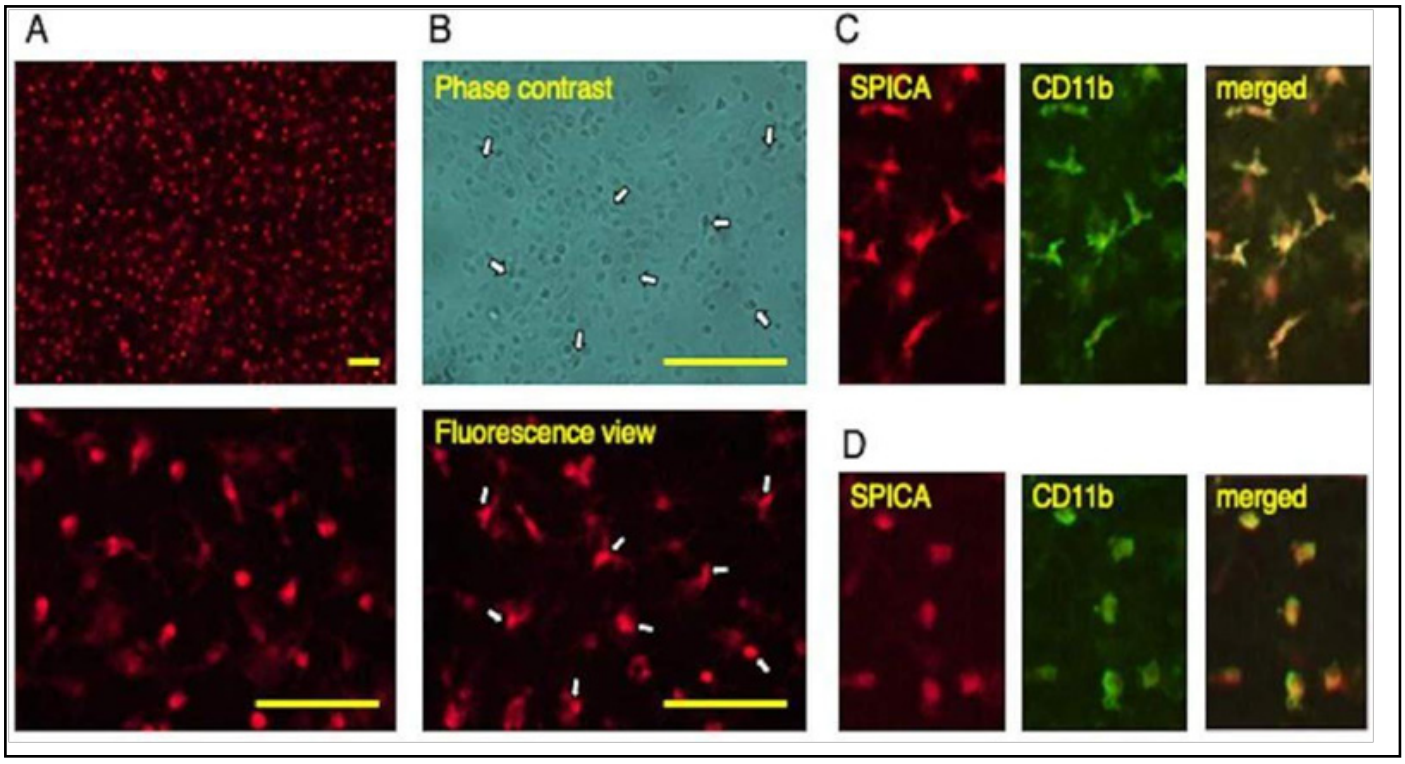

Fig.5: Detectionofmicrogliainratbrainprimarycultures.Primarycellculturesderivedfromnewborn rat brain were prepared as described in the Materials and Methods. Primary cells on the coverslips were incubated with SPICA-Iba1 antibody for 3 h (A). The primary cells were observed in a phase- contrastview(B,upperpanel)andfluorescenceview(B,lowerpanel).Scalebar:100mm.Theprimary cells were dually stained by SPICA-Iba1 antibody (red; left) and anti-CD11b antibody (green; center). The merged image is shown at the right (C,D).

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