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Astrocytes are involved in long-term facilitation of neuronal excitation in the anterior cingulate cortex of mice with inflammatory pain

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ABSTRACT

Neuronal plasticity in the pain-processing pathway is thought to be a mechanism underlying pain hypersensitivity and negative emotions occurring during a pain state. Recent evidence suggests that the activation of astrocytes in the anterior cingulate cortex (ACC) contributes to the development of negative emotions during pain hypersensitivity after peripheral inflammation. However, it is unknown whether these activated astrocytes contribute to neuronal plasticity in the ACC. In this study, by using optical imaging with voltage- and Ca^{2+}-sensitive dyes, we examined the long-term facilitation of neuronal excitation induced by high-frequency conditioning stimulation (HFS) in ACC slices of control mice and mice with peripheral inflammation induced by the injection of complete Freund adjuvant (CFA) to the hind paw. Immunoreactivity of glial fibrillary acidic protein in laminae II–III of the ACC in the CFA-injected mice was higher than in the control mice. Neuronal excitation in ACC slices from the CFA-injected mice was gradually increased by HFS, and the magnitude of this long-term facilitation was greater than in the control mice. The long-term facilitation in the CFA-injected mice was inhibited by the astroglial toxin, the *N*-methyl-D-aspartate (NMDA) receptor antagonist and NMDA receptor glycine binding site antagonist. The increase of intracellular Ca^{2+} concentration in astrocytes during HFS was higher in the CFA-injected mice than in the control mice and was inhibited by L-α-aminoadipate (L-α-AA). These results suggest that the activation of astrocytes in the ACC plays a crucial role in the development of negative emotions and LTP during pain hypersensitivity after peripheral inflammation.

1. Introduction

Long-term potentiation (LTP), the activity-dependent increase in synaptic transmission, has been proposed to contribute to information storage in the brain [2,12,18]. LTP in the pain-related regions of the central nervous system is also thought to play important roles in chronic pain [5,14–16,20,45].

Pain is a subjective multidimensional conscious experience that includes a sensory component, that is, the perception of the severity and location of the pain and a negative and unpleasant affective-motivational component [27,31,38]. The anterior cingulate cortex (ACC) has been identified as a critical area involved in the processing of the affective components of nociception in morphological, electrophysiological, neuroimaging, and behavioral studies [3,6,17,22,30–32,45]. Recent studies have revealed that LTP in the ACC is a mechanism for persistent affective changes in people with chronic pain after nerve injury or peripheral inflammation [1,36,37,42,43,45].

The contribution of astrocytes to chronic pain has been reported in behavioral, histochemical, and electrophysiological studies. In the spinal cord, activated astrocytes, which overexpress glial fibrillary acidic protein (GFAP) and exhibit hypertrophy, have been observed following peripheral inflammation and nerve injury [7,34]. Inhibition of astroglial function in the spinal cord attenuates nerve injury- and peripheral inflammation-induced mechanical allodynia [26,44]. Although the contribution of astrocytes to chronic pain has been studied primarily in the spinal dorsal horn, astroglial activation has also been reported in supraspinal areas such as the rostral ventromedial medulla after chronic constriction injury of the rat infraorbital nerve [9,41] and in the forebrain after the injection of complete Freund adjuvant (CFA) [33]. In the ACC, pain-related aversion increased the expression of GFAP mRNA and protein, and an intra-ACC injection of L-α-aminoadipate (L-α-AA), an astroglial toxin, inhibited escape/avoidance behavior but did not affect the paw withdrawal threshold at 3 d following CFA injection [4,23]. However, it remains unknown whether peripheral

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inflammation-activated astrocytes contribute to the induction of neuronal plasticity in the ACC.

In this study, we attempted to reveal whether astrocyte activation induced by peripheral inflammation contributes to neuronal plasticity in the ACC. That is, by using optical imaging with voltage- and Ca^{2+}-sensitive dyes, we examined the contribution of astrocytes to the long-term facilitation of neuronal excitation induced by high-frequency conditioning stimulation (HFS) in ACC slices from control mice and mice with peripheral inflammation induced by CFA injection to the hind paw.

2. Methods

2.1. Animals

All animal studies were undertaken using protocols approved by the University of Fukui animal ethics committee. We used 25- to 30-day-old male and female ICR mice in all experiments. To induce inflammatory pain, 30 μL of 50% CFA (Sigma-Aldrich, St. Louis, MO, USA) were injected subcutaneously into the dorsal surface of the left hind paw. To induce neuropathic pain, spared nerve injury was performed. An incision was made into the skin on the lateral surface of the left thigh, followed by a section through the biceps femoris muscle to expose the sciatic nerves. The common peroneal and tibial nerves were then tightly ligated with 6-0 silk thread, sectioned distal to the ligation, and removed 2 mm from the distal nerve stump. All experiments were performed at 3 d after these treatments.

2.2. Forced swimming test

The mice were placed individually in a plastic cylinder (40 cm high, 25 cm in diameter) containing 15 cm of water maintained at 23–25 °C, and were left there for 5 m. A mouse was regarded as immobile when it remained floating on the water, making only small movements to keep its head above the surface. The total duration of immobility was recorded.

2.3. Elevated plus-maze test

A wooden plus-maze apparatus, which was elevated to a height of 40 cm and consisted of 2 open arms (40 × 8 cm), 2 closed arms (40 × 8 cm), and 20-cm-high walls), was arranged so that the 2 arms of each type were opposite each other. The time spent in an open arm during a 5-m period was recorded.

2.4. Immunohistochemistry

The mice were anesthetized deeply and perfused intracardially with 0.01 M phosphate-buffered saline followed by cold 4% buffered paraformaldehyde. The brain was then removed immediately, postfixed at 4 °C overnight in the same fixative, and then cryoprotected in 20% sucrose in phosphate-buffered saline (pH 7.4) for 48 h and sectioned at 40 μm. Nonspecific antibody binding was inhibited by incubating the slices in 3% normal goat serum. The slices were then incubated for 48 h at 4 °C with an antibody against GFAP (anti-GFAP, 1:400; Sigma-Aldrich), an astrocyte marker. After incubation, the tissue sections were washed and incubated for 3 h at room temperature with a secondary antibody solution (anti-rabbit IgG-conjugated Alexa Fluor 488, 1:400; Molecular Probes, Eugene, OR, USA). Immunofluorescence intensity measurements were obtained using a confocal microscope (Axiovert200 M; Carl Zeiss, Oberkochen, Germany). For image analysis, the free image analysis software ImageJ 1.40f (National Institutes of Health, Bethesda, MD, USA) was used to measure the density of GFAP-immunoreactivity. A quantitative assessment was made by determining the intensity of immunofluorescence within a fixed area of laminae II–III in the ACC (200 μm^2), and the mean intensity of this area was recorded. The intensity of the background fluorescence of each section was also determined and subtracted from the values obtained.

2.5. Surgery

Mice were anesthetized with 2,2,2-tribromoethanol anesthesia (0.2 g kg^{-1}, intraperitoneally, Wako Pure Chemical Industries, Osaka, Japan) and then positioned in a stereotaxic frame with blunt-tipped ear bars. A midline incision was made and holes were drilled on each side of the rostral ACC area (AP = 0.5, mL = 0.3 from the Bregma). A glass pipette (tip diameter 5 μm) was lowered directly into the rostral ACC (DV = 2.0), and a volume of 0.6 μL (saline or L-α-aminoadipate) (Sigma) of L-α-AA was injected. The syringe remained in place for 10 m after each injection to prevent the spread of the agent to the surface of the brain. This procedure was then repeated in the opposite hemisphere.

2.6. Membrane potential imaging with a voltage-sensitive dye

The mice were anesthetized using diethyl ether and then decapitated rapidly. The brains were removed and transferred to an ice-cold aerated solution (95% O_2 and 5% CO_2) containing (in mM): 120 choline chloride, 2.4 KCl, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.2 ascorbic acid, and 15 glucose. Coronal slices (300 μm thick) containing the rostral ACC (rACC) were prepared using a vibrating microtome. Each slice was stained in a bath filled with the voltage-sensitive dye RH-482 (0.003 mg/mL; 20 m) and set in a chamber on an inverted microscope equipped with a 120-W halogen lamp. Each slice was perfused with Ringer solution containing (in mM): 127 NaCl, 2 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose (oxygenated with 95% O_2 and 5% CO_2) at room temperature (25 ± 2 °C). The light absorption change, at a wavelength of 700 ± 32 nm, in a 0.83 mm^2 area of the rACC was recorded using an imaging system (Deltalon 1700; Fujifilm, Tokyo, Japan) with 128 × 128 pixel photo sensors at a frame rate of 0.6 ms. Stimulation was delivered by a bipolar tungsten stimulating electrode placed in layer V of the rACC. Sixteen single pulses were given at a constant interval of 15 s. Starting at 10 ms before each stimulus, the image sensor took 64 consecutive frames of light-absorption images at a sampling interval of 0.6 ms. A reference frame, which was taken immediately before each series of 64 frames, was subtracted from the subsequent 64 frames. Sixteen series of such difference images were averaged and stored in the system memory. We determined the initial frame by averaging the first 15 frames of the difference image and then subtracting this average from each of the 64 frames of the image data on a pixel-by-pixel basis in order to eliminate the effects of noise contained in the reference frame. The ratio image was then calculated by dividing the image data by the reference frame.

2.7. Ca^{2+} imaging

To identify astrocytes, we used sulforhodamine 101 (SR101), which is known as a fluorescent marker of astrocytes that does not damage or interfere with normal brain functions. SR101 was dissolved in 0.9% NaCl at the concentration of 10 mg/mL. A syringe was inserted into the tail vein and SR101 (20 mg/kg) (Molecular Probes) was injected intravenously. One h after injection, slice preparation was performed as described above. Each slice was