

15-Deoxy- $\Delta^{12,14}$ -Prostaglandin J₂ Biphasically Regulates the Proliferation of Mouse Hippocampal Neural Progenitor Cells by Modulating the Redox State^[S]

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ABSTRACT

The activity of neural progenitor cells (NPCs) is regulated by various humoral factors. Although prostaglandin (PG) D₂ is known to mediate various physiological brain functions such as sleep, its actions on NPCs have not been fully understood. In the process of investigating the effects of PGD₂ on NPCs, we found that 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), an endogenous metabolite of PGD₂, exhibits a novel regulation of the proliferation of NPCs derived from mouse hippocampus. 15d-PGJ₂ showed biphasic effects on epidermal growth factor-induced proliferation of NPCs; facilitation at low concentrations (~0.3 μ M) and suppression at higher concentrations (0.5–10 μ M) *in vitro*. 2-Chloro-5-nitrobenzanilide (GW9662), an inhibitor of peroxisome proliferator-activated receptor γ , known to be a molecular target for 15d-PGJ₂, failed to abolish the effects of

15d-PGJ₂. 9,10-dihydro-15d-PGJ₂ (CAY10410), a structural analog of 15d-PGJ₂ lacking the electrophilic carbon in the cyclopentenone ring, did not show 15d-PGJ₂-like actions. Treatment with 15d-PGJ₂ increased the levels of reactive oxygen species and decreased endogenous GSH levels. Furthermore, supplementation with a membrane-permeable analog of glutathione, GSH ethyl ester (2 mM), diminished the biphasic effects of 15d-PGJ₂. Finally, cell division in the dentate gyrus of postnatal mice was increased by injection of low-dose (1 ng *i.c.v.*) 15d-PGJ₂ and suppressed by high-dose (30 ng) 15d-PGJ₂. These results suggest that 15d-PGJ₂ regulates the proliferation of NPCs via its electrophilic nature, which enables covalent binding to molecules such as GSH.

Recent extensive studies have elucidated that three cell types in the brain (neurons, astrocytes, and oligodendrocytes) originate from immature precursor cells referred to as neural progenitor cells (NPCs) (Weiss et al., 1996; Gage, 2000). During development, NPCs primarily undergo extensive self-renewal and then generate neurons; later, they sequentially generate astrocytes and oligodendrocytes (Miller and Gau-

thier, 2007). NPCs are located not only in the developing mammalian brain but also in the adult brain and are especially abundant in the anterior subventricular zone and subgranular zone of the hippocampal dentate gyrus (DG) (Eriksson et al., 1998). The self-renewal and multipotential activities of NPCs are dynamically regulated by various humoral factors under physiological and pathophysiological conditions, such as ischemia (Nakatomi et al., 2002), seizure (Parent et al., 1997), and sleep (Guzmán-Marín et al., 2003). The elucidation of signaling molecules regulating NPC activity may contribute not only to the understanding of neurogenesis but also to the development of new therapies against nervous system disorders; however, the cellular mechanisms underlying regulation of NPC activity have not been fully understood.

Prostaglandins (PGs) are a group of 20-carbon fatty acids

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ABBREVIATIONS: NPC, neural progenitor cell; DG, dentate gyrus; PG, prostaglandin; 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; MHM, media-hormone mix; EGF, epidermal growth factor; BWA868C, 3-[[2-(cyclohexyl-2-hydroxyethyl)amino]-2,5-dioxo-1-(phenylmethyl)-4-imidazolidineheptanoic acid; CAY10410, 9,10-dehydro-15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; GW9662, 2-chloro-5-nitrobenzanilide; BrdU, bromodeoxyuridine; IgG, immunoglobulin G; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; GFAP, anti-glial fibrillary acidic protein; GSH-EE, GSH reduced ethyl ester; WST-8; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PPAR, peroxisome proliferator-activated receptor; PMA, phorbol 12,13-myristate acetate; PFA, paraformaldehyde; PBS, phosphate-buffered saline; PBSGT, PBS containing 1% normal goat serum and 0.3% Triton X-100; PB, phosphate buffer; ANOVA, analysis of variance; MCI-186, edaravone; DP1, prostaglandin D₂ receptor type 1; DP2, prostaglandin D₂ receptor type 2; Trx, thioredoxin; LPS, lipopolysaccharide.

that are produced within cells via the cyclooxygenase pathway from arachidonic acid in response to a variety of extrinsic stimuli (Smith, 1989, 1992), and some PGs, such as PGE₂, are reported to regulate the activity of NPCs (Uchida et al., 2002). Prostaglandin D₂ (PGD₂) is considered to be a molecular candidate regulating NPCs, because it is the most abundant PG in the brain (Ogorochi et al., 1984) and is known to play critical roles in sleep (Hayaishi, 2002; Herlong and Scott, 2006; Huang et al., 2007), which drastically alters NPC activity (Guzmán-Marín et al., 2003). In the process of investigating the effects of sleep-related factors on the proliferation of mouse hippocampal NPCs, we observed that PGD₂ showed biphasic actions on NPC proliferation (Supplemental Fig. 1). Furthermore, we found that 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), a nonenzymatic metabolite of PGD₂, exhibited a novel regulation of the proliferation of NPCs. 15d-PGJ₂ is endogenously produced from PGD₂ through spontaneous nonenzymatic dehydration followed by isomerization and is an endogenous ligand for peroxisome proliferator-activated receptor γ (PPAR γ) (Forman et al., 1995), which plays a critical role in the regulation of cell differentiation and metabolism (Debril et al., 2001; Walczak and Tontonoz, 2002). On the other hand, it is also reported that 15d-PGJ₂ covalently binds with nucleophilic molecules such as GSH via an electrophilic carbon at position 9 in its cyclopentenone ring (Rossi et al., 2000; Straus et al., 2000; Oliva et al., 2003). GSH is known to be a major endogenous antioxidant that protects cells from reactive oxygen species (ROS) such as free radicals and peroxides (Shibata et al., 2003); thus, decreases in GSH levels would lead to the elevation of ROS levels. At present, little is known about the effects of 15d-PGJ₂ on the activity of NPCs. In this study, we examined the mode of 15d-PGJ₂ actions on the proliferation of mouse hippocampal NPCs *in vitro* and *in vivo*.

Materials and Methods

Animals. Pregnant female and young male (5 weeks old) ICR mice (SLC, Shizuoka, Japan) were used. All mice were housed in polypropylene cages (31 × 22 × 14 cm) with wood shavings and were maintained in an environment with a controlled temperature (23 ± 2°C) and light (12-h light/dark cycle). Food and water were available *ad libitum*. All animal housing and surgical procedures were performed in accordance with the guidelines of the Japanese Pharmacological Society and were approved by the Institutional Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University.

Cell Culture. NPCs were isolated and propagated by a neurosphere method as reported previously (Reynolds et al., 1992) with minor modifications (Moriya et al., 2007). Fetuses on embryonic day 15.5 (embryonic day 0 is defined as midnight on the day of overnight mating) were isolated from their mothers under deep anesthesia with diethyl ether and placed into an ice-cold 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient (Invitrogen, Carlsbad, CA). The hippocampal tissues were carefully micro-dissected using a stereomicroscope and triturated with a 1-ml plastic pipette to obtain a single cell suspension in media-hormone mix (MHM) (Dulbecco's modified Eagle's medium and Ham's F-12 nutrient supplemented with 0.34% glucose, 23 μ g/ml insulin, 92 μ g/ml transferrin, 55 μ M putrescine, 27.5 nM sodium selenite, 20 nM progesterone, 50 U/ml penicillin, and 50 μ g/ml streptomycin). The viable dissociated cells at a density of 4×10^5 cells/ml (total 12 ml) in MHM containing 20 ng/ml epidermal growth factor (EGF; Invitrogen) and 20 ng/ml fibroblast growth factor 2 (PeproTech EC, London, UK)

were seeded into uncoated T75 culture flasks (BD Biosciences, Franklin Lake, NJ) and were maintained in a humidified incubator at 37°C with 95% atmospheric air and 5% CO₂. The cells were fed 3 ml of fresh medium every second day and were incubated for 5 days to form a sufficient number of neurospheres.

Reagents. 15d-PGJ₂, BWA868C, and CAY10410 were purchased from Cayman Chemical (Ann Arbor, MI) and were dissolved in ethanol. Ramatroban was kindly provided by Bayer Yakuhin (Osaka, Japan) and was dissolved in ethanol. GW9662 was purchased from ALEXIS Biochemicals (San Diego, CA) and was dissolved in ethanol. Bromodeoxyuridine (BrdU) was purchased from Nacalai Tesque (Kyoto, Japan). Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 568-conjugated goat anti-rabbit IgG, Alexa Fluor 568-conjugated goat anti-rat IgG, and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) were purchased from Invitrogen. Mouse anti- β -tubulin type III (Tuj1), rabbit anti-gial fibrillary acidic protein (GFAP), GSH reduced ethyl ester (GSH-EE), β -NADPH, 5,5'-dithio-bis[2-nitrobenzoic acid], sulfosalicylic acid (Dayer et al.), and GSH reductase were purchased from Sigma-Aldrich (St. Louis, MO). Mouse anti-nestin monoclonal antibody was purchased from Chemicon (Temecula, CA).

Experimental Procedure. The effects of 15d-PGJ₂ on the proliferation of NPCs were evaluated by two methods: 1) indirect measurement of the number of viable cells by WST-8 assay; and 2) a BrdU incorporation assay. We did not adopt direct and simple counting of cell number because 1) NPCs form colonies (spheres) that prevent us from measuring cell number without enzymatic dissociation, and 2) dead cells especially appeared under EGF-free conditions or in the presence of a high concentration of 15d-PGJ₂, and these could not be discriminated from viable cells. As described under *WST-8 Assay*, although the WST-8 assay is widely used for measuring relative numbers of NPCs (Kanemura et al., 2002; Jiang et al., 2005; Moriya et al., 2007), it measures the metabolic activity of cells. Therefore, we also examined DNA synthesis activity using a BrdU incorporation assay to confirm the results of the WST-8 assay. Single-cell suspensions from primary neurospheres were prepared by centrifugation (300g, 5 min) followed by enzymatic dissociation with collagenase (1 mg/ml). The cells were then allowed to pass through a 40- μ m nylon net and were seeded in nontreatment 96-well plates (BD Biosciences) at a density of 1×10^5 cells/ml, and incubated for 12 h in the absence of EGF. Thereafter, they were stimulated with or without various concentrations of 15d-PGJ₂ (0.01–10 μ M), PGD₂ (0.1–10 μ M), or CAY10410 (0.01–10 μ M) in the presence (2 or 20 ng/ml) or absence of EGF. In some experiments, cells were pretreated with the DP1 antagonist BWA868C (10 μ M), the DP2 antagonist ramatroban (10 μ M) or the PPAR γ antagonist GW9662 (0.1 μ M) for 1 h before 15d-PGJ₂ or PGD₂ stimulation.

WST-8 Assay. The number of viable cells was indirectly estimated by the WST-8 assay using a Cell Counting Kit-SF (Nacalai Tesque), which has been used for to estimate the proliferative activity of NPSs cultured by the neurosphere method (Kanemura et al., 2002; Jiang et al., 2005; Moriya et al., 2007). WST-8 is reduced by the mitochondrial enzyme, NAD-dependent succinate dehydrogenase, to form a colored formazan product, which is soluble in the culture medium. The amount of formazan dye generated by the activity of the dehydrogenases in cells is known to be directly proportional to the number of living cells (Jiang et al., 2005). It has been reported that indirect measurements of viable cells based on metabolic activity are effective and reproducible ways to determine the numbers of viable cells, especially of viable NPCs present within intact neurospheres, without breaking the neurospheres (Kanemura et al., 2002). After NPCs were treated as described above for 48 h, 5 μ l of the Cell Counting Kit solution was added to each well and incubated for an additional 5 h at 37°C. The absorbance at 450 nm with a reference wavelength of 595 nm was measured using a microplate reader (TECAN, Männedorf, Switzerland), and the net absorbance subtracting the values of cell-free wells was calculated.

BrdU Incorporation Assay. After incubation in the absence of EGF for 12 h, NPCs were treated with or without various concentrations of 15d-PGJ₂ in the presence or absence of EGF for 48 h. BrdU (1 μ M) was added to the culture medium for the appropriate length of time. Subsequently, cells were fixed with 4% paraformaldehyde (PFA) for 15 min, washed with phosphate-buffered saline (PBS), and then permeabilized by incubation with 2 N HCl at 37°C for 5 min. After removal of HCl, they were washed with 0.15 M sodium borate, pH 8.5, at room temperature for 10 min. They were then incubated with PBS containing 1% normal goat serum and 0.3% Triton X-100 (PBSGT) for 2 h, and incubated overnight with rat anti-BrdU antibody (1:200; Oxford Biotechnology, Kidlington, UK) in PBSGT at 4°C. After removal of the primary antibody solution, cells were washed with PBS and incubated with secondary antibody (Alexa Fluor 568-conjugated goat anti-rat IgG; 1:200) and 1 μ g/ml Hoechst 33258 for nuclear counterstaining, for 2 h at room temperature under light-shading conditions. After washing cells with PBS, fluorescent images were automatically obtained using the CelaView imaging system (Olympus, Tokyo, Japan), and the numbers of BrdU-labeled cells and Hoechst-stained nuclei were automatically counted by the Scan system (Olympus).

TUNEL Staining. TUNEL staining was performed using the In Situ Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany) according to the manufacturer's instructions. In brief, the dispersed NPCs from the primary neurospheres were seeded into Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) precoated with 30 μ g/ml poly-L-lysine (Sigma) and 15 μ g/ml laminin at a density of 4×10^5 cells/ml for a 12-h incubation period in the absence of EGF. After NPCs were treated with or without of various concentrations of 15d-PGJ₂ in the presence of EGF (2 ng/ml) for 48 h, they were fixed with 4% PFA for 15 min and then washed with PBS. After permeabilization with 0.1% citrate buffer containing 0.1% Triton X-100 for 2 min on ice, the cells were incubated with TUNEL mix for 1 h at 37°C, and then washed with PBS. Thereafter, Hoechst 33258 was added to cells at a concentration of 1 μ g/ml. After washing with PBS, the coverslips were mounted on glass slides. The images in nine randomly selected areas (each $350 \times 350 \mu$ m) for each well were obtained using a fluorescence microscope (10 \times objective lens; IX70; Olympus Tokyo, Japan), and the numbers of TUNEL-positive cells and total cells stained with Hoechst dye were counted by an observer without knowledge of the treatment.

ROS Measurement. ROS levels in NPCs were measured using the fluorescence probe H₂DCFDA. After incubation in the absence of EGF for 12 h, NPCs were treated with or without various concentrations of 15d-PGJ₂, CAY10410, phorbol 12,13-myristate acetate (PMA) and/or GSH-EE in the presence of EGF (2 ng/ml) for 2 h. H₂DCFDA (10 μ M) was added to cells during the last 30 min of the incubation period. Immediately after washing cells with MHM, fluorescent images were obtained using a fluorescence microscope (Olympus IX70) and the numbers of H₂DCFDA-positive cells and the level of fluorescence intensity per cell were evaluated by Scion Image (Scion Corporation, Frederick, MD).

Total Intracellular GSH Measurement. NPCs were treated with or without various concentrations of 15d-PGJ₂ in the presence of EGF (2 ng/ml) for 12 h. The cells were lysed in 5% 5-sulfosalicylic acid solution. The samples were incubated with working solution (6 units/ml GSH reductase and 1.5 mg/ml 5,5'-dithiobis[2-nitrobenzoic acid] in 0.1 M potassium phosphate buffer with 1 mM EDTA) for 5 min followed by addition of 0.16 mg/ml β -NADPH. The total GSH levels were determined by measuring the absorbance at 412 nm with a TECAN microplate reader and the concentration of total GSH (nanomoles per milliliter) was calculated based on a reduced glutathione standard curve. The protein concentrations of the cell lysates were determined by DC protein assay and the total contents of GSH, including both reduced glutathione and oxidized glutathione (nanomoles per milligram of protein), were calculated.

Differentiation Assay. NPCs were seeded into 96-well plates (Nalge Nunc International) precoated with 30 μ g/ml poly-L-lysine

followed by 15 μ g/ml laminin and incubated for 12 h in the presence of EGF (2 ng/ml). Then, NPCs were treated with or without various concentrations of 15d-PGJ₂ in the absence of EGF for 96 h. Thereafter, NPCs were fixed with 4% PFA for 15 min and then washed with PBS. They were incubated with PBS containing 0.5% bovine serum albumin and 0.3% Triton X-100 for 2 h and incubated overnight with primary antibody (mouse anti-Tuj1, 1:200; rabbit anti-GFAP, 1:200) in PBS containing 0.5% bovine serum albumin and 0.3% Triton X-100 at 4°C. After removal of the primary antibody solution, cells were washed with PBS and incubated with secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG, 1:200; Alexa Fluor 568-conjugated goat anti-rabbit IgG, 1:200) and 1 μ g/ml Hoechst 33258 for nuclear counterstaining, for 2 h at room temperature under light-shading conditions. After washing cells with PBS, fluorescent images were automatically obtained using the CelaView imaging system (Olympus) and the numbers of Tuj1- and GFAP-positive cells as well as the numbers of Hoechst-stained nuclei were counted by the Scan system (Olympus).

Nestin Staining. NPCs were seeded in Lab-Tek chamber slides precoated with 30 μ g/ml poly-L-lysine followed by 15 μ g/ml laminin. Then, NPCs were treated with medium in the absence of EGF for 2 h. Thereafter, NPCs were fixed with 4% PFA for 15 min and washed with PBS. The cells were stained using primary antibody (mouse anti-nestin; 1:300) and secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG; 1:200) and 1 μ g/ml Hoechst 33258 as described above.

Schedule of Drug Treatment and Sampling in Vivo. Male ICR mice at 5 weeks of age were administered vehicle or 15d-PGJ₂ (0–30 ng/3 μ l saline per mouse i.c.v.) and were injected with BrdU (50 mg/kg i.p.), 5 or 24 h after 15d-PGJ₂ injection. Two hours after BrdU injection, mice were deeply anesthetized with diethyl ether and then perfused intracardially with 25 ml of chilled saline followed by 25 ml of 4% PFA in 0.1 M phosphate buffer (PB). Thereafter, their brains were quickly removed.

Immunohistochemistry. The brains were fixed with 4% PFA in 0.1 M PB overnight at 4°C, followed by immersion in 20% sucrose in 0.1 M PB for 48 h. The brains were cut into 40- μ m sections from the rostral to the caudal edge of the dentate gyrus using a cryostat (HM560; Mikron Instrument, Inc., San Marcos, CA) and the sections were alternately divided into two groups. For BrdU immunohistochemistry, one group of sections was treated with HCl (2 N) at 37°C for 20 min, then neutralized twice with sodium borate buffer (0.15 M, pH 8.5) at room temperature for 10 min. After three washes with PBS, the sections were incubated with rat anti-BrdU antibody (1:200) diluted with PBSGT at 4°C overnight, followed by Alexa Fluor 568-conjugated goat anti-rat IgG (1:200) and 1 μ g/ml Hoechst 33258, diluted with PBSGT at room temperature for 2 h.

Quantification of the Number of BrdU-Labeled Cells. The numbers of BrdU-labeled cells were counted using a 10 \times objective (IX70; Olympus, Tokyo, Japan) throughout the rostrocaudal extent of the dentate gyrus. We counted the number of BrdU-labeled cells in the subgranular zone, which was defined as a two-cell body wide zone (approximately 10 μ m) along the border of the GCL and the hilus. Because we stained every second section, resulting numbers were then multiplied by two to obtain the estimated total number of BrdU-labeled cells per dentate gyrus. The counting was performed by an observer with no knowledge of the experimental groups.

Statistical Analysis. Results are expressed as the means \pm S.E.M. The statistical significance of differences was determined by one-way ANOVA followed by Dunnett's test or Fisher's protected least significant difference test. Results were considered to be significant if the *p* value was less than 0.05.

Results

The Effect of 15d-PGJ₂ on EGF-Induced Proliferation of Cultured Hippocampal NPCs. We expanded NPCs from embryonic mouse hippocampus using the neurosphere

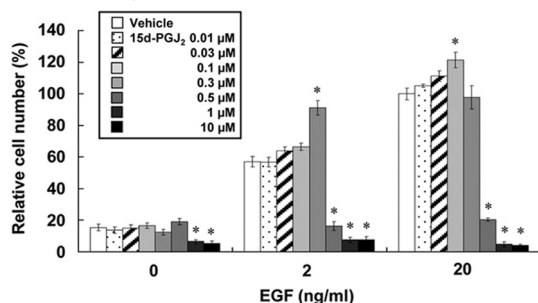
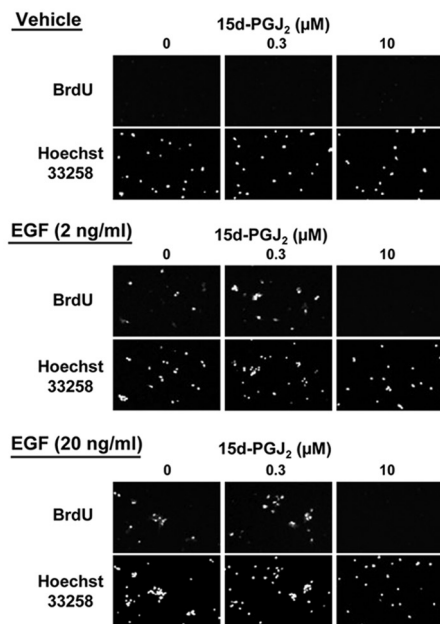
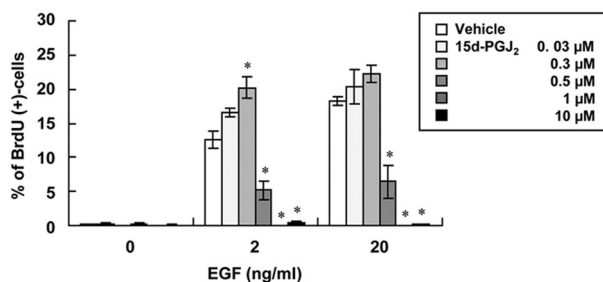
A WST-8 assay**B BrdU incorporation****(a) Representative images****(b) Quantified data**

Fig. 1. The effect of 15d-PGJ₂ on EGF-induced proliferation of cultured NPCs. Dispersed NPCs from primary neurospheres were seeded in non-treatment 96-well plates at a density of 10^5 cells/ml and cultured for 12 h in the absence of EGF. Subsequently, they were treated with or without various concentrations of 15d-PGJ₂ (0.01–10 μ M) in the presence or absence of EGF (0, 2, or 20 ng/ml) for 48 h. The number of viable cells and the DNA synthesis activity were assessed by WST-8 assay (A), and BrdU incorporation assay (B), respectively. A, in the WST-8 assay, WST-8 solution was added to each well and incubated for an additional 5 h. The value of the vehicle group in the presence of 20 ng/ml EGF was set to 100%. The data are means \pm S.E.M. of four to six wells. *, significant difference from vehicle control in each concentration of EGF ($p < 0.05$, one-way ANOVA followed by Dunnett's test). B, in the BrdU incorporation assay, cells were labeled with BrdU (1 μ M) 12 h before fixation, and BrdU-labeled cells were stained by immunocytochemistry. Representative images (a) and the proportion of BrdU-labeled cells among all cells (b) are shown. The data are means \pm S.E.M. of four to five wells. *, significant difference from vehicle control in each concentration of EGF ($p < 0.05$, one-way ANOVA followed by Dunnett's test). Scale bar, 200 μ m.

method and confirmed that a large proportion of neurosphere cells ($95.3 \pm 0.73\%$) expressed nestin, a marker for NPCs under our experimental conditions. In our pilot study investigating the effects of sleep-related compounds on the proliferation of cultured NPCs from embryonic mouse hippocampus, we observed that PGD₂ exhibited biphasic actions on the proliferation of NPCs. In examining the effects of PGD₂ and 15d-PGJ₂, we used not only the most popular concentration of EGF (20 ng/ml), but also a relatively low concentration of EGF (2 ng/ml) as well as EGF-free conditions, because it has been reported that the growth-promoting activity of EGF on embryonic brain-derived neurospheres shows a concentration dependence between 0.5 and 20 ng/ml (Tropepe et al., 1999), and a submaximal concentration of 2 ng/ml was considered to be optimal for finding the accelerative and/or suppressive potential of PG on the proliferation of NPCs. As shown in Supplemental Fig. 1, PGD₂ increased the number of NPCs when present at a concentration of approximately 2 μ M but decreased the number when present at concentrations of more than 3 μ M in the presence of EGF (2 and 20 ng/ml). The maximum increase by PGD₂ was greater in the 2 ng/ml EGF condition (190%) than in the 20 ng/ml condition (133%) (Supplemental Fig. 1). Under the EGF-free condition, PGD₂ increased cell number at concentrations of 2 to 3 μ M but did not decrease it at high concentrations (Supplemental Fig. 1). Because PGD₂ has been reported to be a ligand for

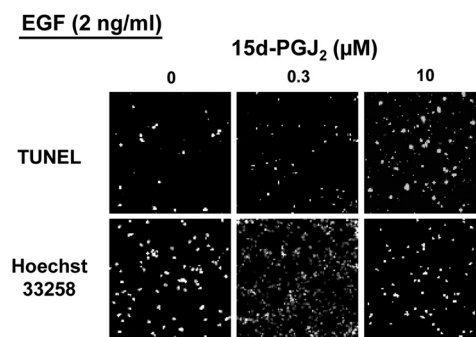
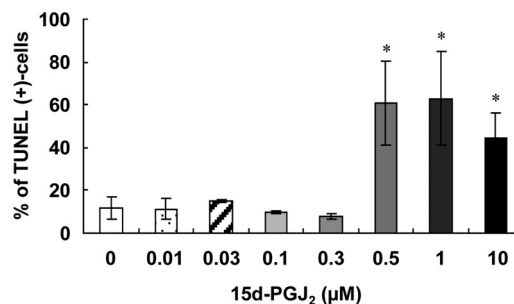
A Representative images**B Quantified data**

Fig. 2. The effect of 15d-PGJ₂ on the cell death of NPCs. Dispersed NPCs from primary neurospheres were seeded in Lab-Tek chamber slides pre-coated with poly-L-lysine, followed by laminin, at a density of 4×10^5 cells/ml and cultured for 12 h in the absence of EGF. Subsequently, they were treated with or without various concentrations of 15d-PGJ₂ (0.01–10 μ M) in the presence of EGF (2 ng/ml) for 48 h. After cells were fixed with PFA, the dead cells were visualized by TUNEL staining. Representative images (A) and the proportion of TUNEL (+)-cells (B) are shown. The data are means \pm S.E.M. of three wells. *, significant difference from vehicle control ($p < 0.05$, one-way ANOVA followed by Dunnett's test). Scale bar, 200 μ m.

two types of G-protein-coupled receptors (DP1 and DP2), we investigated the effects of a DP1 antagonist (BWA868C) and a DP2 antagonist (ramatroban) on the regulation of proliferation by PGD₂ (Supplemental Fig. 1). Although there were some significant differences between the vehicle and antagonist groups at each concentration of PGD₂ (BWA868C, 1 μ M in 2 ng/ml EGF; 3 and 5 μ M in 20 ng/ml EGF; ramatroban, 0.1 μ M in 0 ng/ml EGF; 0.1, 0.3, and 2 μ M in 2 ng/ml EGF; and 5 μ M in 20 ng/ml EGF, $p < 0.05$, Student's t test, significance marks not shown in figure), the biphasic actions of PGD₂ on proliferation of NPCs were still evident in each BWA868C and ramatroban group (Supplemental Fig. 1). These results suggest that neither DP1 nor DP2 mediates the effects of PGD₂ on the proliferation of NPCs.

We next examined the effect of 15d-PGJ₂ on the self-renewal ability of cultured hippocampal NPCs, because PGD₂ is reported to be nonenzymatically converted to 15d-PGJ₂ even in culture medium (Shibata et al., 2002). We employed the WST-8 assay, which indirectly measures the viable cell number based on their metabolic activity. EGF increased the number of viable cells in a concentration-dependent manner (Fig. 1A). In the presence of EGF (2 and 20 ng/ml), 15d-PGJ₂ at low concentrations (~ 0.3 μ M) significantly increased the number of viable cells, whereas at high concentrations (0.5–10 μ M), it significantly reduced the number of viable cells. In contrast to PGD₂, 15d-PGJ₂ at lower concentrations failed to increase the cell number under EGF-free conditions; however, it reduced the number of viable cells at high concentrations (1–10 μ M). These results show that 15d-PGJ₂ exhibits biphasic actions on EGF-induced increase in viable

NPC number. The increase in NPC number induced by 15d-PGJ₂ was more marked in the presence of a low concentration of EGF (2 ng/ml) than in the presence of a high concentration of EGF (20 ng/ml), whereas the suppression of NPC number by 15d-PGJ₂ was evident even in the presence of a high concentration of EGF (20 ng/ml). To determine whether the regulation of viable cell number by 15d-PGJ₂ is due to an action on cell division, we next examined the effect of 15d-PGJ₂ on DNA synthesis activity of NPCs using a BrdU incorporation assay. We first investigated the time dependence of BrdU incorporation to determine the plateau reached after labeling the whole cycling population under our experimental condition. BrdU (1 μ M) was added to the culture medium during the last 12 h (36–48 h after EGF stimulation), 24 h (24–48 h), 36 h (12–48 h), or 48 h (0–48 h) of the exposure period, and cells were fixed and processed for BrdU immunocytochemistry. As shown in Supplemental Fig. 2, the proportion of BrdU-labeled cells increased in EGF concentration- and exposure time-dependent manners and reached a plateau after 36 h of labeling time. The maximum proportion of BrdU-labeled cells (25–33%) was comparable with that reported by another laboratory using the neurosphere method (approximately 40% of BrdU-labeled cells after 16 h of exposure) (Palazuelos et al., 2006), suggesting that the population of cells undergoing cell cycling is approximately 30 to 40% in neurosphere culture conditions. Based on this observation, we adopted the last 12 h of the BrdU-labeling protocol to examine accelerative and/or suppressive potential of 15d-PGJ₂ on the proliferation of NPCs. EGF increased the proportion of BrdU-labeled cells in a concentration-dependent

A Representative images

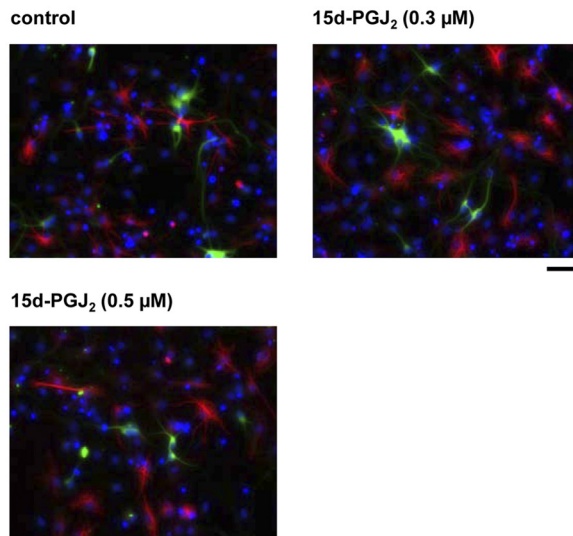
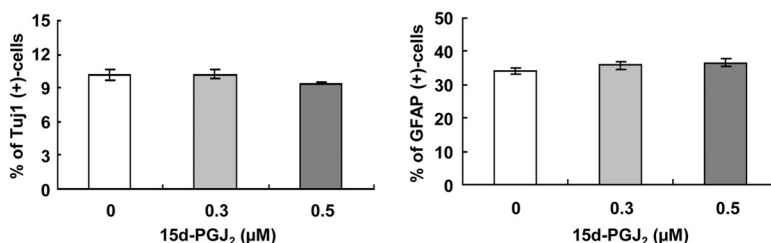


Fig. 3. The effect of 15d-PGJ₂ on the multipotency of cultured NPCs. NPCs were treated with or without various concentrations of 15d-PGJ₂ in the absence of EGF for 96 h. Differentiation was evaluated by fluorescence-based immunocytochemistry using a mouse monoclonal antibody against Tuj1 (green), a neural marker, a rabbit polyclonal antibody against GFAP (red), an astroglial marker, and Hoechst 33258 (blue). Representative immunofluorescence images (A) and the proportion of Tuj1 (+)-cells (left) and GFAP (+)-cells (right) (B) are shown. The data are means \pm S.E.M. of four wells. Scale bar, 50 μ m.

B Quantified data



manner (Fig. 1B). In the presence of EGF (2 and 20 ng/ml), 15d-PGJ₂ at a low concentration (0.3 μ M) significantly increased the amount of BrdU incorporation, whereas at a high concentration (over 0.5 μ M), it significantly suppressed the amount of BrdU incorporation (Fig. 1B).

The Effect of 15d-PGJ₂ on the Cell Death of NPCs. We next investigated whether 15d-PGJ₂ induced cell death using TUNEL staining, which enabled us to detect apoptosis-associated DNA strand breaks (Gavrieli et al., 1992). As shown in Fig. 2, the proportion of TUNEL-positive cells in control group was $11.62 \pm 5.06\%$. Treatment with 15d-PGJ₂ at high concentrations (1–10 μ M) caused an approximately 3.8- to

5.4-fold increase in the proportion of TUNEL-positive cells, whereas low concentrations (0.01–0.3 μ M) of 15d-PGJ₂ failed to affect this proportion. Likewise, PGD₂ at high concentrations (3–10 μ M) significantly increased the proportion of TUNEL-positive cells (Supplemental Fig. 3). We noticed that the proportion of TUNEL-positive cells induced by 15d-PGJ₂ was greater than by that induced by PGD₂.

The Effect of 15d-PGJ₂ on the Multipotency of Cultured NPCs. Because NPCs are known to differentiate into neural and glial cells in the absence of growth factor, we examined the effect of 15d-PGJ₂ on the multipotency of cultured NPCs using immunocytochemistry with antibodies to neuronal (Tuj1) or astroglial (GFAP) markers. There were no significant changes in the proportions of Tuj1-positive and GFAP-positive cells after treatment with 15d-PGJ₂ at the examined concentrations (0.3–0.5 μ M) (Fig. 3). Treatment with 1 μ M 15d-PGJ₂ caused abundant cell death in the absence of EGF (data not shown).

The Effect of PPAR γ Antagonist on the Action of 15d-PGJ₂ on the Proliferation of NPCs. Because 15d-PGJ₂ is known to act as a natural ligand for PPAR γ , we next examined the effect of the PPAR γ -irreversible antagonist GW9662 on the regulation of proliferation by 15d-PGJ₂ using the WST-8 assay. GW9662 alone did not affect basal or EGF-induced proliferation in the absence of 15d-PGJ₂ (Fig. 4). Even in the presence of GW9662 (0.1 μ M), 15d-PGJ₂ at low concentrations (\sim 0.3 μ M) facilitated EGF-induced proliferation of NPCs, whereas at high concentrations (1–10 μ M), it suppressed EGF-induced proliferation of NPCs (Fig. 4). Pretreatment with GW9662 also failed to affect the suppression of the viable cell number induced by 15d-PGJ₂ at high concentrations (1 and 10 μ M) in the absence of EGF. Although it has been reported that GW9662 shows some cell toxicity at concentrations higher than 1 μ M (Ray et al., 2006), the biphasic actions of 15d-PGJ₂ on EGF-induced proliferation of NPCs were still observed in the presence of a high concentration of GW9662 (10 μ M) (data not shown). In the same way, we investigated the effects of GW9662 on the regulation of proliferation by PGD₂ (Supplemental Fig. 4). Although there were some significant differences between vehicle and

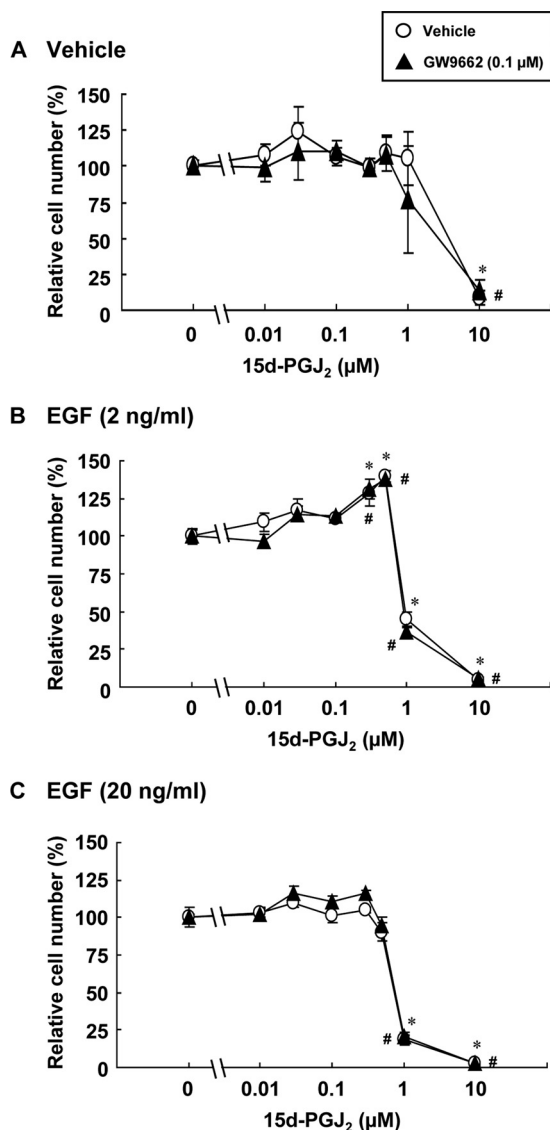


Fig. 4. The effect of PPAR γ antagonist on the regulation of NPC proliferation by 15d-PGJ₂. Dispersed NPCs from primary neurospheres were seeded in nontreatment 96-well plates at a density of 10^5 cells/ml and cultured for 12 h in the absence of EGF. After pretreatment with or without PPAR γ antagonist GW9662 (0.1 μ M) for 1 h, cells were stimulated with or without various concentrations of 15d-PGJ₂ (0.01–10 μ M) in the presence or absence of EGF (0, 2, or 20 ng/ml) for 48 h. The number of viable cells was assessed by WST-8 assay. The value of control group was set to 100%. The data are means \pm S.E.M. of three to four wells. *, significant difference from 15d-PGJ₂-free (0 μ M) in vehicle group; #, significant difference from 15d-PGJ₂-free (0 μ M) in GW9662 group ($p < 0.05$, one-way ANOVA followed by Dunnett's test). Note that there was no significant difference between the vehicle and GW9662 groups.

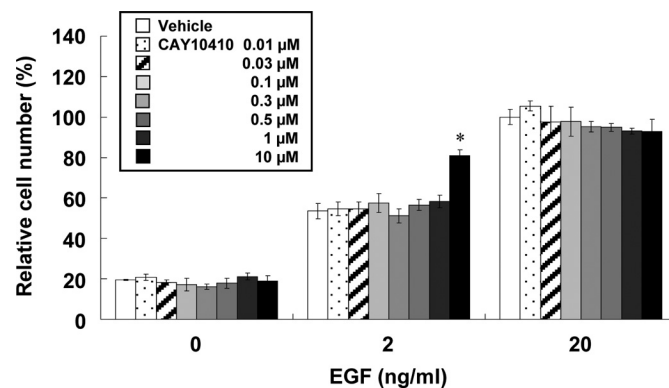


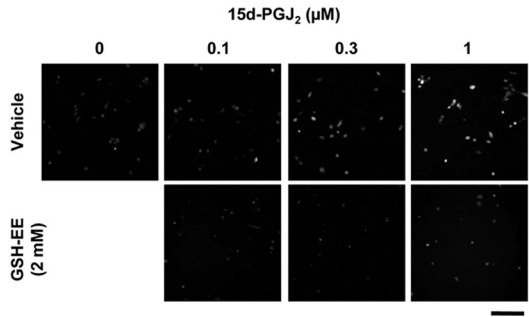
Fig. 5. The effect of CAY10410 on EGF-induced proliferation of NPCs. Dispersed NPCs from primary neurospheres were seeded in nontreatment 96-well plates at a density of 10^5 cells/ml and cultured for 12 h in the absence of EGF. Subsequently, they were treated with or without various concentrations of CAY10410 (0.01–10 μ M) in the presence or absence of EGF (0, 2, and 20 ng/ml) for 48 h. WST-8 solution was added to each well and incubated for an additional 5 h. The value of the vehicle group at 20 ng/ml EGF was set to 100%. The data are means \pm S.E.M. of three to four wells. *, significant difference from the vehicle control in 2 ng/ml EGF group ($p < 0.05$, one-way ANOVA followed by Dunnett's test).

GW9662 groups at some concentrations of PGD₂ (3 μM in the absence of EGF, 2 and 3 μM in the presence of 2 ng/ml EGF, and 1, 2, and 3 μM in the presence of 20 ng/ml EGF, *p* < 0.05, Student's *t* test, significance marks not shown in figure), the biphasic actions of PGD₂ on proliferation of NPCs were still observed in the GW9662-treated group (Supplemental Fig. 4).

The Effect of CAY10410 on EGF-Induced Proliferation of NPCs. To investigate whether the electrophilic property of 15d-PGJ₂ is necessary for its action, we examined the effect of CAY10410, a structural analog of 15d-PGJ₂ with PPARγ agonistic activity but lacking the electrophilic carbon at position 9 in the cyclopentenone ring (Ray et al., 2006). We found that CAY10410 at concentrations up to 1 μM failed to influence EGF-induced proliferation of NPCs (Fig. 5). However, CAY10410 at 10 μM significantly increased the viable cell number in the presence of 2 ng/ml EGF. These results suggest that the electrophilic carbon at position 9 in 15d-PGJ₂ is critical for its biphasic effects on the proliferation of NPCs.

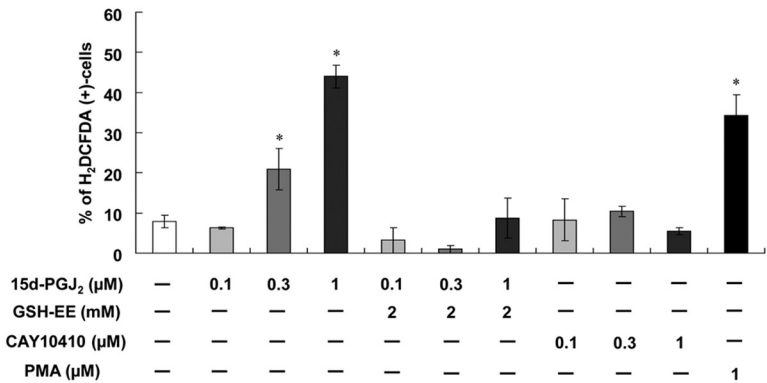
The Effect of 15d-PGJ₂ on ROS Levels in NPCs. Because PPARγ was not significantly involved in the regulation of the proliferation by 15d-PGJ₂, we further investigated the mechanisms underlying such regulation by 15d-PGJ₂. It has been reported that the intracellular ROS levels in SH-SY5Y cells were elevated by 15d-PGJ₂ in a concentration-dependent manner (Shibata et al., 2003). To determine whether 15d-PGJ₂ affects ROS levels in NPCs, we used a ROS-detecting probe, H₂DCFDA. This cell-permeant indicator is nonfluorescent and contains acetate groups that can be hydrolyzed by intracellular esterase, which enables it to react with oxidants to generate detectable fluorescence (Hempel et al., 1999; Ray et al., 2006). As shown in Fig. 6, 15d-PGJ₂ increased the proportion of H₂DCFDA-fluorescence-positive cells as well as the level of fluorescence intensity per cell in a concentration-dependent manner, indicating that 15d-PGJ₂ elevated intracellular ROS levels. By contrast, CAY10410 failed to affect the intracellular ROS level at concentrations of up to 1 μM. Furthermore, pre-

A Representative images



B Quantified data

(a) Proportion of positive cells



(b) Averaged intensity levels

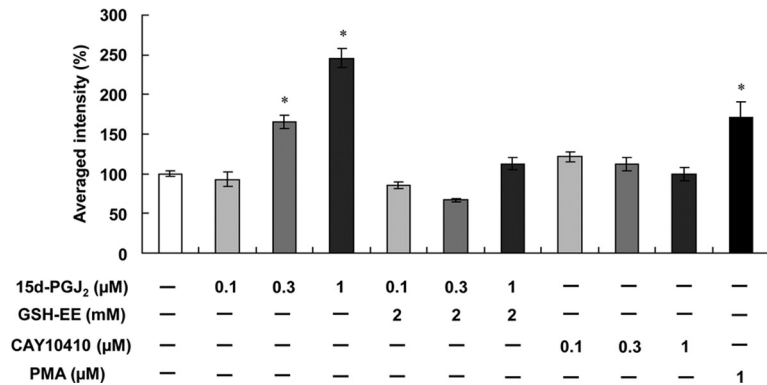


Fig. 6. The effect of 15d-PGJ₂ on ROS levels in NPCs. After incubation in the absence of EGF for 12 h, NPCs were treated with or without various concentrations of 15d-PGJ₂, GSH-EE, CAY10410, or PMA in the presence of EGF (2 ng/ml) for 2 h. Immediately after the loading of H₂DCFDA (10 μM) during the last 30 min of the incubation period, cells were washed with MHM and fluorescent images were obtained using a fluorescence microscope (A). The proportion of positive cells (a) and the level of fluorescence intensity per cell (b) were calculated with the use of Scion Image (B). The data are means ± S.E.M. of three to six wells (a) or 65 to 312 cells. *, significant difference from the vehicle control (*p* < 0.05, one-way ANOVA followed by Fisher's protected least significant difference test). Scale bar, 200 μm.

treatment with GSH-EE, a membrane-permeant analog of the endogenous antioxidant glutathione, suppressed the increase in ROS level induced by 15d-PGJ₂. Phorbol ester PMA, used as a positive control reagent, also increased the ROS level in NPCs.

The Effects of 15d-PGJ₂ on Endogenous GSH Levels and the Effect of GSH-EE Treatment on the Regulation of Proliferation by 15d-PGJ₂. Because it has been reported that 15d-PGJ₂ binds covalently to endogenous GSH and decreases its intracellular level, we examined the effects of 15d-PGJ₂ on the intracellular GSH level in NPCs. The level of total intracellular GSH (both reduced and oxidized forms) was measured 12 h after exposure to 15d-PGJ₂. As shown in Fig. 7A, 15d-PGJ₂ at 0.3 μ M did not change the GSH level; however, 15d-PGJ₂ at concentrations of 0.5, 1, and 10 μ M significantly decreased the level of GSH to 50 to 60% of the level in the vehicle group.

If the decrease in GSH levels followed by ROS elevation is indispensable for the biphasic action of 15d-PGJ₂, treatment with an antioxidant such as GSH would reverse the effects of 15d-PGJ₂ on the proliferation of NPCs. To test this hypothesis, we next investigated whether supplementation with GSH-EE could prevent the effects of 15d-PGJ₂ on the proliferation of NPCs using WST-8 assay. NPCs were pretreated with GSH-EE (2 mM) for 1 h and then treated with or without various concentrations of 15d-PGJ₂ (0.01–10 μ M) in the presence of EGF (2 and 20 ng/ml) for 48 h. In the presence of a low concentration of EGF (2 ng/ml), treatment with GSH-EE prevented the facilitatory actions of 0.1 and 0.3 μ M 15d-PGJ₂ and the inhibitory actions of 0.5, 1, and 10 μ M 15d-PGJ₂ (Fig. 7B). Likewise, in the presence of a high concentration of EGF (20 ng/ml), GSH-EE suppressed the facilitatory and inhibitory effects of 15d-PGJ₂ (Fig. 7B). Similar to these effects on 15d-PGJ₂ actions, pretreatment with GSH-EE (2 mM) also blunted the regulation of NPC proliferation by PGD₂ (Supplemental Fig. 5). These results suggest that glutathione plays an important role in the actions of 15d-PGJ₂.

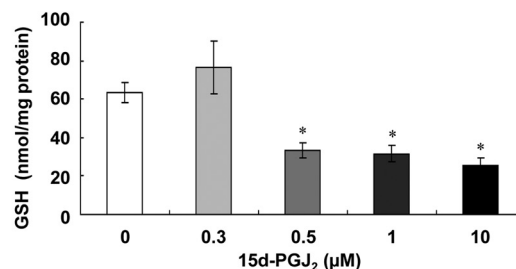
Effect of Central Injection of 15d-PGJ₂ on the Proliferation of NPCs Located in the DG of Postnatal Mice. To substantiate the relevance of our *in vitro* observations, we next examined the effect of various doses of 15d-PGJ₂ (0.3–30 ng) on the proliferation of neural stem/progenitor cells in the DG *in vivo* using BrdU immunohistochemistry. Mice were given BrdU (50 mg/kg *i.p.*) 5 or 24 h after 15d-PGJ₂ injection. When BrdU was injected 5 h after 15d-PGJ₂, the number of BrdU-labeled cells in the DG was significantly decreased by only the highest dose of 15d-PGJ₂ (30 ng) (Fig. 8A). By contrast, when BrdU was injected 24 h after 15d-PGJ₂, 15d-PGJ₂ at a low dose (1 ng) significantly increased the number of BrdU-labeled cells to 148% of the level in the control group, whereas 15d-PGJ₂ at a high dose (30 ng) decreased the number of BrdU-labeled cells to 71% of the level in the control group (Fig. 8, A and B). These results suggest that 15d-PGJ₂ biphasically regulates the proliferation of neural stem/progenitor cells in the DG of postnatal mice *in vivo*.

Discussion

In the present study, we examined the mode of action of 15d-PGJ₂ on the proliferation of mouse hippocampal NPCs *in vitro* and *in vivo*. Using assays assessing the relative viable cell number as well as DNA synthesis activity, we found that 15d-PGJ₂ showed biphasic effects on the EGF-

induced proliferation of NPCs, facilitation at low concentrations (\sim 0.3 μ M), and suppression at higher concentrations (0.5–10 μ M) *in vitro*. GW9662, an inhibitor of PPAR γ , failed to abolish the effects of 15d-PGJ₂. We also found that the electrophilic carbon at position 9 is critical for the action of 15d-PGJ₂ on proliferation, because CAY10410, a structural analog of 15d-PGJ₂ lacking the electrophilic carbon, did not show 15d-PGJ₂-like actions. Treatment with 15d-PGJ₂ at low or high concentrations increased ROS levels and decreased endogenous GSH levels. Furthermore, supplementation with GSH-EE (2 mM) diminished the biphasic effects of 15d-PGJ₂. Finally, similar biphasic effects of 15d-PGJ₂, facilitation at

A Intracellular GSH level



B WST-8 assay

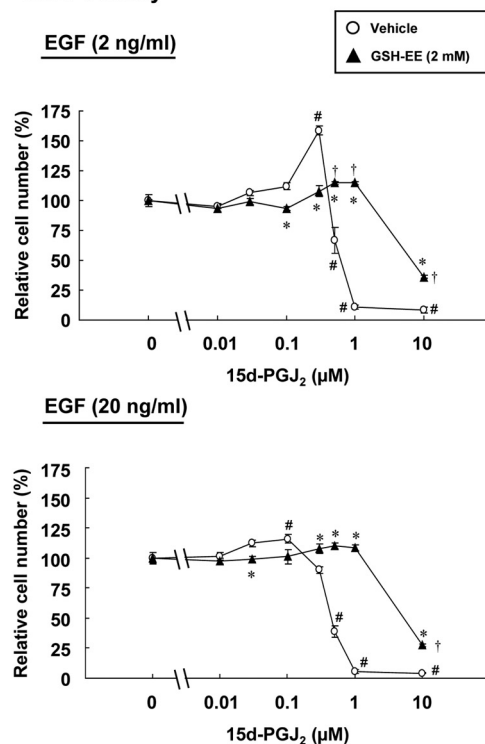


Fig. 7. The effect of 15d-PGJ₂ on the intracellular GSH level (A). NPCs were treated with or without various concentrations of 15d-PGJ₂ in the presence of EGF (2 ng/ml) for 12 h, and the total GSH level was measured using enzymatic reducing cycle system. The data are means \pm S.E.M. of four experiments. *, significant difference from vehicle control ($p < 0.05$, one-way ANOVA followed by Dunnett's test). B, the effect of pretreatment with GSH-EE on the regulation of the NPC proliferation by 15d-PGJ₂. The details are same as those in Fig. 4 legend. The value of the control group was set to 100%. The data are means \pm S.E.M. of three to four wells. #, significant difference from 15d-PGJ₂-free (0 μ M) in the vehicle group; †, significant difference from 15d-PGJ₂-free (0 μ M) in the GSH-EE group ($p < 0.05$, one-way ANOVA followed by Dunnett's test). *, significant difference between the vehicle and GSH-EE groups at each concentration of 15d-PGJ₂ ($p < 0.05$, Student's *t* test).

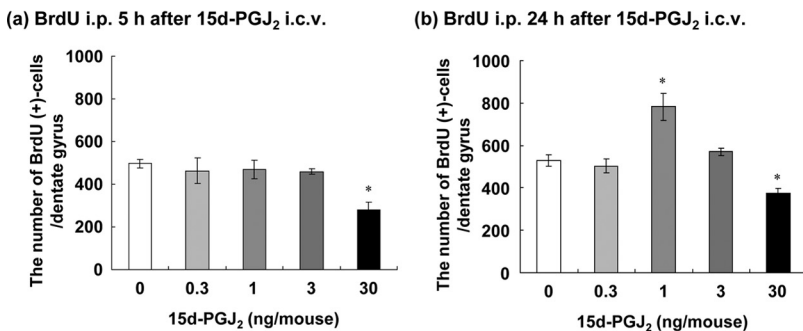
low dose and suppression at high dose, were observed in the dentate gyri of postnatal mice in vivo.

15d-PGJ₂ contains a reactive electrophilic carbon in the cyclopentenone ring and changes the redox state by covalently modifying cysteine residues in their target proteins (Rossi et al., 2000; Straus et al., 2000). We previously reported that MCI-186, which is known as an antioxidant, suppressed EGF-induced proliferation of NPCs derived from mouse ganglionic eminence and increased subsequent neural differentiation (Moriya et al., 2007). Moreover, it has been reported that the level of ROS is significantly higher in neural precursor cells compared with other primary and transformed cell lines and would be drastically changed by cell density (Limoli et al., 2004). These authors demonstrated that ROS levels were significantly elevated under low-density conditions compared with high-density conditions, and higher ROS levels found at lower cell densities were associated with elevated proliferation (Limoli et al., 2004). These lines of evidence suggest that the cellular redox state plays important roles in regulating the proliferative activity of NPCs. In the present study, we found that 15d-PGJ₂ increased ROS levels and that supplementation with GSH-EE partially suppressed the effects of 15d-PGJ₂ on the proliferative activity of NPCs. At higher concentrations (1–10 μ M), 15d-PGJ₂ decreases the amount of GSH, probably by covalently scavenging intracellular GSH, and strongly increases cellular oxidation over the physiological ranges, resulting in cell cycle arrest followed by apoptosis of NPCs. Likewise, 15d-PGJ₂ at high concentrations (5–20 μ M) was reported to in-

duce apoptosis in human B lymphocytes by increasing intracellular ROS levels in a PPAR γ -independent manner (Ray et al., 2006). Taken together, these findings suggest that 15d-PGJ₂ extraordinarily increases intracellular ROS levels and subsequently causes cytotoxicity by inducing cellular oxidative stress.

On the other hand, 15d-PGJ₂ at a lower concentration (0.3 μ M) failed to influence the amount of GSH, but modestly increased the intracellular ROS level; this was associated with the facilitation of NPC proliferation. The mechanism underlying the GSH-independent increase in ROS levels elicited by low concentrations of 15d-PGJ₂ is not clear at present. We speculate that redox-related substances other than GSH may be involved in the effects of a low concentration of 15d-PGJ₂. For example, thioredoxin (Trx), an important redox regulatory molecule, is reported to be a target protein of 15d-PGJ₂ (Shibata et al., 2003). 15d-PGJ₂ covalently modifies cysteine residues and inhibits the activity of Trx, which has a role in removing intracellular ROS, leading the rupture of intracellular redox state. Because supplementation with GSH-EE partly suppressed the effects of 15d-PGJ₂ on the self-renewal ability of NPCs, 15d-PGJ₂ can covalently modify other redox regulatory molecules such as Trx. In addition, the signaling pathways downstream of ROS elevation that are involved in 15d-PGJ₂ actions are not clear at present. It has been reported that H₂O₂, a ROS, rapidly activated the nuclear factor- κ B pathway in a human T cell line (Schreck et al., 1991). Schreck et al. demonstrated that reactive oxygen directly or indirectly released the inhibitory subunit I κ B from nuclear factor- κ B (Schreck et al., 1991). To elucidate the

A Quantified data



B Representative images

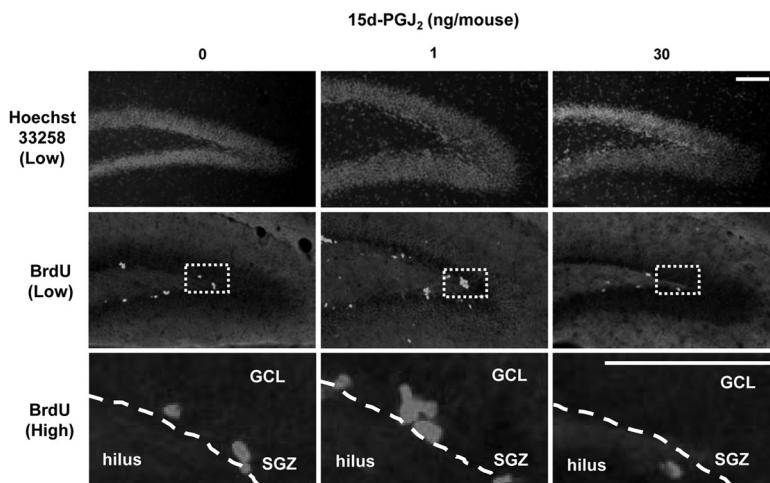


Fig. 8. The effect of the central injection of 15d-PGJ₂ on the proliferation of neural stem/progenitor cells in the DG of postnatal mice. A, the number of BrdU-labeled cells in the DG of mice that were given BrdU 5 h (a) and 24 h (b) after 15d-PGJ₂ injection. Five hours (a) or 24 h (b) after intracerebroventricular injection of 15d-PGJ₂ or vehicle, animals were injected with BrdU (50 mg/kg i.p.). Two hours after BrdU injection, animals were sacrificed and coronal brain sections were processed for BrdU immunohistochemistry. The data are means \pm S.E.M. of three to five mice. *, significant difference from the vehicle control ($p < 0.05$, Dunnett's test). B, representative immunofluorescence images of BrdU-labeled cells in the DG of mice that were given BrdU 24 h after 15d-PGJ₂ or vehicle injection. The dashed rectangles in the low-power image indicate the area shown in the high-power image. GCL, granule cell layer; SGZ, subgranular zone. Scale bar, 100 μ m.

signaling molecule(s) mediating 15d-PGJ₂-induced ROS elevation, further experiments will be required.

15d-PGJ₂ has been identified as a natural ligand for PPAR γ , which belongs to the nuclear receptor superfamily. It has been reported that 15d-PGJ₂ causes the differentiation of adipocytes via the activation of PPAR γ (Forman et al., 1995). On the other hand, in the human leukemia cell line THP-1, 15d-PGJ₂ had biphasic effects on proliferation in a PPAR γ -independent manner (Azuma et al., 2004), similar to our present observation. As shown in Fig. 5, only 10 μ M CAY10410 increased the viable cell number in the presence of 2 ng/ml EGF. It has been reported that CAY10410, a structural analog of 15d-PGJ₂, lacks the electrophilic carbon at position 9, but shows PPAR γ agonistic activity (Shiraki et al., 2005). These authors showed that CAY10410 at 10 μ M could act as an agonist of PPAR γ ; however, it did not react with PPAR γ at concentrations lower than 1 μ M. Taken together, these findings suggest that PPAR γ may be involved in the facilitatory effects of CAY10410 at 10 μ M on the proliferation of NPCs in our study.

The DG of the hippocampus is one of two neurogenesis-rich regions in the adult mammalian brain (Altman and Das, 1965; Dayer et al., 2003). We examined whether 15d-PGJ₂ influenced the proliferative activity of neural stem/progenitor cells in the mouse DG in vivo. We observed that intracerebroventricular injection of 30 ng of 15d-PGJ₂ significantly decreased the number of BrdU-labeled cells in the DG when BrdU was administered 5 or 24 h after 15d-PGJ₂ injection, suggesting that the suppressive or toxic effects of 15d-PGJ₂ appear quickly. By contrast, the increase in the number of BrdU-labeled cells induced by a low dose of 15d-PGJ₂ was observed only when BrdU was administered 24 h after 15d-PGJ₂ injection, not 5 h after, suggesting that the facilitating action of 15d-PGJ₂ requires a long period for action (at least 24 h). Furthermore, the difference in the time for the appearance of 15d-PGJ₂ effects between facilitation and suppression implies that the biphasic action of 15d-PGJ₂ may involve different intracellular mechanisms.

Growing evidence indicates that neurogenesis in adulthood is influenced by certain types of the central diseases, such as neuroinflammation. We have previously shown that lipopolysaccharide (LPS) impairs the survival of newly generated cells derived from neural stem/progenitor cells in the DG in vivo, and these effects of LPS were presumably mediated by cyclooxygenase-2 expression in the DG (Bastos et al., 2008). It is noteworthy that the central infusion of LPS drastically increases the brain levels of PGD₂, a major metabolite of arachidonic acid in the central nervous system (Rosenberger et al., 2004). Because the doses of 15d-PGJ₂ used in this study were considered to induce a situation similar to that of intracerebral hemorrhage (Zhao et al., 2006), PGD₂, as well as its metabolite 15d-PGJ₂, is a feasible candidate molecule regulating neurogenesis under pathological conditions such as neuroinflammation.

In conclusion, the present study shows that 15d-PGJ₂ exhibits a novel regulation of the proliferation of NPCs, and supplementation with GSH prevents the effects of 15d-PGJ₂. Our pharmacological and biochemical analyses show that ROS elevation, with or without depletion of endogenous GSH levels, might mediate the facilitatory and inhibitory actions of 15d-PGJ₂. Further experiments will be required to clarify

the downstream signaling pathways underlying the regulation of NPC proliferation by 15d-PGJ₂.

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