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Resveratrol improves cognitive function in mice by increasing production of insulin-like growth factor-I in the hippocampus

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Abstract

We examined whether resveratrol increases insulin-like growth factor-I (IGF-I) production in the hippocampus by stimulating sensory neurons in the gastrointestinal tract, thereby improving cognitive function in mice. Resveratrol increased calcitonin gene-related peptide (CGRP) release from dorsal root ganglion (DRG) neurons isolated from wild-type (WT) mice. Increases in tissue levels of CGRP, IGF-I, and IGF-I mRNA and immunohistochemical expression of IGF-I were observed in the hippocampus at 3 weeks after oral administration of resveratrol in WT mice. Significant enhancement of angiogenesis and neurogenesis was observed in the dentate gyrus of the hippocampus in these animals ($P<.01$). Improvement of spatial learning in the Morris water maze was observed in WT mice after administration of resveratrol. None of the effects of resveratrol observed in WT mice were seen after resveratrol administration in CGRP-knockout ($CGRP^{-/-}$) mice. Although red wine containing 20 mg/L of resveratrol produced effects similar to those of resveratrol administrationl in WT mice, neither red wine containing 3.1 mg/L of resveratrol nor white wine exhibited such effects in WT mice. Resveratrol was undetectable in the hippocampus of WT mice administered resveratrol and red wine containing 20 mg/L of resveratrol. These observations strongly suggest that resveratrol increases hippocampal IGF-I production via sensory neuron stimulation in the gastrointestinal tract, thereby improving cognitive function in mice.

Keywords: Resveratrol; Sensory neurons; Calcitonin gene-related peptide; Insulin-like growth factor-I; Hippocampus; Neurogenesis; Cognitive function

1. Introduction

Epidemiological evidence suggests that moderate consumption of red wine reduces the incidence of dementia [1]. However, the mechanism underlying this effect is not fully understood.

Resveratrol (3,5,4-trihydroxystilbene), a polyphenol of the phytoalexin family, is found in the seeds of various plant species including grapes, peanuts, and constitutes one of the components of red wine [2]. Resveratrol has been shown to have significant antioxidant properties and found to be neuroprotective against excitotoxicity, ischemia and hypoxia in both in vitro and in vivo models [3–5]. Although the antioxidant activities of resveratrol have been shown to play critical roles in its neuroprotective effects, such activities may not fully explain the beneficial effects of resveratrol [6].

Insulin-like growth factor-I (IGF-I) is a basic peptide composed of 70 amino acids with rather a ubiquitous distribution in various tissues and cells, and mediates the growth-promoting effects of growth hormone (GH), thereby playing important roles in postnatal and adolescent growth [7]. IGF-I has been shown to enhance excitatory synaptic transmission in the CA_1 region of the hippocampus [8] and to improve spatial learning by inducing neurogenesis in the hippocampus [9,10]. The impaired spatial learning in mice with low serum levels of IGF-I is reversed by exogenous administration of IGF-I [11]. Furthermore, a close relationship has been demonstrated between plasma IGF-I levels and cognitive function in older individuals [12]. These observations strongly suggest that IGF-I may improve cognitive function by increasing plasticity and neurogenesis in the hippocampus.

The angiogenetic factor vascular endothelial growth factor (VEGF) plays an important role in coupling angiogenesis with neurogenesis in the brain [13]. IGF-I promotes angiogenesis via a VEGF-dependent mechanism in the brain [14]. Since vascular elements are thought to be an essential feature of the stem-cell niche in the hippocampus [15], it is possible that IGF-I promotes hippocampal neurogenesis by promoting angiogenesis.

IGF-I has been shown to stimulate β-amyloid release from neurons and promote brain amyloid clearance, and also to inhibit tau-hyperphosphorylation by inhibiting activation of glycogen synthase kinase-3β [16].

These observations strongly suggest that the decrease of IGF-I production is deeply related to the development of cognitive decline in the elderly and in patients with Alzheimer's disease.

Capsaicin-sensitive sensory neurons are nociceptive neurons found in many tissues within the lining epithelia, around blood vessels, and associated with nonvascular smooth muscle and

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myocardium of the atria [17]. These sensory neurons release calcitonin gene-related peptide (CGRP) following stimulation with a wide variety of types of noxious physical and chemical stimulation of the transient receptor potential vanilloid 1 (TRPV1) expressed on them [18], thereby exerting sensory-efferent functions. CGRP, a 37-amino acid neuropeptide produced by alternative splicing of the calcitonin gene, is widely distributed in the central and peripheral nervous systems [19] and is believed to have diverse physiological functions [20]. We previously reported that CGRP rapidly increases IGF-I production via increase in its transcription in various tissues including the brain in mice administered capsaicin [21,22].

Capsaicin-sensitive sensory neurons containing CGRP are also present in the gastrointestinal (GI) tract and these neurons are predominantly spinal in origin [23]. We demonstrated that nociceptive information arising from stimulation of sensory neurons in the gastrointestinal tract was transmitted to the hippocampus via the spino-parabrachial circuits including the solitary tract nucleus (NTS) as a relay point, thereby increasing IGF-I production in the astrocytes via increases of CGRP levels in the mouse hippocampus [24].

Based on these observations, we hypothesized that resveratrol contained in red wine could improve cognitive function by increasing IGF-I production in the hippocampal astrocytes through stimulation of sensory neurons in the GI tract. To test this hypothesis, we examined the effect of resveratrol on sensory neuron stimulation in vitro and examined the effect of oral administration of resveratrol on the hippocampal IGF-I production and cognitive function in wild-type (WT) and CGRP knockout ($CGRP^{-/-}$) mice in vivo. Furthermore, we compared effects of oral administration of red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol and white wine with those of oral administration of resveratrol in mice to determine whether the resveratrol contained in red wine improves cognitive function by increasing the hippocampal IGF-I production in mice.

2. Methods

2.1. Generation of αCGRP deficient mice

The generation of αCGRP deficient ($CGRP^{-/-}$) mice was described previously [25]. The mouse CT/αCGRP genomic DNA was cloned from a BALB/c mouse genomic library in EMBL3 using synthetic oligonucleotide probes derived from the mouse CT/αCGRP cDNA sequence. A 7.0-kb fragment containing exons 3–5 of the mouse CT/αCGRP gene was subcloned into pBluescript (Stratagene). A targeting vector was constructed by replacing the 1.6-kb XbaI-XbaI fragment encom- passing exon 5, which is specific for αCGRP, with the neomycin resistance gene and flanking the thymidine kinase gene. This plasmid was linearized with NotI and introduced into 129/Sv-derived SM-1 ES cells by electroporation, after which the cells were selected in medium containing G418 (300 μg/ml) and ganciclovir (2 μM). Homologous recombinants were identified by polymerase chain reaction (PCR) and Southern blot analysis. Targeted ES cell clones were injected into C57BL/6 mouse blastocysts to generate chimeric mice. Male chimeras were then crossbred with C57BL/6 females and germline transmission was achieved. Littermates obtained by breeding heterozygotes with the genetic background of the 129/SvXC57BL/6 hybrid were used for phenotypic analysis. Only males were used in this study.

2.2. Genotype determination of $CGRP^{-/-}$ pups

Genomic DNA was extracted from tails of mice as previously described [25] and was used for PCR analysis. PCR was performed using the external primers of the replaced gene fragment. The wild-type allele and the mutant allele gave different band sizes. Primer sequences and PCR conditions have been described [25].

2.3. Chemicals

Resveratrol, capsazepine (CPZ), an inhibitor of TRPV1 activation [26], and 5-bromo-2′-deoxyuridine (BrdU) were purchased from Sigma Chemical (St. Louis, MO, USA). KT5720, an inhibitor of protein kinase A (PKA), was purchased from Alexis (Basel, Switzerland). The red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol, and white wine were kindly supplied by Mercian (Fujisawa, Japan). Concentration of total polyphenols of the red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol, and white wine were 2252, 1336, and 236 mg/L, respectively. Percentage of ethanol of the red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol, and white wine were 11.9, 11.5, and 11.3, respectively. All other reagents were of analytical grade.

2.4. Isolation and culture of dorsal root ganglion (DRG) neurons

DRG neurons from the lumber, cervical and thoracic region were dissected from WT mice as described previously [27]. In brief, DRG were placed ice-cold sterile calcium-, and magnesium-free Dulbecco's phosphate-buffered saline (PBS) (Gibco, Grand Island, NY, USA). Ganglia were chopped and incubated at 37°C for 15 min in Dulbecco's PBS containing 20 U/ml papain (Worthington Biochemical Corporation, Lakewood, NJ, USA). The tissue was then incubated at 37°C for 15 min in Dulbecco's PBS containing 4 mg/ml collagenase type II (Worthington Biochemical Corporation). The tissue was incubated for a further 30 min in Dulbecco's PBS containing 2000 U/ml dispase I (Godo Shusei, Tokyo, Japan) at 37°C. Individual cells were then dissociated by trituration through a fire-polished Pasture pipette. After centrifugation at 250×g for 5 min, the resultant pellet was washed twice in serum free Ham's F-12 medium (Hycone, Logan, UT, USA). Cells were plated on 60 mm polystyrene dish precoated with Vitrogen (Cohesion Technologies, Palo Alto, CA, USA) in Ham's F-12 medium containing 10% supplemented calf serum, 2 mM glutamine and 50 ng/ml mouse 2.5S nerve growth factor (Upstate Biotechnology, Lake Placid, NY). After 24 h, the culture medium was removed and replaced every 2 d.

2.5. Release of CGRP from DRG neurons in culture and cyclic adenosine monophosphate (cAMP) measurement

After 5 days in culture, the medium was aspirated gently and washed with serum free Ham's F-12 medium. Cells were incubated with resveratrol (0.1–10 μM) or vehicle for 30 min in Ham's F-12 medium containing 1% supplemented calf serum without nerve growth factor. After incubation, supernatants were sampled and stored at −20°C for CGRP measurement. To determine whether resveratrol increased CGRP release from DRG neurons via TRPV1 activation, we examined the effect of CPZ, an inhibitor of TRPV1 activation [26], on resveratrol-induced CGRP release from DRG neurons. CGRP levels were determined using specific enzyme immunoassay kit (SPI-BIO, Massy, France). Recent studies demonstrated that cAMP plays a critical role in CGRP release from sensory neurons by phsphorylating TRPV1 through activation of PKA [26], and cAMP-dependent PKA activation is critically involved in CGRP production in DRG neurons [26]. Therefore, we measured the intracellular cAMP levels in DRG neurons. We examined the effect of KT5720 on CGRP release from DRG neurons at a concentration of 10 μM, as described previously [27]. After collection of supernatants, plates were placed on ice, media were removed and cells were washed by ice-cold PBS. Thereafter, ice-cold 65% ethanol were added to each well and placed on ice. Ethanol were collected and dried under nitrogen gas. Intracellular levels of cAMP were determined with an enzyme immunoassay kit (GE Healthcare, Buckinghamshire, UK) according to the manufacture's instructions.

2.6. Animal model

Age-matched (10–12 weeks old, 21–24 g) male C57BL/6 WT (Nihon SLC, Hamamatsu, Japan) and $CGRP^{-/-}$ mice were used in each experiment. They were maintained under standard conditions of temperature (23–25°C) and on a 12 h light/dark cycle. Food and water were provided ad libitum. The care and handling of the animals were in accordance with the National Institute of Health guidelines. All the experimental procedures described below were approved by the Nagoya City University Animal Care and Use Committee. The animals were sacrificed at the end of each treatment period. Mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg).

Separate sets of animals were used for each measurement in the present study.

Administration of resveratrol, the red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol and white wine.

Resveratrol was dissolved in 1% Tween 20/1% ethanol (1%) with normal saline (20 mg/L) and was administered orally at a volume of 200 μl once a day for 3 weeks. Two hundred microliters of the red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol and white wine were administered orally once a day for 3 weeks.

KT5720 and CPZ were dissolved in 10% Tween 20/10% methanol (10%) with normal saline. Solutions were prepared immediately just before the experiments.

2.7. Determination of hippocampus tissue levels of CGRP

Tissue levels of CGRP were determined in animals as described previously [24]. The concentration of CGRP was assayed by using a specific enzyme immunoassay kit (SPI-BIO).

2.8. Determination of hippocampus IGF-I level

Tissue levels of IGF-I were determined in animals as described previously [24]. The concentration of IGF-I was assayed by using a specific enzyme immunoassay kit (Diagnostic Systems Laboratories, Webster, TX, USA).

2.9. Quantitative mRNA analysis

Quantitative mRNA analysis was performed as previously described [24]. Known concentrations of serially diluted IGF-I and β-actin cDNA generated by PCR were used as standards for quantitation of sample cDNA. Copy numbers of cDNA for IGF-I was standardized those for β-actin from same sample.

2.10. Determination of hippocampal tissue levels of resveratrol

Hippocampal tissue levels of *trans*- and *cis*-resveratrol were determined in WT mice administered resveratrol and red wine containing 20 mg/L of resveratrol for 3 weeks according to the methods as described previously [28]. In brief, the hippocampus was minced and homogenized in a polytron-type homogenizer (two times of 15 s) using 1 ml of methanol. The homogenates was then centrifuged at 4500×*g* for 10 min at 4°C. The supernatant was separated and evaporated under stream of nitrogen gas and the residue was reconstituted in 200 μl methanol before high-performance liquid chromatography (HPLC). The HPLC system used for analysis was a Shimadzu LC-10A gradient liquid chromatograph quipped with a Shimadzu SPD-M6A photodiode array ultraviolet (UV)-visible detector. A Micosorb C_{18} Short-One column (RAININ) with the size of 4.6×100 mm was used, with a particle size of 3 μm in diameter. Samples were eluted by the following gradient time program with solution A (0.4%, v/v, phosphoric acid) and B (80%, v/v, acetonitrile and 20% of Solution A): 0–5 min, 0% of Solution B; 20 min, 15% of Solution B; 35 min, 22.5% of Solution B; 65 min, 62.5% of solution B. The other conditions were as follows: flow rate, 1.0 ml/min; detection, UV at 303 nm; injection volume, 5 μl.

2.11. Immunohistochemical staining of IGF-I and glial fibrillary acidic protein in hippocampi

The double labeling of immunofluorescent technique was used for immunohistochemical staining of various tissues with anti-IGF-I antibody as described previously [24]. Sections (18 μm thick) were rinsed in PBS, and then incubated for 1 hour with PBS, 0.2% Triton X-100, and 0.5% blocking reagent (Roche Diagnostics, Basel, Switzerland) at room temperature. They were incubated for overnight at 4°C with mouse anti-IGF-I monoclonal antibody (1:200; Upstate Biotechnology) and rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:1000; Dako, Glostrup, Denmark). The sections were treated with secondary antibodies Alexa Fluor 568 anti-rabbit IgG for GFAP, Alexa Fluor 488 anti-mouse IgG for IGF-I (1:500; Invitrogen) for 1 h at room temperature. The number of both IGF-I and GFAP-positive cells was counted in the granule cell layer and in the hilus. The number of cells was counted using one 18-μm-thick section per animals.

2.12. c-fos immunohistochemistry

Immunohistochemical staining of c-*fos* in mice brain was performed according to the method as previously described [24]. Sections were rinsed in PBS, and then incubated for 1 h with PBS, 0.2% Triton X-100 and 0.5% blocking reagent (Roche Diagnostics) at room temperature. They were incubated for overnight at 4°C with rabbit anti- c-*fos* polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were treated with secondary antibodies Alexa Fluor 568 anti-rabbit IgG (1:500; Invitrogen) for 1 h at room temperature. After staining, samples were examined in a Fluorescence microscope (Axio Imager A1, Carl Zeiss).

2.13. Double stain immunohistochemistry for CD31, GFAP or calbindin-D28k with BrdU

We used BrdU labeling to monitor proliferation and double immunofluorescent labeling for BrdU and cell-specific markers to determine the phenotype of the progenitor progeny. During the last 5 d of each treatment period, the animals for determination of BrdU-labeled nuclei received a daily intraperitoneal injection of BrdU at dosage of 50 mg/kg. Angiogenesis and neurogenesis in the mouse brains was monitored by BrdU incorporation into the nuclei of dividing cells as previously described [9]. Samples were treated for DNA denaturation in the following manner: tissues were incubated in 50% formamide in 2X SSC buffer (1X SSC, 0.3M NaCl and 0.03M sodium citrate) at 65°C for 2 h, rinsed in PBS, and then incubated for 30 min with 2 N HCl at 37°C. They were rinsed for 10 min at room temperature in 0.1 M boric acid (pH 8.4). The tissue was rinsed in PBS three times, followed by incubation in PBS, 0.2% Triton X-100, and 0.5% blocking reagent (Roche Diagnostics) for 1 h and then with primary rat anti-CD31 monoclonal antibody (1:200; BD Biosciences PharMingen, San Diego, CA, USA) with mouse anti-BrdU monoclonal antibody (1:100; Invitrogen), or rabbit anti-GFAP polyclonal antibody (1:1000; Dako), or mouse anti-calbindin-D28k monoclonal antibody (1:200; Abcam, Cambridge, UK) with rat anti-BrdU monoclonal antibody (1:400; Abcam) overnight at 4°C. The sections were treated with secondary antibodies Alexa Fluor 594 anti-rat IgG for CD31, Alexa Fluor 488 anti-mouse IgG for BrdU (anti-mouse), Alexa Fluor 568 anti-rabbit IgG for GFAP, Alexa Fluor 488 anti-mouse IgG for calbindin-D28k, and Alexa Fluor 594 anti-rat IgG for BrdU (anti-rat) (1:500; Invitrogen) for 1 h at room temperature. After staining samples were examined in a fluorescence microscope (Axio Imager A1, Carl Zeiss). BrdU-positive cells were counted in the granule cell layer and in the hilus. The number of cells was counted using one 18-μm-thick section per animal.

2.14. Morris water maze task

Behavioral testing was conducted as described previously [29]. We used a circular pool (150 cm diameter). The pool was filled with water at 30°C and contained a round-shaped transparent acrylic platform (10 cm diameter). In the task, the platform was submerged 1 cm below the surface of the water and located in the southeast quadrant of the pool throughout the trials. After a mouse was put into the pool, each had a maximum of 90 s to locate and climb onto the platform (one trial). When a mouse located the platform, it was allowed to stay on it for 20 s. Mouse that did not find the platform in the allowed time was placed on it by the experimenter and left there for 20 s. Latency to reach the platform was monitored. Each mouse was subjected to one trial per day. The task consisted of 5 d of trials. Two hours after the last trial, the probe test was carried out. For this test, the platform was removed from the pool and the trial was performed with the cutoff time of 90 s. The time spent in the target area (zone radius: 30 cm, three times the target diameter) was recorded as a percentage of the trial time in the pool.

2.15. Statistical analysis

Data are expressed as the mean±S.D. The results were compared using an analysis of variance followed by Scheffé's post hoc test. A level of $P<.05$ was considered statistically significant.

3. Results

3.1. Effect of resveratrol on CGRP release and cellular cAMP levels in DRG neurons isolated from WT mice

Resveratrol, at concentrations of 0.1, 1 and 10 μM, increased CGRP release from DRG neurons isolated from WT mice (Fig. 1A).

Cyclic AMP has been shown to play an important role in the release of CGRP from the sensory neurons upon activation [26]. Resveratrol, at concentrations of 0.1, 1 and 10 μM, increased the cellular cAMP levels in DRG neurons (Fig. 1B). Pretreatment with CPZ

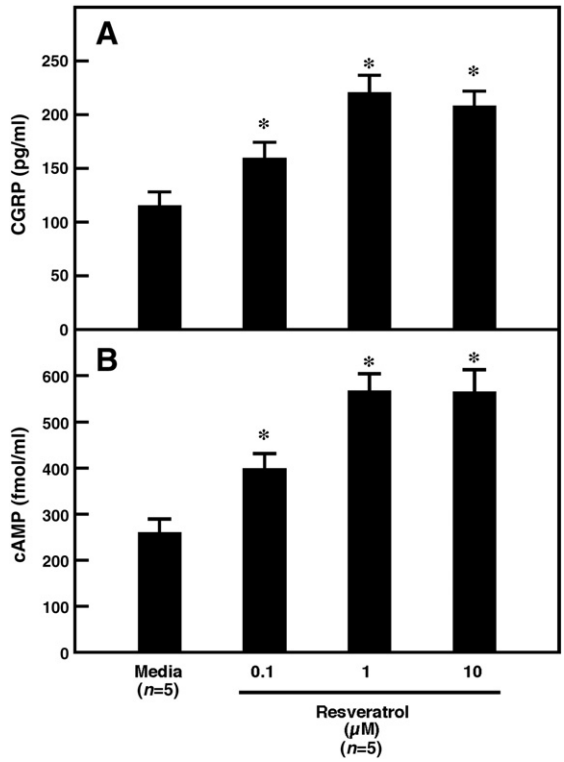

Fig. 1. Effects of resveratrol on CGRP release from DRG neurons (A) and intracellular cAMP levels (B) in DRG neurons isolated from WT mice. DRG neurons were incubated with resveratrol (0.1–10 μM) for 30 min. Supernatants were sampled and CGRP levels were measured by enzyme immunoassay. Intracellular cAMP levels were measured by enzyme immunoassay. Each value represents the mean±S.D. derived from five experiments. *$P<.01$ versus media.

reversed the resveratrol-induced increase of CGRP release from DRG neurons (Fig. 2). TRPV1 is activated by phosphorylation through cAMP-dependent PKA [26]. We hypothesized that resveratrol might increase CGRP release from sensory neurons by sensitizing TRPV1 activation thorough activation of PKA. To examine this hypothesis, we analyzed the effect of pretreatment with KT5720, an inhibitor of PKA, on the resveratrol-induced CGRP release from DRG neurons isolated from WT mice. As shown in Fig. 2, the resveratrol-induced increase of CGRP release from DRG neurons was completely reversed by pretreatment with KT5720.

3.2. Effects of resveratrol, red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol and white wine on tissue levels of CGRP, IGF-I, and IGF-I mRNA in the hippocampus of WT and CGRP$^{-/-}$ mice

To examine whether resveratrol contained in red wine increases tissue levels of CGRP, IGF-I and IGF-I mRNA in the hippocampus through sensory neuron stimulation in mice, we determined tissue levels of these substances in the hippocampus after oral administration of resveratrol, red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol and white wine for 3 weeks in WT and CGRP$^{-/-}$ mice. At baseline, tissue levels of CGRP, IGF-I and IGF-I mRNA in the hippocampus of WT mice were significantly higher than those in the hippocampus of CGRP$^{-/-}$ mice ($P<.01$) (Fig. 3). Administration of resveratrol significantly increased hippocampal tissue levels of CGRP, IGF-I and IGF-I mRNA in WT mice ($P<.01$), whereas no such increases were observed in the hippocampus of CGRP$^{-/-}$ mice (Fig. 3). Although administration of red wine containing 20 mg/L of resveratrol produced effects similar to those induced by resveratrol administration in WT and CGRP$^{-/-}$ mice (Fig. 3), administration of neither red wine containing 3.1 mg/L of resveratrol nor white wine showed any effects in WT mice (Fig. 3) and CGRP $^{-/-}$ mice (data not shown).

3.3. Effects of resveratrol, red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol and white wine on the number of cells expressed both IGF-I and GFAP in the hippocampus of WT and CGRP$^{-/-}$ mice

Increase of the immunohistochemical expression of IGF-I in the dentate gyrus (DG) was observed after administration of resveratrol

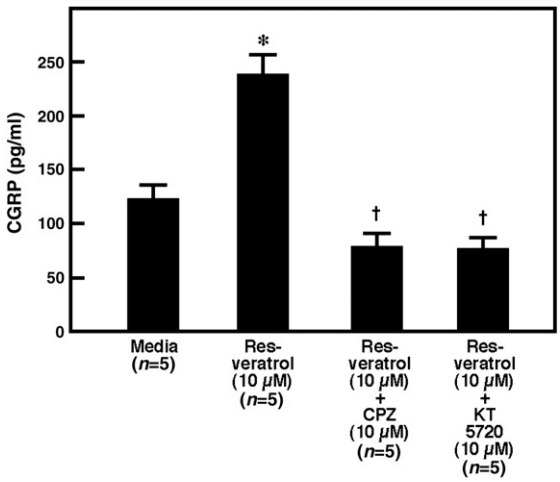

Fig. 2. Effects of CPZ and KT5720 on resveratrol-induced increase in CGRP release from DRG neurons isolated from WT mice. DRG neurons were incubated with resveratrol (10 μM) for 30 min in the presence or absence of the inhibitor of TRPV1 activation CPZ (10 μM), or the PKA inhibitor KT5720 (10 μM), Each value represents the mean±S.D. from 5 experiments. *$P<.01$ versus media; †$P<.01$ versus resveratrol.

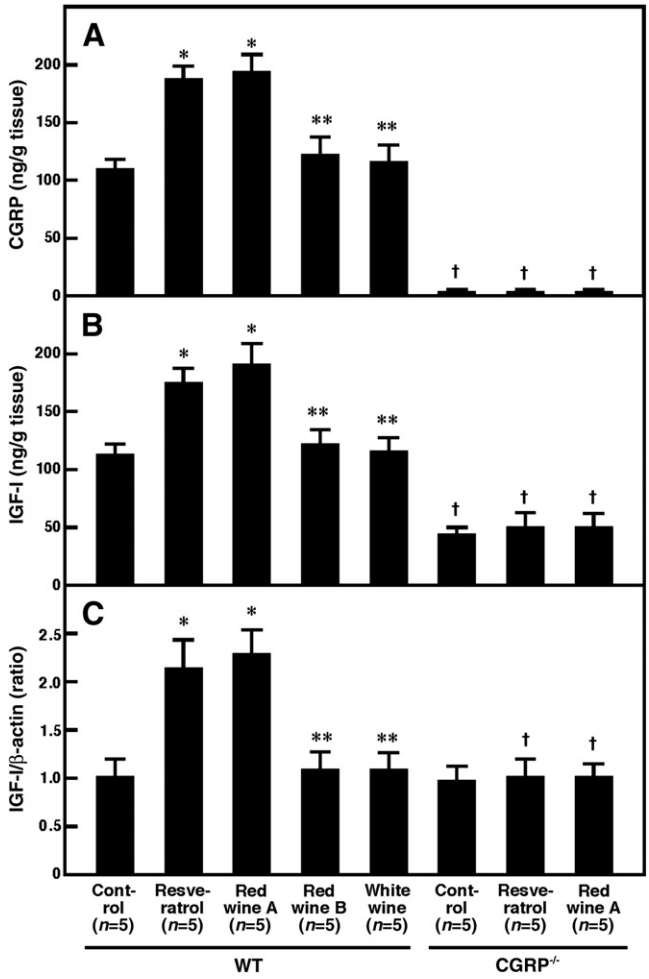

Fig. 3. Effects of resveratrol, red wine containing 20 mg/L of resveratrol (red wine A), red wine containing 3.1 mg/L of resveratrol (red wine B), and white wine on hippocampal levels of CGRP (A) IGF-I (B) and IGF-I mRNA (C) in WT mice and CGRP$^{-/-}$ mice. All tissues were removed at 3 weeks after oral administration of resveratrol, red wine A, red wine B or white wine. Values are means±S.D. derived experiments using 5 animals. *$P<.01$ versus control; **$P<.01$ versus resveratrol; †$P<.01$ versus WT.

in WT mice (Fig. 4), but not in CGRP$^{-/-}$ mice (data not shown). The IGF-I immunoreactivity was co-localized with the immunoreactivity for the astrocyte marker GFAP in WT mice and increase of the IGF-I immunoreactivity colocalized with GFAP immunoreactivity was observed following resveratrol administration in WT mice (Figs. 4 and 5).

Administration of red wine containing 20 mg/L of resveratrol produced effects similar to those induced by resveratrol administration in WT mice (Figs. 4 and 5) and CGRP$^{-/-}$ mice (data not shown), while administration of neither red wine containing 3.1 mg/L of resveratrol nor white wine showed any effects in WT mice (Figs. 4 and 5) and in CGRP$^{-/-}$ mice (data not shown).

3.4. Effects of resveratrol, red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol and white wine on the c-fos expression in the spinal and supraspinal nervous tissues in WT and CGRP$^{-/-}$ mice

To analyze the mechanism and pathway of the relay system that leads to the increase of the hippocampal IGF-I production in WT mice administered resveratrol, we determined the c-*fos* expression in the spinal and supraspinal nervous tissues of WT and CGRP$^{-/-}$ mice after

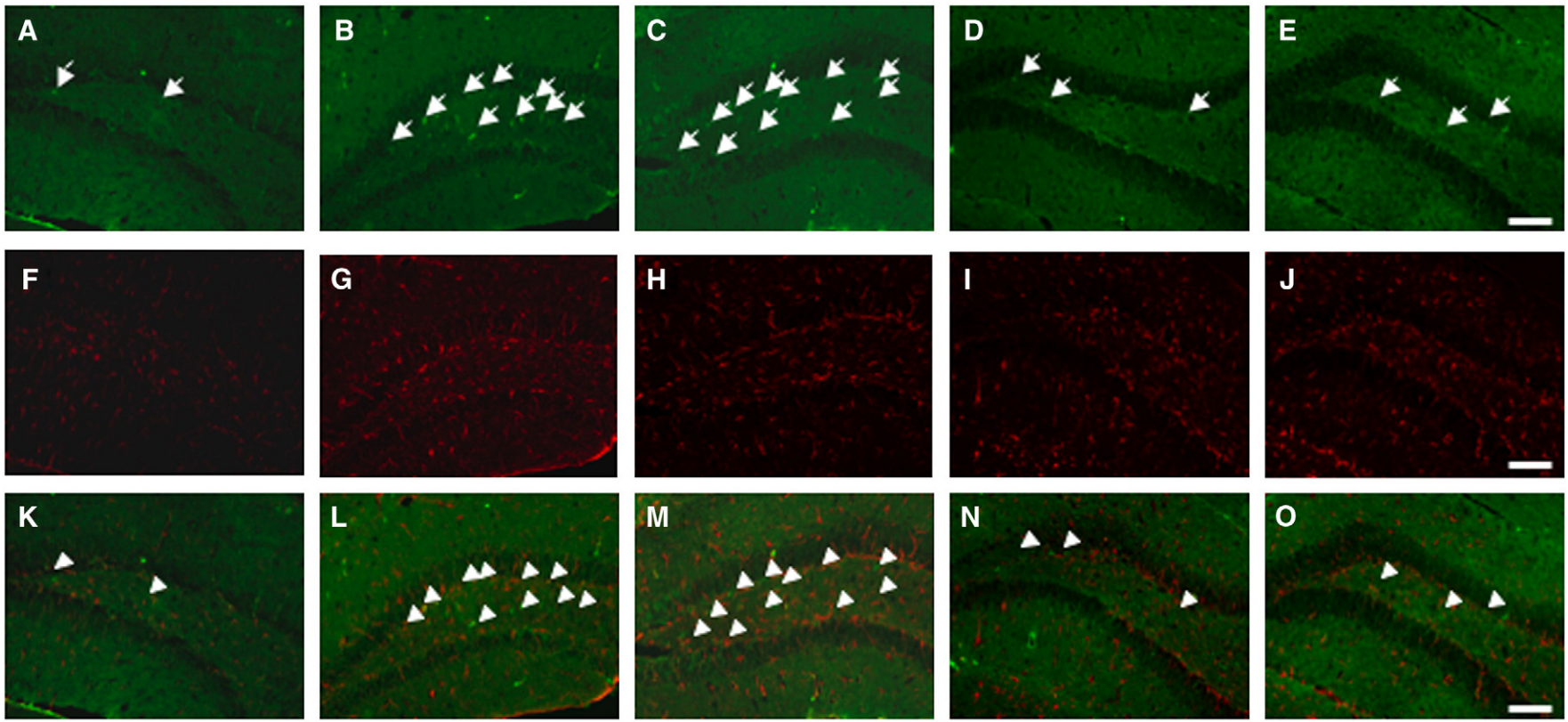

Fig. 4. Effects of resveratrol, red wine containing 20 mg/L of resveratrol (red wine A), red wine containing 3.1 mg/L of resveratrol (red wine B) and white wine on immunohistochemical expression of IGF-I in the hippocampus of WT mice. Fluorescent photomicrographs of cryostat sections show IGF-I expression in the hippocampus of WT mice. Double labeling of IGF-I (green) and GFAP (red) showed the localization of IGF-I in the hippocampus. Immunohistochemical expression of the DG in a control (A, F and K), a resveratrol-treated animal (B, G and L) a red wine A-treated animal (C, H and M), a red wine B-treated animal (D, I and N) and a white wine-treated animal (E, J and O) is shown in the insets. Five animals in each group were examined and typical results are shown. Arrows indicate IGF-I-positive staining. Arrowheads indicate co-localization of IGF-I and GFAP-positive staining. Scale bars=50 μm.

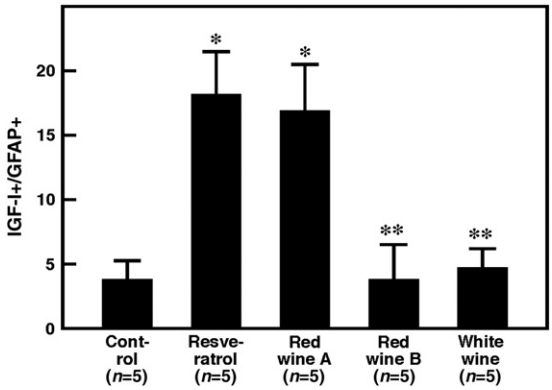

Fig. 5. Effects of resveratrol, red wine containing 20 mg/L of resveratrol (red wine A), red wine containing 3.1 mg/L of resveratrol (red wine B) and white wine on the number of cells showing co-localization of IGF-I- and GFAP-positive staining in the hippocampus of WT mice. Values are means±S.D. derived from 5 animal experiments. *P<.01 versus control; **P<.01 versus resveratrol.

oral administration of resveratrol for 3 weeks (Figs. 6 and 7). In WT mice administered resveratrol, increase of the c-*fos* expression was observed in the dorsal horns (lamina I-II) of the spinal cord and, supraspinally, in the NTS, parabrachial nuclei (PBN) and the hippocampus (Fig. 7). In contrast, no increase of c-*fos* expression was observed in the same tissues after resveratrol administration in CGRP$^{-/-}$ mice (data not shown).

Administration of red wine containing 20 mg/L of resveratrol produced effects similar to those induced by resveratrol administration in WT mice (Fig. 7) and CGRP$^{-/-}$ mice (data not shown), while administration of neither red wine containing 3.1 mg/L of resveratrol nor white wine showed any effects in WT mice (Fig. 7) and CGRP$^{-/-}$ mice (data not shown).

3.5. Effects of resveratrol, red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol, and white wine on the hippocampal angiogenesis and neurogenesis in WT and CGRP$^{-/-}$ mice

The number of BrdU-immunoreactive cells in the DG was significantly higher in WT mice than in CGRP$^{-/-}$ mice (P<.01) (Fig. 8A). Further significant increase in the number of BrdU-immunoreactive cells was observed in the DG of WT mice after administration of resveratrol for 3 weeks (P<.01), while no such increase was observed in CGRP$^{-/-}$ mice (Fig. 8A).

Colocalization of BrdU immunoreactivity with immunoreactivity for the vascular endothelial cell marker CD31, the granule cell marker calbindin-D28k and the astrocyte marker GFAP was examined to determine the phenotype of the progenitor cell progeny in the DG after resveratrol administration in WT and CGRP$^{-/-}$ mice (Fig. 8B–D). The number of BrdU+/CD31+ cells, BrdU+/calbindin-D28k+ cells, and BrdU+/GFAP+ cells was significantly higher in the DG of WT mice than in that of CGRP$^{-/-}$ mice (P<.01)(Fig. 8, B, C, and D). Significant increase of the number of BrdU+/CD31+ cells and BrdU+/calbindin-D28k+ cells, but not of BrdU+/GFAP+ cells, in the DG was observed after administration of resveratrol for 3 weeks in WT mice (Fig. 8B–D). Increase of the number of these cells in the DG was not observed after resveratrol administration in CGRP$^{-/-}$ mice (Fig. 8B–D).

Although administration of red wine containing 20 mg/L of resveratrol produced effects similar to those induced by resveratrol administration in WT and CGRP$^{-/-}$ mice (Fig. 8), administration of neither red wine containing 3.1 mg/L of resveratrol nor white wine showed any effects in WT mice (Fig. 8) and CGRP$^{-/-}$ mice (data not shown).

3.6. Effects of resveratrol, red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol and white wine on the spatial learning in WT and CGRP$^{-/-}$ mice

To determine whether resveratrol contained in red wine improves the cognitive function in mice by stimulating sensory neurons, we examined the effects of resveratrol, red wine containing 20 mg/L of resveratrol, red wine containing 3.1 mg/L of resveratrol, and white wine on the spatial learning in WT and CGRP$^{-/-}$ mice using the

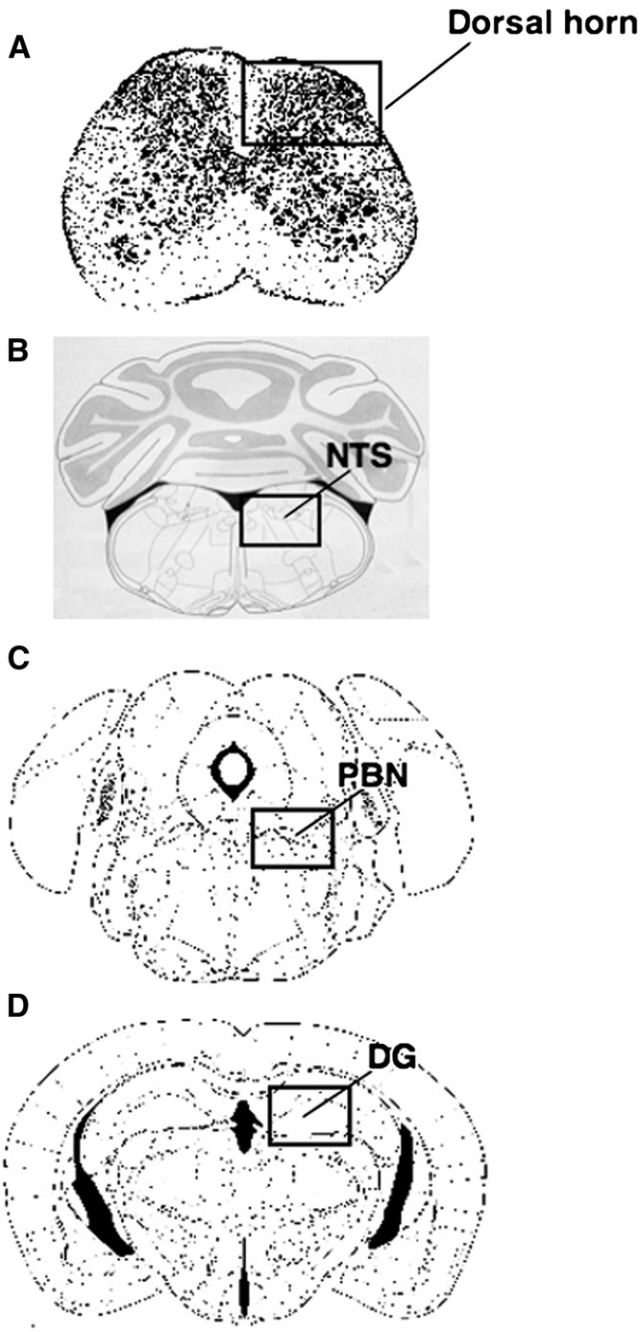

Fig. 6. Schematic diagrams of spinal cord (A) and brain sections (B, C and D) adapted from the atlas of the mouse spinal cord [48] and the mouse brain atlas [49], respectively, where select spinal cord, and brain regions were analyzed for c-*fos* expression. The location and approximate size of the analyzed area for each region is indicated. (A) dorsal horn of spinal cord. (B) Solitary tract nucleus. (C) Parabrachial nucleus; (D) Granular cell layer of the DG.

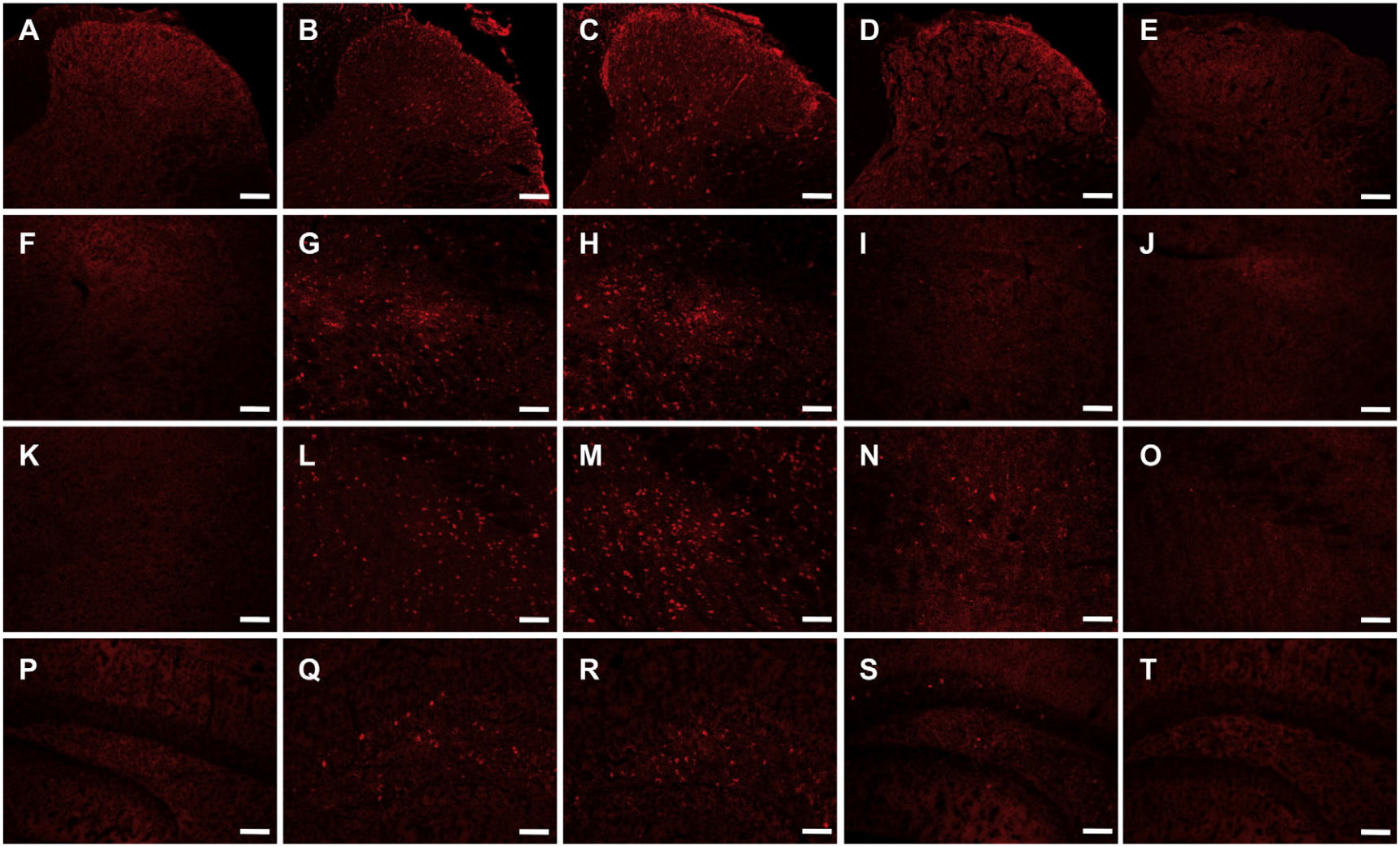

Fig. 7. Effect of resveratrol, red wine containing 20 mg/L of resveratrol (red wine A), red wine containing 3.1 mg/L of resveratrol (red wine B), and white wine on c-*fos* expression in spinal and supraspinal tissue levels in WT mice. Immunohistochemical expression of c-*fos* in a control (A, F, K, and P), a resveratrol-treated animal (B, G, L and Q), a red wine A-treated animal (C, H, M and R), a red wine B-treated animal (D, I, N and S), and a white wine-treated animal (E, J, O and T). Immunohistochemical expression of c-*fos* in the dorsal horn of the spinal cord indicated in Fig. 6A (A, B, C, D and E), solitary tract nucleus indicated in Fig. 6B (F, G, H, I and J), medial parabrachial nucleus indicated in Fig. 6C (K, L, M, N and O), and granular cell layer of the DG indicated in Fig. 6D (P, Q, R, S and T). Three animals in each group were examined, and typical results are shown. Scale bars=50 μm.

Morris water maze test for 5 consecutive days. In control animals, significant improvement of spatial learning on Days 3, 4 and 5 as compared with that on Day 1 was observed in WT mice ($P<.02$) (Fig. 9A); on the other hand, no such improvement was noted through the 5 days in CGRP$^{-/-}$ mice (Fig. 9B). In the WT mice, the improvement of spatial learning on Days 2, 3, 4 and 5 was significantly enhanced in animals treated with resveratrol as compared with that in control animal (Fig. 9A). However, resveratrol did not produce any improvement of the spatial learning through the 5 days in CGRP$^{-/-}$ mice (Fig. 9B).

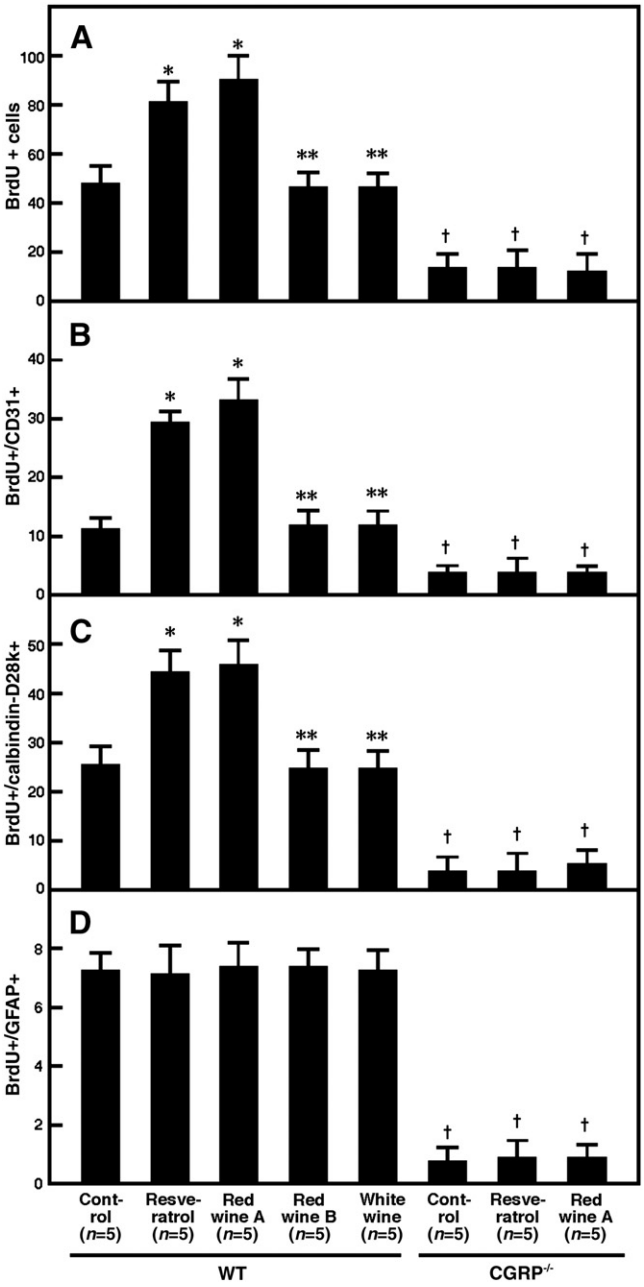

Fig. 8. Effects of resveratrol, red wine containing 20 mg/L of resveratrol (Red wine A), red wine containing 3.1 mg/L of resveratrol (Red wine B) and white wine on the hippocampal angiogenesis and neurogenesis in WT mice and CGRP$^{-/-}$ mice. Resveratrol enhanced neurovascular progenitors in the DG. Angiogenesis was examined by the colocalization of the endothelial marker CD31 and the proliferation marker BrdU. Neurogenesis was examined by the colocalization of the neuronal marker calbindin-D28k and the proliferation marker BrdU. All tissues were removed 5 days after BrdU injections. In each experiment, data were obtained from five separate experiments with 1 animal in each treatment condition. Values are expressed as means ±S.D. derived from five animal experiments. *$P<.01$ versus control; **$P<.01$ versus resveratrol; †$P<.01$ versus WT.

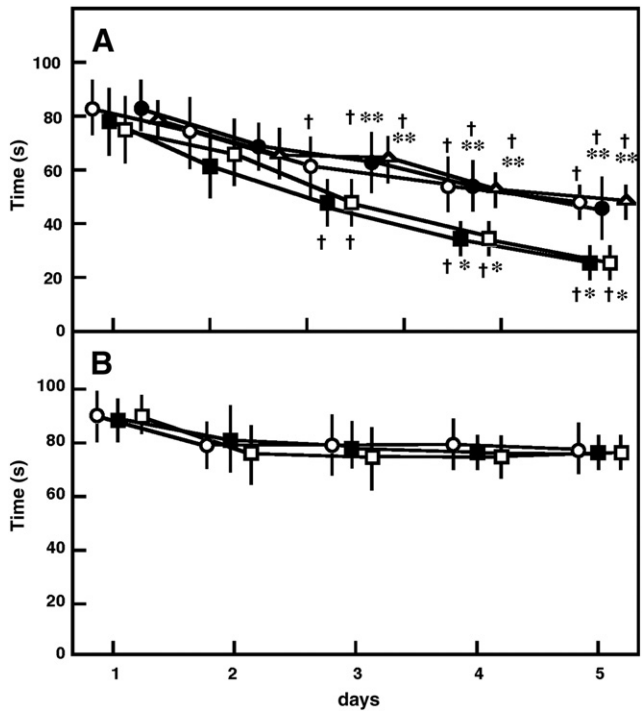

Fig. 9. Effects of resveratrol, red wine containing 20 mg/L of resveratrol (red wine A), red wine containing 3.1 mg/L of resveratrol (red wine B) and white wine on the spatial learning function in WT mice (A) and CGRP $^{-/-}$ mice (B). Each value is expressed as the mean±S.D. derived from five animal experiments. (A) Open circles: control; closed squares: resveratrol; open squares: red wine A; closed circles: red wine B; open triangles: white wine. (B) Open circles: control; closed squares: resveratrol; open squares: red wine A. (A) †$P<.01$ vs. Day 1; *$P<.01$ versus control; **$P<.01$ versus resveratrol.

Administration of red wine containing 20 mg/L of resveratrol produced effects similar to those induced by resveratrol administration in WT and CGRP$^{-/-}$ mice (Fig. 9), while administration of neither red wine containing 3.1 mg/L of resveratrol or white wine did not show any effects in WT mice (Fig. 9A) and CGRP$^{-/-}$ mice (data not shown).

Similar effects were observed with time spent in the target area (probe test). Administration of resveratrol improved the performance in the probe test in WT mice but not in CGRP$^{-/-}$ mice (data not shown). Administration of red wine containing 20 mg/L of resveratrol produced effects similar to those induced by resveratrol administration in WT and CGRP$^{-/-}$ mice (data not shown), while administration of neither red wine containing 3.1 mg/L of resveratrol or white wine did not show any effects in WT and CGRP$^{-/-}$ mice (data not shown).

3.7. Hippocampal tissue levels of resveratrol in WT mice administered resveratrol and red wine containing 20 mg/L of resveratrol

Hippocampal tissue levels of trans- and cis-resveratrol were determined in WT mice administered resveratrol and red wine containing 20 mg/L of resveratrol. Consequently, neither *trans*- nor *cis*-resveratrol was detectable in the hippocampus of these animals (data not shown).

4. Discussion

In the present study, we demonstrated that resveratrol increased CGRP release from DRG neurons isolated from WT mice in vitro.

Pretreatment with CPZ, an inhibitor of TRPV1 activation, reversed the resveratrol-induced increase of CGRP release from DRG neurons. These observations suggest that TRPV1 activation may be critically involved in the increase of CGRP release from sensory neurons treated with resveratrol. However, the mechanisms by which resveratrol activates TRPV1 are still not fully understood. Activation of PKA has been shown to induce phosphorylation of TRPV1, thereby sensitizing sensory neurons to activation by endogenous agonists such as anandamide [30,31]. Consistent with this hypothesis, we demonstrated that resveratrol increased cellular cAMP levels in DRG neurons and that the increase of CGRP release from DRG neurons treated with resveratrol was completely inhibited by the PKA inhibitor KT5720. These observations suggest that resveratrol may increase CGRP release from sensory neurons by activating PKA through increases of cellular cAMP levels. Consistent with this hypothesis, resveratrol has been shown to increase cAMP levels by activating adenylyl-cyclase, thereby activating PKA in human breast cancer cells [32].

Hippocampal tissue levels of IGF-I and IGF-I mRNA in CGRP$^{-/-}$ mice were significantly lower than those in WT mice. Oral administration of resveratrol increased hippocampal tissue levels of CGRP, IGF-I, IGF-I mRNA, and the immunohistochemical expression of IGF-I in WT mice, but not in CGRP$^{-/-}$ mice. These observations strongly suggest that oral administration of resveratrol may stimulate sensory neurons, thereby increasing the transcription and production of IGF-I in the hippocampus through increases of hippocampal CGRP levels. Consistent with this notion, we demonstrated that oral administration of the therapeutic agent for Alzheimer's disease donepezil that is capable of stimulating sensory neurons increased the transcription and production of IGF-I by increasing CGRP levels in the hippocampus of WT mice [24].

IGF-I immunoreactivity was colocalized with immunoreactivity for the astrocyte marker GFAP in the DG of WT mice administered resveratrol, suggesting that astrocytes may produce IGF-I in the hippocampus of WT mice administered ersveratrol. This notion is consistent with previous reports showing that astrocytes are capable of producing IGF-I in the hippocampus [33]. To examine the mechanisms by which sensory afferent information arising from stimulation by resveratrol in the GI tract is transmitted to the hippocampus, we examined c-*fos* expression in the spinal and supraspinal nervous tissues in WT and CGRP$^{-/-}$ mice after 3-week administration of resveratrol. In WT mice, increase of c-*fos* expression was observed in the dorsal horns (lamina I-II) of the spinal cord and, supraspinally, in the NTS, PBN and hippocampus after resveratrol administration. These observations strongly suggest that nociceptive information arising from stimulation with resveratrol in the GI tract may be transmitted via the spino-parabrachial circuits including NTS as a relay point [34–36].

In contrast to the observations in WT mice administered resveratrol, induction of c-*fos* expression was not observed in either the spinal or supra-spinal nervous tissues in CGRP$^{-/-}$ mice after resveratrol administration. These observations suggest that CGRP may function as a transmitter in the pathway involved in this sensory nervous relay system. Consistent with this hypothesis, CGRP has been shown to be expressed at synaptic contacts between the primary afferent sensory neurons and spinothalamic tract neurons in the dorsal horn of the spinal cord [37], in spinothalamic tract cells [38] and in nerve fibers originating from the PBN [19].

The number of CD31+, calbindin-D28k+ and GFAP+ cells among BrdU-immunoreactive cells of the DG in CGRP$^{-/-}$ mice was significantly lower than that in WT mice, suggesting that CGRP and/or IGF-I may be deeply related to neural stem cell proliferation in the mouse hippocampus. Consistent with this hypothesis, IGF-I has been shown to be necessary for angiogenesis in the mouse adult brain [14], and also for neural stem cell proliferation in collaboration with activities of epidermal growth factor and fibroblast growth factor-2 [39].

Administration of resveratrol increased the number of BrdU+ cells and that of both BrdU+ and calbindin-D28k+ double-positive cells, but not that of both BrdU+ and GFAP+ double-positive cells in the DG of WT mice. On the other hand, administration of resveratrol had no effect on the number of these cells in the DG of CGRP$^{-/-}$ mice. Peripheral infusion of IGF-I was shown to selectively induce angiogenesis via a VEGF-dependent mechanism in the adult mouse brain [14] and neurogenesis in the adult rat hippocampus [9]. These observations strongly suggest that stimulation of sensory neurons by resveratrol may induce angiogenesis and neurogenesis via induction of IGF-I production by increasing CGRP levels in the mouse hippocampus. Furthermore, since angiogenesis has been shown to offer a favorable environment for neuronal stem cell proliferation via activation of an VEGF-dependent mechanism [15], the hippocampal neurogenesis induced by resveratrol administration in the WT mice might be mediated at least in part by angiogenesis.

IGF-I exerts beneficial effects against the decline in cognitive function by inducing neurogenesis in the hippocampus [9,40], suggesting that resveratrol administration may improve cognitive function by inducing IGF-I production through promotion of CGRP release in the mouse hippocampus. Consistent with this hypothesis, resveratrol administration significantly improved spatial learning function in WT mice, but not CGRP$^{-/-}$ mice. These observations suggest that stimulation of sensory neurons by resveratrol may increase the release of CGRP, inducing IGF-I production in the mouse hippocampus, thereby improving cognitive function.

In the present study, both angiogenesis and neurogenesis were observed after 3-week administration of resveratrol. It is known that it takes approximately 2–4 weeks are required before newly generated neurons are functionally integrated and begin to modify active hippocampal circuits [39]. IGF-I has been shown to exert critical enhancing effects on synaptic transmission and plasticity within minutes [8]. These observations suggest that the resveratrol-induced improvement of cognitive function is mainly dependent on the latter effect induced by IGF-I in mouse hippocampus.

The average concentration of resveratrol in red wine is reported to be 4.7 mg/L [41]. Administration of red wine containing 20 mg/L of resveratrol produced effects similar to those induced by resveratrol administration, while administration of neither red wine containing 3.1 mg/L of resveratrol nor white wine showed such effects. Thus, although ethanol has been shown to enhance TRPV1 activation [42], the effects of administration of red wine containing 20 mg/L of resveratrol might be mainly dependent of the action of resveratrol.

The relationship between moderate consumption of red wine and the low risk of dementia has been believed to be due principally to the antioxidant effect of resveratrol [43]. However, the resveratrol concentrations reached by drinking within safe limits of some red wines might be just within or below the therapeutic range, as determined by isolated tissue experiments in vitro [44]. Thus, it is likely that some unknown mechanisms other than those hitherto proposed, such as anti-oxidant effects, operates and contributes to lowering the risk of dementia in vivo. Resveratrol was undetectable in the hippocampus of WT mice administered resveratrol and red wine containing 20 mg/L of resveratrol, suggesting that resveratrol might not directly increase tissue levels of CGRP and IGF-I in the hippocampus of these animals. These observations strongly suggest that resveratrol may stimulate sensory neurons in the GI tract, thereby increasing IGF-I production in the mouse hippocampus via activation of the nociceptive pathway.

Sensory nerve endings have been shown to be located close to the lumen of the GI tract, and thus are capable of responding to the chemical composition of luminal contents [23]. Concentrations of resveratrol in various red wines range from 4 to 79 μM [44]. The concentration of free resveratrol is about 10% of the total resveratrol in red wine [41]. Thus, concentrations of free resveratrol may reach

0.1 μM, a concentration capable of stimulating sensory neurons in vitro, in the GI tract even when ingested red wine is diluted several-fold by other foods and drinks. Consistent with this notion, the GI tract has been shown to be a major site of activities of polyphenols, since much higher concentrations of polyphenols are achieved in the GI tract than in plasma or other tissues [45].

Red wine has been shown to lower the relative risk of dementias including Alzheimer's disease [1,46,47]. In the present study, oral administration of red wine containing 20 mg/L of resveratrol for 3 weeks improved cognitive function by increasing IGF-I production, while that of red wine containing 3.1 mg/L of resveratrol for 3 weeks did not. It is thus likely that habitual drinking of red wines containing relatively higher concentrations of resveratrol contributes to improvement of cognitive function in humans. However, the resveratrol concentration varies substantially among wines [44]. It is thus possible that drinking red wines with regular concentrations of resveratrol for long periods lowers the risk of age-associated cognitive decline. This possibility requires examination by in vivo animal experiments.

Taken together, observations in the present study strongly suggest that sensory neuron stimulation by resveratrol in the GI tract may increase the IGF-I production and promote angiogenesis and neurogenesis in the hippocampus, thereby improving cognitive function in mice. This may explain at least in part the beneficial effects of moderate consumption of red wine on cognitive function in humans.

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