migration, whereas astrocyte-derived cues also play a critical role in the pathological process by forming boundaries and retarding axonal outgrowth (Powell *et al.* 2001).

It has been documented recently that astrocytes in the neostriatum show hypertrophy and proliferation upon treatment with methamphetamine (METH) at neurotoxic doses in mice (Pu and Vorhees 1995). Astrocytic morphological changes can also be induced by the administration of cocaine in mice (Fattore *et al.* 2002). These findings indicate that astrocytes may play an important role in the development of dependence on psychostimulants. However, relatively little is known about the mechanism that underlies psychostimulant-induced astrocytic responses, even if astrocytes are considered to play a critical role in long-term synaptic plasticity in the CNS (Ullian *et al.* 2001; Song *et al.* 2002). In the present study, we investigated the mechanism of METH-induced astrocytic activation in cultured cortical astrocytes and cortical neuron/glia co-cultures. We also documented whether morphine (MRP) could directly regulate astrocytic responses to cultures.

METH and cocaine are strongly addictive psychostimulants that dramatically affect the CNS, and they are highly abused drugs worldwide. Abuse of psychostimulants leads to the development of psychotic symptoms that resemble those of paranoid schizophrenia (Synder 1974). In rodents, it has been shown consistently that repeated exposure to psychostimulants results in a progressive and enduring enhancement in the motor stimulant effect elicited by a subsequent drug challenge, which termed behavioral sensitization (Vanderschuren and Kalivas 2000). Accumulating evidence suggests that the behavioral sensitization induced by psychostimulants may be accompanied by long-lasting neural plasticity (Robinson and Kolb 1999) that may involve structural modifications in the dopaminergic (Steketee 2003) and/or glutamatergic system (Sripada *et al.* 2001). Here we report for the first time that chronic treatment with METH causes a long-lasting PKC-dependent behavioral sensitization related to the enhanced astrocytic responses, whereas MRP produces a reversible behavioral sensitization.

Materials and methods

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Tissue processing

Purified cortical astrocytes were grown as follows: cerebral cortices were obtained from newborn ICR mice (Tokyo Laboratory Animals, Tokyo, Japan), minced, and treated with trypsin (0.025%, Invitrogen, Carlsbad, CA, USA) dissolved in phosphate-buffered saline (PBS) solution containing 0.02% L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) monohydrate, 0.5% glucose (Wako Pure Chemicals, Osaka, Japan) and 0.02% bovine serum albumin (Wako Pure Chemicals). After enzyme treatment at 37°C for 15 min, cells were dispersed by gentle agitation through a pipette and plated on a flask. One week after seeding in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% precolostrum newborn calf serum (FBS, Invitrogen), 5% heat-inactivated (56°C, 30 min) horse serum (HS, Invitrogen), 10 U/mL penicillin and 10 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, the flask was shaken for 12 h at 37°C to remove nonastrocytic cells. The cells were seeded at a density of 1×10^5 cells/cm^2. The cells were maintained for 3–10 days in DMEM supplemented with 5% FBS, 5% HS, 10 U/mL penicillin and 10 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Cortical neuron/glia co-cultures were grown as follows: cerebral cortex was obtained from newborn ICR mice (Tokyo Laboratory Animals Science), minced, and treated with papain (9 U/mL, Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% bovine serum albumin. After enzyme treatment at 37°C for 15 min, cells were seeded at a density of 2×10^6 cells/cm^2. The cells were maintained for 7 days in DMEM supplemented with 10% FBS, 10 U/mL penicillin and 10 μg/mL streptomycin. On day 8, the cells were treated with drugs.

Drug treatment and immunohistochemistry

At day 3–7 *in vitro*, the cells were treated with either normal medium, methamphetamine hydrochloride (METH, 0.01–1000 μM, Dainippon Pharmaceutical, Osaka, Japan), a selective protein kinase C (PKC) inhibitor, chelerythrine chloride (CHE, 10 nM, Sigma-Aldrich) + METH (1–100 μM), morphine hydrochloride (MRP, 1–1000 μM, Sankyo, Tokyo, Japan) or MRP (1–100 μM) + CHE (10 nM). The treatments lasted for 1–3 days. The cells were then identified by immunofluorescence using rabbit anti-glial fibrillary acidic protein antibody (GFAP, 1 : 1000; Chemicon International, Inc., Temecula, CA, USA), mouse anti-GFAP antibody (1 : 1000, Chemicon, International, Inc.), rabbit anti-phosphorylated-protein kinase C antibody (p-PKC, 1 : 400; Cell Signaling Technology Inc., Beverly, MA, USA), or rabbit anti-cleaved caspase-3 antibody (1 : 100, Cell Signaling Technology Inc., Beverly, MA, USA), followed by incubation with Alexa 488-conjugated goat anti-rabbit antibody (1 : 4000) or Alexa 546-conjugated goat anti-rabbit antibody (1 : 4000) for GFAP, Alexa 488-conjugated goat anti-rabbit antibody (1 : 1000) for p-PKC, and Alexa 488-conjugated goat anti-rabbit antibody (1 : 10000) for cleaved caspase-3. Images were collected using a Radiance (2000) laser-scanning microscope (Bio-Rad, Richmond, CA, USA).

The density of GFAP-like immunoreacivity was measured with a computer-assisted system (NIH IMAGE). The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity to provide an image with immunoreactive material appearing in black pixels, and non-immunoreactive material as white pixels. The area and density of pixels within the threshold value representing immunoreactivity were calculated.

Evaluation of astrocytic stellation

In order to evaluate the astrocytic stellation, purified cortical astrocytes were cultured on 24-well plates and treated with METH

(0.01–100 μM) or METH (1–100 μM) + CHE (10 nM) for 1–3 days. The cells were fixed in 4% paraformaldehyde and stained with cresyl violet (0.1%, ICN Biomedicals, Aurora, OH, USA) to determine the percentage of stellate cells in cultures. Cells with processes longer than their perinuclear diameters were defined as stellate cells. Stained cells were mounted on glass slides and viewed under transmitted light using a microscope with a 10× objective lens (IX 70, Olympus Optical, Tokyo, Japan). For each coverslips, four randomly chosen fields were counted (about 170 cells in each field), and the percentage of stellate cells was determined. Each experimental condition was repeated from four independent culture preparations. The percentage of stellate cells was expressed as average ± SE. Student's *t*-test was used for statistical analysis.

Confocal Ca^{2+} imaging

Purified cortical astrocytes were loaded with 10 μM fluo-3 acetoxymethyl ester (Dojindo Molecular Technologies, Inc., Gaithersburg, MD, USA) for 90 min at room temperature. After a further 20–30 min of de-esterification with the acetoxymethyl ester, the coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance 2000, Bio-Rad). Fluo-3 was excited with the 488 nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths > 515 nm, and average baseline fluorescence (F_0) of each cells was calculated. To compensate for the uneven distribution of fluo-3, self-ratios were calculated (Ratio: $R_s = F/F_0$).

Dopamine (1–100 μM, Sigma-Aldrich) or glutamate (1–100 μM, Sigma-Aldrich) was perfused for 30 sec at 5 mL/min at room temperature in cultured cortical astrocytes followed by superfusion of basal salt saline (BSS, pH 7.4) containing 150 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethaneslufonic acid and 10 mM D-glucose.

Locomotor assay for METH and MRP

Male ICR mice (20–25 g) were housed at a room temperature of 23 ± 1°C with a 12-h light : 12-h dark cycle (lights on 08:00–20:00). Food and water were available *ad libitum*.

The locomotor activity of mice was measured by an ambulometer (ANB-M20, O'Hara, Tokyo, Japan) as described previously (Narita *et al.* 1993). Briefly, a mouse was placed in a tilting-type round activity cage of 20 cm in diameter and 19 cm in height. Any slight tilt of the activity cage caused by horizontal movement of the animal was detected by micro-switches. Total activity counts in each 10-min segment were automatically recorded for 30 min prior to the injections and for 180 min following METH administration.

According to previous reports (Kuribara 1996; Narita *et al.* 2002), a repeated injection paradigm was used in which animals were treated with an injection of METH (2 mg/kg, s.c.) or MPR (10 mg/kg, s.c.) every 96 h to induce sensitization to METH- or MRP-induced hyper-locomotion. Total activity was counted for 3 h after each treatment.

To investigate the implication of PKC in the development of sensitization to METH-induced hyper-locomotion, mice were pretreated with saline or a selective PKC inhibitor *S*-2,6-diamino-*N*-[(1-[1-oxotridecyl]-2-piperidinyl)methyl]hexamide dihydorochloride (NPC-15437: 1 mg/kg, s.c., Sigma-Aldrich) 30 min prior to METH (2 mg/kg, s.c.) treatment.

Immunohistochemistry using brain-slice sections

Twenty-four hours after the last METH treatment, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M PBS. Then, the brains were removed quickly after perfusion and thick coronal section of the forebrain including the caudate putamen, nucleus accumbens, and cingulate cortex region was initially dissected using brain blocker. The coronal section of the midbrain was post-fixed in 4% PFA for 2 h. After the brains were permeated with 20% sucrose for 2 days and 30% sucrose for 2 days, they were frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at – 30°C until used. Frozen coronal sections (8 μm) were cut in a cryostat, and thaw-mounted on poly-L-lysine-coated glass slides.

Each primary antibody was diluted in 0.01 M PBS containing 10% normal horse serum [1 : 10 GFAP (Nichirei Co., Tokyo, Japan)] and was incubated twice overnight at 4°C. The antibodies were then rinsed and incubated with each secondary antibody for 2 h at room temperature. For each labeling, Alexa 488-conjugated goat anti-rabbit antibody for GFAP was diluted 1 : 1200 in PBS containing 10% NHS.

Statistical analysis

The data are presented as the mean ± SEM. The statistical significance of differences between groups was assessed by an analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test or Student's *t*-test.

Results

METH-induced astrocytic activation

Treatment with METH (10 μM) for 3 days caused a robust activation of cultured cortical astrocytes, as detected by a stellate morphology and an increase in the level of GFAP-like immunoreactivity (GFAP-IR) compared to that in normal medium-treated cells (Fig. 1A). As shown in Fig. 1B, treatment with METH (1–100 μM) for 1–3 days significantly increased the number of stellate astrocytes in cultured cortical astrocytes. Although 1 day treatment with METH caused a significant increase in stellate astrocytes in cultured astrocytes, 3 day treatment with METH (10–100 μM) showed a drastic increase in stellate astrocytes. In addition, this activation of astrocytes was partially reversed by treatment with the specific protein kinase C (PKC) inhibitor chelerythrine chloride (CHE, Fig. 1), indicating the possible implication of PKC in this event. Immunohistochemical staining with an anti-phosphorylated PKC (p-PKC) antibody confirmed that treatment with METH increased the immunoreactiviy of p-PKC in astrocytes (Fig. 1D). These results suggest that astrocytic PKC is involved in METH-induced astrocytic activation (Fig. 1C). Treatment with METH (10–100 μM) for 3 days also caused a robust astrocyte activation in cortical neuron/glia co-cultures, and this activation was reversed by cotreatment with CHE (Fig. 2).

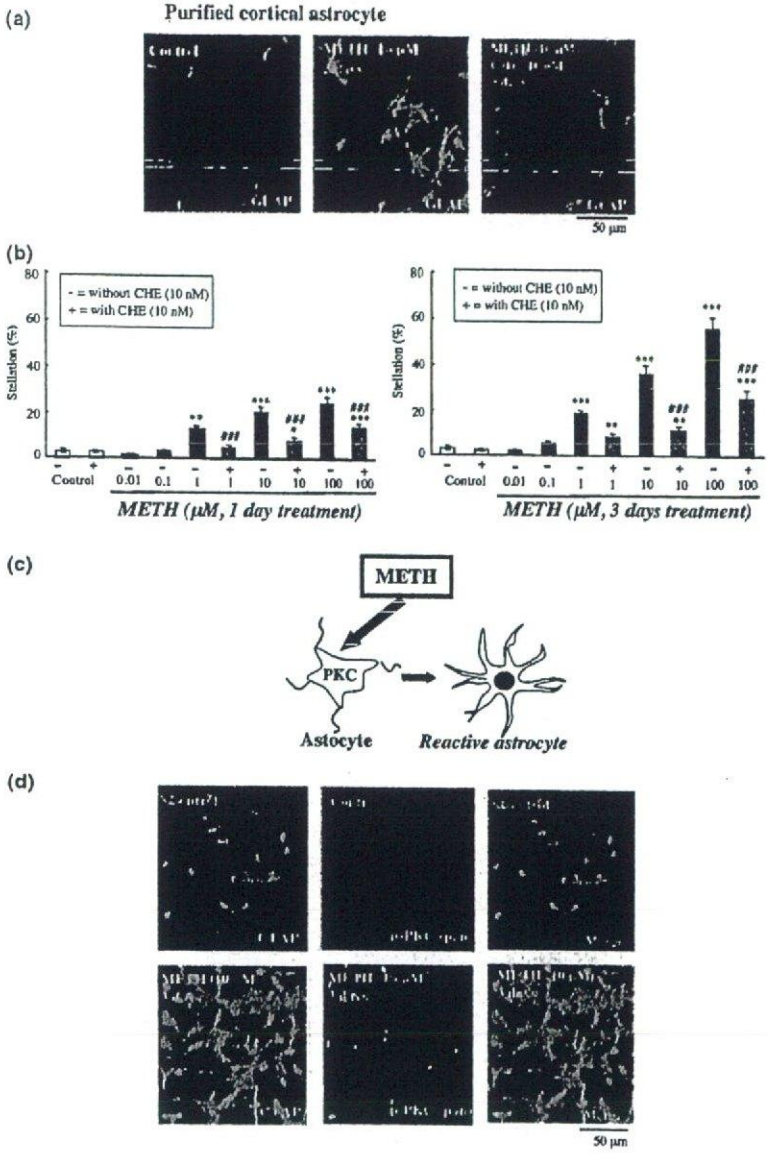


Fig. 1 Treatment with methamphetamine (METH) causes astrocytic activation in purified cortical astrocytes. (a) Purified cortical astrocytes were incubated with normal medium, METH (10 μM) or METH + chelerythrine (CHE, 10 nM) for 3 days. The cells were stained with a rabbit polyclonal antibody to GFAP. (b) Purified cortical astrocytes were incubated with normal medium, METH (0.01–100 μM) or METH (1–100 μM) + CHE (10 nM) for 1–3 days. Astrocytic activation as shown by a stellate morphology with processes longer than their perinuclear diameters was evaluated. Data represent the mean ± SEM of 139–250 cells from four separate observations. $^{**}p < 0.01$ and $^{***}p < 0.001$; control, $^{###}p < 0.001$; vs. cells without CHE. (c) Proposed scheme showing the mechanism of METH-induced atrocytic activation. (d) The green labeled for p-PKC (pan) stained with a rabbit polyclonal antibody and the red labeled for GFAP stained with a mouse polyclonal antibody are mostly overlapped as yellow in METH-treated astrocytes.

We next investigated whether treatment with METH could induce any functional changes in astrocytes. Astrocytes are known to express a variety of neurotransmitters and/or hormone receptors, including dopamine (DA) and glutamate (GLU) receptors. As shown in Fig. 3 2, either DA (1–100 μM) or GLU (1–100 μM) produced a transient increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured cortical astrocytes. The Ca^{2+} responses to DA and GLU in astrocytes were significantly enhanced by 3 days of treatment with METH (10 μM, 3 days).

Morphine-induced astrocytic activation

Opioid agonists such as morphine (MRP) modulate several physiological processes including a rewarding effect by stimulating opioid receptors. To compare its effects with those of METH, we investigated the effect of MRP in astrocytes. Unlike METH, treatment with MRP (1–1000 μM) for 1–3 days did not produce morphological changes in the activation of purified cortical astrocytes (Figs 4a and b). In contrast to MRP treatment in purified astrocytes, treatment with MRP (10–100 μM) for 3 days activated GFAP-positive astrocytes in cortical neuron/glia co-cultures (Figs 4c and d), and this activation was partially attenuated by CHE (10 nM).

Different maintenance of METH- and MRP-induced astrocytic activation

We next investigated the difference between METH and MRP in the maintenance of astrocytic activation. As shown

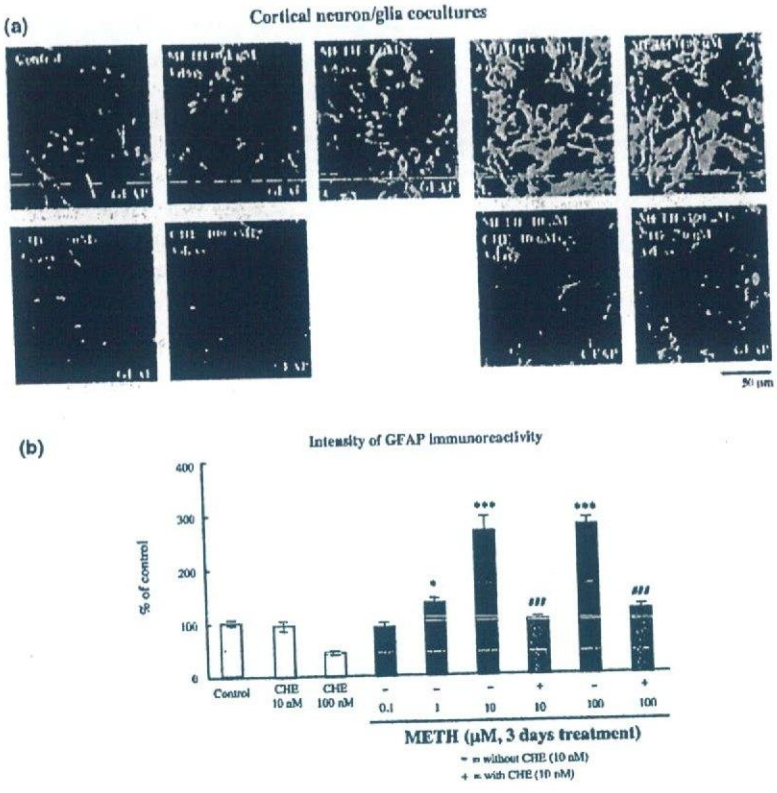


Fig. 2 Treatment with methamphetamine (METH) for 3 days causes astrocytic activation in cortical neuron/glia co-cultures. (a) Cortical neuron/glia co-cultures were with normal medium, METH (0.1–100 μM) or METH (1–100 μM) + CHE (10 nM) for 3 days. The cells were stained with a rabbit polyclonal antibody to GFAP. (b) The density of GFAP-like immunoreactivity of each image was measured using NIH IMAGE. The level of GFAP like immunoreactivity on MEHT- and METH + CHE-treated cells is expressed as a percent increase (mean ± SEM) with respect to that on control cells. $^{*}p < 0.05$, $^{***}p < 0.001$; vs. control cells. $\#\#\#p < 0.001$; vs. METH-treated cells.

in Fig. 5a, treatment with METH (10 μM) for 1–3 days caused the activation of GFAP-positive astrocytes in cortical neuron/glia co-cultures. The METH-contained medium was then switched to normal medium, and the cells were cultured for additional 2 days. It is of interest to note that the METH-induced increase in the level of GFAP-IR still remained after an additional 2 days of cultured with normal medium. Treatment with MRP (10 μM) for 1–3 days also caused the activation of GFAP-positive astrocytes in cortical neuron/glia co-cultures (Fig. 5b). The MRP-containing medium was then switched to normal medium, and the cells were cultured for additional 2 days. Unlike METH, the MRP-induced increase in the level of GFAP-IR was reversed after an additional 2 days of cultured with normal medium.

Long-lasting maintenance of behavioral sensitization to METH, but not MRP

The repeated administration of psychostimulant drugs results in a progressive and enduring elevation in the motor response elicited, which may be accompanied by a long-lasting neural plasticity. Therefore, we hypothesized that the psychostimulant-induced astrocytic activation may be related to behavioral sensitization. Based on the data and the hypothesis presented above, we next investigated whether repeated *in vivo* treatment with METH could cause a long-lasting maintenance of behavioral sensitization to METH-induced hyper-locomotion.

To clarify the development of sensitization to METH- or MRP-induced hyper-locomotion, mice were given five treatments [METH (2 mg/kg, s.c.) or MRP (10 mg/kg, s.c.)] every 96 h. As shown in Fig. 6, repeated injection of either METH or MRP produced a progressive elevation of the METH- or MRP-induced locomotor-enhancing effect, indicating the development of sensitization to METH- or MRP-induced hyper-locomotion ($p < 0.01$, first session vs. fifth session). Intriguingly, the METH-induced sensitization to hyper-locomotion was maintained even after 2 months withdrawal following intermitted METH administration (Fig. 6a). However, the MRP-induced sensitization was reversed by 2 months withdrawal following intermitted MRP administration (Fig. 6b).

METH-induces neuronal cell death

METH has been recognized as a drug of abuse that induces nerve terminal degeneration and neuronal apoptosis in the mammalian brain (Jiménez *et al.* 2004). We therefore investigated whether *in vitro* treatment with high concentration of METH or MRP could induce neuronal cell death. As shown in Fig. 7, treatment with

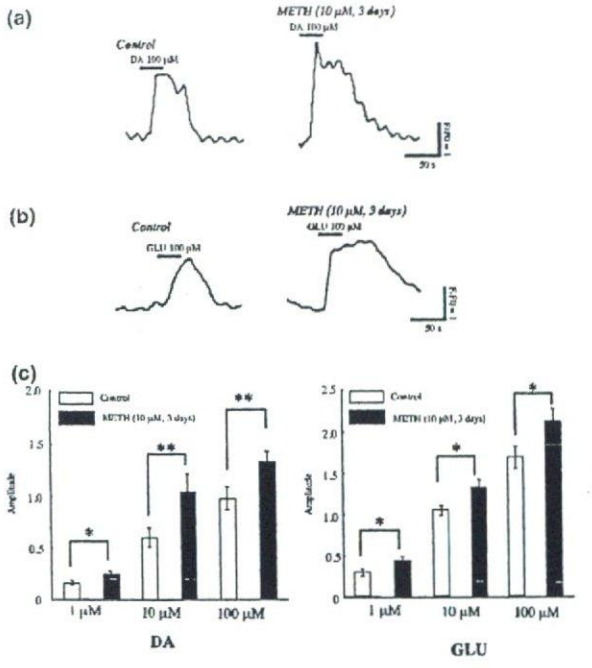


Fig. 3 The Ca^{2+} responses to dopamine and glutamate in astrocytes were significantly enhanced by 3 days of treatment with METH. (a) Traces show the dopamine (DA, 100 μM)-evoked increase in the intracellular Ca^{2+} concentration in control and METH-treated astrocytes. (b) Traces show the glutamate (GLU, 100 μM)-evoked increase in the intracellular Ca^{2+} concentration in control and METH-treated astrocytes. (c) The Ca^{2+} responses to DA and GLU in control and METH-treated astrocytes are summarized. Data represent the mean ± SEM of 54–72 cells. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control astrocytes.

METH (100–1000 μM) for 3 days in cortical neuron/glia co-cultures caused the robust activation of cleaved caspase-3, which is a marker of neuronal death. However, unlike METH, a high concentration of MRP failed to produce the caspase-3 activation.

In vivo astrocytic responses by METH

Finally, we investigated *in vivo* astrocytic responses in the development of METH-induced sensitization. In order to investigate the direct involvement of PKC in the development of sensitization to METH-induced hyperlocomotion, mice were given intermittently METH (2 mg/kg, s.c..) in combination with a specific PKC inhibitor NPC-15437 (1 mg/kg, s.c.). As shown in Fig. 8a, intermittent co-administration of NPC-15437 abolished the development of sensitization to METH-induced hyperlocomotion.

We also confirmed that repeated *in vivo* treatment with METH under the present schedule failed to cause the neuronal cell death; the present schedule of treatment with METH had no effect on the caspase-3 activity in the caudate putamen (data not shown).

Immunohistochemical studies were also performed in order to investigate the change in GFAP-IR levels in the cingulate cortex and nucleus accumbens following intermittent treatment with METH. As shown in Fig. 8, the GFAP-IR level was clearly increased in METH-sensitized mice compared to those in mice that had been repeatedly treated with saline. This increase in GFAP-IR level in METH-sensitized mice was completely abolished by intermittent coadministration of NPC-15437.

Discussion

Recently, astrocytes have been reported to induce synapse formation and/or stabilize CNS synapses (Barres and Smith 2001) and may be capable of integrating neuronal inputs and modulating synaptic activity. The morphological changes that occur in astrocytes produce what are collectively known as reactive astrocytes that are characterized by specific changes such as the accumulation of intermediate-filament GFAP and hyperplasia (Ridet *et al.* 1997). In the present study, we observed morphological changes in astrocytes by treatment with either METH or MRP in cortical neuron/glia co-cultures. On the other hand, a difference was noted between the effects of METH and MRP in purified cortical astrocytes: while METH markedly activated astrocytes with phosphorylation of PKC, MRP had no such effect.

In the present study, we found for the first time that treatment with METH (10 μM) for 3 days increased the sensitivity of cortical astrocytes to dopamine and glutamate; this can be responsible for rewarding effects of psychostimulants and opioids (Carlezon and Nestler 2002; Wise 2002). Many lines of evidence support the idea that the enhanced Ca^{2+} signaling in astrocytes is not restricted to single cells as Ca^{2+} can cross cell borders via gap junctions, resulting in intracellular Ca^{2+} waves traveling from one astrocyte to another, and the induction of Ca^{2+} responses in neurons (Verkhratsky and Kettenmann 1996). Taken together, these findings suggest that treatment with METH may cause the functional up-regulation of neuroactive substances in astrocytes. It is also possible that the increase in astrocytic Ca^{2+} signaling induced by dopamine and glutamate following chronic exposure to METH may result from an enhancement of astrocytic dopamine and glutamate receptor functions induced by METH.

A study of cultures of newborn rodent CNS cells has shown that heterogeneous subpopulations of astrocytes can express one or more type of opioid receptor (Ridet *et al.* 1997). It has been reported that preferential μ-opioid receptor agonists can interfere with neuronal cell division (Stiene-Martin *et al.* 2001). In the present study, we found that the μ-opioid receptor agonist MRP had no effect on astrocytic activation in cortical purified astrocytes, whereas it caused astrocytic activation in cortical neuron/glia co-cultures. These findings constitute evidence that MRP might activate

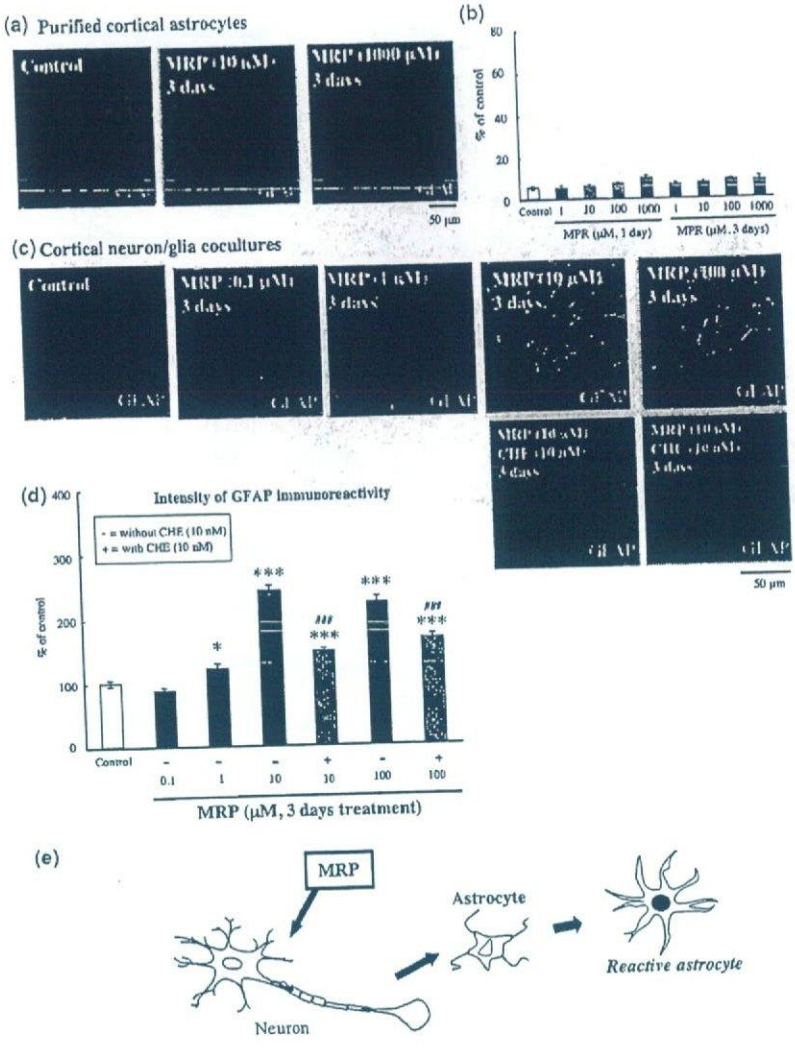


Fig. 4 Morphine (MRP) causes astrocytic activation in cortical neuron/glia co-cultures, but not in cortical purified astrocytes. (a) Purified cortical astrocytes were incubated with normal medium or MRP (10–1000 μM) for 3 days. The cells were stained with a rabbit polyclonal antibody to GFAP. (b) Purified cortical astrocytes are incubated with normal medium and MRP (1–1000 μM) for 1–3 days. Astrocytic activation as shown by a stellate morphology with processes longer than their perinuclear diameters was evaluated. Data represent the mean ± SEM of 175–230 cells from four separate observations. (c) Cortical neuron/glia co-cultures were incubated with normal medium, MRP (0.1–100 μM) or MRP (10–100 μM) + CHE (10 nM) for 3 days. The cells were stained with a rabbit polyclonal antibody to GFAP. (d) The density of GFAP-like immunoreactivity of each image was measured using NIH IMAGE. The level of GFAP like immunoreactivity on MRP- and MRP + CHE-treated cells is expressed as a percent increase (mean ± SEM) with respect to that on control cells. $^{*}p < 0.05$, $^{***}p < 0.001$; vs. control cells. $^{\#\#\#}p < 0.001$; vs. MRP-treated cells. (e) Proposed scheme showing the mechanism of MRP-induced atrocytic activation in mouse cortical neuron/glia co-cultures.

astrocytes via neurons. Furthermore, the present results raise the possibility that METH and MRP may differentially regulate long-term changes in neuron–glia communication.

Here we demonstrated that the astrocytic activation in cortical neuron/glia co-cultures induced by either METH or MRP was blocked by treatment with a specific PKC inhibitor. PKC is a key regulatory enzyme that modulates both presynaptic and postsynaptic neuronal function, the synthesis and release of neurotransmitters, and the regulation of receptors (Narita *et al.* 2001). Several lines of evidence have suggested that *in vitro* neurite outgrowth on several cell adhesion and matrix molecules (Walsh and Doherty 1996; Powell *et al.* 2001), including fibronectin (Kuhn *et al.* 1995), laminin and collagen (Bixby and Jhabvala 1992), are reduced by the specific inhibition of PKC, suggesting that PKC plays an important role in regulating the direction of neurite growth. In our preliminary study, we found that direct PKC activation by phorbol 12,13-dibutyrate induced a robust astrocytic activation in purified cortical astrocytes (data not shown). These findings, along with those in the present study, suggest that PKC is probably one of the most important factors in modulating the synaptic plasticity induced by METH and MRP.

We also found the difference between METH and MRP in the maintenance of astrocytic activation; METH produced prolonged astrocytic activation, whereas MRP caused a reversible activation of astrocytes in cortical neuron/glia co-cultures. Astrocytic activation has been considered for a long time as the major impediment to axonal regrowth after an injury in the CNS (Ridet *et al.* 1997). However, there is increasing evidence that astrocytes play a dynamic role in regulating synaptic strength, synaptogenesis and neurogenesis (Hama *et al.* 2004). Although the exact function of METH- and MRP-induced astrocytic activation remains

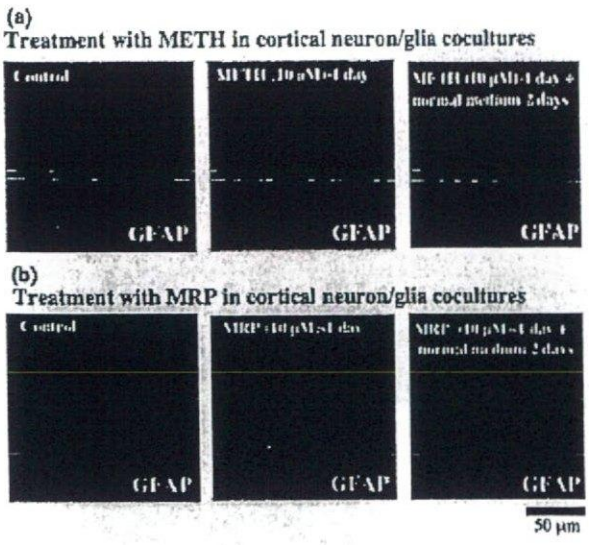


Fig. 5 METH, but not MRP, causes a long-lasting astrocytic activation in cortical neuron/glia co-cultures. (a) Cortical neuron/glia co-cultures were incubated with normal medium or METH (10 μM) for 1 day or 3 days, and cells were cultured with normal medium for additional 2 days. (b) Cortical neuron/glia co-cultures were incubated with MRP (10 μM) for 1 day or 3 day, and then, cells were cultured with normal medium for additional 2 days. All cells were stained with a rabbit polyclonal antibody to GFAP.

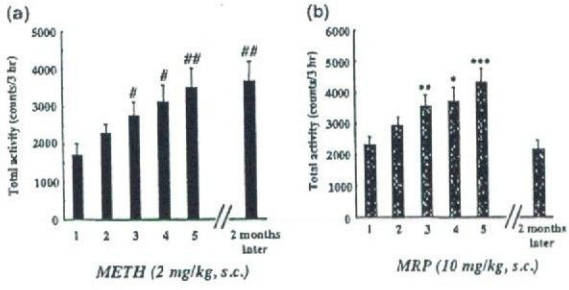


Fig. 6 The difference between METH and MRP in the maintenance of behavioral sensitization in mice. (a) Mice were treated with METH (2 mg/kg, s.c.) every 96 h for five sessions. Mice were then administered with METH (2 mg/kg, s.c.) after 2 months withdrawal. Total activity was counted for 3 h after each treatment (1, 5, 9, 13, 17 days and after 2 months withdrawal). #$p < 0.05$, ##$p < 0.01$, vs. the 1st administration. (b) Another group of mice were given five intermittent treatments morphine (10 mg/kg, s.c.) every 96 h. Mice were then administered with morphine (10 mg/kg, s.c.) after 2 months withdrawal. Total activity was counted for 3 h after the treatment (1, 5, 9, 13, 17 days and after 2 months withdrawal). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the 1st administration.

unclear at this time, it may positively modulate synaptic activity by directly controlling synaptic strength, leading to synaptic plasticity in the CNS.

Although cultured cells used in the present *in vitro* study represents a simplication relative to the state of neuronal–glial communication in the CNS, additional interactions with these cells and matrix components in *in vivo* system are likely to reflect the behavioral change such as behavioral sensitization. In fact, one of the most important aspects of the present study was that the METH-induced behavioral sensitization was maintained even after a long period of abstinence, while the MRP-induced sensitization was reversible. This may be consistent with the evidence that METH, but not MRP, produced long-lasting astrocytic activation in cortical neuron/glia co-cultures. It is therefore worthwhile in future studies to identify the precise molecular steps associated with astrocyte–neuron signaling on a long-lasting maintenance of METH-induced behavioral sensitization.

Another key finding of the present study was that the levels of GFAP in the mouse cingulate cortex and nucleus accumbens were clearly increased by repeated *in vivo* administration of METH; this was related to behavioral sensitization (Fig. 8). These results suggest the repeated *in vivo* treatment of METH could produce the astrocytic activation in the cingulated cortex and nucleus accumbens. Central dopamine systems have been implicated in mediating reward-related behaviors. In particular, the nucleus accumbens of the mesolimbic dopamine pathway plays an important role in regulating the rewarding effects of many stimuli including drugs of abuse (Wise and Hoffman 1992). It has been also recognized that the cingulate cortex is responsible for stimulus-reward learning (Allman *et al.* 2001). Taken together, these studies suggest the possibility that METH-induced atrocytic activation in these areas modulates the development of METH-induced behavioral sensitization.

Furthermore, the development of behavioral sensitization to METH with astrocytic activation was abolished by cotreatment with the PKC inhibitor, NPC-15437. Although further experimentation is still required, these findings indicate that activated PKC-dependent astrocytic response in the cingulate cortex and nucleus accumbens by intermittent METH treatment may be implicated in the development of sensitization to the METH-induced hyper-locomotion.

Finally, we investigated the neurotoxic effects of METH and MRP; METH markedly induced neuronal cell death in cortical neuron/glia co-cultures, while MRP had no such effect. Glial activation is thought to be neuroprotective (Ridet *et al.* 1997), however, excess activation can be deleterious in the brain (Ahlemeyer *et al.* 2002). In fact, overexpression of astrocyte-derived neurotrophic protein S100β has been shown to induce neuronal cell death through nitric oxide released from astrocytes (Hu *et al.* 1997). Taken together, the present findings support the idea that the direct effect induced by high concentration of METH on astrocytes may lead to a dynamic change in neuron–glia network, resulting in the neurotoxicity.

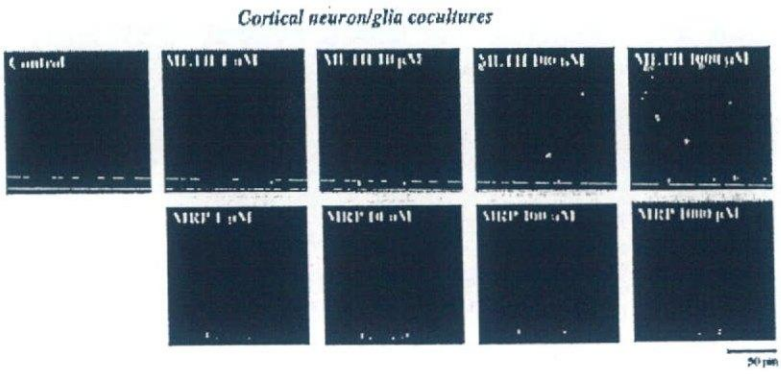
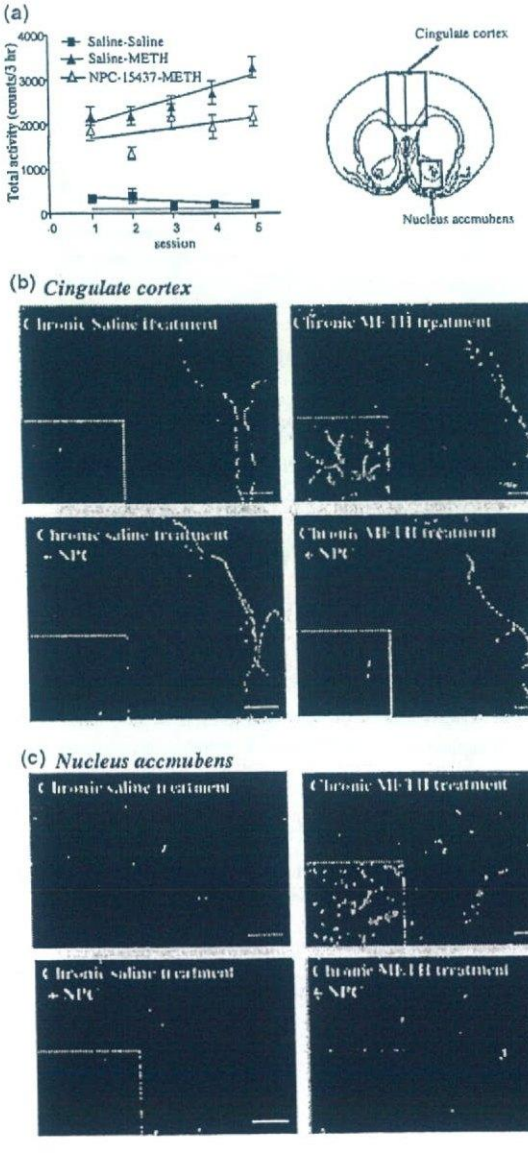


Fig. 7 High concentration of METH, but not MRP, causes a neuronal cell death in cortical neuron/glia co-cultures. (a) Cortical neuron/glia co-cultures were incubated with normal medium or METH (1–1000 μM) for 3 days. (b) Cortical neuron/glia co-cultures were incubated with MRP (1–1000 μM) for 3 days. All cells were stained with a rabbit polyclonal antibody to cleaved caspase-3.



In conclusion, the present data clearly provide direct evidence for the distinct mechanisms between METH and MRP on the astroglial and neuronal responses. Nevertheless opioids, such as MRP, have been used worldwide to control chronic pain, the appearance of opioid addiction following chronic administration of opioids seriously limits their use for the relief of moderate to severe pain. The information of the reversibility of astroglial response and behavioral sensitization with no neuronal cell death induced by MRP could break through the definition of 'opioid addiction' and the misleading of concept that morphine is dangerous. Furthermore, the long-lasting maintenance of behavioral sensitization to METH and neuronal cell death by high concentration of METH observed in this study strongly support the idea for the high risk of the psychostimulant use in humans.

Fig. 8 GFAP-like immunoreactivity (IR) in the cingulate cortex and nucleus accumbens of mice by repeated *in vivo* treatment with METH with or without cotreatment with a specific PKC inhibitor NPC-15437. (a) METH (2 mg/kg, s.c.) or saline was repeatedly given to mice every 96 h, and the total activity was counted for 3 h after each treatment. Repeated injection of METH produced a progressive elevation of the METH-induced locomotor-enhancing effect, indicating the development of sensitization to METH-induced hyper-locomotion ($F_{1,190}$ = 430.20, $p < 0.001$ vs. Saline–Saline). Another group of mice were given METH intermittently in combination with NPC (1 mg/kg, s.c.) every 96 h. NPC or saline was pretreated at 30 min before METH administration (2 mg/kg, s.c.). Intermittent co-administration of NPC-15437 significantly suppressed the development of sensitization to METH-induced hyper-locomotion ($F_{1,90}$ = 20.54, $p < 0.001$ vs. Saline–METH). There were no significant differences between the 1st and 5th administration in NPC-15437-treated mice. The density of GFAP-IR was increased in the cingulate cortex (b) and nucleus accumbens (c) of mice during the development of sensitization to METH. There were no changes in the density of GFAP-IR in the cingulate cortex (b) and nucleus accumbens (c) of mice treated with METH in combination with NPC-15437 as compared to saline treatment. Scale bar (unbroken line): 100 μm; scale bar (broken line): 20 μm (b) or 50 μm (c).

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Involvement of β1 Integrin in Microglial Chemotaxis and Proliferation on Fibronectin: Different Regulations by ADP Through PKA

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KEY WORDS
PKA; ATP; ADP; purinergic; glia; extracellular matrix; migration

ABSTRACT
Microglia are immune cells in the brain; their activation, migration, and proliferation have pivotal roles in brain injuries and diseases. Microglia are known to attach firmly to fibronectin, the upregulation of which is associated with several pathological conditions in the CNS, through β1 integrin and become activated. Extracellular nucleotides can serve as potent signaling molecules. Recently, ATP and ADP were revealed to possess chemoattractive properties to microglia via Gi-coupled P2Y receptors. In the present study, we report that the ADP-induced chemotaxis of microglia is mediated by P2Y12/13 receptors and is β1 integrin-dependent in the presence of fibronectin. Signals from P2Y12/13 receptors also cause β1 integrin translocation to the membrane ruffle regions, but this redistribution was lost when the intracellular cyclic AMP (cAMP) was increased by forskolin or dibutyryl cAMP. This inhibitory effect of cAMP-elevating agents did not appear when microglia were co-incubated with a protein kinase A (PKA) inhibitor, KT-5720, suggesting that PKA is a negative regulator of the β1 integrin translocation. We also show that the engagement of β1 integrin enhanced microglial proliferation. Signals from P2Y12/13 receptors attenuated the proliferation, whereas ADP itself had no effect on microglial growth. Furthermore, β1 integrin-induced proliferation is positively regulated by the cAMP-dependent PKA. Together, these results indicate the involvement of β1 integrin in microglial proliferation and chemotaxis, both of which have clinical importance. The data also suggest that PKA is inversely involved in these two cellular functions.

INTRODUCTION

Microglia are considered to act as brain macrophages. They participate in brain injuries and diseases (Nakajima and Kohsaka, 1993), in which their motility, aberrant activation, and proliferation are known to play crucial roles. Microglia quickly respond to numerous inflammatory mediators by migrating to the source of the mediators, where they become activated and exert their neuroprotective effects (Hanisch, 2002; Streit, 2002). Unfortunately, their hyperactivation often leads to neurotoxicity instead, and several pathological conditions in the CNS are, in fact, accompanied by an excess proliferation of microglia (Gehrmann et al., 1995). Thus, better understanding of the regulation of microglial chemotaxis and proliferation may have important therapeutic implications.

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits; they mediate cell–cell and cell–extracellular matrix (ECM) interactions. At present, 16 α and 8 β chains have been identified, and at least 22 different complexes are known. β1 integrin, the most ubiquitous β subunit, pairs with at least 10 different α chains to comprise receptors for a wide variety of ECM proteins. Within the CNS, β1 integrin is expressed on many different cell types, including neurons, glial cells, and endothelial cells (Pinkstaff et al., 1999). As shown previously by other investigators, microglia express several integrin receptors of β1 and β2 families that are upregulated following microglial activation in vitro (Hailer et al., 1996; Yu et al., 1998; Kloss et al., 2001; Milner and Campbell, 2003). In vivo, integrin expression is found to be increased on activated microglia in Alzheimer's disease (Akiyama and McGeer, 1990), after nerve injuries (Coyle, 1998; Kloss et al., 1999; Tsuda et al., 2003) and in multiple sclerosis lesions (Bo et al., 1996). Integrins serve not only as adhesive molecules but also as signaling receptors, and they regulate numerous cellular functions (Hemler, 1998) including cell migration and proliferation. β1 integrin is closely associated with the regulation of cell motility and growth in many cell types (Hynes, 1992; Jones and Watt, 1993; Howlett et al., 1995), but its role in microglial chemotaxis and proliferation remains unclear.

Fibronectin is one of the ECM molecules; it is a large, multidomain glycoprotein that exists both as a cell surface protein and in plasma. The expression of ECM molecules is regionally and developmentally regulated in the brain, and their presence is relatively minor in the

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normal CNS. Some ECM molecules, including fibronectin, however, are upregulated after adult CNS injury. Fibronectin also exists at high concentrations in the blood plasma and a breakdown of the blood-brain barrier should result in an increase in its local concentration in the CNS. Fibronectin induces firm adhesion and activation of microglia (Milner and Campbell, 2002, 2003). It is a major ligand of the β1 integrin family, but recently Mac-1 was also reported to play a role in the adhesion of leukocytes to fibronectin (Lishko et al., 2003).

Previously, Honda et al. (2001) demonstrated that extracellular ATP and ADP could induce the chemotactic migration of microglia. Several lines of evidence so far have indicated that extracellular nucleotides serve as signaling molecules (Bodin and Burnstock, 2001). ATP, and possibly other nucleotides, are released from damaged cells or secreted via nonlytic mechanisms and activate microglia. In the work by Honda et al. (2001), extracellular ATP and ADP induced chemotaxis as well as membrane ruffling, which was possibly mediated by Gi-coupled P2Y receptors. The P2Y12 receptor is a recently cloned Gi-coupled P2 receptor (Hollopeter et al., 2001), and expressed on platelets and exclusively in microglia in the brain (Sasaki et al., 2003). The P2Y13 receptor is another Gi-coupled P2 receptor that was recently identified (Communi et al., 2001; Zhang et al., 2002). Its messenger RNA is expressed at highest levels in the brain and immune tissues, particularly the spleen (Zhang et al., 2002), suggesting its roles in neuron and in immune systems. P2Y12 and P2Y13 receptors present a very similar pharmacological profile. Both receptors show high affinities for ADP and 2MeSADP (Hollopeter et al., 2001; Zhang et al., 2002) and are selectively blocked by ARC-67085 and ARC-69931 (Ingall et al., 1999). No specific agonists/antagonists are known currently to distinguish these two receptors pharmacologically.

The involvement of β1 integrin in chemotaxis and proliferation is already well characterized. Its role in microglial chemotaxis and proliferation, however, has not been well studied, and its correlation with purinoceptors such as P2Y12/13 receptor is still unclear. We provide new evidence that (1) ADP-induced chemotaxis through P2Y12/13 receptors involves β1 integrin in the presence of fibronectin, (2) ADP induces β1 integrin redistribution which colocalizes with membrane ruffling on microglia, and PKA functions as a negative regulator of this translocation, (3) β1 integrin mediates microglial proliferation through positive regulation of PKA, and (4) signals from P2Y12/13 receptors abrogate the proliferative effect of β1 integrin. Taken together, these results indicate that β1 integrin is crucially involved in both the proliferation and chemotaxis of microglia, which are under the inverse regulation of PKA.

Abbreviations

ATP	adenosine 5′-triphosphate
ADP	adenosine 5′-diphosphate
cAMP	adenosine 2′:3′-cyclic monophosphate
CNS	central nervous system
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
CX_3CR	CX_3C chemokine receptor
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
GPCR	G-protein-coupled receptor
IL-8	interleukin-8
MAP	mitogen-activated protein
2MeSADP	dimethylthioadenosine 5′-diphosphate
PKA	protein kinase A
VLA	very late antigen

MATERIALS AND METHODS

Isolation of Microglia

Rat primary cultures were derived from the cerebral cortex of neonatal Wistar rats. In brief, the rat cortices were separated from the meninges, minced, treated with trypsin and DNase, and then centrifuged to remove dead cells. The pellet was resuspended in DMEM, filtrated and cultured in medium with 10% fetal bovine serum for 12–23 days. Microglia were isolated on day 10 and day 15 by gently shaking of the flasks for 2 min and were attached to appropriate dishes or coverslips. One flask (75 cm^2) yielded 1–2 × 10^6 microglial cells by this preparation, and the cultures were of >98% purity. The purity of microglial culture was determined by immunostaining for Ox-42 and Eva-1.

Immunofluorescence Staining of Cell Surfaces

Microglia were washed once with ice-cold staining buffer (phosphate-buffered saline [PBS] 1% fetal calf serum [FCS], 0.1% NaN_3) and then Fc blocked for 15 min on ice. After washing, they were incubated with Ha2/5 (PharMingen) for 30 min on ice. They were washed once, incubated with mouse anti-hamster IgM (PharMingen) for 30 min on ice, and then washed again. Finally, cells were incubated with Alexa-Fluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) for 30 min on ice in the dark, washed twice, resuspended in the staining buffer, and the fluorescence intensity of the labeled microglia was analyzed with FACScan (Becton Dickinson).

Immunocytochemistry

Coverslips were briefly treated with hydrochloric acid and extensively washed with PBS. They were then coated with fibronectin (Sigma, St. Louis, MO) at 10 μg/ml overnight and washed with PBS immediately before use. Microglia were plated on coverslips and kept at 37°C for 1 h, and unattached cells were washed off gently with warm DMEM. After 1-h serum starvation, the cells were stimulated with ADP (50 μM) for 5 min at 37°C. The attached cells were then fixed in 3.7% formaldehyde in PBS for 5 min and then washed with PBS. The cells were permeabilized with 0.1% Triton-X in PBS for 5 min, washed again with PBS, and then blocked for 30 min with ACE blockase (Yukijirushi, Ltd.) with 3% goat serum at room temperature. To visualize β1 integ-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

rin and membrane ruffling, the cells were stained with Ha2/5 mAb, mouse anti-hamster IgM Ab, Alexa-Fluor 488-conjugated goat anti-mouse IgG, or Texas Red-X phalloidin (Molecular Probes) and observed under a fluorescent microscope.

Defining Membrane Rufflings

To the best of our knowledge, there are no methods to quantify the degree of membrane ruffling. Therefore, we tried to define membrane ruffling by analyzing the pictures from immunocytochemistry. First we pictured cells that were stained with Texas Red-X phalloidin, and then a line was drawn across the cell and the intensity of the Texas Red-X phalloidin labeling along the line was analyzed using computer software. Membrane ruffling is characterized by the sharp, strong labeling of Texas Red-X phalloidin, which reaches >200 a.u. (out of 256) of intensity, whereas cells with no ruffling show less intense, blurred, and dispersed staining. We defined membrane ruffling as a wave-like structure that is stained with phalloidin and the labeling intensity of which reaches >200 a.u.. Cells satisfying these criteria were construed as bearing membrane ruffling morphology.

Chemotaxis Assay

Chemotaxis assays were performed using a direct-viewing Dunn chemotaxis chamber (Weber Scientific International, Teddington, UK). The details concerning this apparatus and its use are given in Webb et al. (1996) and Zicha et al. (1991). In brief, microglia were attached to coverslips that had been coated with fibronectin. After 1-h incubation, the cells were washed with warm DMEM and cultured in the absence of FCS for an additional 1 h. The coverslips were then inverted onto the slide, the inner and the outer wells of which were filled with DMEM. The edges of the coverslip were tightly sealed with adhesive tape except for one on which a thin filling slit was left. Using a needle and a syringe, the medium in the outer well was gently replaced with DMEM containing 100 μM ADP. The filling slit was quickly sealed with adhesive tape and the chamber was carefully set on the stage of a microscope equipped with a 10× phase-contrast objective. Microglia adhered to the coverslip were exposed to the ADP gradient and then monitored in the Dunn chemotaxis chamber for a period of 1 h. One region of the bridge was viewed directly via a CCD video camera and the data were recorded every 30 s during the 1-h observation using image software. After recording, cells were randomly selected from a set area of the field and the straight distance they migrated was plotted against x,y coordinates on scatter diagrams. The x-axis was parallel to the outer ring while the y-axis was vertical. Recording of the cell migration usually started within 30 min of assembling the chamber, by which time a linear diffusion gradient had been established (Webb et al., 1996).

Proliferation Assay

In this study, 96-well plates (Corning) were incubated with fibronectin (10 μg/ml), anti-β1 integrin Ab (10 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit serum (Sigma) overnight at 4°C and washed withPBS before use. For anti-β1 integrin Ab and normal rabbit serum coatings, the wells were pre-coated with goat anti-rabbit IgG (Sigma) for 2 h at 37°C and washed with PBS. Microglia were attached to the wells and cultured in DMEM 4% FCS for 24 h at 37°C. After the incubation, cell survival and proliferation were assayed using an MTT cell growth kit (Chemicon) according to the manufacturer's instructions and also by direct cell counting.

RESULTS

ADP-Induced Chemotaxis of Microglia Is Mediated by P2Y12/13 Receptor and Is Blocked by an Elevation of cAMP

In the previous study by Honda et al. (2001), extracellular ADP induced microglial chemotaxis through Gi-coupled P2Y receptors and the ADP-induced membrane ruffling was inhibited by a P2Y12- and P2Y13-selective antagonist, ARC-69931. First, to make the system closer to pathological conditions in the CNS, we performed the chemotaxis assay on fibronectin substrates. To examine whether microglial chemotaxis toward ADP on fibronectin substrate is also mediated by the P2Y12/13 receptor, ARC-69931 was used in the chemotaxis experiment (Fig. 1). Cultured microglia were adhered to the fibronectin-coated coverslips and their chemotaxis against the ADP concentration gradient was studied with the Dunn chemotaxis chamber (see materials and methods for details). In the absence of the ADP gradient, microglia did not move on the fibronectin-coated coverslips (Fig. 1b). When ADP 100 μM was applied to the system, however, the cells migrated toward the stimulant (Fig. 1a,c). Pretreatment of microglia with ARC-69931 at 10 μM totally blocked the ADP-induced microglial chemotaxis (Fig. 1d), suggesting that the P2Y12/13 receptor is responsible for the microglial chemotaxis toward ADP. Since the activation of P2Y12/13 receptors inhibits adenylate cyclase, the effect of changes in the intracellular cAMP level on microglial chemotaxis was tested. Forskolin is a potent adenylate cyclase activating agent and dibutyryl cAMP is a membrane permeable AMP analogue that activates cAMP protein kinases. When microglia were pretreated with forskolin (Fig. 1e) or dibutyryl cAMP (Fig. 1f), their chemotaxis toward ADP was greatly attenuated.

F1

β1 Integrin Is Involved in the Microglial Migration Toward ADP on Fibronectin Substrate

To study the function of β1 integrin in the P2Y12/13-receptor mediated migration of microglia, we first examined its expression on cultured microglia. Flow cyto-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

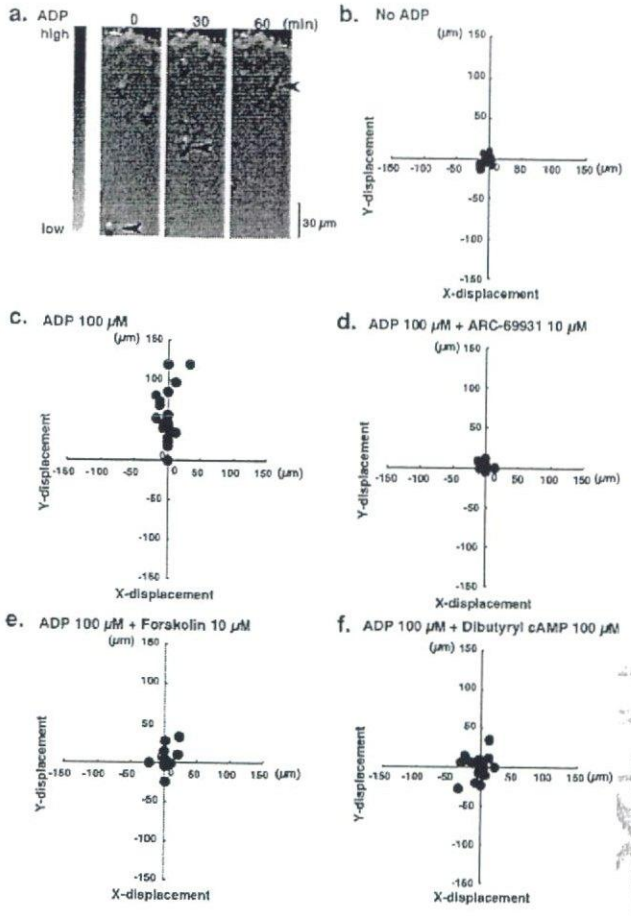


Fig. 1. ADP-induced chemotaxis of microglia was mediated through P2Y12/13 receptors and blocked by an elevation of intracellular cAMP. Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were assayed for migration toward ADP in the Dunn chemotaxis chamber. **a:** Typical chemotactic responses of microglia toward ADP. Arrowheads depict the position of a single microglial cell at the indicated time, showing the kinetics of chemotaxis. Microglia were almost static on fibronectin in the absence of the stimulant (**b**). Microglia, however, showed chemotactic responsiveness to ADP (100 μM) (**c**), which was completely blocked by the P2Y12/13 receptor antagonist ARC-69931 (**d**), confirming that the chemotaxis to ADP is mediated by P2Y12/13 receptor. Forskolin pretreatment (**e**) and dibutyryl cAMP pretreatment (**f**) attenuated the ADP-induced chemotaxis of microglia. The data represent three independent experiments.

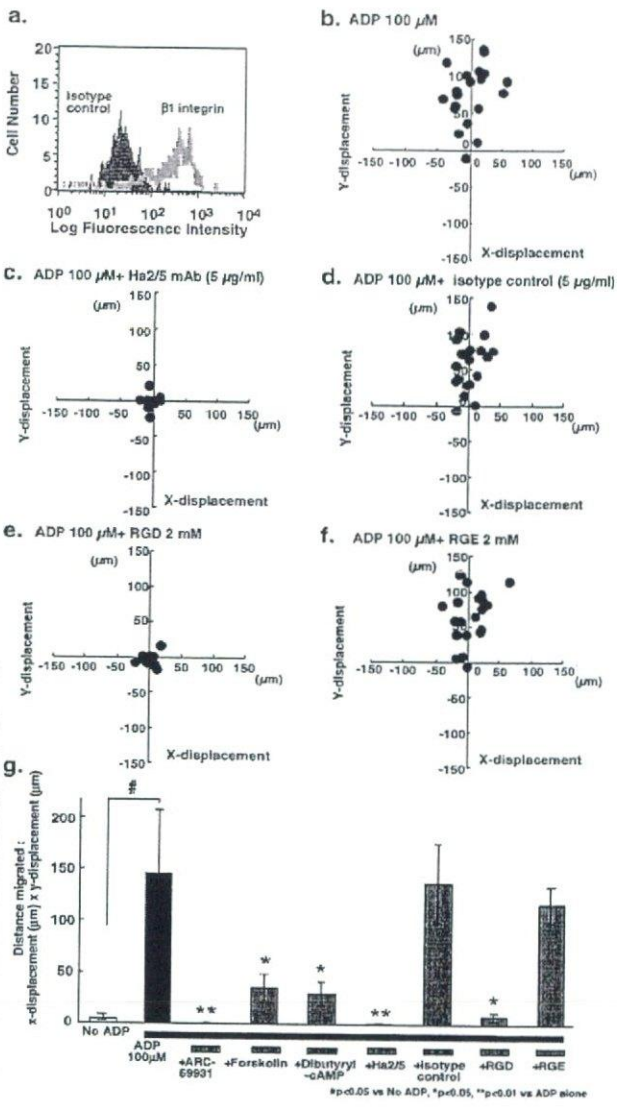


Fig. 2. β1 integrin is highly expressed on cultured microglia, and it is involved in the ADP-induced chemotaxis of microglia on the fibronectin substrate. Cultured microglia were labeled with anti-β1 integrin antibody (Ha2/5) (gray) or with isotype-matched control antibody (black). The labeling was detected by Alexa-Fluor 488-conjugated antibody and the cells were subjected to flow cytometry analysis (**a**). **b–f:** Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were assayed for migration toward ADP in the Dunn chemotaxis chamber. The microglia migration toward ADP (100 μM) (b) was totally inhibited by Ha2/5 (c) and RGD peptide (e). In contrast, the migration was not affected by isotype-matched control (d) and RGE peptide (f). Data (a–f) represent three independent experiments. Each chemotaxis was quantified by calculating the x displacement (μm) multiplied by the y-displacement (μm) (**g**). Data (g) are mean ± SE of three separate experiments. #Greater than No ADP ($P < 0.05$, Student's *t*-test); *Smaller than ADP 100 μM ($P < 0.05$, Student's *t*-test); **Smaller than ADP 100 μM ($P < 0.01$, Student's *t*-test).

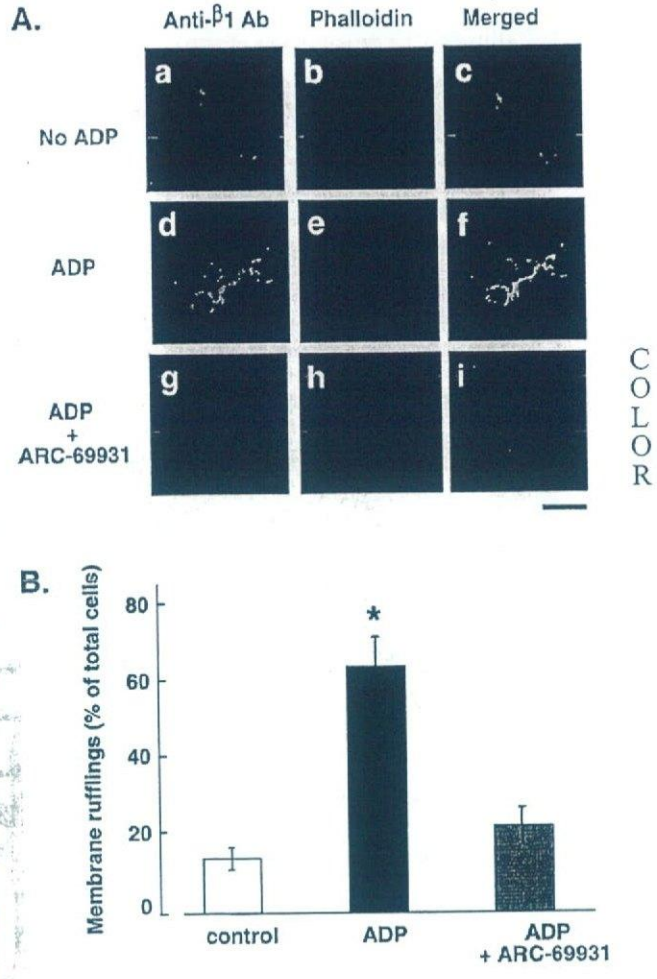
metric analysis confirmed that the cultured microglia expressed significant amounts of the β1 integrin subunit (Fig. 2a). Next, to assess the role of β1 integrin in the cell migration toward ADP, a chemotaxis assay was performed with the Dunn chemotaxis chamber in the presence of a monoclonal antibody specific for the β1 integrin subunit (Ha2/5) and RGD peptide (Fig. 2b–f). Treatment of microglia with Ha2/5 antibody at 5 μg/ml suppressed the ADP-induced chemotaxis (Fig. 2c), indicating that β1 integrin is required for this process. An isotype-matched control antibody did not interfere with the microglial chemotaxis toward ADP (Fig. 2d). Treatment with RGD peptide at 2 mM also perturbed the microglial migration toward ADP (Fig. 2e), whereas control RGE peptide did not inhibit the migration (Fig. 2f), suggesting that the RGD sequence is important. The RGD (Arg-Gly-Asp) sequence is present in several extra-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

cellular matrix components including fibronectin, and many integrins show RGD sequence-dependent binding to their ligands. Therefore, these results demonstrate that β1 integrin was responsible for the motility of microglia on the fibronectin substrate and its interaction with fibronectin involves the RGD sequence. Each chemotaxis was quantified by calculating the x displacement multiplied by the y displacement (Fig. 2g), and thus the dot-plotted data in Figures 1 and 2 were all evaluated for statistical significance.

Stimulation of P2Y12/13 Receptor Causes β1 Integrin Redistribution That Colocalizes With Membrane Ruffling

The involvement of β1 integrin in the ADP-induced chemotaxis was further studied using immunofluorescence (Fig. 3). Extracellular ATP and ADP have been reported to induce a membrane structure called membrane ruffling (Honda et al., 2001). Membrane ruffling is a unique, wave-like structure of the plasma membrane. It is the actin polymerization seen as a projection from the cell membrane, and the most conventional way of visualizing this unique structure is to stain the cells with phalloidin. In our experiments, membrane ruffling was construed as labeling intensity that reached >200 a.u. (see Materials and Methods). Microglia were attached to fibronectin-coated coverslips, stimulated with ADP at 50 μM for 5 min, fixed and permeabilized, and then the appearance of membrane ruffling and the cellular localization of β1 integrin were studied using Texas Red-X phalloidin and Ha2/5 antibody, respectively. Without ADP stimulation, microglia showed no membrane ruffling and the β1 integrin subunit was dispersed over the plasma membrane of the microglia (Fig. 3a–c). In response to ADP stimulation at 50 μM, membrane ruffling appeared within 5 min after stimulation (Fig. 3e) and β1 integrin was redistributed to the cell edge (Fig. 3d). A merged image revealed that β1 integrin colocalized with the membrane ruffling (Fig. 3f). Treatment of microglia with ARC-69931 abrogated the membrane ruffling formation (Fig. 3h) (Honda et al., 2001), β1 integrin redistribution (Fig. 3g) and thus the colocalization of these two (Fig. 3i), suggesting that these responses were mediated by P2Y12/13 receptors. The numbers of cells with membrane ruffling were directly counted among control, ADP, and ADP with ARC-69931 populations (Fig. 3B). The result again indicated that ADP induces membrane ruffling which is inhibited by ARC-69931.



COLOR

Fig. 3. P2Y12/13 receptor-mediated microglial β1 integrin redistribution that colocalized with membrane ruffling in response to ADP stimulation. **A:** Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were stimulated with ADP (50 μM) for 5 min, fixed and permeabilized. Then, for the immunofluorescence studies, the cells were incubated with anti-β1 antibody Ha2/5 (a,d,g) and phalloidin (b,e,h). a–c: Controls, i.e., PBS alone. β1 integrin redistribution and membrane ruffling occur in response to ADP stimulation (d,e), but they disappear in the presence of ARC-69931 (g,h). Merged image (f) shows that β1 integrin redistributes and colocalizes with membrane ruffling in response to ADP (50 μM). **B:** Cultured microglia on fibronectin-coated coverslips were stimulated with ADP (50 μM) for 5 min, fixed and permeabilized. The cells were labeled with Texas Red-X phallodin to visualize membrane rufflings. Total cells and cells undergoing membrane ruffling were counted respectively, and the percentage was calculated. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test). Scale bar = 10 μm in A.

An Increase in the Intracellular cAMP Level Abrogated Colocalization of Membrane Ruffling and β1 Integrin, and the PKA Inhibitor KT-5720 Restored the β1 Integrin Redistribution and Membrane Ruffling

We next investigated whether an increase in the intracellular concentration of cAMP in microglia would have any effect on the appearance of the membrane ruffling and redistribution of β1 integrin. Microglia were adhered to fibronectin-coated coverslips and pretreated either with forskolin or dibutyryl cAMP. The cells were then stimulated with ADP at 50 μM for 5 min and stu-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

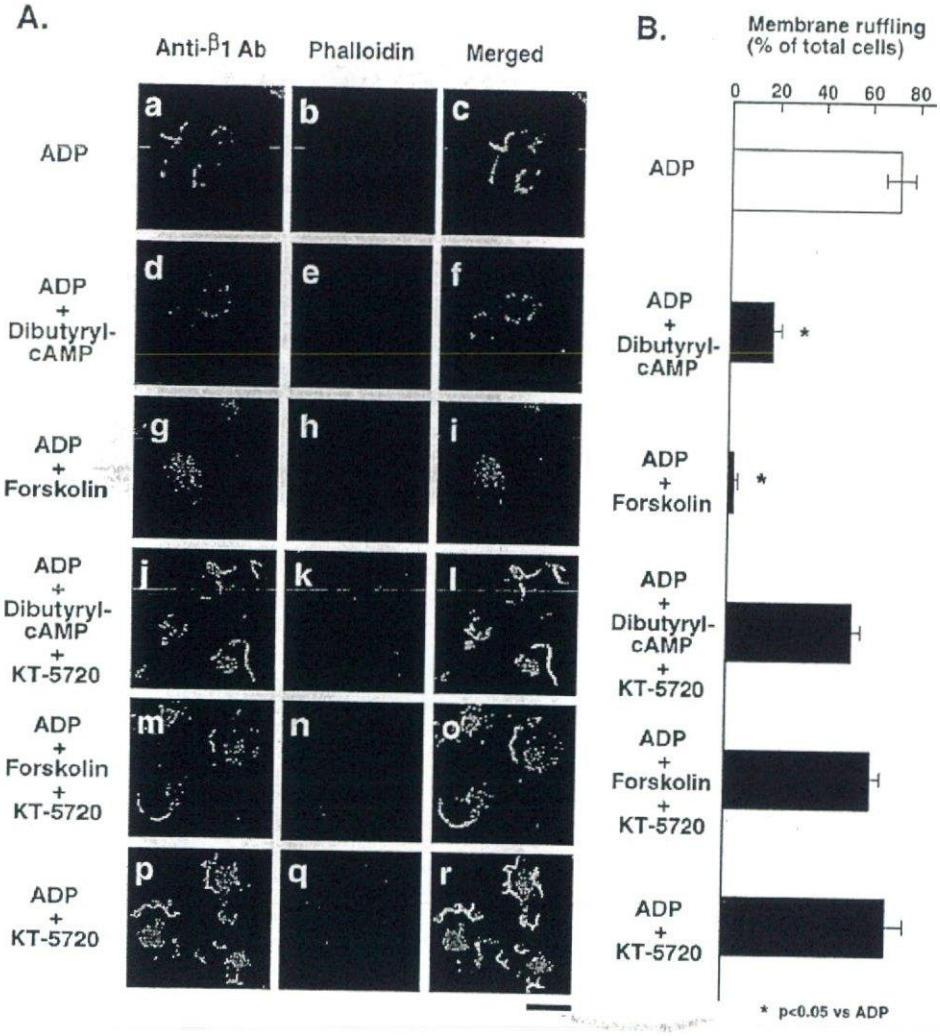


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Fig. 4. Elevation of intracellular cAMP abrogated the β1 integrin redistribution and membrane ruffling in response to ADP stimulation. The PKA inhibitor KT-5720 restored the ADP-induced β1 integrin redistribution and membrane ruffling on microglia. **A:** Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were stimulated with ADP (50 μM) for 5 min in the presence of dibutyryl cAMP (d–f) or forskolin (g–i) to study the involvement of the intracellular cAMP concentration. The elevation of intracellular cAMP abrogated the β1 integrin redistribution (d,g) and membrane ruffling (e,h) in response to ADP (50 μM). PKA involvement was examined by pretreating the cells with KT-5720 at 5 μM (j–o). KT-5720 treatment itself did not affect the morphology of the cells (data not shown) and KT-5720 pretreated cells responded normally to ADP at 50 μM (p–r). Blocking PKA by pretreatment with KT-5720 restored β1 integrin clustering and increased membrane ruffling even in the presence of dibutyryl cAMP (j–l) or forskolin (m–o). **B:** Cultured microglia on fibronectin-coated coverslips were stimulated as in A for 5 min, fixed and permeabilized. The cells were labeled with Texas Red-X phallodin to visualize membrane rufflings. Total cells and cells undergoing membrane ruffling were counted respectively, and the percentage was calculated. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's *t*-test).

F4 died by immunofluorescence. As seen in Figure 4Ad–i, neither the β1 integrin redistribution nor the membrane ruffling appeared on the surface of microglia when the intracellular cAMP level was elevated. These results indicate that an elevation of intracellular cAMP by dibutyryl cAMP or by forskolin inhibited the β1 integrin accumulation and membrane ruffling in response to ADP. PKA is located downstream of the cAMP elevation, and its negative regulation of the β2 integrin avidity and the integrin-mediated adhesion of lymphocytes has been described in other studies (Laudanna et al., 1997; Jones, 2002). Thus, since the PKA activity appears to regulate the integrin function, we next investigated whether PKA activation is responsible for the loss of the β1 integrin redistribution and the attenuation of the ADP-induced chemotaxis of microglia. Microglia were pretreated with forskolin or with dibutyryl cAMP, both of which were in the presence of the PKA inhibitor KT-5720. When these cells were stimulated with ADP at 50 μM, the integrin redistribution was restored (Fig. 4Aj–o). The number of cells with membrane ruffling was directly counted for each population (Fig. 4B), and the result confirmed the effect of dibutyryl cAMP, forskolin, and KT-5720 on membrane ruffling.

β1 Integrin Mediates Microglial Proliferation

β1 integrin regulates cellular proliferation, migration, survival, and differentiation via outside-in signaling. β1 integrin is involved in the proliferation of many cell types (Jones and Watt, 1993; Howlett et al., 1995), but its effect on microglial proliferation has not been clarified. To study the function of β1 integrin in microglial proliferation, an MTT assay and direct cell counting were used in this study. Figure 5 shows that microglia F5 cultured on fibronectin substrate proliferate more than 1.5-fold after 24-h incubation as compared to the control. Microglia plated on anti-β1 integrin antibody showed similar results, suggesting that cross-linking of β1 integ-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

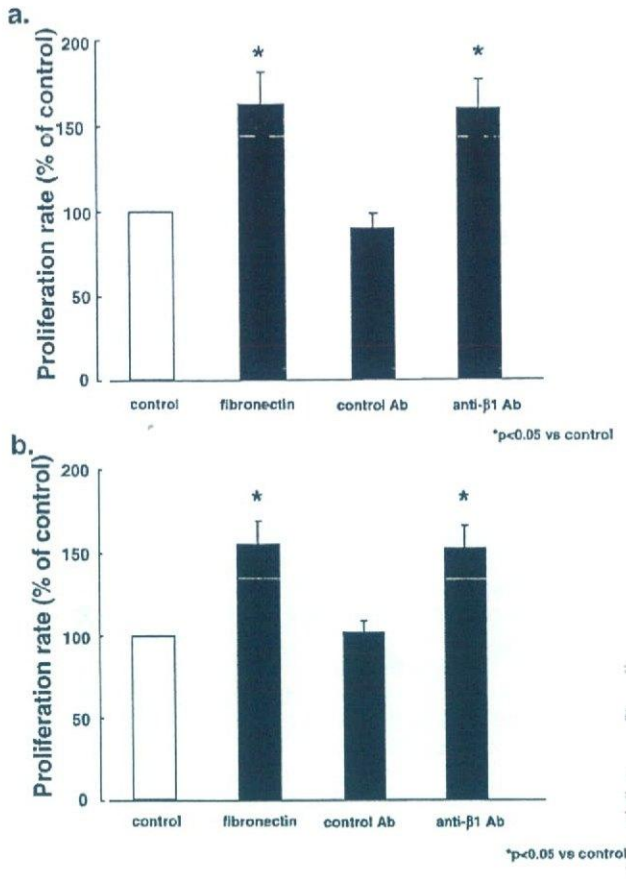

Fig. 5. β1 integrin mediates microglial proliferation. Cultured microglia were adhered to uncoated, fibronectin-coated, anti-β1 antibody-coated or control antibody-coated plates and cultured for 24 h at 37°C. After the incubation, the cell's viability was measured by (**a**) MTT assay or (**b**) direct cell counting. The data are shown as percentage of proliferation of microglia on uncoated culture plates after 24-h incubation. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test).

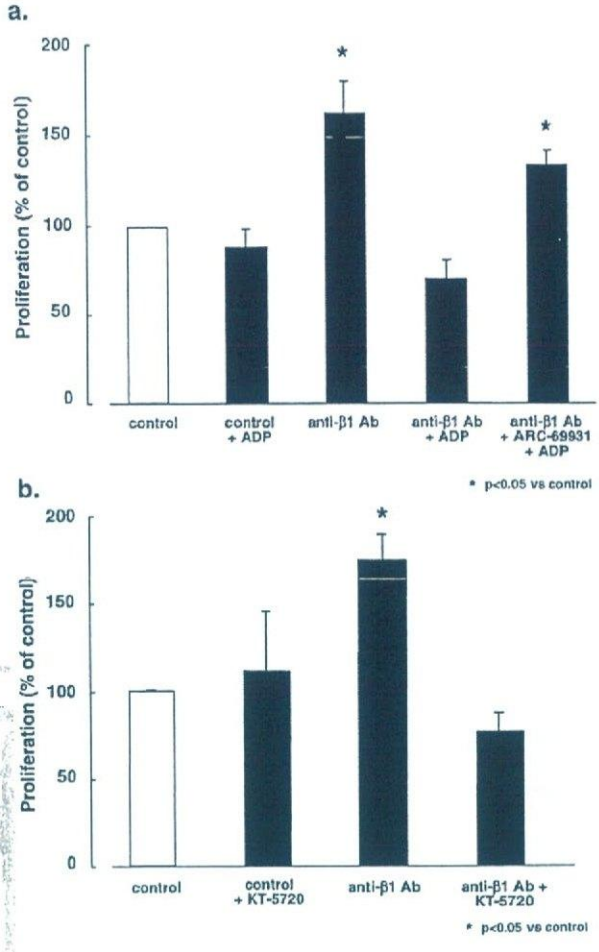

Fig. 6. β1 integrin-mediated proliferation of microglia is inhibited by ADP. PKA is a positive regulator of β1 integrin-mediated proliferation. Cultured microglia were adhered to uncoated or anti-β1 integrin antibody-coated plate in the presence of ADP (100 μM) and ARC-69931 (10 μM) (**a**) or KT-5720 (10 μM) (**b**) and incubated for 24 h at 37°C. The cells' viability was then tested by MTT assay. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test).

rins leads to microglial proliferation. Recent reports have shown that integrin engagements, either with ligands or with antibodies, are capable of transducing signals (Miyamoto et al., 1995). An isotype-matched control antibody did not induce microglial proliferation. In this way, it is clear from these results that microglial β1 integrin mediates the outside-in signal, which promotes the proliferation of the cell.

ADP Inhibits β1 Integrin-Mediated Proliferation of Microglia and the Mechanism Involves PKA Activation

Chemokines act through Gi-coupled GPCRs to attract the target cells (Neptune and Bourne, 1997; Rollins, 1997), and several of them are known to induce the proliferation of cells. Several chemokine receptors including IL-8R, CXCR1-4, CCR2, CCR3, CCR5, and CX_3CR1 are detected on microglia (Hanisch, 2002; Abbadie et al., 2003) and most of them are reported to induce microglial proliferation as well. Since P2Y12/13 is also a Gi-coupled receptor, we studied its effect on cell growth. When microglia were stimulated with ADP alone at 100 μM, they did not increase in number (Fig. 6a), suggesting that ADP itself does not cause microglial proliferation. As mentioned earlier, microglia with β1 integrin cross-linking showed marked proliferation, but the integrin-induced high proliferation rate was no longer observed in the presence of ADP (Fig. 6a). The addition of ARC-69931 restored the high proliferation rate, suggesting that the ADP signal from the P2Y12/13 receptor did not synergize the proliferative signal from β1 integrin, but rather counteracted it. F6

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

We next investigated whether PKA, a major intracellular target for cAMP in mammalian cells, is involved in this process. Microglial proliferation was studied in the presence of the PKA inhibitor KT-5720 (Fig. 6b). As seen in the control, the addition of KT-5720 at 10 μM did not affect the cell's viability after 24-h incubation. When KT-5720 was added to microglia with anti-β1 integrin antibody, however, the proliferation rate was significantly reduced. These results indicate that the activation of PKA positively regulates the microglial proliferation signal from β1 integrin, in sharp contrast to its effect on P2Y12/13-induced β1 integrin translocation.

DISCUSSION

Microglia are resident tissue macrophages in the brain, exhibiting ramified morphologies in the quiescent state. Once activated by numerous soluble molecules, including cytokines, growth mediators, and nucleotides, however, they quickly respond by transforming into an amoeboid phenotype, migrating to the source of mediators, proliferating and upregulating the expression of various inflammatory cytokines (Nakajima and Kohsaka, 1993; Hanisch, 2002; Inoue, 2002). In the study described herein, we investigated the role of β1 integrin in microglial chemotaxis and proliferation, and characterized its regulation by signals delivered from P2Y12/13 receptors.

Our observation that P2Y12/13 receptors mediate the chemotaxis of microglia on the fibronectin substrate was compatible with the earlier finding by Honda et al. that membrane ruffling is cause by ADP stimulation (Honda et al., 2001). ADP is a potent agonist of P2Y1, P2Y12, and P2Y13. The purine and pyrimidine receptors known to be expressed in microglia include P2X4, P2X7, P2Y2, and P2Y12 (Inoue, 2002; Sasaki et al., 2003; Tsuda et al., 2003), and possibly P2Y13, due to its abundant mRNA in the brain and the immune system (Zhang et al., 2002). P2Y12 and P2Y13 are recently identified Gi-coupled receptors that share the same agonists and antagonists, making it very difficult to pharmacologically differentiate between these two receptors. Whether the P2Y12 and/or P2Y13 receptor was/were truly responsible for the chemotaxis and/or inhibition of proliferation awaits the development of a selective agonist or antagonist.

To mimic damages in the CNS, we performed chemotaxis assays on coverslips coated with an ECM molecule that is known to be expressed in the real pathological situation and thus chose fibronectin, which is upregulated following adult CNS injury (Jones, 1996). In the present study, we demonstrated that β1 integrin is highly expressed in microglia and is crucially involved in the ADP-induced chemotaxis of microglia on fibronectin, and that signals from P2Y12/13 receptors recruit β1 integrin to the membrane ruffle regions. When non-coated coverslips that were pretreated with hydrochloric acid were used in the assays, microglia neither adhered well to the coverslips nor migrated against the ADP gradient (data not shown). These results also indicate that β1 integrin is crucially involved in the adhesion to fibronectin and ADP-induced chemotaxis in the presence of fibronectin in the CNS. β1 integrin couples with α1 integrin through α6 integrin to form VLA-1 through VLA-6, respectively, and with αv integrin to form αvβ1. Among these pairs, VLA-3, VLA-4, VLA-5, and αvβ1 have fibronectin as their ligand, and microglia are known to express α4, α5, and αv chains (Kloss et al., 2001; Milner and Campbell, 2003). The ADP-induced translocation of β1 integrin may contribute to the subsequent formation of focal complexes and then focal adhesions, both of which are important for cell migration.

We have shown that ADP induced β1 integrin translocation to the membrane ruffles by lowering the intracellular cAMP via the P2Y12/13 receptor, and that β1 integrin is important for microglial chemotaxis mediated by the same receptor. Chemotaxis is an integrated process consisting of multiple steps (Lauffenburger and Horwitz, 1996). Integrins are essential for cell migration not only because they mediate adhesion directly, but also because they regulate intracellular signaling pathways required for the cell locomotion (Hood and Cheresh, 2002). When fibroblasts migrate, integrins and other molecules form focal complexes and assemble at the leading edge of the cell. These complexes evolve into highly organized focal adhesions (Laukaitis et al., 2001), which in turn generate a signal to cause actin cytoskeletal reorganization resulting in cell motility (Lauffenburger and Horwitz, 1996). However, details of the signaling pathway(s) generated by β1 integrin translocation at the membrane ruffles and the effect of this outside-in signaling on microglial chemotaxis remain unclear. Although the P2Y12/13 receptor-mediated decrease in cAMP is an important step for microglial migration, it is interesting to note that excess inhibition as well as hyperactivation of cAMP/PKA pathways also inhibits cell migration (Edin et al., 2001; O'Connor and Mercurio, 2001). Thus, an integrin-mediated outside-in signaling may function as a fine and local tuning device to control such signaling cascades for more efficient cell migration.

In the present study, we showed that cross-linking of β1 integrin resulted in a dramatic increase of microglia. CNS injuries and diseases are often accompanied by microglial proliferation (Gehrmann et al., 1995) and our results indicate that this may be due to the β1 integrin engagement by newly upregulated ECM molecules such as fibronectin. The MAP kinase cascade is an essential component of pathways that regulate cell proliferation (Widmann, 1999). In microglia, ERKs are involved in the proliferative response to granulocyte-macrophage colony-stimulating factor (Liva et al., 1999) or corticotropin-releasing hormone (Wang et al., 2003), and the importance of Jak/STAT (Liva et al., 1999) and p38 (Tikka et al., 2001) cascades in proliferation has also been suggested. Integrin engagement also causes the activation of MAP kinases (Chen et al., 1994; Zhu and Assoian, 1995; Schlaepfer and Hunter, 1998) and, therefore, it is likely that the cross-linking of β1 integrin trig-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

gered the proliferation signal of MAP kinases within the cell in our experiments. In view of other evidence that integrin engagement also mediates cytokine production (Miyake et al., 1993; Walzog et al., 1999) and that the proliferation of adherent cells usually requires coordinated signals from growth factor receptors and integrin, which anchors the cell to the ECM (Renshaw, 1997; Aplin et al., 1999), we cannot exclude the possibility that β1 integrin mediated an upregulation of cytokines, which then stimulated the cell in an autocrine fashion during the 24-h incubation.

We demonstrated that ADP canceled out the proliferative effect of β1 integrin engagement, although ADP alone did not influence the cell's viability. The suppression by ADP disappeared when ARC-69931 was added, suggesting that signals from P2Y12/13 receptors are responsible. β1 integrin-mediated proliferation seems to involve PKA, since the proliferation rate was markedly reduced by the downregulation of the cAMP/PKA signaling pathway by ADP or KT-5720. Taken together, we concluded that cAMP positively regulates the β1 integrin-mediated proliferation of microglia through the activation of PKA. In microglia, PKA is known to activate MEKK1/MEK/JNK (Delgado, 2002). Moreover, PKA activity favors the nuclear translocation of ERK1/2 in PC12 and hippocampal neurons, as well as presynaptic sensory neurons from *Aplysia* (Impey et al., 1998; Martin et al., 1998, Yao et al., 1998). These reports support our hypothesis that the β1 integrin engagement promotes microglial proliferation via PKA/MAP kinase pathways.

The finding that the P2Y12/13 receptor-mediated translocation of β1 integrin and membrane ruffle formation were negatively regulated by PKA was in marked contrast to the role of in microglial proliferation. It is AQ1 intriguing to hypothesize that signals from P2Y12/13 receptors impaired the proliferation by shifting integrins from the firm adhesion mode that leads to cell proliferation to the mobile mode, which is more suitable for dynamic movement. Further studies will be necessary to address the functional relationship between β1 integrin and the intracellular PKA concentration in proliferation and chemotaxis.

In summary, both microglial chemotaxis and proliferation clearly consist of numerous and complicated processes in which integrins are fundamentally involved. The results presented show that both functions are tightly linked, possibly through integrins and PKA, and that PKA likely serves as an opposite regulator between the two cellular functions. Elucidation of such mechanisms might reveal new therapeutic strategies for conditions in which the activation of microglia is detrimental such as some neuropathies of the spinal cord (Tsuda et al., 2003).

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Possible Involvement of Increase in Spinal Fibronectin Following Peripheral Nerve Injury in Upregulation of Microglial $P2X_4$, a Key Molecule for Mechanical Allodynia

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ABSTRACT
We have recently demonstrated that the $P2X_4$ receptor, an ATP-gated cation channel, in spinal microglia is a key molecule that mediates the mechanical allodynia induced by peripheral nerve injury. Although microglial $P2X_4$ receptor expression is increased after peripheral nerve injury, the molecular mechanism(s) underlying its upregulation remains largely unknown. Fibronectin is a member of the extracellular matrix molecules and is actively produced in response to injury and diseases in the CNS. Here, we describe the influence of fibronectin on $P2X_4$ receptor expression in microglia and the upregulation of fibronectin after peripheral nerve injury. Microglia that were cultured on fibronectin-coated dishes showed a marked increase in $P2X_4$ receptor expression, both at the mRNA and protein levels, as compared to those cultured on control dishes. Fibronectin also enhanced the microglial Ca^{2+} responses mediated by $P2X_4$ receptors. Moreover, Western blot examination of the spinal cord from a rat with spinal nerve injury indicated that fibronectin was upregulated on the ipsilateral side. Interestingly, intrathecal injection of ATP-stimulated microglia to the rat lumber spinal cord revealed that microglia cultured on fibronectin-coated dishes was more effective in the induction of allodynia than microglia cultured on control dishes. Taken together, our results suggest that spinal fibronectin is elevated after the peripheral nerve injury and it may be involved in the upregulation of the $P2X_4$ receptor in microglia, which leads to the induction of neuropathic pain.

INTRODUCTION

Extracellular nucleotides act as signaling molecules in numerous tissues. Two groups of purinoceptors with distinct signal transduction mechanisms are known to exist. P2X purinoceptors are ligand-gated ion (cation) channels, whereas P2Y purinoceptors are members of the superfamily of G protein-coupled receptors. The P2X family consists of seven different subunits that can form homo- or hetro-oligomeric assemblies, and each subunit has two transmembrane regions with intracellular N- and C-termini. The $P2X_4$ receptor has a broad expression pattern in the periphery, and it predominates in the CNS (Le et al., 1998; Soto et al., 1996). With regard to the physiological and pathological importance of $P2X_4$ in the CNS, we have recently showed that $P2X_4$ receptors in the spinal cord are upregulated after peripheral nerve injury, which is responsible for the induction of mechanical allodynia in rats (Tsuda et al., 2003). Interestingly, the $P2X_4$ receptor is upregulated in microglia but not in neurons in the spinal cord. Allodynia is a form of neuropathic pain that is caused by normally innocuous stimuli, such as touch, and although the symptom has been recognized for over a century, its cellular mechanisms are largely unknown. Microglial $P2X_4$ receptors in the spinal cord could be a key molecule that induces the mysterious neuropathic pain, allodynia.

Microglia are brain-specific macrophages, and their activation is a general response to pathological processes in the CNS. They are in a quiescent state in the normal brain, but become rapidly activated upon brain injury, inflammation, or diseases, transforming from ramified microglia into an amoeboid macrophage-like phenotype. Microglia are known to attach firmly to fibronectin, the upregulation of which is associated with several pathological conditions in the CNS, through β1 integrin and become activated (Milner and Campbell, 2002, 2003). Fibronectin is one of the extracellular matrix (ECM) molecules, and it is a large, multi-domain glycoprotein existing both as a cell surface protein and in plasma. Fibronectin is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion (Adams and Watt, 1993; Hynes, 1992; Raghow, 1994). The expression of ECM molecules is regionally and developmentally regulated in the brain, and their presence is relatively minor in the normal CNS. Some ECM molecules including fibronectin, however, are upregulated following adult CNS injury (Jones, 1996). These

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