

VOLUME 282 (2007) PAGES 23892–23898

The CB₁ cannabinoid receptor mediates excitotoxicity-induced neural progenitor proliferation and neurogenesis.

Tania Aguado, Eva Romero, Krisztina Monory, Javier Palazuelos, Michael Sendtner, Giovanni Marsicano, Beat Lutz, Manuel Guzmán, and Ismael Galve-Roperh

In this study, to block the degradation of the endocannabinoid 2-arachidonoylglycerol (2AG), we used a compound (URB754) that had been previously claimed to be a selective inhibitor of the 2AG-hydrolyzing enzyme monoacylglycerol lipase (MAGL) (Makara, J. K., Mor, M., Fegley, D., Szabo, S. I., Kathuria, S., Astarita, G., Duranti, A., Tontini, A., Tarzia, G., Rivara, S., Freund, T. F., and Piomelli, D. (2005) *Nat. Neurosci.* **8**, 1139–1141). However, recent reports have raised concerns about the selectivity of URB754 (Vandevoorde, S., Jonsson, K. O., Labar, G., Persson, E., Lambert, D. M., and Fowler, C. J. (2007) *Br. J. Pharmacol.* **150**, 186–191; Saario, S. M., Palomaki, V., Lehtonen, M., Nevalainen, T., Jarvinen, T., and Laitinen, J. T. (2006) *Chem. Biol.* **13**, 811–814). In addition, a corrigendum of the original article by Makara *et al.* (2005) reported that the original batches of this compound contain an impurity that seems to constitute the molecular entity responsible for MAGL inhibition (Makara, J. K., Mor, M., Fegley, D., Szabo, S. I., Kathuria, S., Astarita, G., Duranti, A., Tontini, A., Tarzia, G., Rivara, S., Freund, T. F., and Piomelli, D. (2007) *Nat. Neurosci.* **10**, 134). Our study showed that exogenously added 2AG and URB754 increase neural progenitor proliferation and fibroblast growth factor-2 production, with both actions being prevented by the CB₁ cannabinoid receptor antagonist rimonabant. It is therefore conceivable that our observations can be explained by the MAGL inhibitory action of the contaminant present in the batches of commercial URB754.

VOLUME 282 (2007) PAGES 34913–34920

The serine protease domain of hepatitis C viral NS3 activates RNA helicase activity by promoting the binding of RNA substrate.

Rudolf K. F. Beran, Victor Serebrov, and Anna Marie Pyle

The numbers below the construct diagram in Fig. 1B (Page 34915) should read as follows (from left to right): 1, 166, 188, and 631.

VOLUME 282 (2007) PAGES 36024–36036

The intercalated disc protein, mXin α , is capable of interacting with β -catenin and bundling actin filaments.

Sunju Choi, Elisabeth A. Gustafson-Wagner, Qinchuan Wang, Shannon M. Harlan, Haley W. Sinn, Jenny L.-C. Lin, and Jim J.-C. Lin

There was an error in the title of the article in the printed Journal. The correct title is shown above and is correct in the on-line Journal.

We suggest that subscribers photocopy these corrections and insert the photocopies in the original publication at the location of the original article. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

The CB₁ Cannabinoid Receptor Mediates Excitotoxicity-induced Neural Progenitor Proliferation and Neurogenesis*[§]

Received for publication, January 24, 2007, and in revised form, May 4, 2007. Published, JBC Papers in Press, June 7, 2007, DOI 10.1074/jbc.M700678200

Tania Aguado^{†1}, Eva Romero^{‡2}, Krisztina Monory[§], Javier Palazuelos[‡], Michael Sendtner[¶], Giovanni Marsicano^{§||}, Beat Lutz[§], Manuel Guzmán[‡], and Ismael Galve-Roperh^{‡3}

From the [†]Department of Biochemistry and Molecular Biology I, School of Biology, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Complutense University, 28040 Madrid, Spain, the [§]Department of Physiological Chemistry, Johannes Gutenberg University Mainz, Duesbergweg 6, 55099 Mainz, Germany, the [¶]Institute of Clinical Neurobiology, Würzburg University, Versbacher Str. 5, 97078 Würzburg, Germany, and the ^{||}U862 INSERM, Institute Francois Magendie, 146, rue Léo Saigat, 33076 Bordeaux, France

Endocannabinoids are lipid signaling mediators that exert an important neuromodulatory role and confer neuroprotection in several types of brain injury. Excitotoxicity and stroke can induce neural progenitor (NP) proliferation and differentiation as an attempt of neuroregeneration after damage. Here we investigated the mechanism of hippocampal progenitor cell engagement upon excitotoxicity induced by kainic acid administration and the putative involvement of the CB₁ cannabinoid receptor in this process. Adult NPs express kainate receptors that mediate proliferation and neurosphere generation *in vitro* via CB₁ cannabinoid receptors. Similarly, *in vivo* studies showed that excitotoxicity-induced hippocampal NPs proliferation and neurogenesis are abrogated in CB₁-deficient mice and in wild-type mice administered with the selective CB₁ antagonist rimonabant (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide; SR141716). Kainate stimulation increased basic fibroblast growth factor (bFGF) expression in cultured NPs in a CB₁-dependent manner as this response was prevented by rimonabant and mimicked by endocannabinoids. Likewise, *in vivo* analyses showed that increased hippocampal expression of bFGF, as well as of brain-derived neurotrophic factor and epidermal growth factor, occurs upon excitotoxicity and that CB₁ receptor ablation prevents this induction. Moreover, excitotoxicity increased the number of CB₁⁺bFGF⁺ cells, and this up-regulation preceded NP proliferation. In summary, our results show the involvement of the CB₁ cannabinoid receptor in NP proliferation and neurogenesis induced by excitotoxic injury and support a role for bFGF signaling in this process.

In the adult brain, generation of new neurons is restricted to discrete areas including the subventricular zone and the subgranular zone of the dentate gyrus (1, 2). Newly generated neurons have the ability to become functional and integrate into established brain circuits (3), and thus, they may contribute to cognitive functions. In addition, the observation of increased neurogenesis after brain injury has been proposed to constitute an endogenous neuroprotective response aimed at reducing brain damage (2). Thus, ischemia induces neurogenesis in the hippocampus and the subventricular zone (4–6), and similarly, neuronal replacement from endogenous subventricular zone progenitors occurs in the striatum after brain stroke (7). Despite the described mobilization from neurogenic areas of endogenous neural progenitors (NPs)⁴ after brain injury, the signaling factors involved in this protective response are still unclear. Therefore, the characterization of the endogenous factors that positively regulate this process is a matter of intense research (2).

Besides classical cell fate signaling systems such as growth factors and cytokines, neuronal activity plays a significant role in neurogenesis (3). Likewise, different neurotransmitters (*e.g.* Glu, γ -aminobutyric acid, and dopamine) and neuromodulators such as opioids regulate progenitor cell proliferation and differentiation (3, 8). The CB₁ cannabinoid receptor exerts an important neuromodulatory action by regulating synaptic transmission in different brain areas (9, 10). Moreover, CB₁ receptor activation is also involved in the control of neural cell fate (11, 12) and exerts a neuroprotective action in different *in vivo* models of brain injury, including excitotoxicity and ischemia (13–15). Recent studies have shown that the endocannabinoid (eCB) signaling system is expressed in NPs (16, 17) and participates in the control of progenitor cell proliferation (17–19) and differentiation (20, 21) in the normal brain. However, the role of the eCB system in the response of NPs that ensues upon brain injury is as yet unknown. In the present study, we therefore investigated the possible involvement of the CB₁ can-

* This work was supported by grants from Comunidad Autónoma de Madrid (Grants S-SAL/0261/2006 and 950344), Fundación de Investigación Médica Mutua Madrileña Automovilística, Santander Complutense (Grant PR27/05-13988), Deutsche Forschungsgemeinschaft (Grant LU755-4), and the AVENIR Program of INSERM (in partnership with the Fondation Bettencourt-Schueller). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains two supplemental tables and a figure.

[†] Supported by Comunidad Autónoma de Madrid.

[‡] Present address: AVENIR INSERM, Institute Francois Magendie, 146, rue Léo Saigat, 33076 Bordeaux, France.

³ To whom correspondence should be addressed. Tel.: 34-913944668; Fax: 34-913944672; E-mail: igr@quim.ucm.es.

⁴ The abbreviations and trivial names used are: NP, neural progenitor; eCB, endocannabinoid; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; 2AG, 2-arachidonoylglycerol; AEA, anandamide; KA, kainic acid; BrdUrd, bromodeoxyuridine; GFAP, glial fibrillary acidic protein; BDNF, brain-derived neurotrophic factor; rimonabant, *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide.

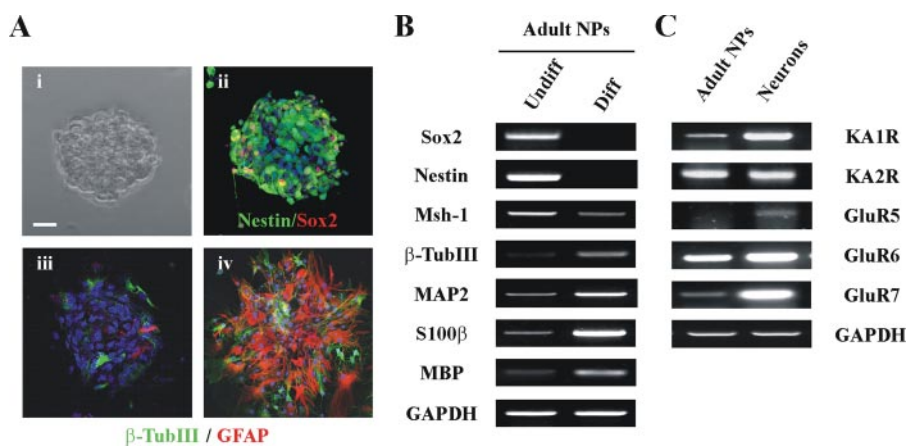


FIGURE 1. Characterization of adult neural progenitors and kainate receptor expression. A, phase-contrast image of an adult hippocampus-derived neurosphere (panel i). The expression of the indicated neural progenitor (panel ii) and differentiation markers (panels iii and iv) was determined by immunofluorescence before (panels i–iii) and after differentiation (panel iv). Scale bars are as follows: 15 in panel i, 30 in panels ii and iii, and 50 μ m in panel iv. β -TubIII, β -tubulin III. B, reverse transcription-PCR expression analysis of the indicated neural lineage markers in undifferentiated (Undiff) and differentiated (Diff) neural progenitors. MBP, myelin basic protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, expression of KA receptors in adult NPs as evidenced by reverse transcription-PCR analysis. Differentiated primary neurons were employed as controls.

nabinoid receptor in NP proliferation in the context of the neurogenerative response that follows excitotoxic brain injury.

EXPERIMENTAL PROCEDURES

Materials—Rimonabant (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide; SR141716) was kindly provided by Sanofi-Aventis (Montpellier, France), and polyclonal anti-mouse CB₁ receptor antibody was kindly provided by K. Mackie. Mouse monoclonal anti-nestin and anti-Sox2 antibodies as well as polyclonal anti-musashi-1 antibody were from Chemicon (Temecula, CA). URB597 (cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl ester), URB754 (6-methyl-2((4-methylphenyl)amino)-4H-3,1-benzoxazin-4-one), and 2-arachidonoylglycerol (2AG) were from Cayman Chemicals (Ann Arbor, MI). Recombinant human epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were from R&D Systems (Minneapolis, MN). Accutase, anandamide (AEA), kainic acid (KA), and mouse monoclonal anti- β -tubulin III and anti-gial fibrillary acidic protein (GFAP) antibodies were from Sigma.

Neural Progenitor Cell Culture—Multipotent hippocampal adult NPs were obtained from 8-week-old mice after dissociation of the tissue located between 1.2 and 3.0 mm, relative to bregma, from which the midbrain was removed, and then the hippocampus, as a whole, was separated gently from the corpus callosum. The tissue was digested by papain and DNase I treatment and further cultured in chemically defined medium consisting of Dulbecco's modified Eagle's and F12 media supplemented with N2 (Invitrogen), 0.6% glucose, non-essential amino acids, 50 mM Hepes, 2 μ g/ml heparin, 20 ng/ml EGF, and 20 ng/ml bFGF. Primary neurospheres were cultured at clonal density (1,000 cells/ml) and employed for *in vitro* experiments. Neurosphere generation experiments (see Fig. 2A) were performed in 96-well dishes with 100 μ l of medium, and the number of wells with neurospheres was quantified. Cell proliferation (see Fig. 2B) was determined by quantifying 5-bro-

mo-2'-deoxyuridine (BrdUrd)-positive cells. Neurospheres were dissociated by incubation with Accutase for 10 min and subsequent mild mechanical dissociation, treated for 16 h with 10 μ g/ml BrdUrd and, after plating in polyornithine-coated wells, immunostained with rat monoclonal anti-BrdUrd antibody from AbCam (Cambridge, MA). NPs were characterized for neural markers (see Fig. 1) in proliferating conditions (as above), and after differentiation as ensued by growth factor deprivation of adherent NPs in chemically defined medium, supplemented with 1% fetal calf serum and 20 ng/ml brain-derived neurotrophic factor (BDNF). Analyses of multipotenti-

ality (supplemental Table 1) were performed in 35 single cell-derived neurospheres after differentiation in growth factor-deprived chemically defined medium supplemented with 1% fetal calf serum and the action of 500 nM KA or 20 ng/ml BDNF when compared with vehicle.

Animals and Drug Treatment—CB₁ knock-out mice generation has been described previously (22). CB₁^{-/-} mice (8 weeks old) and their respective wild-type littermates were treated with vehicle or KA (15 mg/kg, intraperitoneal), injected with 50 mg/kg of BrdUrd daily for 5 days, and perfused 1, 7, or 30 days later ($n = 4$, $n = 4$, $n = 6$ for each group, respectively). In other experiments, rimonabant (1 mg/kg, intraperitoneal) or the corresponding vehicle (100 μ l of phosphate-buffered saline supplemented with 0.5 mg of fatty acid-free bovine serum albumin and 4% dimethyl sulfoxide) was co-administered with BrdUrd to wild-type mice ($n = 3$ for each group). Animal procedures were performed according to the European Union guidelines (86/609/EU) for the use of laboratory animals.

Immunostaining and Confocal Microscopy—Brains were perfused, and immunostaining was performed in 30- μ m coronal free-floating sections (16, 21). Sections were incubated with rat monoclonal anti-BrdUrd, mouse monoclonal anti-NeuN (Sigma), and rabbit polyclonal anti-S100 β (Swant, Bellinzona, Switzerland) antibodies followed by secondary staining for rat, mouse, and rabbit IgGs with highly cross-adsorbed Alexa Fluor 594, Alexa Fluor 488, and Alexa Fluor 647 secondary antibodies (Invitrogen), respectively. A minimum of five coronal sections per animal was examined. A 1-in-6 series of hippocampal sections located between 1.3 and 2.1 mm posterior to bregma was analyzed using the TCS-SP2 Leica software (Wetzlar, Germany) and SP2 AOBS microscope with $\times 63$ objective, two passes with a Kalman filter, and a 1,024 \times 1,024 collection box. Positive cells were normalized to the dentate gyrus area determined with $\times 10$ objective. The absolute number of positive cells was considering the total hippocampal volume as determined by the sum of the areas of the sampled sections multiplied by the distances between them. Additional

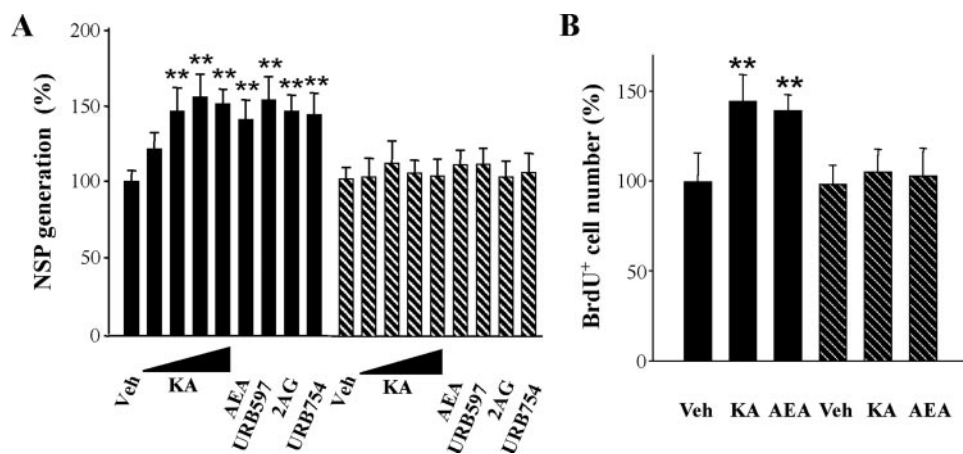


FIGURE 2. Kainate-induced neural progenitor proliferation is mediated by the CB₁ cannabinoid receptor. A, neurosphere (NSP) generation was determined in the presence of increasing KA concentrations (100 nM, 10 μM, 100 μM, and 500 μM), the endocannabinoids AEA and 2AG (1 μM), or the respective inhibitors of their degradation, URB597 (30 nM) and URB754 (30 nM). The involvement of CB₁ receptors in KA-induced NSP generation was assessed in the presence of rimonabant (1 μM; dashed bars). Veh, vehicle. B, NP proliferation was determined by quantification of BrdUrd⁺ (BrdU⁺) cells after 16 h of stimulation with KA (100 μM) or AEA in the absence or presence of rimonabant (as above). The percentage of generated neurospheres and cell proliferation is referred to that of vehicle-treated cultures. Significantly different from vehicle-treated cells: **, *p* < 0.01.

ofluorescences were also performed with polyclonal EGF antibody (Upstate Biotechnology – Millipore) and rabbit antiserum against recombinant mouse BDNF. Sections were co-stained with DNA-intercalating agent TOTO-3 (Invitrogen). Growth factor immunoreactivity was determined with Metamorph-Offline software (Universal Imaging, Downingtown, PA). *In vitro* immunofluorescence was performed with antibodies against neural progenitor (nestin, Sox2, musashi-1), neuronal (β-tubulin III), and the corresponding secondary fluorescent antibodies (as above). In addition, Cy3-conjugated glial (GFAP) antibody was employed. Total cells were counterstained with Hoechst 33258. Confocal immunofluorescence and image analyses were performed with the support and assistance of the Microscopy Imaging Research Unit (Complutense University, Madrid, Spain) and the Confocal Microscopy Unit (Centro Biología Molecular Severo Ochoa, Universidad Autónoma, Madrid, Spain).

mRNA Extraction, Reverse Transcription-PCR, and Quantification—mRNA was extracted with the RNeasy Protect kit (Qiagen) using the RNase-free DNase kit. cDNA was subsequently obtained using the SuperScript first-strand cDNA synthesis kit (Roche Applied Science, Welwyn Garden City, UK), and amplification of cDNA was performed with primers for mouse neural genes and KA receptor primers (supplemental Table 2). The following PCR conditions were used for most of the analyzed genes: 35 cycles (30 s at 94 °C, 30 s at 58 °C, and 75 s at 72 °C). β-Tubulin III and S100β were performed with an extension temperature of 55 and 65 °C, respectively, whereas glyceraldehyde-3-phosphate dehydrogenase was determined with 20 cycles (as above). Finally, after a final extension step at 72 °C for 5 min, PCR products were separated on 1.5% agarose gels. Real-time quantitative PCR was performed with TaqMan probes for bFGF, EGF, and BDNF obtained from Applied Biosystems (Foster City, CA). Ampli-

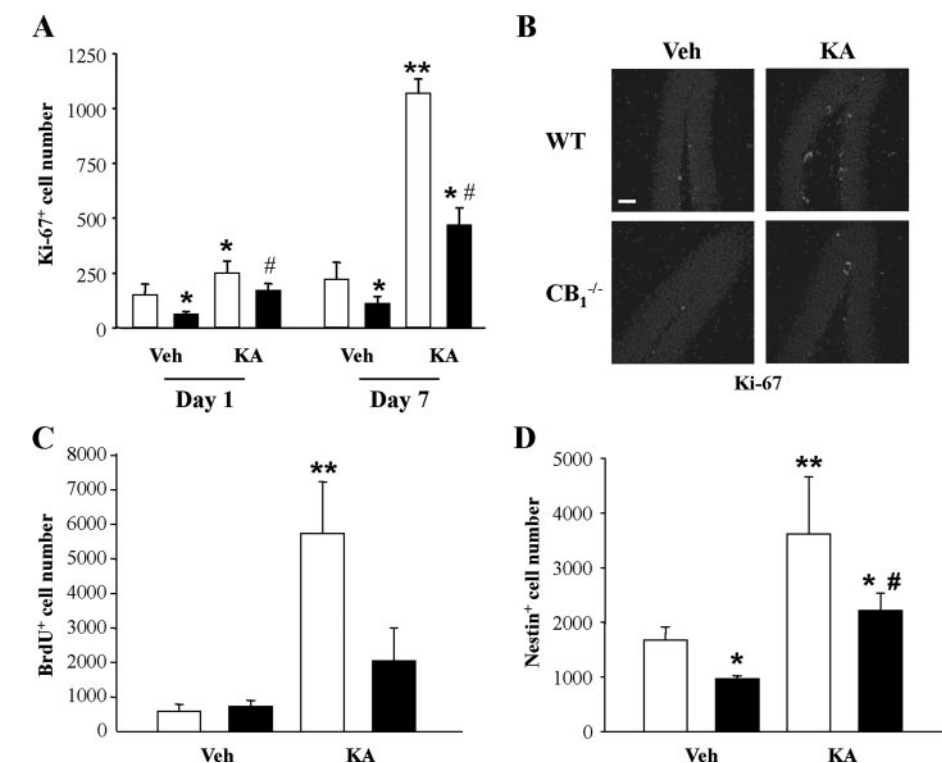


FIGURE 3. Excitotoxicity-induced neural progenitor proliferation is impaired in CB₁-deficient mice. A, NP proliferation was quantified by determining Ki-67⁺ cells in the subgranular zone of the dentate gyrus in wild-type (white bars) and CB₁^{-/-} (black bars) mice after 1 or 7 days (left and right, respectively) of vehicle (Veh) or KA treatment. The absolute number of positive cells was quantified considering the total hippocampal volume as determined by the sum of the areas of sampled sections multiplied by the distances between them. B, representative images are shown with Ki-67 (red) and total cells co-stained with TOTO-3 (blue). Scale bar: 45 μm. WT, wild type. C, quantification of BrdUrd⁺ (BrdU⁺) cells in the hippocampus of CB₁^{-/-} and wild-type littermates perfused at day 7. D, quantification of nestin⁺ cells at day 7 after KA treatment. Significantly different from vehicle-treated wild-type mice: *, *p* < 0.05; **, *p* < 0.01; #, *p* < 0.05 versus vehicle-treated CB₁^{-/-} mice.

immunofluorescence experiments were performed with rabbit monoclonal anti-Ki-67 (clone SP6) antibody (LabVision, Fremont, CA) and monoclonal anti-bFGF clone bFM2 antibody (Upstate Biotechnology – Millipore, Dundee UK). Immun-

tion step at 72 °C for 5 min, PCR products were separated on 1.5% agarose gels. Real-time quantitative PCR was performed with TaqMan probes for bFGF, EGF, and BDNF obtained from Applied Biosystems (Foster City, CA). Ampli-

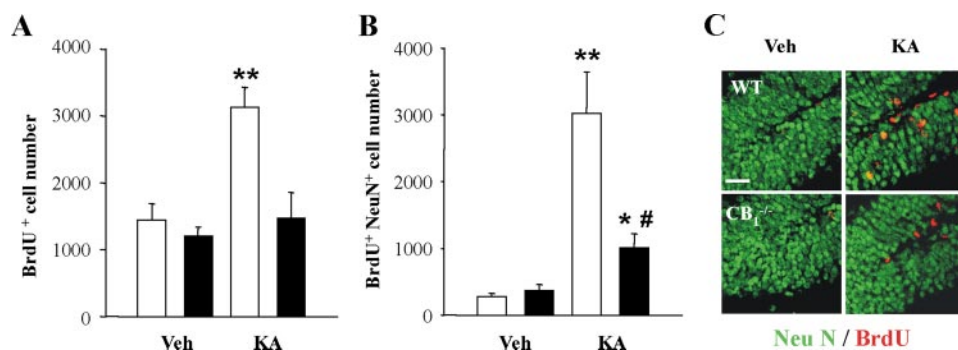


FIGURE 4. Excitotoxicity-induced neurogenesis is mediated by the CB₁ cannabinoid receptor. *A*, quantification of the number of newly formed cells, identified as BrdUrd⁺ (BrdU⁺), 30 days after vehicle (Veh) or KA treatment of wild-type (white bars) and CB₁^{-/-} (black bars) mice. *B*, neurogenesis determined by BrdUrd (red)-NeuN (green) double-positive cell counting in the same animals. *C*, representative images are shown. Scale bar: 20 μm. The absolute number of positive cells was quantified considering the total hippocampal volume as determined by the sum of the areas of sampled sections multiplied by the distances between them. Significantly different from vehicle-treated wild-type (WT) mice: *, *p* < 0.05, **, *p* < 0.01; #, *p* < 0.05 versus vehicle-treated CB₁^{-/-} mice.

was made by the Student-Neuman-Keuls test. *In vivo* data were analyzed by an unpaired Student *t* test.

RESULTS

The CB₁ Cannabinoid Receptor Is Involved in Kainate-induced Neural Progenitor Proliferation—

To investigate the mechanism involved in the regulation of NPs upon excitotoxicity, we cultured and characterized adult NPs, cells known to express cannabinoid receptors (16–21, 23). Hippocampal progenitors formed primary neurospheres and expressed a variety of progenitor markers including nestin, Sox2, and musashi-1 (Fig. 1, *A* and *B*). After differentiation in the presence of serum and BDNF, NPs gave rise to the different neural cell lineages and expressed neuronal (β-tubulin III and MAP2) and glial markers (GFAP, S100β, and myelin basic protein) (Fig. 1, *A* and *B*), whereas neurotrophin deprivation abolished their neuronal potential (supplemental Table 1). Next, we characterized the expression of KA receptors in NPs, which showed the presence of KA1R, KA2R, GluR6, and GluR7 but not GluR5 (Fig. 1*C*). Neurosphere generation assays were then performed in the presence of KA, and the role of the CB₁ receptor was investigated. KA increased neurosphere generation in a dose-dependent manner, and blockade of CB₁ receptors with the selective antagonist rimonabant prevented this action (Fig. 2*A*). NPs were subsequently incubated in the presence of the eCBs AEA and 2AG, as well as the selective inhibitors of their degradation URB597 and URB754, respectively (24, 25). eCB stimulation increased neurosphere generation in a CB₁-dependent manner (Fig. 2*A*). Results derived from neurosphere generation assays were confirmed in cell proliferation experiments by the quantification of

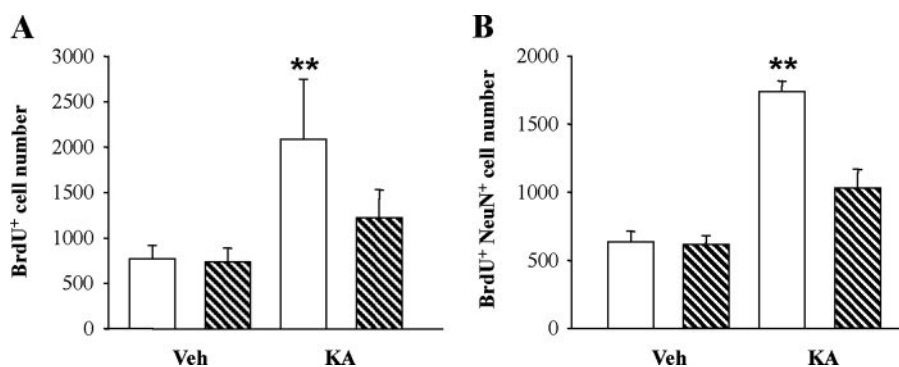


FIGURE 5. CB₁ antagonist rimonabant inhibits kainate-induced cell proliferation and neurogenesis. *A*, NP proliferation was quantified 30 days after vehicle (Veh) or KA treatment of wild-type mice co-injected with vehicle (plain bars) or the CB₁ antagonist rimonabant (dashed bars). BrdU⁺, BrdUrd⁺. *B*, neurogenesis was determined as above in vehicle- and rimonabant-treated mice. Significantly different from vehicle-treated wild-type mice: **, *p* < 0.01.

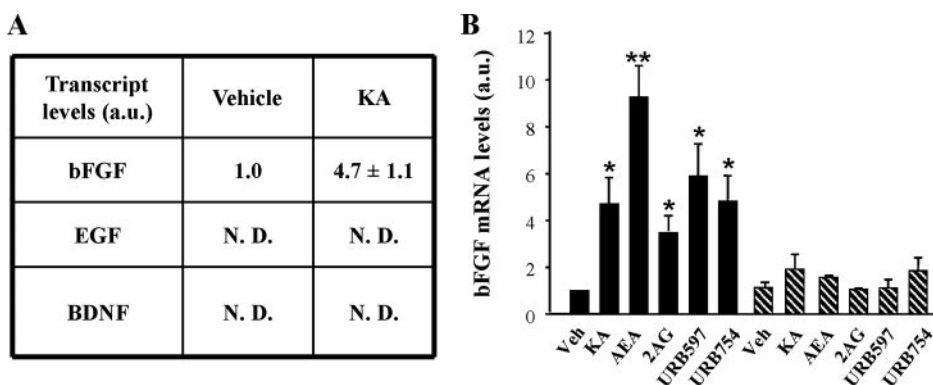


FIGURE 6. Growth factor expression in cultured neural progenitor cells. *A*, real-time PCR quantification of the indicated growth factors after treatment of NPs with KA or vehicle for 24 h. Transcript levels were normalized to 18 S RNA expression and are given in arbitrary units (a.u.). N.D., non-detectable. *B*, the involvement of the CB₁ cannabinoid receptor in bFGF expression after excitotoxicity was investigated by incubation of NPs with the indicated agents for 24 h. Results correspond to three independent experiments. Significantly different from vehicle-treated wild-type mice: *, *p* < 0.05, **, *p* < 0.01.

fications were run in a 7700 real-time PCR system (Applied Biosystems), and obtained values were adjusted using 18 S RNA levels as reference.

Statistical Analysis—Results shown represent the means ± S.D. of the number of experiments indicated in every case. Statistical analysis was performed by analysis of variance. A post hoc analysis

BrdUrd incorporation. Thus, both KA and AEA increased NP proliferation (Fig. 2*B*).

The CB₁ Cannabinoid Receptor Is Involved in Excitotoxicity-induced Neurogenesis—As the aforementioned data point to the involvement of CB₁ receptor activation in KA-induced NP proliferation *in vitro*, we investigated the involvement of the

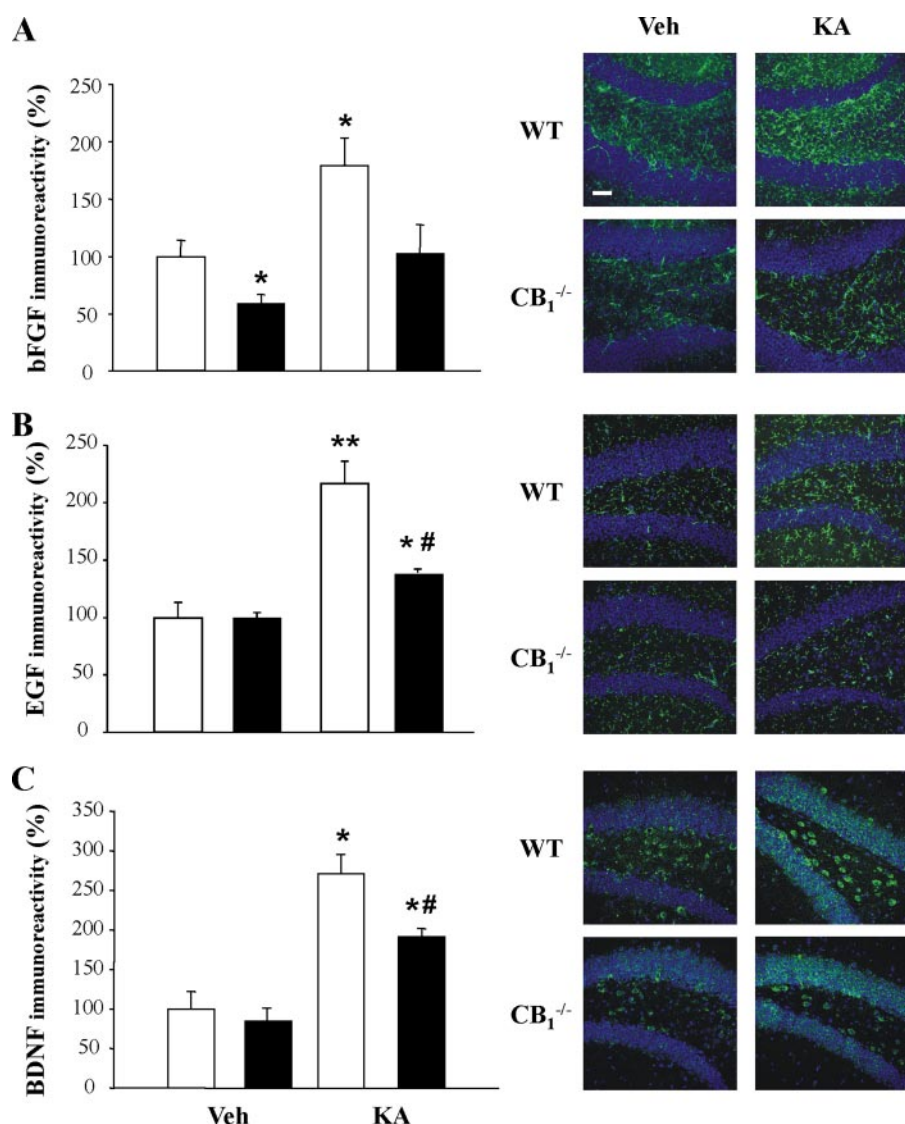


FIGURE 7. Increased bFGF expression after excitotoxicity involves the CB₁ cannabinoid receptor. *A*, quantification of bFGF immunofluorescence in the hippocampus 1 day after vehicle (Veh) or KA treatment in wild-type (WT) (white bars) and CB₁^{-/-} (black bars) mice. *B* and *C*, EGF (*B*) and BDNF (*C*) expression was determined under the same conditions. Significantly different from vehicle-treated cells: *, $p < 0.05$, **, $p < 0.01$; #, $p < 0.05$ versus vehicle-treated CB₁^{-/-} mice. Representative images of growth factor expression (green) in wild-type and CB₁-deficient mice are shown. Total cells were co-stained with TOTO-3 (blue). Scale bar: 45 μ m.

eCB system in adult neurogenesis in an *in vivo* model of excitotoxic epileptiform seizures induced by KA injection. The response of hippocampal NPs was analyzed at different time points by quantifying the number of cells in the subgranular zone of the dentate gyrus expressing the endogenous proliferative marker Ki-67. Increased NP proliferation was observed in wild-type mice 24 h and 7 days after KA injection (Fig. 3, *A* and *B*), and this increase was, however, no longer detectable at day 30 after excitotoxicity (data not shown). The excitotoxicity-induced proliferative response was severely impaired in CB₁-deficient mice (Fig. 3, *A* and *B*). This observation was corroborated by quantification of BrdUrd incorporation, which further supported that CB₁^{-/-} mice have lost injury-induced hippocampal proliferation (Fig. 3*C*). Consistently, the induction of nestin, a characteristic neuroepithe-

lial progenitor cell marker, was blunted in CB₁-deficient mice after KA treatment (Fig. 3*D*).

To determine whether CB₁-dependent hippocampal proliferation results in effective neurogenesis, immunostaining for the presence of BrdUrd-labeled cells that co-express the phenotypic markers NeuN and S100 β for mature neurons and astroglia, respectively, was performed one month after injury. Newly generated cells induced by excitotoxicity were detectable at this time point (Fig. 4*A*), and increased neurogenesis (NeuN-BrdUrd double-positive cells) was observed in KA-treated wild-type animals (Fig. 4, *B* and *C*). In contrast, this neurogenic response was mostly lost in CB₁-deficient mice. Excitotoxicity also induced astrogliogenesis (S100 β -BrdUrd double-positive cells), and CB₁ deletion prevented this response (data not shown). Further support for the role of the CB₁ receptor in KA-induced neurogenesis was obtained by pharmacological regulation with the administration of rimonabant to wild-type mice. In line with the data derived from genetically modified mice, KA-induced hippocampal NP proliferation (Fig. 5*A*) and neurogenesis (Fig. 5*B*) were strongly inhibited by CB₁ pharmacological blockade. Overall, these findings support the existence of a CB₁-mediated regulatory process that promotes neurogenesis after brain excitotoxicity.

CB₁ Cannabinoid Receptor-mediated Cell Proliferation Involves bFGF Production—To investigate

the mechanism of the CB₁ receptor proliferative action after excitotoxicity, we determined the production of different growth factors (bFGF, EGF, and BDNF) that are involved in the regulation of NP cell proliferation (1, 2) and that have been shown to cross-talk with the eCB system (15, 26, 27). Real-time PCR analyses showed that bFGF was produced at significant levels in adult NP clonal cultures, whereas BDNF and EGF were not present at detectable amounts (Fig. 6*A*). Likewise, KA and eCBs increased bFGF transcript levels in a CB₁ receptor-dependent manner (Fig. 6*B*), whereas BDNF and EGF remained undetectable. The involvement of bFGF in the CB₁-mediated response of NPs to excitotoxicity was analyzed in vehicle and KA-treated mice. Under basal conditions, bFGF expression was lower in CB₁^{-/-} mice than in their wild-type littermates, and excitotoxicity increased bFGF expression in wild-type animals

but not in CB₁-deficient mice (Fig. 7A). EGF and BDNF expression *in vivo* was also up-regulated after excitotoxicity, but the reduction of their protein levels in CB₁-deficient mice was minor when compared with bFGF repression (Fig. 7, B and C, and supplemental Fig. 1). These observations suggest that bFGF is involved in CB₁-dependent cell proliferation upon excitotoxicity. We quantified CB₁ expression after excitotoxicity and observed a significant up-regulation (Fig. 8A) that correlated with an increased number of double-labeled CB₁⁺-bFGF⁺ hippocampal cells after KA administration (Fig. 8, B and C). The quantification of bFGF⁺-proliferating (Ki-67⁺) progenitor cells supported that bFGF expression precedes NP proliferation (Figs. 3A and 8D). In addition, the involvement of the CB₁ receptor in bFGF induction in progenitor cells was revealed by the analysis of CB₁^{-/-} mice in which KA-triggered increase of bFGF⁺-Ki-67⁺ cells was impaired (Fig. 8D).

DISCUSSION

Brain injury-induced neurogenesis requires the existence of complex regulatory mechanisms in which different signaling factors are required to coordinate NP cell proliferation, differentiation, survival, and migration (1, 2). Fostering cell proliferation after injury requires the activation of progenitor cells in a process that, besides endogenous progenitor features, relies on extracellular signaling systems (1–3). Here we addressed the question of whether the CB₁ cannabinoid receptor might be involved in this process by regulating hippocampal progenitor cell proliferation and neurogenesis. For this purpose, we employed an excitotoxicity injury model in which the CB₁ receptor is known to be neuroprotective (15) and in which increased eCB ligands are produced (15, 28). CB₁ receptor-deficient mice exhibited impaired hippocampal NP proliferation and neurogenesis after excitotoxicity. Likewise, CB₁ receptor blockade by rimonabant administration to wild-type mice effectively blocked excitotoxicity-induced neurogenesis. eCBs are produced by NPs upon intracellular calcium increase (9), and via CB₁ receptor activation, they promote hippocampal NP proliferation (17, 18) and neurogenesis (20). Our results support that the eCB system is involved in excitotoxicity-induced neurogenesis, in line with its participation in the control of neural cell fate and the modulation of the balance between cell proliferation, death, and survival (11, 12, 23). Thus, the CB₁ cannabinoid receptor plays a neuroprotective role against brain excitotoxicity and stroke (13–15) and in different neurodegenerative disorders (11).

The existence of an injury-induced neurogenic process is considered to constitute an attempt for neuroregeneration that is well documented in ischemia and excitotoxicity models (4–7) but, however, usually fails to assess full recovery of brain functionality (2). bFGF is known to play a crucial role in progenitor proliferation in normal (29, 30) and excitotoxicity-damaged brain (5). Nonetheless, other growth factors as EGF (30, 31), vascular endothelial growth factor (32), and stem-derived supporting factor (33) have also been implicated in NP proliferation in both normal and pathological brain conditions. In the present study, bFGF was up-regulated after kainate treatment of cultured progenitor cells, whereas we did not observe EGF and BDNF expression under the same conditions. *In vivo*

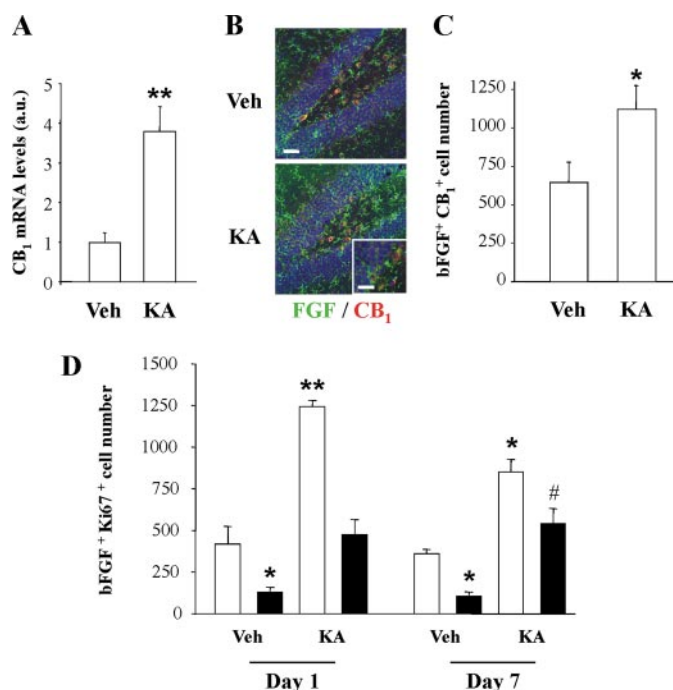


FIGURE 8. The CB₁ cannabinoid receptor, bFGF expression, and cell proliferation are up-regulated after excitotoxicity. A, real-time PCR quantification of CB₁ transcript levels in the hippocampus 1 day after vehicle (Veh) or KA treatment in wild-type mice. B, immunofluorescence confocal images are shown with bFGF (green) and CB₁ receptor (red). Total cells co-stained with TOTO-3 are shown in blue. Inset shows a higher magnification image. Scale bars: 45 and 30 μ m. C, quantification of bFGF-CB₁ double-positive hippocampal cells in vehicle- or KA-treated wild-type mice. D, quantification of bFGF-Ki-67 double-positive cells after 1 or 7 days of KA treatment (left and right, respectively). Significantly different from vehicle-treated wild-type mice: *, $p < 0.05$, **, $p < 0.01$; #, $p < 0.05$ versus vehicle-treated CB₁^{-/-} mice.

analyses showed that bFGF expression was lower in CB₁^{-/-} than in wild-type mice and that excitotoxicity increased bFGF expression in wild-type mice but not in CB₁-deficient littermates. EGF and BDNF expression also varied upon excitotoxicity, but the magnitude of the changes was lower when compared with those of bFGF. Our findings point to the involvement of the CB₁ receptor in hippocampal NP proliferation by increasing bFGF production, although the contribution of additional factors cannot be excluded. Previous evidences support a positive cross-talk between the eCB system and growth factor-mediated signaling pathways (15, 26, 27). BDNF expression is also regulated by the CB₁ receptor (34, 35) and may be indeed important for appropriate neuronal maturation and positioning of newly generated cells. In addition, the CB₁ receptor antagonist rimonabant has been reported to inhibit axonal growth stimulated by bFGF, and 2AG production was implicated in this bFGF action (26). Finally, cannabinoid regulation of neurite outgrowth can be mediated by intracellular signaling pathways coupled directly to CB₁ receptor activation (16, 36).

The CB₁ receptor is ideally located to regulate neural cell fate decisions as it is present along the neural lineage from progenitor to differentiated cells (9, 23), its expression increases after injury in various *in vivo* models (37, 38), and its activation regulates neural cell survival and proliferation (11, 12), migration, and axonal growth (23). In addition, the synthesis of the endogenous ligands AEA and 2AG is increased after different types of

brain insults including excitotoxicity and during neuronal activity (9, 10). Although the neurogenic response to injury may be of benefit for neural activity (2), seizure-induced neurogenesis may contribute as well to aberrant hippocampal reorganization (39). In this context, chronic pharmacological administration of the synthetic cannabinoid HU-210 induces neurogenesis, and this has been associated with improvement in anxiety and depression tests (20). Likewise, selective activation of CB₂ cannabinoid receptors, which is devoid of undesirable psychoactive actions (9, 40, 41), results in increased progenitor proliferation (19), and this might be of benefit for therapeutic manipulation of neural stem cells after brain injury. Our results indicate that similarly to other extracellular lipid signaling systems (e.g. sphingosine-1-phosphate and lysophosphatidic acid) that act via G-protein-coupled Edg receptors (42, 43), eCBs via the CB₁ receptor exert a regulatory role on NP cell proliferation and differentiation. Overall, the functionality of the eCB system in adult brain neurogenesis, together with the ability of cannabinergic drugs to modulate neurogenesis after brain damage, may open new strategies aimed at improving the insufficient neurogenic response of endogenous NPs to excitotoxicity or ischemia.

Acknowledgments—We are indebted to our laboratory colleagues for enthusiasm and encouragement, to Z. Kokaia and A. Martinez-Serrano for critical comments and advice, to A. Egia and E. Resel for excellent technical support, and to B. Julien and M. Papantonopoulou for expert assistance.

REFERENCES

1. Alvarez-Buylla, A., and Lim, D. (2004) *Neuron* **41**, 683–686
2. Lie, D. C., Song, H., Colamarino, S. A., Ming, G., and Gage, F. H. (2004) *Annu. Rev. Pharmacol. Toxicol.* **44**, 399–421
3. Lledo, P. M., Alonso, M., and Grubb, M. S. (2006) *Nat. Rev. Neurosci.* **7**, 179–193
4. Jin, K., Minami, M., Lan, J. Q., Mao, X. O., Bateur, S., Simon, R. P., and Greenberg, D. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 4710–4715
5. Yoshimura, S., Takagi, Y., Harada, J., Teramoto, T., Thomas, S. S., Waeber, C., Bakowska, J., Breakefield, X. O., and Moskowitz, M. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5874–5879
6. Nakatomi, H., Kuriu, T., Okabe, S., Yamamoto, S., Hatano, O., Kawahara, N., Tamura, A., Kirino, T., and Nakafuku, M. (2002) *Cell* **110**, 429–441
7. Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. (2002) *Nat. Neurosci.* **5**, 963–970
8. Kim, E., Clark, A. L., Kiss, A., Hahn, J. W., Wesselschmidt, R., Coscia, C. J., and Belcheva, M. M. (2006) *J. Biol. Chem.* **281**, 33749–33760
9. Piomelli, D. (2003) *Nat. Rev. Neurosci.* **4**, 873–884
10. Chevaleyre, V., Takahashi, K. A., and Castillo, P. E. (2006) *Annu. Rev. Neurosci.* **29**, 37–76
11. Mechoulam, R., Spatz, M., and Shohami, E. (2002) *Science's STKE* **129**, RE5
12. Guzmán, M. (2003) *Nat. Rev. Cancer* **3**, 745–755
13. Nagayama, T., Sinor, A. D., Simon, R. P., Chen, J., Graham, S. H., Jin, K., and Greenberg, D. A. (1999) *J. Neurosci.* **19**, 2987–2995
14. Parmentier-Batteur, S., Jin, K., Mao, O., Xie, L., and Greenberg, D. A. (2002) *J. Neurosci.* **22**, 9771–9775
15. Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., Azad, S. C., Cascio, M. G., Ortega-Gutierrez, S., Van der Stelt, M., López-Rodriguez, M. L., Casanova, E., Schütz, G., Zieglgänsberger, W., Di Marzo, V., Behl, C., and Lutz, B. (2003) *Science* **302**, 84–88
16. Rueda, D., Navarro, B., Martínez-Serrano, A., Guzmán, M., and Galve-Roperh, I. (2002) *J. Biol. Chem.* **277**, 4645–4650
17. Jin, K., Xie, L., Kim, S. H., Parmentier-Batteur, S., Sun, Y., Mao, X. O., Childs, J., and Greenberg, D. A. (2004) *Mol. Pharmacol.* **66**, 204–208
18. Aguado, T., Monory, K., Palazuelos, J., Stella, N., Cravatt, B., Lutz, B., Marsicano, G., Kokaia, Z., Guzmán, M., and Galve-Roperh, I. (2005) *FASEB J.* **19**, 1704–1706
19. Palazuelos, J., Aguado, T., Egia, A., Mechoulam, R., Guzmán, M., and Galve-Roperh, I. (2006) *FASEB J.* **20**, 2405–2407
20. Jiang, W., Zhang, Y., Xiao, L., Van Cleemput, J., Ji, S., Bai, G., and Zhang, Z. (2005) *J. Clin. Investig.* **115**, 3104–3116
21. Aguado, T., Palazuelos, J., Monory, K., Stella, N., Cravatt, B., Lutz, B., Marsicano, G., Kokaia, Z., Guzmán, M., and Galve-Roperh, I. (2006) *J. Neurosci.* **26**, 1551–1561
22. Marsicano, G., Wotjak, C. T., Azad, S. C., Bisogno, T., Rammes, G., Cascio, M. G., Hermann, H., Tang, J., Hofmann, C., Zieglgänsberger, W., Di Marzo, V., and Lutz, B. (2002) *Nature* **418**, 530–534
23. Harkany, T., Guzmán, M., Galve-Roperh, I., Berghuis, P., Devi, L. A., and Mackie, K. (2007) *Trends Pharmacol. Sci.* **28**, 83–92
24. Kathuria, S., Gaetani, S., Fegley, D., Valiño, F., Duranti, A., Tontini, A., Mor, M., Tarzia, G., La Rana, G., Calignano, A., Giustino, A., Tattoli, M., Palmery, M., Cuomo, V., and Piomelli, D. (2003) *Nat. Med.* **9**, 76–81
25. Makara, J. K., Mor, M., Fegley, D., Szabo, S. I., Kathuria, S., Astarita, G., Duranti, A., Tontini, A., Tarzia, G., Rivara, S., Freund, T. F., and Piomelli, D. (2005) *Nat. Neurosci.* **8**, 1139–1141
26. Williams, E., Walsh, F. S., and Doherty, P. (2003) *J. Cell Biol.* **160**, 481–486
27. Hart, S., Fischer, O. M., and Ullrich, A. (2004) *Cancer Res.* **15**, 1943–1950
28. Guan, X. L., He, X., Ong, W., Yeo, W. K., Shui, G., and Wenk, M. R. (2006) *FASEB J.* **20**, 1152–1161
29. Palmer, T. D., Markakis, E. A., Willhoite, A. R., Safar, F., and Gage, F. H. (1999) *J. Neurosci.* **19**, 8487–8497
30. Tropepe, V., Sibilio, M., Ciruna, B. G., Rossant, J., Wagner, E. F., and van der Kooy, D. (1999) *Dev. Biol.* **208**, 166–188
31. Reynolds, B. A., and Weiss, S. (1996) *Dev. Biol.* **175**, 1–13
32. Zhu, Y., Jin, K., Mao, X. O., and Greenberg, D. A. (2003) *FASEB J.* **17**, 186–193
33. Toda, H., Tsuji, M., Nakano, I., Kobuke, K., Hayashi, T., Kasahara, H., Takahashi, J., Mizoguchi, A., Houtani, T., Sugimoto, T., Hashimoto, N., Palmer, T. D., Honjo, T., and Tashiro, K. (2003) *J. Biol. Chem.* **278**, 35491–35500
34. Berghuis, P., Dobszay, M. B., Wang, X., Spano, S., Ledda, F., Sousa, K. M., Schulte, G., Ernfors, P., Mackie, K., Paratcha, G., Hurd, Y. L., and Harkany, T. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 19115–19120
35. Khaspekov, L. G., Brenz Verca, M. S., Frumkina, L. E., Hermann, H., Marsicano, G., and Lutz, B. (2004) *Eur. J. Neurosci.* **19**, 1691–1698
36. Jordan, J. D., He, J. C., Eungdamrong, N. J., Gomes, I., Ali, W., Nguyen, T., Bivona, T. G., Philips, M. R., Devi, L. A., and Iyengar, R. (2005) *J. Biol. Chem.* **280**, 11413–11421
37. Jin, K., Mao, X. O., Goldsmith, P. C., and Greenberg, D. A. (2000) *Ann. Neurol.* **48**, 257–261
38. Unzicker, C., Erberich, H., Moldrich, G., Woldt, H., Bulla, J., Mechoulam, R., Ehrenreich, H., and Siren, A. (2005) *Neurochem. Res.* **30**, 1305–1309
39. Parent, J. M., Yu, T. W., Leibowitz, R. T., Geschwind, D. H., Sloviter, R. S., and Lowenstein, D. H. (1997) *J. Neurosci.* **17**, 3727–3738
40. Mackie, K. (2006) *Annu. Rev. Pharmacol. Toxicol.* **46**, 101–122
41. Fernandez-Ruiz, J., Romero, J., Velasco, G., Tolon, R. M., Ramos, J. A., and Guzman, M. (2007) *Trends Pharmacol. Sci.* **28**, 39–45
42. Harada, J., Foley, M., Moskowitz, M. A., and Waeber, C. (2004) *J. Neurochem.* **88**, 1026–1039
43. Svetlov, S. I., Ignatova, T. N., Wang, K. K., Hayes, R. L., English, D., and Kukekov, V. G. (2004) *Stem Cells Dev.* **13**, 685–693