



## NEUROBIOLOGY

# Activation of Cannabinoid CB2 Receptor—Mediated AMPK/CREB Pathway Reduces Cerebral Ischemic Injury

In-Young Choi,<sup>\*</sup> Chung Ju,<sup>\*</sup> Angela M.A. Anthony Jalin,<sup>\*</sup> Da In Lee,<sup>†</sup> Paul L. Prather,<sup>‡</sup> and Won-Ki Kim<sup>\*</sup>

From the Department of Neuroscience,<sup>\*</sup> College of Medicine, Korea University, Seoul, South Korea; the Department of Biological Sciences,<sup>†</sup> Columbia College, Columbia University, New York, New York; and the Department of Pharmacology and Toxicology,<sup>‡</sup> University of Arkansas for Medical Sciences, Little Rock, Arkansas

**CME Accreditation Statement:** This activity ("ASIP 2013 AJP CME Program in Pathogenesis") has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education (ACCME) through the joint sponsorship of the American Society for Clinical Pathology (ASCP) and the American Society for Investigative Pathology (ASIP). ASCP is accredited by the ACCME to provide continuing medical education for physicians.

The ASCP designates this journal-based CME activity ("ASIP 2013 AJP CME Program in Pathogenesis") for a maximum of 48 AMA PRA Category 1 Credit(s)<sup>™</sup>. Physicians should only claim credit commensurate with the extent of their participation in the activity.

**CME Disclosures:** The authors of this article and the planning committee members and staff have no relevant financial relationships with commercial interests to disclose.

Accepted for publication  
November 26, 2012.

Address correspondence to  
Won-Ki Kim, Ph. D.,  
Department of Neuroscience,  
College of Medicine, Korea  
University, Anamdong-5-ga,  
Seongbuk-gu, Seoul 136-705,  
South Korea. E-mail: [wonki@korea.ac.kr](mailto:wonki@korea.ac.kr).

The type 2 cannabinoid receptor (CB2R) was recently shown to mediate neuroprotection in ischemic injury. However, the role of CB2Rs in the central nervous system, especially neuronal and glial CB2Rs in the cortex, remains unclear. We, therefore, investigated anti-ischemic mechanisms of cortical CB2R activation in various ischemic models. In rat cortical neurons/glia mixed cultures, a CB2R agonist, *trans*-caryophyllene (TC), decreased neuronal injury and mitochondrial depolarization caused by oxygen-glucose deprivation/re-oxygenation (OGD/R); these effects were reversed by the selective CB2R antagonist, AM630, but not by a type 1 cannabinoid receptor antagonist, AM251. Although it lacked free radical scavenging and antioxidant enzyme induction activities, TC reduced OGD/R-evoked mitochondrial dysfunction and intracellular oxidative stress. Western blot analysis demonstrated that TC enhanced phosphorylation of AMP-activated protein kinase (AMPK) and cAMP responsive element-binding protein (CREB), and increased expression of the CREB target gene product, brain-derived neurotrophic factor. However, TC failed to alter the activity of either Akt or extracellular signal-regulated kinase, two major CB2R signaling pathways. Selective AMPK and CREB inhibitors abolished the neuroprotective effects of TC. In rats, post-ischemic treatment with TC decreased cerebral infarct size and edema, and increased phosphorylated CREB and brain-derived neurotrophic factor expression in neurons. All protective effects of TC were reversed by co-administration with AM630. Collectively, these data demonstrate that cortical CB2R activation by TC ameliorates ischemic injury, potentially through modulation of AMPK/CREB signaling, and suggest that cortical CB2Rs might serve as a putative therapeutic target for cerebral ischemia. (*Am J Pathol* 2013, 182: 928–939; <http://dx.doi.org/10.1016/j.ajpath.2012.11.024>)

Ischemic brain injury emerges as a consequence of complex pathological cascades, including excitotoxicity, mitochondrial depolarization, oxidative stress, and inflammation.<sup>1</sup> Much effort has been made to develop neuroprotective drugs able to interrupt these cascades and demonstrate efficacy, while producing tolerable adverse effects when tested in clinical trials. Recent studies suggest a protective role of the cannabinoid signaling system in cerebral ischemia.<sup>2</sup> For example, during ischemic injury, endocannabinoids accumulate, cannabinoid receptors are up-regulated, and

treatment with cannabinoid agonists (either endocannabinoids or phytocannabinoids/synthetic cannabinoids) protects neurons against damage resulting from ischemic stroke.<sup>2,3</sup>

Supported by a Brain Research Center of the 21st Century Frontier Research Program grant 2011K000267 (W.-K.K.), the Bio & Medical Technology Development Program grant 2011-0019440 (W.-K.K.), and the Basic Science Research Program grant 2011-0014968 (I.-Y.C.) through the National Research Foundation of Korea, funded by the Ministry of Science and Technology, Republic of Korea.

I.-Y.C. and C.J. contributed equally to this work.

The effects of cannabinoids are mainly mediated by action at G-protein—coupled type 1 cannabinoid receptors (CB1Rs) and type 2 cannabinoid receptors (CB2Rs). CB2Rs were initially thought to be expressed primarily in activated microglia and peripheral immune cells, regulating antigen presentation, cytokine/chemokine production, and cell migration.<sup>4</sup> Considering the critical role of inflammation in ischemic pathophysiological characteristics<sup>5</sup> and the protective effects observed in several peripheral organs<sup>6–8</sup> by administration of CB2R agonists during ischemia-reperfusion injury, selective activation of the CB2R has drawn much attention as a potential therapeutic target for treatment of cerebral ischemia, without producing psychoactive adverse effects associated with CB1R activation.<sup>9,10</sup> For example, recent studies demonstrate that CB2R agonists produce neuroprotective effects, and mice lacking CB2Rs are more sensitive to cerebral ischemic injury.<sup>11–15</sup> CB2R expression is also up-regulated in microglia/macrophages associated with ischemic brain lesions.<sup>13,16</sup> The most commonly proposed mechanisms responsible for the anti-inflammatory actions underlying the anti-ischemic effects of CB2R activation include attenuation of leukocyte rolling and adhesion,<sup>11,12</sup> limitation of neutrophil recruitment,<sup>14</sup> and reduction of pro-inflammatory mediator production.<sup>15</sup> Recent studies, however, suggest that functional CB2Rs exist within immune cells present in the central nervous system (CNS) and in the neurons and glial cells. Selective CB2R agonists alter drug abuse behaviors in *CB1R*-knockout mice but not in *CB2R*-knockout mice, and a possible association of genetic variants of the *CB2R* gene and depression in a human population has been identified.<sup>17–19</sup> Furthermore, expression of CB2R mRNA and protein in CNS neurons, including cortical, hippocampal, and cerebellar neurons, and glial cells has been reported.<sup>20–25</sup> Although much evidence indicates the presence of functional CB2Rs in the CNS, potential neuroprotective properties of these CB2Rs and the molecular mechanisms underlying the effects associated with their activation during ischemic injury are not yet understood.

A bicyclic sesquiterpene, *trans*-caryophyllene (TC), has been reported to be a CB2R-selective agonist ( $K_i$  values of 155 nmol/L for human CB2Rs, with no significant affinity for binding to CB1Rs), leading to activation of the  $G_i/G_o$  subtype of G proteins.<sup>26</sup> Interestingly, although TC is a major cannabinoid derived from the essential oil of *Cannabis sativa* L, it has a fundamentally different structure from the classic cannabinoids, due to an unusual cyclobutane-containing scaffold.<sup>26</sup> Similar to other CB2R agonists, TC inhibits inflammation, edema formation, and the expression of inflammatory mediators *in vitro* and *in vivo*.<sup>26–33</sup> TC was also found to reduce oxygen-glucose deprivation (OGD)—evoked cell death in neuroblastoma cells *in vitro*.<sup>34</sup> However, the anti-ischemic effect of TC in cerebral ischemia and its underlying mechanism has yet to be elucidated.

In the present study, TC was found to produce neuroprotective effects in *in vitro* and *in vivo* ischemic models.

The anti-ischemic effects of TC in mixed cortical cultures is mediated by activation of CB2Rs, most likely leading to an increase in the levels of AMP-activated protein kinase (AMPK) and phosphorylation of one of its substrates, cAMP responsive element-binding protein (CREB). In an *in vivo* ischemic rat model, it was further found that post-ischemic treatment of TC increases the levels of phosphorylated CREB and brain-derived neurotrophic factor (BDNF), one of the target gene products of CREB.

## Materials and Methods

### Reagents

TC was purchased from Sigma-Aldrich (St. Louis, MO). AM251 and AM630 were purchased from Tocris (Ellisville, MO), and compound C (CC) and CREB inhibitor were obtained from Calbiochem (Darmstadt, Germany). Tetramethylrhodamine methyl ester (TMRM), 5-(and 6-)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H<sub>2</sub>DCF-DA), and secondary antibodies (conjugated Alexa fluorophores) were obtained from Molecular Probes (Eugene, OR). The following primary antibodies were used: CB1R (Cayman Chemicals, Ann Arbor, MI); CB2R (an N-terminus antibody from Cayman Chemicals); mitogen-activated protein (MAP)-2 (Sigma-Aldrich); neuronal nuclei (NeuN; Chemicon, Temecula, CA); phosphorylated AMPK (pAMPK), AMPK, phosphorylated CREB (pCREB), and CREB antibodies (Cell Signaling, Danvers, MA); and BDNF (Abcam, Cambridge, MA). Neurobasal medium and B27 supplement were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum was obtained from Hyclone (Logan, UT). All other chemicals were purchased from Sigma-Aldrich.

### Primary Mixed Culture of Cortical Neurons/Glia

Dissociated cultures of rat cortical neurons/glia were prepared from embryonic 17- to 18-day-old Sprague-Dawley rat embryos. In brief, meninges-free cortices were dissociated by triturating through a Pasteur pipette (Poulten & Graf GmbH, Wertheim, Germany). Neuronal cells ( $1.5 \times 10^3$  cells/mm<sup>2</sup>) were initially plated in plates precoated with poly-D-lysine (100 µg/mL) and laminin (4 µg/mL) in neurobasal medium containing 10% fetal bovine serum. Cells were then maintained in a B27-supplemented neurobasal medium in humidified 95% air/5% CO<sub>2</sub> at 37°C. Experiments were performed on cultures 14 to 15 days after initial plating. Cultures contain 40% to 50% of neurons, as assessed by immunostaining with cell type—specific markers.

### Oxygen-Glucose Deprivation and Re-Oxygenation

To induce ischemic insult *in vitro*, cells were replenished with glucose-free Dulbecco's modified Eagle's medium and transferred to an anaerobic chamber containing 5% CO<sub>2</sub> and 10% H<sub>2</sub> atmosphere, balanced with N<sub>2</sub> (partial pressure of

oxygen, <2 mm Hg), for 1.5 hours at 37°C. OGD was terminated by returning plates to the original growth conditions, in oxygenated Dulbecco's modified Eagle's medium supplemented with 25 mmol/L glucose under normoxic conditions. TC was applied immediately before initiation of OGD and maintained throughout OGD/re-oxygenation (OGD/R). In a subset of experiments to determine a potential mechanism of action for TC, cells were exposed to a 30-minute pretreatment with AM251 (a selective CB1R antagonist), AM630 (a selective CB2R antagonist), CC (an AMPK inhibitor), or CREB—CREB-binding protein interaction inhibitor (CREB signaling inhibitor) before the addition of TC and maintained throughout OGD/R. Five hours after re-oxygenation, lactate dehydrogenase (LDH) release was measured to assess cell injury or death.

### Assessment of Cell Injury or Death

Cell injury or death was assessed by morphological examination using a phase-contrast microscope (DM IL; Leica, Nussloch, Germany) or by measuring the amount of LDH released into the culture medium using a diagnostic kit (Sigma-Aldrich), as previously described.<sup>35</sup> Absorbance readings were measured at 450 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA) and are expressed as the percentage of total LDH release, which was derived from sister cultures subjected to repeated freeze/thaw cycles.

### Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 15 minutes and permeabilized with 0.2% Triton X-100. After blocking in 10% normal horse serum for 30 minutes, cells were incubated with primary antibodies at 4°C overnight: mouse anti-MAP2 (diluted to 1:300) and either rabbit anti-CB1R (1:200) or rabbit anti-CB2R (1:200). Cells were then washed three times with PBS and incubated with goat secondary antibodies conjugated with Alexa fluorophores (1:200). After mounting, immunoreactivity was observed under a confocal laser microscope (Zeiss LSM510; Zeiss, Oberkochen, Germany), with a 488- and 543-nm laser used for excitation and 505- to 530-nm band-pass and 560-nm long-pass filters used for emission.

### Measurement of Intracellular Oxidative Stress: DCF-DA Assay

As previously described, intracellular oxidative stress precedes neuronal injury, quickly increasing after re-oxygenation.<sup>36</sup> At 1 hour after re-oxygenation, cells were loaded with 30  $\mu$ mol/L CM-H<sub>2</sub>DCF-DA. CM-H<sub>2</sub>DCF-DA diffuses through cell membranes and is hydrolyzed by intracellular esterases to the non-fluorescent analogue, dichlorofluorescein. Dichlorofluorescein then reacts with intracellular free radicals, such as peroxyl radical, peroxynitrite, or hydrogen peroxide, to form dichlorofluorescein, a green fluorescent dye. Two hours after

loading, cells were washed with Earle's balanced salt solution buffer containing 0.1% bovine serum albumin and 2.5 mmol/L probenecid. Fluorescent intensities were then measured at 488 nm of excitation wavelength and 525 nm of emission wavelength using a fluorescence microscope (DM IL HC Fluo; Leica) equipped with a digital camera (DFC420C; Leica). The intensity of fluorescence was quantified by an image analyzer (TOMORO ScopeEye 3.5; Techsan Digital Imaging, Seoul, Korea).

### Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured by quantifying the accumulation of TMRM, as previously described.<sup>37</sup> In brief, cells were loaded with 10 nmol/L TMRM at 37°C, after 1 hour of re-oxygenation; 2 hours after loading, residual TMRM was removed by washing and the mitochondrial uptake of TMRM was measured by a fluorescence microscope (DM IL HC Fluo). Fluorescent intensity was analyzed by using an imaging analyzing program (TOMORO ScopeEye 3.5).

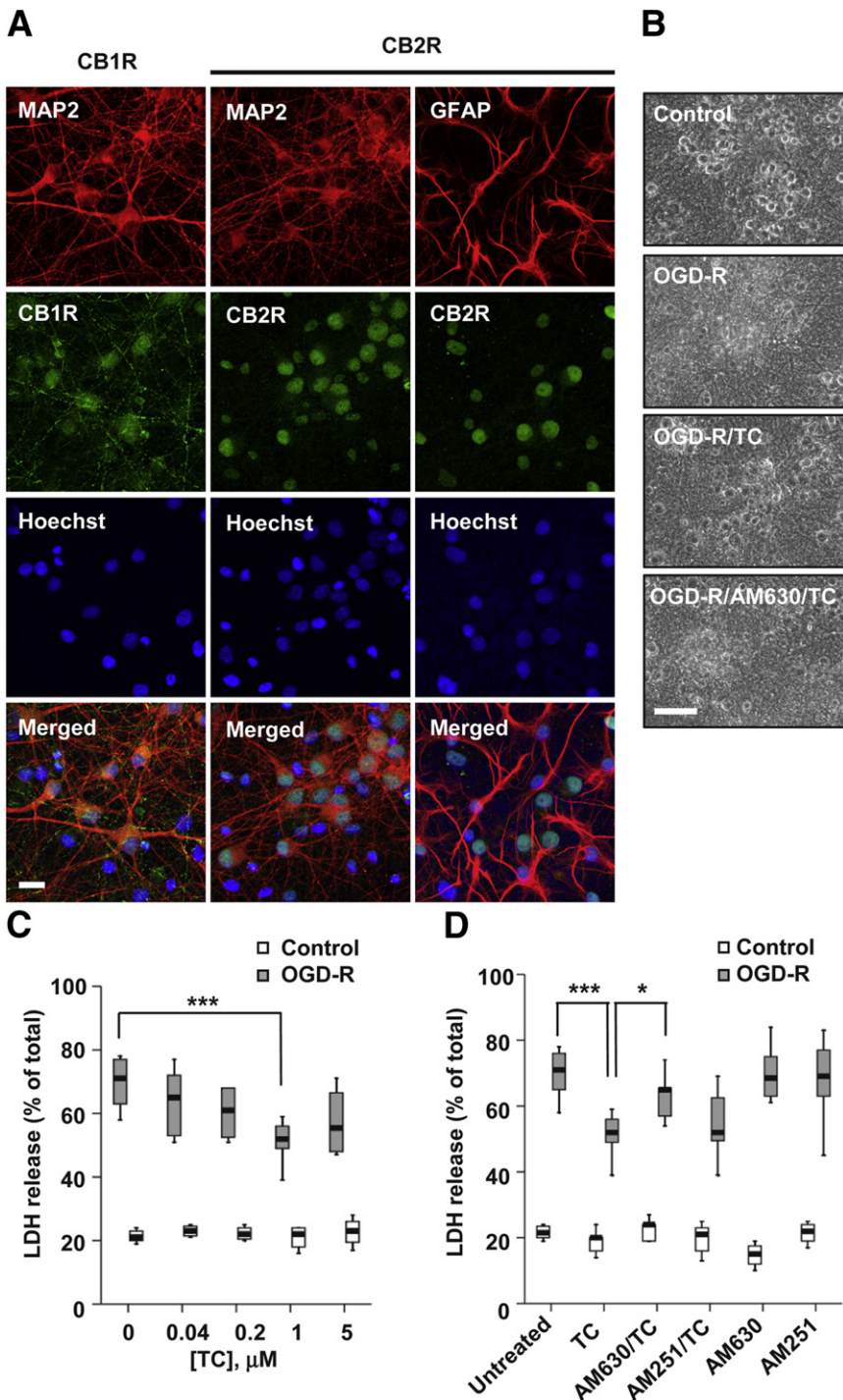
### Measurement of Free Radical Scavenging Activities: DPPH and ORAC Assays

Direct free radical scavenging activities were measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays, as previously described.<sup>38,39</sup> In brief, for the DPPH reduction assay, an organic nitrogen radical generator, DPPH (23.6  $\mu$ g/mL in ethanol), was incubated for 30 minutes at 37°C in the presence of test drugs at various concentrations. The decrease in absorbance was then measured at 517 nm by a microplate reader (SPECTRAMax 340PC; Molecular Devices). The scavenging activity of free radicals was expressed as the percentage of maximum inhibition obtained from a standard curve generated by using vitamin C. For the ORAC assay, various concentrations of antioxidants react to peroxyl radicals generated from 2,2'-azobis-(2-methylpropionamide)-dihydrochloride (60 mmol/L) in a competitive manner with a fluorescent indicator, fluorescein (50 nmol/L). A fluorescence decay curve was measured every 5 minutes for 3 hours at 37°C by a fluorescence microplate reader (SpectraMax GeminiEM; Molecular Devices) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. For quantification of scavenging capacity of the peroxyl radical, the area under the curve (AUC) was calculated based on kinetic curves:  $AUC = (0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \dots + f_{n-2}/f_0 + f_{n-1}/f_0 + f_n/f_0) \times 5$ , where  $f_i$  is the fluorescence reading at time  $i$  (in minutes). The net AUC = AUC<sub>sample</sub> - AUC<sub>blank</sub>.

### Western Blot Analysis

To examine putative downstream signaling molecules of TC, the amount of total and phosphorylated proteins for AMPK,





**Figure 1** TC inhibits OGD/R-induced neuronal injury via CB2R in rat cortical cultures. **A:** Expression of CB1Rs and CB2Rs in cortical mixed neuronal/glial cultures. Representative confocal microscopy analyses with anti-MAP2 (a neuronal somatodendritic marker) and anti-glial fibrillary acidic protein (GFAP; an astrocyte marker) antibodies and nuclear counterstaining with Hoechst 33258. Scale bar = 50  $\mu\text{m}$ . **B–D:** Cortical cultures were exposed to OGD (1.5 hours) and subsequent re-oxygenation (5 hours). TC, at indicated concentrations (**C**) or 1  $\mu\text{mol/L}$  (**D**), was applied immediately before OGD and maintained throughout re-oxygenation in the presence or absence of either 1  $\mu\text{mol/L}$  AM630 or 100 nmol/L AM251. Neuronal injury was assessed by morphological characteristics (**B**) or measurement of LDH release into the culture media (**C** and **D**). **B:** Representative phase-contrast images. Scale bar = 50  $\mu\text{m}$ . **C:** LDH release. Data were expressed as percentage of total LDH release measured in parallel cultures exposed to repeated freeze/thaw cycles. Horizontal bar, median; vertical box, interquartile ranges (Q1 to Q3); and whiskers, minimum/maximum. Data were analyzed with the Kruskal-Wallis test, followed by the *U*-test.  $N = 4$  to 15.  $***P < 0.001$ , significantly different from the untreated OGD/R group. **D:** AM630 abolishes the neuroprotective effect of TC.  $N = 5$  to 15.  $*P < 0.05$ ,  $***P < 0.001$ , significantly different between the indicated groups.

CREB, or BDNF was analyzed using Western blot analysis, as previously described.<sup>40</sup> In brief, 30  $\mu\text{g}$  per lane of cell extracts was electrophoresed on 7.5% or 10% Tris-glycine SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After blocking with Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk, the membranes were incubated overnight at 4°C using the previously described primary antibodies (1:3000 in blocking buffer). A standard electrochemiluminescence prime

detection procedure was then used for final visualization (Amersham Biosciences, Buckinghamshire, UK).

## Animals

Male Sprague-Dawley rats, weighing 260 to 270 g, were purchased from Charles River Laboratories (Seoul, Korea) and kept on a 12-hour light/dark cycle with ad libitum access to food and water. Rats were acclimated to environments

before use in experiments. All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Korea University Institutional Animal Care and Use Committee.

### Focal Cerebral Ischemia

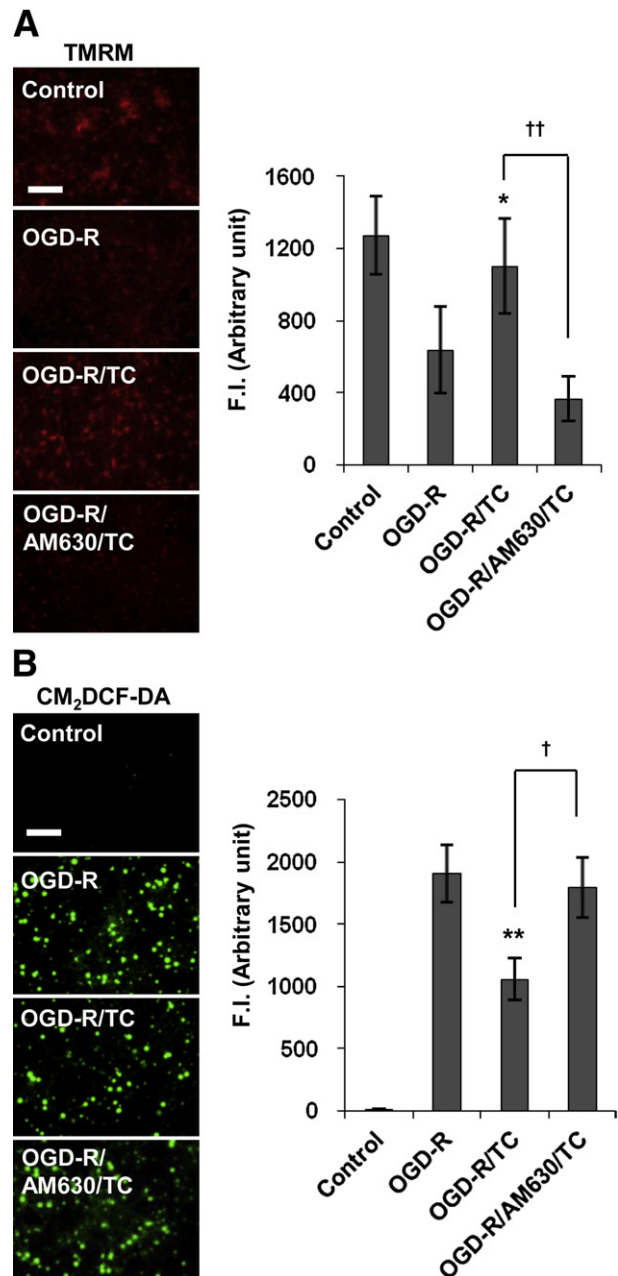
For induction of cerebral ischemia, rats were initially anesthetized via face mask with 3% isoflurane in a 70% N<sub>2</sub>O and 30% O<sub>2</sub> (v/v) mixture. Anesthesia was maintained with 2% isoflurane. A rectal temperature probe was introduced, and body temperature was maintained at 37°C during the entire surgical period. Focal cerebral ischemia was achieved by right-sided endovascular middle cerebral artery occlusion (MCAO), as previously described.<sup>35</sup> After 1.5 hours of ischemia, the occlusion was released and animals were allowed to recover. TC, 10 mg/kg, was initially dissolved in dimethyl sulfoxide, further diluted (5%) in sterile saline with 10% Cremophor EL (polyoxy-35 hydrogenated castor oil; Merck KGaA, Darmstadt, Germany), and administered i.p. as a post-ischemic treatment (3 hours after initiation of MCAO) in the presence or absence of AM630 (1 mg/kg).

### Measurement of Infarct Volume

Rats were anesthetized with chloral hydrate and decapitated 24 hours after MCAO. Rat brains embedded in a rat brain matrix (Ted Pella, Redding, CA) were cut into coronal sections (2 mm thick). Brain sections were then incubated with 2% triphenyltetrazolium chloride at room temperature for 30 minutes to reveal ischemic infarctions. The cross-sectional area of an infarction between the bregma levels of 4 mm (anterior) and -6 mm (posterior) was determined with the aid of a computer-assisted image analysis program (OPTIMAS 5.1; BioScan Inc., Edmonds, WA). Cerebral edema was determined by calculating the percentage increase of the ipsilateral ( $V_I$ )/contralateral ( $V_C$ ) hemisphere area: % Edema Volume =  $[(V_I - V_C)/V_C] \times 100$ . The total volume of the infarct was quantified by integrating six sections while compensating for brain edema, as previously described<sup>41</sup>: Infarct Volume (mm<sup>3</sup>) =  $IV_d \times (V_C/V_I)$ , where  $IV_d$  is the ipsilateral volume obtained by direct measurement;  $V_I$ , ipsilateral hemisphere area; and  $V_C$ , contralateral hemisphere area.

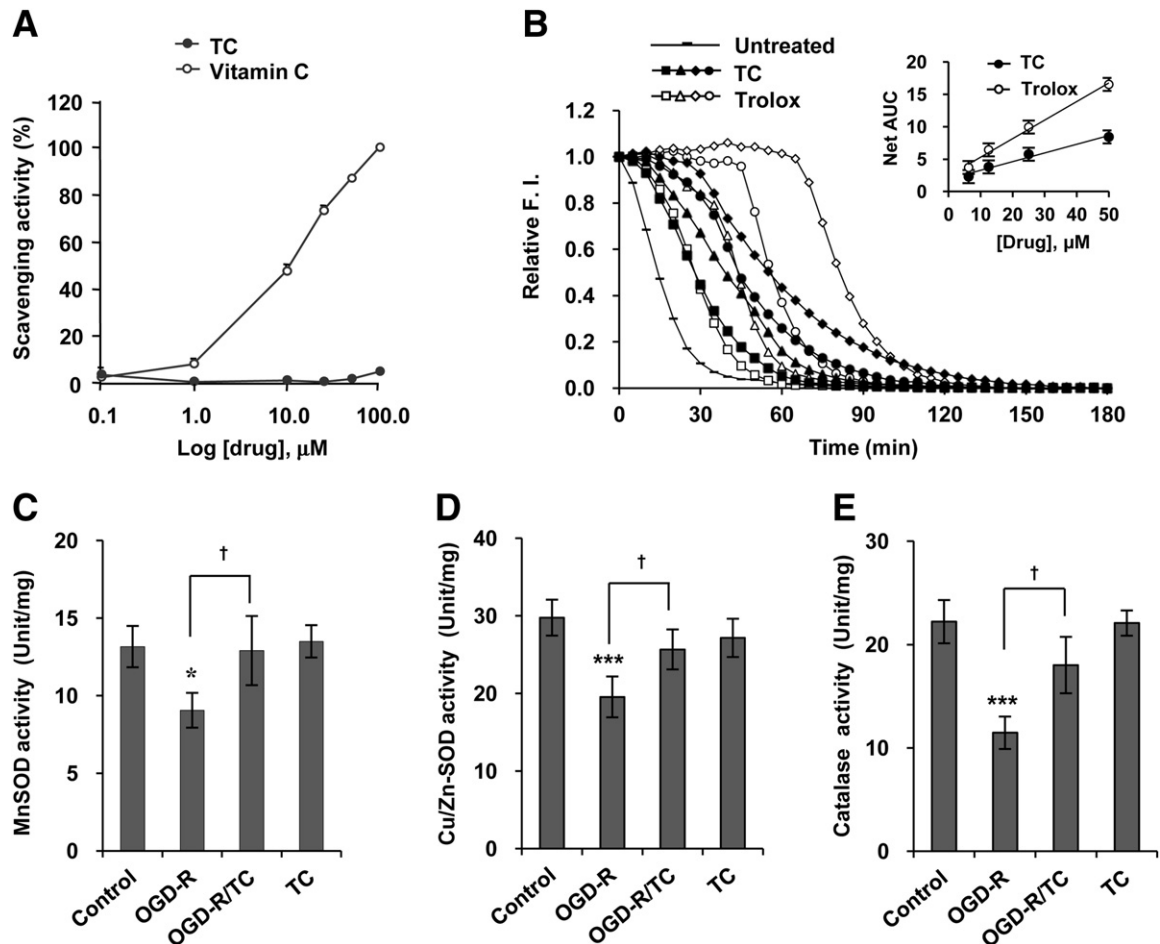
### Immunohistochemistry

Immunohistochemical (IHC) detection of pAMPK, pCREB, and BDNF in neurons of ischemic lesions was performed as previously described.<sup>40</sup> In brief, rats were sacrificed 24 hours after MCAO and perfused transcardially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Brains were postfixed overnight and cryoprotected. Serial coronal sections (30  $\mu$ m thick) were incubated in blocking buffer (10% normal horse serum—supplemented PBS) for 30 minutes. Sections were then incubated overnight at room



**Figure 2** TC reduces OGD/R-evoked mitochondrial depolarization and intracellular oxidative stress. Cortical cultures were exposed to OGD (1.5 hours)/R (3 hours) in the presence or absence of 1  $\mu$ mol/L TC and/or AM630. **A:** Mitochondrial depolarization assessed by the decrease in TMRM fluorescence. TMRM, 10 nmol/L, was loaded at 1 hour after R. Representative images (left panel) and quantification of fluorescence intensities (F.I.s; right panel). Scale bar = 50  $\mu$ m. **B:** Intracellular oxidative stress measured by dichlorofluorescein (DCF). CM-H<sub>2</sub>DCF-DA was loaded 1 hour after R, and fluorescence was measured at 3 hours after R. Data are given as representative images and quantified. Data are expressed as means  $\pm$  SD from four independent experiments each. \* $P$  < 0.05, \*\* $P$  < 0.01, significantly different from the OGD/R group. † $P$  < 0.05, †† $P$  < 0.01, significantly different between the indicated groups.

temperature with primary antibodies: rabbit anti-CB2R (1:50), mouse anti-NeuN (1:300), rabbit anti-pCREB (1:100), or rabbit anti-BDNF (1:25) antibodies in PBS containing 0.3% Triton X-100 and 3% normal horse serum. After



**Figure 3** TC neither scavenges free radicals nor increases SOD or catalase activity. **A:** 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The scavenging activity of TC against nitrogen radical generated from DPPH was measured by a decrease in absorbance and expressed as percentage of scavenging activity compared with a standard curve using vitamin C. Data are expressed as means  $\pm$  SD.  $N = 4$ . **B:** Oxygen radical absorbance capacity (ORAC) assay. 2,2'-Azobis-(2-methylpropionamide)-dihydrochloride (AAPH)-induced decay in fluorescein fluorescence intensity (F.I.) was measured in the presence of TC or Trolox at the indicated concentrations (white and black squares, 6.25  $\mu\text{mol/L}$ ; white and black triangles, 12.5  $\mu\text{mol/L}$ ; white and black circles, 25  $\mu\text{mol/L}$ ; and white and black diamonds, 50  $\mu\text{mol/L}$ ) and measured as relative F.I. A representative curve from four independent experiments is shown. **Inset**, best fit lines between net area under the curve (AUC) and concentrations of TC or Trolox. **C–E:** For an antioxidant enzyme assay, cells were exposed to either normoxia or OGD (1.5 hours)/R (3 hours) in the presence or absence of 1  $\mu\text{mol/L}$  TC. Data were calculated as units per mg protein and expressed as means  $\pm$  SD.  $N = 4$ . **C:** MnSOD activity assay. **D:** Cu/ZnSOD activity assay. **E:** Catalase assay.  $N = 4$  each. \* $P < 0.05$ , \*\*\* $P < 0.001$ , significantly different from the control group. <sup>†</sup> $P < 0.05$ , significantly different from the OGD/R group.

washing with PBS, sections were further incubated with Alexa fluorophore-conjugated secondary antibodies for 2 hours at room temperature (1:100 or 1:300). After mounting, a digitalized image of each section was obtained by using a confocal microscope (Zeiss LSM510) with a 488- and 543-nm laser used for excitation and 505- to 530-nm band-pass and 560-nm long-pass filters for emission.

### Statistical Analysis

Data were expressed as means  $\pm$  SD and analyzed for statistical significance using an analysis of variance, followed by *post hoc* analysis using Tukey's test for multiple comparisons. Before analysis of variance, the normality of data and the  $P$  value of Levene's Test for Equality of Variances were confirmed ( $P > 0.05$ ). Otherwise, data were expressed as medians  $\pm$  interquartile ranges from quartile 1

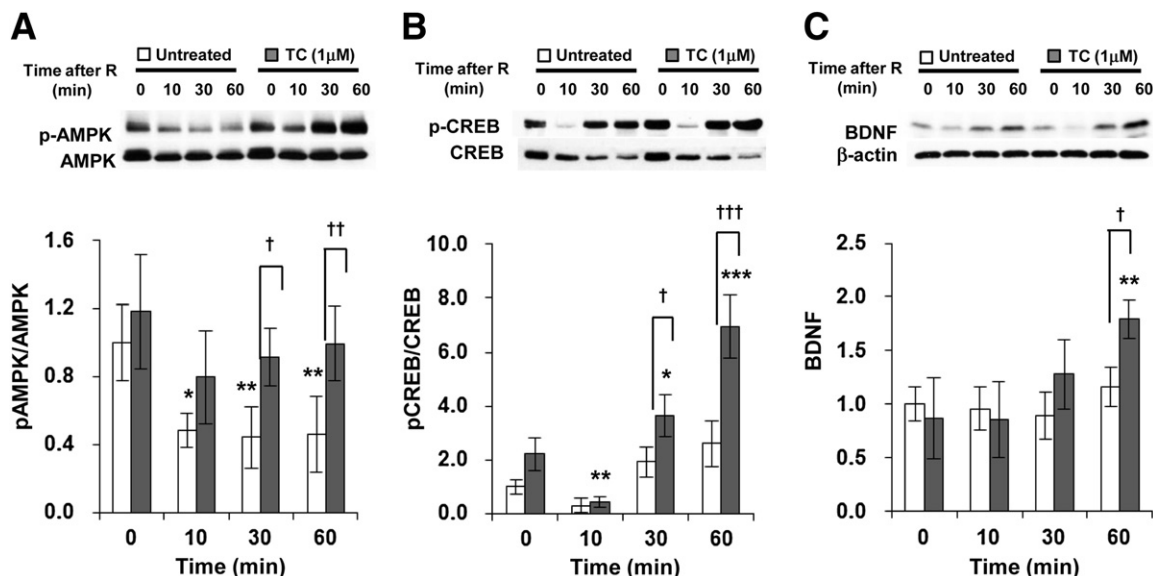
(Q1) to quartile 3 (Q3) and analyzed by the Kruskal-Wallis test, followed by the  $U$ -test.  $P < 0.05$  was considered significant after Bonferroni's correction.

## Results

### TC Inhibits Neuronal Ischemic Death via CB2R Activation

Double immunofluorescence of CBRs and a somatodendritic neuronal marker, MAP2, showed that both CB1Rs and CB2Rs were expressed in cortical neurons (Figure 1A and Supplemental Figure S1).<sup>22,40,42–44</sup> Although CB1Rs were predominantly expressed in cell bodies and axons of cortical neurons, CB2R immunoreactivity was observed in intracellular compartments of neurons, including cell bodies and apical dendrites, as previously described.<sup>42,43</sup> Although relatively weak, CB2R immunoreactivity was





**Figure 4** TC up-regulated phosphorylation of AMPK and CREB and expression of BDNF. Cortical cultures were subject to OGD (1.5 hours) in the presence or absence of 1  $\mu$ mol/L TC. At the indicated time (in minutes) after re-oxygenation after OGD, cell lysates were collected and subjected to Western blot analysis of pAMPK and AMPK (A), phosphorylated CREB at Ser133 (pCREB) and CREB (B), and BDNF (C). **Top panel**, immunoblots. **Bottom panel**, densitometric analyses of four to six independent experiments. Data were expressed as either ratios of phosphorylated to total proteins (A and B) or total amount (C) after normalization to  $\beta$ -actin. Each box represents the means  $\pm$  SD. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, significantly different from matched control groups at 0 minutes after re-oxygenation. † $P$  < 0.05, †† $P$  < 0.01, and ††† $P$  < 0.001, significantly different from the untreated group.

also detected in cortical glia (Figure 1A and Supplemental Figure S1). Other immune cells, including microglia, were not detected in mixed cortical cultures under our culture conditions (data not shown). The specificity of the anti-CB2R antibody used in our study was previously confirmed in CB2R-deficient mice<sup>20,21</sup> and by use of a blocking peptide.<sup>18,20,21</sup>

We first examined whether TC could inhibit OGD/R-evoked neuronal injury. TC significantly attenuated morphological deterioration and LDH release in mixed cortical cultures exposed to OGD/R (Figure 1, B and C). Although a selective CB2R antagonist, AM630, abolished the protective effect of TC, the CB1R antagonist, AM251, had no effect (Figure 1, B and D, and Supplemental Figure S2A). The released amount of LDH reflected neuronal injury, because glial cells, such as astrocytes, were resistant to OGD/R-evoked injury under our experimental conditions (data not shown), as previously reported.<sup>36,45</sup> TC maximally reduced OGD-evoked neuronal injury when cotreated at the onset of OGD (Supplemental Figure S2B). The neuroprotective effects were then gradually decreased with an increased delay in treatment, but the effect of TC was still significant when treated 30 minutes after OGD.

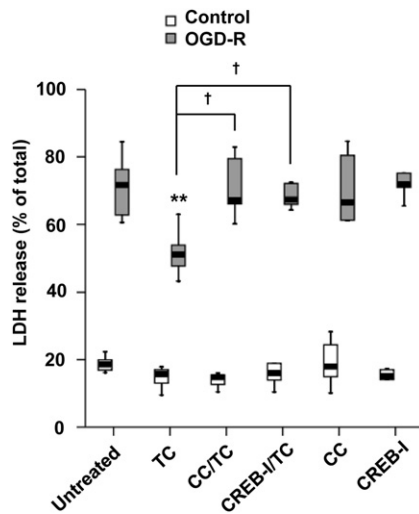
#### CB2R Activation Is Involved in the Ability of TC to Reduce OGD/R-Evoked Mitochondrial Depolarization and Intracellular Oxidative Stress

Mitochondrial dysfunction and oxidative stress have been shown to be critical determinants of ischemic neuronal death and survival.<sup>46,47</sup> TC significantly reduced mitochondrial

depolarization (Figure 2A) and intracellular oxidative stress (Figure 2B) in cortical cultures exposed to OGD/R. We also found that TC reduced OGD/R-evoked release of a mitochondrial protein, cytochrome *c* (Supplemental Figure S2C). The inhibitory effect of TC was significantly blocked by AM630, a CB2R antagonist (Figure 2, A and B). We further found that TC did not directly scavenge free radicals, such as nitrogen or peroxy radicals (Figure 3, A and B). Although TC attenuated OGD/R-evoked depletion of mitochondrial Mn superoxide dismutase (SOD), cytosolic Cu/Zn-SOD, and catalase activity in cortical cultures, it failed to induce those same antioxidant enzyme activities itself (Figure 3, C and D). *N*-methyl-D-aspartic acid (NMDA) receptor-mediated excitotoxicity is a well-established major factor producing mitochondrial dysfunction and oxidative stress through  $\text{Ca}^{2+}$  overload.<sup>46</sup> However, TC did not inhibit NMDA-evoked neuronal cell death (data not shown).

#### TC Potently Activates the AMPK and CREB Pathway

The pathways involved in CB2R signaling demonstrate great diversity, depending on cell type and cellular context,<sup>4</sup> and have not been fully elucidated in neurons. In neurons or neuron-like PC12 cells, activation of CB2Rs can trigger signaling cascades involving MAP kinase [eg, extracellular signal-regulated kinase 1/2 (ERK1/2) and p38]/c-Jun N-terminal kinase, phosphoinositide 3-kinase/Akt, inositol 1,4,5-trisphosphate receptor-mediated  $\text{Ca}^{2+}$  release or  $\text{Cl}^-$  channel activity, and AMPK.<sup>24,42,48–51</sup> Accordingly, we investigated phosphorylation/expression of putative candidates affected by TC. TC significantly



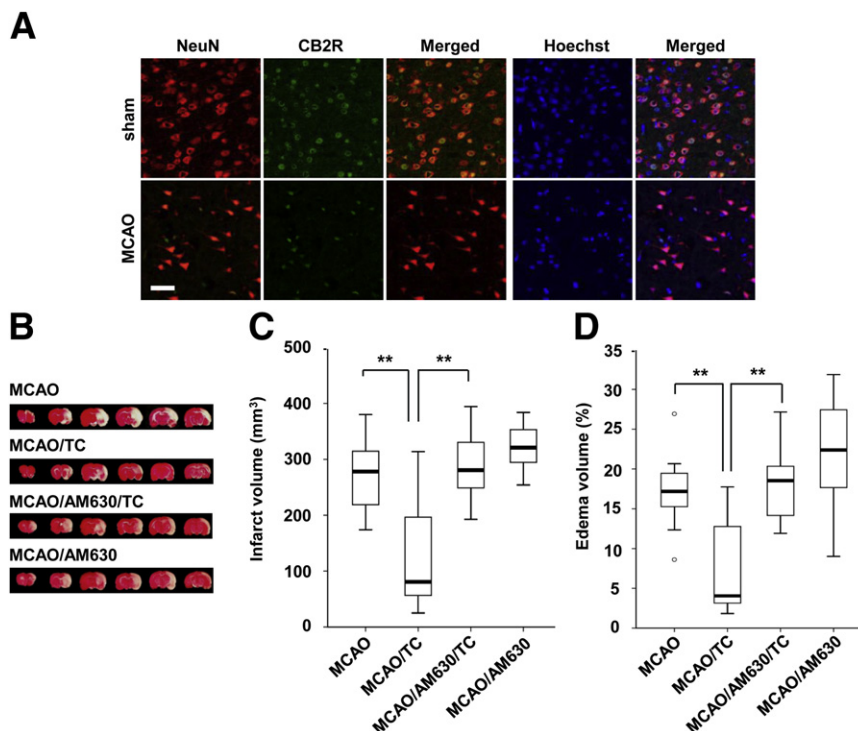
**Figure 5** Pharmacological inhibition of AMPK and CREB interferes with the protective effect of TC against OGD-evoked neuronal injury. Cortical neurons were subjected to OGD/R in the presence or absence of 1  $\mu\text{mol/L}$  TC. Either 5  $\mu\text{mol/L}$  CC (AMPK inhibitor) or 1  $\mu\text{mol/L}$  CREB–CREB-binding protein interaction inhibitor (CREB-I) was pretreated 30 minutes before OGD. Five hours after re-oxygenation, neuronal injury was assessed by LDH assay. Data were calculated as percentage of total LDH. Horizontal bar, median; vertical box, interquartile ranges (Q1 to Q3); and whiskers, minimum/maximum.  $N = 5$  to 10.  $**P < 0.01$ , significantly different from the untreated OGD/R group;  $^\dagger P < 0.05$ , significantly different from the TC-treated group, when analyzed by Kruskal-Wallis test, followed by the Mann-Whitney test.

enhanced AMPK activity, as reflected by an elevated level of phosphorylated proteins in cortical cultures exposed to OGD (Figure 4A). In contrast, TC did not significantly alter the level of phosphorylated ERK and phosphorylated

Akt (data not shown). As might have been predicted, TC also resulted in an increase in phosphorylation of CREB, one of the AMPK targets (Figure 4B). Among proteins transcriptionally controlled by CREB,<sup>52</sup> TC increased the expression level of BDNF (Figure 4C), but not B-cell lymphoma 2 (Bcl-2) and Bcl-X<sub>L</sub> (data not shown). More important, the selective AMPK and CREB inhibitors, CC and CREB–CREB-binding protein interaction inhibitor, reversed the protective effect of TC against OGD-evoked neuronal injury (Figure 5), suggesting that AMPK and CREB mediate the protective effect of TC in cortical neurons. Neither of the inhibitors evoked LDH release by themselves (Figure 5).

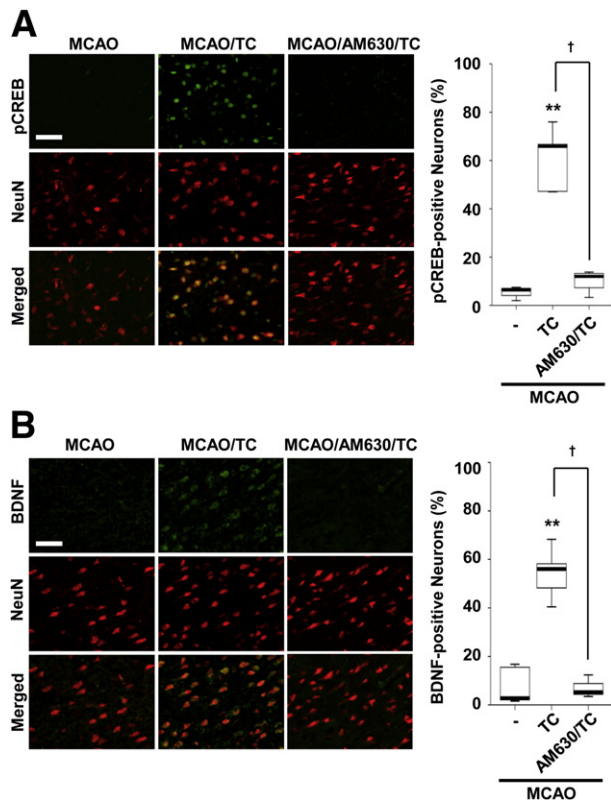
### TC Reduces Infarct Size in Rat Cerebral Ischemia in a CB2R-Dependent Manner

CB2R immunoreactivity was found in the cortex of both control and ischemic brains, mostly in cortical neurons and some glial cells (Figure 6A and Supplemental Figure S1). Post-ischemic treatment with 10 mg/kg TC (i.p.; initiated 3 hours after beginning MCAO) substantially reduced the infarct size and edema in rats subjected to 1.5 hours of MCAO, followed by 24 hours of reperfusion (53.8% and 51.9% inhibition, respectively) (Figure 6, B–D). Co-administration of 1 mg/kg AM630 completely blocked the protective effect of TC, suggesting the role of CB2R. AM630 alone did not significantly change the infarct size and edema volume. TC did not alter body weight or physiological variables, such as arterial blood pressure,  $\text{PaO}_2$ ,  $\text{PaCO}_2$ , pH, and rectal temperature (data not shown).



**Figure 6** Post-ischemic treatment of TC attenuates infarct volume in rat focal cerebral ischemia via CB2R. Rats were subject to MCAO for 1.5 hours and then reperfusion for 24 hours. TC, 10 mg/kg, was i.p. administered once at 3 hours after initiation of MCAO in the presence or absence of 1 mg/kg AM630. **A:** Representative confocal images of CB2R (green) and a neuronal marker, NeuN (red), immunostaining in cortical penumbra and merged images. Scale bar = 50  $\mu\text{m}$ . **B:** Representative coronal sections stained with 2% triphenyltetrazolium chloride. Quantification of infarct volume (**C**) and edema volume (**D**). Horizontal bar, median; vertical box, interquartile ranges (Q1 to Q3); whiskers, minimum/maximum; and open circles, outliers. Data were analyzed by the Kruskal-Wallis test, followed by the U-test.  $N = 8$  to 23.  $**P < 0.01$ , significantly different between the indicated groups.





**Figure 7** TC enhances phosphorylation of CREB and expression of BDNF in neurons of ischemic lesions via CB2R. **A:** Coronal sections obtained at 24 hours after MCAO were double labeled with anti-NeuN, a neuronal marker, and anti-phosphorylated (pCREB) (**A**) or anti-BDNF (**B**) antibodies. Representative confocal images of pCREB or BDNF (green), NeuN (red) immunostaining in ipsilateral cortical penumbra, and merged images (**left panel**). Scale bar = 50  $\mu$ m. Quantification of percentage of pCREB- or BDNF-immunoreactivity-positive neurons to total number of neurons (**right panel**). Horizontal bar, median; vertical box, interquartile ranges (Q1 to Q3); and whiskers, minimum/maximum. Data were analyzed by the Kruskal-Wallis test, followed by the Mann-Whitney test.  $N = 5$  to  $7$ .  $***P < 0.01$ , significantly different from the untreated MCAO group.  $^\dagger P < 0.05$ , significantly different between the indicated groups.

### CB2R Activation Is Involved in the Ability of TC to Induce CREB Phosphorylation and BDNF Expression in Rat Ischemic Cortexes

CREB phosphorylation increases during cerebral ischemia. CREB phosphorylation in the ischemic core either rapidly decreases to normal levels by 12 hours of reperfusion or slowly decreases in the penumbra and the peri-infarct area.<sup>53</sup> In the present study, TC produced an increase in phosphorylation of neuronal CREB in the ischemic penumbra, which remained significantly elevated for up to 24 hours after reperfusion (Figure 7A). Compared with the brain sections of sham-treated rats, 1.5 hours of MCAO increased the level of BDNF expression in the ischemic brains (data not shown), as previously described.<sup>54</sup> TC further increased expression of BDNF, a CREB target gene product (Figure 7B). Co-administration with AM630 abolished all effects produced by TC,

suggesting that a CB2R-dependent CREB pathway might be involved in neuronal TC signaling *in vivo*. AM630 did not itself significantly induce pCREB and BDNF (data not shown).

### Discussion

In the present study, TC significantly reduced OGD/R-evoked mitochondrial dysfunction and intracellular oxidative stress (Figure 2), two major, but not mutually exclusive, factors contributing to ischemic injury cascades.<sup>46,55</sup> These events can be initiated and exacerbated by NMDA receptor-mediated excitotoxicity and subsequent calcium overload.<sup>1</sup> Previously, a non-psychoactive cannabinoid, HU-211, exhibited neuroprotective activity by exhibiting antioxidant activity and limiting NMDA-induced excitotoxicity.<sup>56,57</sup> However, in the present study, TC did not decrease NMDA-induced neuronal injury (data not shown). TC also did not demonstrate any direct free radical scavenging activity or induction of antioxidant enzyme activity itself (Figure 3). These results indicate that the anti-ischemic effect of TC observed herein is mediated by mechanism(s) distinct from NMDA receptor antagonism or antioxidant activity.

Indeed, the present study demonstrates, for the first time to our knowledge, that TC reduces cerebral ischemic injury via activation of the AMPK-CREB pathway, mediated via activation of cortical CB2Rs. All effects of TC were completely reversed by AM630, a CB2R-selective antagonist, suggesting the importance of CB2R agonist activity in modulating neuronal survival. Although TC has been shown to have a unique structure and interacting geometry for binding to CB2Rs, relative to classic cannabinoids (eg,  $\Delta^9$ -tetrahydrocannabinol),<sup>26</sup> in the present study, TC was shown to bind to, and act as, a full agonist at CB2Rs to exert its anti-ischemic effect. The presence and function of CB2Rs in the cortex has long been debatable.<sup>42,58,59</sup> However, increasing evidence demonstrates CB2R expression in the CNS, including cortical neurons and glial cells.<sup>19–21,23,42</sup> Data reported herein also support the presence of CB2Rs in cortical neurons and astroglia. The expression of CB2Rs in cortical neurons was confirmed by immunocytochemistry (Figure 1). CB2R immune reactivity was also detected in cortical astroglia, but was relatively low compared with that in neurons. Moreover, in cortical cultures lacking microglia or immune cells, it was demonstrated that the inhibition of OGD/R-evoked neuronal injury produced by the CB2R agonist, TC, was reversed by a CB2R antagonist, AM630, but not by a CB1R antagonist, AM251 (Figure 1). These observations strongly indicate an important neuroprotective role for CB2Rs present in cortical neuronal/glial cells rather than peripheral or microglial cells. We further demonstrated that TC enhanced expression of proteins involved in survival signaling (ie, pCREB and BDNF) in cortical cultures (Figure 4). TC also attenuated the decrease of pCREB and BDNF occurring in neurons analyzed from MCAO rats, and these effects were abolished

by AM630 (Figure 7), suggesting that activation of CB2Rs might also mediate the neuroprotective effects of TC observed *in vivo*. The results presented, however, convincingly argue against previous reports suggesting a lack of neuronal CB2R immunoreactivity in ischemic mouse brains subjected to permanent MCAO.<sup>15</sup> Differences between species and experimental models (transient versus permanent MCAO) may contribute to differences between studies. However, a more detailed, systematic comparative study may be required to resolve such issues.

The CB2R belongs to the G-protein–coupled receptor superfamily and is coupled to the inhibitory G<sub>i</sub>/G<sub>o</sub>-subtype of G-proteins. Although the protective role of CB2R activation in ischemic/reperfusion injury has been described for several organs, the signal transduction pathways responsible for mediating these effects are not well characterized. Activation of peripheral CB2Rs in immune cells results in coupling to several principal signaling pathways, including MAP kinase (eg, ERK1/2, p38, and p42/44), c-Jun N-terminal kinase,<sup>14</sup> ERK, and PI3/Akt pathways.<sup>8</sup> At present, CB2R signaling pathways in cortical neurons and astroglia are poorly understood, in pathophysiological (eg, ischemic) and under physiological conditions. In some disease models, CB2Rs have been shown to trigger survival signaling cascades involving MAP kinase/c-Jun N-terminal kinase,<sup>48,49</sup> phosphoinositide 3-kinase/Akt,<sup>24,50,60</sup> inositol 1,4,5-trisphosphate receptor–mediated Ca<sup>2+</sup> release or Cl<sup>−</sup> channel activity,<sup>42</sup> and AMPK.<sup>60</sup> Our study demonstrates that TC-induced CB2R activation up-regulates pAMPK and pCREB in response to neuronal ischemic injury (Figure 4). The importance of AMPK/CREB signaling in CB2R-mediated neuroprotection was further highlighted when the activity of these pathways was selectively inhibited. For example, pretreatment with either the AMPK inhibitor, CC, or the CREB inhibitor, CREB–CREB-binding protein interaction inhibitor, abolished the neuroprotective effect of CB2R activation by TC (Figure 5). Interestingly, although TC has previously been shown to modulate Erk1/2 and p38 signaling in immune cells,<sup>26</sup> it failed to significantly alter either ERK or Akt activity in cortical cultures under the experimental conditions examined herein (data not shown). Such apparent diversity in CB2R signaling between cortical neurons/glia and other types of cells (ie, immune cells) may be due, in part, to distinct cell types examined, specific cellular context evaluated, or disease models investigated. In addition, recent studies report that two distinct isoforms of the CB2R, CB2A and CB2B, appear to be differentially expressed in neurons relative to immune cells.<sup>61</sup>

AMPK, a serine/threonine protein kinase, acts as a key sensor of cellular energy status in various tissues.<sup>62,63</sup> AMPK is activated by AMP binding or phosphorylation at the Thr172 residue on an increase in the cellular AMP/ATP ratio and cytosolic calcium levels. AMPK has been shown to be highly expressed in neurons, and its activity increases during glucose deprivation, ischemia, and hypoxia.<sup>62,63</sup> In cerebral ischemic injury, AMPK activation may result in dual functions concerning neuronal survival, in a context-

specific manner (ie, depending on the tissue, degree of stimulation, or conditions of activation). Because AMPK activation can either exert protective effects<sup>64</sup> or exacerbate injury, inhibition of AMPK may provide protection, depending on the specific cellular context.<sup>65–67</sup> CREB is one of many well-known prosurvival proteins<sup>52,53</sup> and is one of the several targets regulated by members of the AMPK family.<sup>67,68</sup> In the present study, AMPK activation by TC appears to produce neuroprotection in cortical neurons, possibly by increasing phosphorylation of CREB (Figures 4, 5, and 7). Phosphorylation of CREB enhances the expression of various prosurvival genes, *BDNF*, *Bcl-2*, and *Bcl-X<sub>L</sub>*.<sup>52</sup> Among those examined, TC significantly increases the expression of BDNF under both *in vitro* and *in vivo* conditions (Figures 4 and 7).

In conclusion, our data indicate that CB2R activation by TC ameliorates ischemic injury potentially through the AMPK/CREB pathway. Previous studies have focused primarily on the anti-inflammatory actions of peripherally located CB2Rs in immune cells.<sup>11,12,14,15</sup> To our knowledge, novel data presented in this study provide evidence, for the first time, supporting a previously unappreciated role of cortical CB2R, especially neuronal CB2Rs in ischemia, and further demonstration of AMPK/CREB involvement in CB2R-mediated neuroprotection. More important, our study also shows that the anti-ischemic activity of TC can be observed even when treatment is initiated post-ischemically (3 hours after initiation of MCAO). Because TC appears to maintain CB2R agonist activity when administered orally and is a common ingredient found in many food additives and folk medicines,<sup>26</sup> this study suggests that further investigation is warranted to establish the clinical usefulness of TC as a preventative and therapeutic agent for the treatment of stroke.

## Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2012.11.024>.

## References

1. Moskowitz MA, Lo EH, Iadecola C: The science of stroke: mechanisms in search of treatments. *Neuron* 2010, 67:181–198
2. Hillard CJ: Role of cannabinoids and endocannabinoids in cerebral ischemia. *Curr Pharm Des* 2008, 14:2347–2361
3. Zhang M, Martin BR, Adler MW, Razdan RJ, Kong W, Ganea D, Tuma RF: Modulation of cannabinoid receptor activation as a neuroprotective strategy for EAE and stroke. *J Neuroimmune Pharmacol* 2009, 4:249–259
4. Cabral GA, Griffin-Thomas L: Emerging role of the cannabinoid receptor CB2 in immune regulation: therapeutic prospects for neuroinflammation. *Expert Rev Mol Med* 2009, 11:e3
5. Iadecola C, Anrather J: The immunology of stroke: from mechanisms to translation. *Nat Med* 2011, 17:796–808
6. Batkai S, Osei-Hyiaman D, Pan H, El-Assal O, Rajesh M, Mukhopadhyay P, Hong F, Harvey-White J, Jafri A, Hasko G, Huffman JW, Gao B, Kunos G, Pacher P: Cannabinoid-2 receptor

- mediates protection against hepatic ischemia/reperfusion injury. *FASEB J* 2007, 21:1788–1800
7. Rajesh M, Pan H, Mukhopadhyay P, Batkai S, Osei-Hyiaman D, Hasko G, Liaudet L, Gao B, Pacher P: Cannabinoid-2 receptor agonist HU-308 protects against hepatic ischemia/reperfusion injury by attenuating oxidative stress, inflammatory response, and apoptosis. *J Leukoc Biol* 2007, 82:1382–1389
  8. Montecucco F, Lenglet S, Braunersreuther V, Burger F, Pelli G, Bertolotto M, Mach F, Steffens S: CB(2) cannabinoid receptor activation is cardioprotective in a mouse model of ischemia/reperfusion. *J Mol Cell Cardiol* 2009, 46:612–620
  9. Pertwee RG: Emerging strategies for exploiting cannabinoid receptor agonists as medicines. *Br J Pharmacol* 2009, 156:397–411
  10. Pacher P, Hasko G: Endocannabinoids and cannabinoid receptors in ischaemia-reperfusion injury and preconditioning. *Br J Pharmacol* 2008, 153:252–262
  11. Zhang M, Adler MW, Abood ME, Ganea D, Jallo J, Tuma RF: CB2 receptor activation attenuates microcirculatory dysfunction during cerebral ischemic/reperfusion injury. *Microvasc Res* 2009, 78: 86–94
  12. Zhang M, Martin BR, Adler MW, Razdan RK, Jallo JI, Tuma RF: Cannabinoid CB(2) receptor activation decreases cerebral infarction in a mouse focal ischemia/reperfusion model. *J Cereb Blood Flow Metab* 2007, 27:1387–1396
  13. Zhang M, Martin BR, Adler MW, Razdan RK, Ganea D, Tuma RF: Modulation of the balance between cannabinoid CB(1) and CB(2) receptor activation during cerebral ischemic/reperfusion injury. *Neuroscience* 2008, 152:753–760
  14. Murikinati S, Juttler E, Keinert T, Ridder DA, Muhammad S, Waibler Z, Ledent C, Zimmer A, Kalinke U, Schwaninger M: Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment. *FASEB J* 2010, 24:788–798
  15. Zarruk JG, Fernandez-Lopez D, Garcia-Yebenes I, Garcia-Gutierrez MS, Vivancos J, Nombela F, Torres M, Burguete MC, Manzanares J, Lizasoain I, Moro MA: Cannabinoid type 2 receptor activation downregulates stroke-induced classic and alternative brain macrophage/microglial activation concomitant to neuroprotection. *Stroke* 2012, 43:211–219
  16. Ashton JC, Rahman RM, Nair SM, Sutherland BA, Glass M, Appleton I: Cerebral hypoxia-ischemia and middle cerebral artery occlusion induce expression of the cannabinoid CB2 receptor in the brain. *Neurosci Lett* 2007, 412:114–117
  17. Morgan NH, Stanford IM, Woodhall GL: Functional CB2 type cannabinoid receptors at CNS synapses. *Neuropharmacology* 2009, 57: 356–368
  18. Onaivi ES, Ishiguro H, Gong JP, Patel S, Meozzi PA, Myers L, Perchuk A, Mora Z, Tagliaferro PA, Gardner E, Brusco A, Akinshola BE, Hope B, Lujilde J, Inada T, Iwasaki S, Macharia D, Teasenfiz L, Arinami T, Uhl GR: Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects. *PLoS One* 2008, 3:e1640
  19. Xi ZX, Peng XQ, Li X, Song R, Zhang HY, Liu QR, Yang HJ, Bi GH, Li J, Gardner EL: Brain cannabinoid CB2 receptors modulate cocaine's actions in mice. *Nat Neurosci* 2011, 14:1160–1166
  20. Onaivi ES, Ishiguro H, Gong JP, Patel S, Perchuk A, Meozzi PA, Myers L, Mora Z, Tagliaferro P, Gardner E, Brusco A, Akinshola BE, Liu QR, Hope B, Iwasaki S, Arinami T, Teasenfiz L, Uhl GR: Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. *Ann N Y Acad Sci* 2006, 1074:514–536
  21. Gong JP, Onaivi ES, Ishiguro H, Liu QR, Tagliaferro PA, Brusco A, Uhl GR: Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res* 2006, 1071:10–23
  22. Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie KJ, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD, Sharkey KA: Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* 2005, 310:329–332
  23. Lanciego JL, Barroso-Chinea P, Rico AJ, Conte-Perales L, Callen L, Roda E, Gomez-Bautista V, Lopez IP, Lluís C, Labandeira-Garcia JL, Franco R: Expression of the mRNA coding the cannabinoid receptor 2 in the pallidal complex of *Macaca fascicularis*. *J Psychopharmacol* 2011, 25:97–104
  24. Viscomi MT, Oddi S, Latini L, Pasquariello N, Florenzano F, Bernardi G, Molinari M, Maccarrone M: Selective CB2 receptor agonism protects central neurons from remote axotomy-induced apoptosis through the PI3K/Akt pathway. *J Neurosci* 2009, 29: 4564–4570
  25. Sheng WS, Hu S, Min X, Cabral GA, Lokensgard JR, Peterson PK: Synthetic cannabinoid WIN55,212-2 inhibits generation of inflammatory mediators by IL-1beta-stimulated human astrocytes. *Glia* 2005, 49:211–219
  26. Gertsch J, Leonti M, Raduner S, Racz I, Chen JZ, Xie XQ, Altmann KH, Karsak M, Zimmer A: Beta-caryophyllene is a dietary cannabinoid. *Proc Natl Acad Sci U S A* 2008, 105:9099–9104
  27. Tung YT, Chua MT, Wang SY, Chang ST: Anti-inflammation activities of essential oil and its constituents from indigenous cinnamon (*Cinnamomum osmophloeum*) twigs. *Bioresour Technol* 2008, 99: 3908–3913
  28. Passos GF, Fernandes ES, da Cunha FM, Ferreira J, Pianowski LF, Campos MM, Calixto JB: Anti-inflammatory and anti-allergic properties of the essential oil and active compounds from *Cordia verbenacea*. *J Ethnopharmacol* 2007, 110:323–333
  29. Martin S, Padilla E, Ocete MA, Galvez J, Jimenez J, Zarzuelo A: Anti-inflammatory activity of the essential oil of *Bupleurum frutescens*. *Planta Med* 1993, 59:533–536
  30. Loizzo MR, Tundis R, Menichini F, Saab AM, Statti GA: Anti-proliferative effects of essential oils and their major constituents in human renal adenocarcinoma and amelanotic melanoma cells. *Cell Prolif* 2008, 41:1002–1012
  31. Fernandes ES, Passos GF, Medeiros R, da Cunha FM, Ferreira J, Campos MM, Pianowski LF, Calixto JB: Anti-inflammatory effects of compounds alpha-humulene and (-)-trans-caryophyllene isolated from the essential oil of *Cordia verbenacea*. *Eur J Pharmacol* 2007, 569: 228–236
  32. Di Sotto A, Mazzanti G, Carbone F, Hrelia P, Maffei F: Inhibition by beta-caryophyllene of ethyl methanesulfonate-induced clastogenicity in cultured human lymphocytes. *Mutat Res* 2010, 699:23–28
  33. Cho JY, Chang HJ, Lee SK, Kim HJ, Hwang JK, Chun HS: Amelioration of dextran sulfate sodium-induced colitis in mice by oral administration of beta-caryophyllene, a sesquiterpene. *Life Sci* 2007, 80:932–939
  34. Chang HJ, Kim HJ, Chun HS: Quantitative structure-activity relationship (QSAR) for neuroprotective activity of terpenoids. *Life Sci* 2007, 80:835–841
  35. Choi IY, Lee JC, Ju C, Hwang S, Cho GS, Lee HW, Choi WJ, Jeong LS, Kim WK: A3 adenosine receptor agonist reduces brain ischemic injury and inhibits inflammatory cell migration in rats. *Am J Pathol* 2011, 179:2042–2052
  36. Lim JH, Lee JC, Lee YH, Choi IY, Oh YK, Kim HS, Park JS, Kim WK: Simvastatin prevents oxygen and glucose deprivation/reoxygenation-induced death of cortical neurons by reducing the production and toxicity of 4-hydroxy-2E-nonenal. *J Neurochem* 2006, 97:140–150
  37. Choi Y, Kim SK, Choi IY, Ju C, Nam KW, Hwang S, Kim BW, Yoon MJ, Won MH, Park YK, Kim WK: Amelioration of cerebral infarction and improvement of neurological deficit by a Korean herbal medicine, modified Bo-Yang-Hwan-O-Tang. *J Pharm Pharmacol* 2011, 63:695–706
  38. Choi IY, Lim JH, Hwang S, Lee JC, Cho GS, Kim WK: Anti-ischemic and anti-inflammatory activity of (S)-cis-verbenol. *Free Radic Res* 2010, 44:541–551
  39. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Deemer EK: Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated beta-



- cyclodextrin as the solubility enhancer. *J Agric Food Chem* 2002, 50: 1815–1821
40. Cai Y, Cho GS, Ju C, Wang SL, Ryu JH, Shin CY, Kim HS, Nam KW, Jalin AM, Sun W, Choi IY, Kim WK: Activated microglia are less vulnerable to hemin toxicity due to nitric oxide-dependent inhibition of JNK and p38 MAPK activation. *J Immunol* 2011, 187:1314–1321
  41. Golanov EV, Reis DJ: Contribution of cerebral edema to the neuronal salvage elicited by stimulation of cerebellar fastigial nucleus after occlusion of the middle cerebral artery in rat. *J Cereb Blood Flow Metab* 1995, 15:172–174
  42. den Boon FS, Chameau P, Schaafsma-Zhao Q, van Aken W, Bari M, Oddi S, Kruse CG, Maccarrone M, Wadman WJ, Werkman TR: Excitability of prefrontal cortical pyramidal neurons is modulated by activation of intracellular type-2 cannabinoid receptors. *Proc Natl Acad Sci U S A* 2012, 109:3534–3539
  43. Mikasova L, Groc L, Choquet D, Manzoni OJ: Altered surface trafficking of presynaptic cannabinoid type 1 receptor in and out synaptic terminals parallels receptor desensitization. *Proc Natl Acad Sci U S A* 2008, 105:18596–18601
  44. Carlisle SJ, Marciano-Cabral F, Staab A, Ludwick C, Cabral GA: Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *Int Immunopharmacol* 2002, 2:69–82
  45. Almeida A, Delgado-Esteban M, Bolanos JP, Medina JM: Oxygen and glucose deprivation induces mitochondrial dysfunction and oxidative stress in neurones but not in astrocytes in primary culture. *J Neurochem* 2002, 81:207–217
  46. Sims NR, Muyderman H: Mitochondria, oxidative metabolism and cell death in stroke. *Biochim Biophys Acta* 2010, 1802:80–91
  47. Niizuma K, Yoshioka H, Chen H, Kim GS, Jung JE, Katsu M, Okami N, Chan PH: Mitochondrial and apoptotic neuronal death signaling pathways in cerebral ischemia. *Biochim Biophys Acta* 2010, 1802:92–99
  48. Derkinderen P, Valjent E, Toutant M, Corvol JC, Enslen H, Ledent C, Trzaskos J, Caboche J, Girault JA: Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. *J Neurosci* 2003, 23:2371–2382
  49. Sarker KP, Biswas KK, Yamakuchi M, Lee KY, Hahiguchi T, Kracht M, Kitajima I, Maruyama I: ASK1-p38 MAPK/JNK signaling cascade mediates anandamide-induced PC12 cell death. *J Neurochem* 2003, 85:50–61
  50. Molina-Holgado F, Pinteaux E, Heenan L, Moore JD, Rothwell NJ, Gibson RM: Neuroprotective effects of the synthetic cannabinoid HU-210 in primary cortical neurons are mediated by phosphatidylinositol 3-kinase/AKT signaling. *Mol Cell Neurosci* 2005, 28:189–194
  51. Sagar DR, Kelly S, Millns PJ, O'Shaughnessy CT, Kendall DA, Chapman V: Inhibitory effects of CB1 and CB2 receptor agonists on responses of DRG neurons and dorsal horn neurons in neuropathic rats. *Eur J Neurosci* 2005, 22:371–379
  52. Kitagawa K: CREB and cAMP response element-mediated gene expression in the ischemic brain. *FEBS J* 2007, 274:3210–3217
  53. Tanaka K: Alteration of second messengers during acute cerebral ischemia: adenylate cyclase, cyclic AMP-dependent protein kinase, and cyclic AMP response element binding protein. *Prog Neurobiol* 2001, 65:173–207
  54. Kokaia Z, Andsberg G, Yan Q, Lindvall O: Rapid alterations of BDNF protein levels in the rat brain after focal ischemia: evidence for increased synthesis and anterograde axonal transport. *Exp Neurol* 1998, 154:289–301
  55. Niizuma K, Endo H, Chan PH: Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. *J Neurochem* 2009, 109(Suppl 1):133–138
  56. Nadler V, Mechoulam R, Sokolovsky M: Blockade of 45Ca<sup>2+</sup> influx through the N-methyl-D-aspartate receptor ion channel by the non-psychoactive cannabinoid HU-211. *Brain Res* 1993, 622:79–85
  57. Eshhar N, Striem S, Kohen R, Tirosh O, Biegon A: Neuroprotective and antioxidant activities of HU-211, a novel NMDA receptor antagonist. *Eur J Pharmacol* 1995, 283:19–29
  58. Onaivi ES, Ishiguro H, Gu S, Liu QR: CNS effects of CB2 cannabinoid receptors: beyond neuro-immuno-cannabinoid activity. *J Psychopharmacol* 2012, 26:92–103
  59. Atwood BK, Mackie K: CB2: a cannabinoid receptor with an identity crisis. *Br J Pharmacol* 2010, 160:467–479
  60. Dagon Y, Avraham Y, Ilan Y, Mechoulam R, Berry EM: Cannabinoids ameliorate cerebral dysfunction following liver failure via AMP-activated protein kinase. *FASEB J* 2007, 21:2431–2441
  61. Liu QR, Pan CH, Hishimoto A, Li CY, Xi ZX, Llorente-Berzal A, Viveros MP, Ishiguro H, Arinami T, Onaivi ES, Uhl GR: Species differences in cannabinoid receptor 2 (CNR2 gene): identification of novel human and rodent CB2 isoforms, differential tissue expression and regulation by cannabinoid receptor ligands. *Genes Brain Behav* 2009, 8:519–530
  62. Weisova P, Davila D, Tuffy LP, Ward MW, Concannon CG, Prehn JH: Role of 5'-adenosine monophosphate-activated protein kinase in cell survival and death responses in neurons. *Antioxid Redox Signal* 2011, 14:1863–1876
  63. Ramamurthy S, Ronnett GV: Developing a head for energy sensing: AMP-activated protein kinase as a multifunctional metabolic sensor in the brain. *J Physiol* 2006, 574(Pt 1):85–93
  64. Culmsee C, Monnig J, Kemp BE, Mattson MP: AMP-activated protein kinase is highly expressed in neurons in the developing rat brain and promotes neuronal survival following glucose deprivation. *J Mol Neurosci* 2001, 17:45–58
  65. McCullough LD, Zeng Z, Li H, Landree LE, McFadden J, Ronnett GV: Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke. *J Biol Chem* 2005, 280: 20493–20502
  66. Li J, Zeng Z, Viollet B, Ronnett GV, McCullough LD: Neuroprotective effects of adenosine monophosphate-activated protein kinase inhibition and gene deletion in stroke. *Stroke* 2007, 38:2992–2999
  67. Sasaki T, Takemori H, Yagita Y, Terasaki Y, Uebi T, Horike N, Takagi H, Susumu T, Teraoka H, Kusano K, Hatano O, Oyama N, Sugiyama Y, Sakoda S, Kitagawa K: SIK2 is a key regulator for neuronal survival after ischemia via TORC1-CREB. *Neuron* 2011, 69:106–119
  68. Thomson DM, Herway ST, Fillmore N, Kim H, Brown JD, Barrow JR, Winder WW: AMP-activated protein kinase phosphorylates transcription factors of the CREB family. *J Appl Physiol* 2008, 104:429–438