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The endocannabinoid system drives neural progenitor proliferation

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ABSTRACT

The discovery of multipotent neural progenitor (NP) cells has provided strong support for the existence of neurogenesis in the adult brain. However, the signals controlling NP proliferation remain elusive. Endocannabinoids, the endogenous counterparts of marijuana-derived cannabinoids, act as neuromodulators via presynaptic CB_1 receptors and also control neural cell death and survival. Here we show that progenitor cells express a functional endocannabinoid system that actively regulates cell proliferation both in vitro and in vivo. Specifically, NPs produce endocannabinoids and express the CB_1 receptor and the endocannabinoid-inactivating enzyme fatty acid amide hydrolase (FAAH). CB_1 receptor activation promotes cell proliferation and neurosphere generation, an action that is abrogated in CB_1-deficient NPs. Accordingly, proliferation of hippocampal NPs is increased in FAAH-deficient mice. Our results demonstrate that endocannabinoids constitute a new group of signaling cues that regulate NP proliferation and thus open novel therapeutic avenues for manipulation of NP cell fate in the adult brain.

Keywords: cannabinoid • CB_1 receptor • NP

Cannabinoids and their endogenous counterparts, the endocannabinoids (eCBs), exert their actions in the brain through activation of the seven-transmembrane receptor CB_1 (1, 2). Anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2AG), the two major eCBs (3–5), are synthesized in postsynaptic neurons by a regulated process that relies on intracellular Ca^{2+} increase (1). These mediators act as retrograde synaptic neuromodulators that control neurotransmitter release via presynaptic CB_1 receptors (1). AEA action is finished by its reuptake and hydrolysis by a putative specific membrane transport system (1) and FAAH (6), respectively. Besides their neuromodulatory role, eCBs may also take part in the control of cell fate, thereby modulating the balance between neural cell death and

survival (7, 8). eCBs can be generated on demand as a consequence of brain injury (7) and as evidenced in CB_1 knockout mice, CB_1 engagement is responsible for their proposed neuroprotective action (9, 10). Moreover, cannabinoid administration protects neurons from different brain insults such as ischemia, glutamatergic excitotoxicity, oxidative stress, and trauma (7). Cannabinoid actions on neural cell death and survival are not restricted to neurons, as glial cells are also targeted by CB_1 activation (8). For instance, CB_1 activation protects astrocytes from ceramide-induced apoptosis both in vivo and in vitro (11) and prevents oligodendrocyte cell death induced by growth factor deprivation (12).

During mammalian development, the generation of the central nervous system relies on a finely regulated balance of NP proliferation, differentiation, and survival that is controlled by a number of extracellular signaling cues (13, 14). However, the precise mechanisms involved in the regulation of NP cell fate remain to be fully elucidated. Growth factors, such as epidermal and fibroblast-derived growth factor (EGF, FGF-2), act as key regulators of NP cell proliferation and differentiation (13, 14). In the adult brain, the existence of hippocampal neurogenesis (14, 15) has received strong support by the identification of the NP population located in the subgranular zone (16). NPs are characterized as cells expressing the neuroepithelial marker nestin together with glial fibrillary acidic protein (type I cells) and after fate commitment migrate and integrate into the granular cell layer (14–16). These newly generated cells may contribute to different brain functions, including cognitive processes and brain repair (15).

Importantly, the developmentally regulated pattern of CB_1 receptor expression (17) is followed by its presence at high levels in the adult hippocampus (1, 2). In this regard, whereas the function of the eCB system has been extensively studied in differentiated cells, its potential role in NP cells has not been addressed. Altogether, this evidence prompted us to study whether NP cells posses a functional eCB system, and if so what is its role in the regulation of cell proliferation.

MATERIALS AND METHODS

Materials

Nestin-GFP transgenic mice (18) were kindly donated by G. Enikolopov, SR141716 by Sanofi Synthelabo, and anti-mouse CB_1 polyclonal antibody by K. Mackie. URB597 and 2AG were from Cayman Chemicals, mouse monoclonal anti-nestin and rabbit polyclonal anti-FAAH antibodies from Chemicon, and rabbit monoclonal anti-K_i-67 (clone SP6) antibody from LabVision. Recombinant human EGF and FGF-2 were from R&D Systems. WIN-55,212-2, AEA, mouse monoclonal anti-β-tubulin III, anti-GFAP, and anti-α-tubulin antibodies were from Sigma.

Neurosphere and neural progenitor cell culture

Multipotent self-renewing progenitors were obtained from the dissected cortices of 17-day-old rat embryos and grown in chemically defined medium formed by Dulbecco's modified Eagle's and F-12 media supplemented with N2 (Invitrogen), 0.6% glucose, nonessential amino acids, 50 mM HEPES, 2 μg/ml heparin, 20 ng/ml EGF, and 20 ng/ml FGF-2. Clonal neurospheres were cultured at 1,000 cells/ml and dissociated, and experiments were carried out with early (up to 10) passage neurospheres. Neurosphere generation experiments were performed in 96-well dishes

with 100 μl of medium, and the number of wells with neurospheres together with the number of neurospheres per well were quantified. Embryonic NPs from wild-type and CB_1 knockout mice (19) was cultured (10,000 cells/ml) in the continuous presence of cannabinoids for the indicated number of passages (1 passage every 4 days). Adult NPs were obtained from hippocampi of 4-month-old adult mice and cultured as described above.

Endocannabinoid quantification

Cells were incubated in basal medium, and stimulation was stopped by adding an equal volume of ice-cold methanol. eCBs were chloroform-extracted in the presence of 200 pmol of deuterated eCBs, purified by normal-phase HPLC (Hewlett Packard), and quantified by chemical-ionization gas chromatography/mass spectrometry (Varian) (20).

Cell proliferation assays

NP proliferation was determined by quantifying $BrdU^+$ cells 16 h after incubation with 10 μg/ml BrdU, followed by immunostaining. Thymidine incorporation was measured in cells stimulated for 16 h and incubation with 1 μCi [^{3}H]thymidine for 6 h. After being washed, proteins were precipitated with 5% ice-cold trichloroacetic acid and subsequent alkaline wash in 0.5 N NaOH.

Western blot

Western blot analysis was performed as described previously (21). Cleared cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes, and following antibody incubations developed with enhanced chemiluminiscence detection kit. Loading controls were performed with an anti-α-tubulin antibody.

Animals and drug treatment

FAAH knockout mice generation has been described previously (6). $FAAH^{-/-}$ mice (12 wk old) and their respective wild-type littermates were injected intraperitoneally with 50 mg/kg BrdU daily for 5 days and perfused 1 day later. E18.5 mouse embryos from mothers injected twice with 100 mg/kg BrdU (30 min interval between injections) were obtained 1 h after the first injection. 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 μm coronal free-floating sections (22). Sections were incubated with rat monoclonal anti-BrdU (Abcam) and mouse monoclonal anti-NeuN (Sigma) antibodies followed by secondary staining for rat and mouse IgGs with highly cross-adsorbed AlexaFluor 594 and AlexaFluor 488 secondary antibodies (Molecular Probes), respectively. Five coronal sections per animal, located between 1.3 and 2.1 mm posterior to bregma, were examined using the Laser Sharp 2000 software (Bio-Rad) and a Confocal Radiance 2000 coupled to Axiovert S100 TV microscope (Carl Zeiss) with three passes with a Kalman filter and a 1024×1024 collection box. Paraffin-embedded mouse embryo sections were incubated with anti-BrdU antibody together with Yoyo-1 iodide, and positive cells were determined in seven sections per animal.

Statistical analysis

Results are means ± SD of the number of experiments indicated in every case. Statistical analysis was performed by ANOVA. A post hoc analysis was made by the Student-Neuman-Keuls test. In vivo data were analyzed by an unpaired Student *t* test.

RESULTS

Neural progenitors express the endocannabinoid system

To determine whether progenitor cells express a functional eCB system, we generated clonally-expanded neurospheres derived from embryonic NPs and labeled them with antibodies directed against the CB_1 receptor and FAAH. Immunofluorescence revealed that NP cells express these key elements of the eCB system (Fig. 1*A*). CB_1 receptors and FAAH were expressed by actively dividing cells, as identified by 5-bromo-2'-deoxyuridine (BrdU) incorporation and were also coexpressed with nestin. Moreover, triple immunostaining evidenced that proliferating nestin-positive cells expressed the CB_1 receptor (Fig. 1*A*). CB_1 and FAAH immunofluorescence was quantified on single-cell dissociated neurospheres, with 52 ± 8% and 33 ± 6% of $nestin^+$ cells expressing CB_1 and FAAH, respectively. Western blot analysis confirmed CB_1 and FAAH expression in neurospheres, as well as in terminally differentiated neurons and astrocytes (Fig. 1*B*, upper panel). Specific neural cell markers expressed at different stages of differentiation were determined in parallel, and, as expected, nestin was hardly detectable in differentiated cells, whereas β-tubulin III and GFAP levels increased in differentiated neurons and astrocytes, respectively (Fig. 1*B*, lower panel).

Expression of CB_1 and FAAH in NPs prompted us to determine their ability to produce eCBs. Indeed, neurospheres produced AEA and 2AG, 2AG being 50-100 times more abundant than AEA (Fig. 1*C*). Additional putative CB_1 ligands such as *N*-docosatetraenylethanolamine and *N*-homo-γ-linolenylethanolamine (1) were below detection limits. Importantly, AEA and 2AG production by NPs showed a robust response to A23187, a Ca^{2+} ionophore (Fig. 1*C*).

Endocannabinoids promote neural progenitor proliferation and neurosphere generation

To determine whether the eCB system controls NP cell function, cells were incubated with the synthetic cannabinoid agonist WIN-55,212-2 and the selective FAAH inhibitor URB597 (23). Cannabinoid stimulation increased neurosphere generation (Fig. 2*A*), an effect prevented by the selective CB_1 antagonist SR141716, pointing to the involvement of the CB_1 receptor. In addition, WIN-55,212-2 and URB597 increased both the number of neurospheres per well (control: 1.9±0.3; WIN-55,212-2: 13.5±0.2; URB597; 11.3±0.4; n=4; P<0.01) and neurosphere size (Fig. 2*B*, upper panels). The direct impact of cannabinoids on NP proliferation was subsequently examined. WIN-55,212-2, URB597, AEA, and 2AG increased the number of BrdU-incorporating cells in a CB_1-dependent manner (Fig. 2*B* and *C*). Similarly, cannabinoids stimulated [^{3}H]-thymidine incorporation (Fig. 2*D*). NP proliferation was also assessed with K_i-67, an endogenous marker of mitotic cells. Cannabinoids increased the number of K_i-67^+ cells, a finding that correlates with higher number of multipotent $nestin^+$ cells (Fig. 2*C* and *E*).

The relevance of the eCB system in NP proliferation was further investigated by evaluating primary clonal neurosphere generation in CB_1-deficient mice. Such analysis showed a decreased

rate of neurosphere generation (neurospheres per well; $CB_1^{+/+}$: 1.27±0.10, n=4; $CB_1^{-/-}$: 0.96±0.04, n=4; P<0.05). Accordingly, limit dilution analysis (Fig. 3*A*) revealed that the number of cells required to generate at least 1 neurosphere per well was 85 ± 12 for $CB_1^{+/+}$ mice (n=4) and 177 ± 15 for $CB_1^{-/-}$ mice (n=4; P<0.01). The involvement of the CB_1 receptor was proven by the absence of effect of WIN-55,212-2 and URB597 on neurosphere generation in CB_1-deficient NPs (Fig. 3*B*). Cannabinoid impact on NP self-renewal was determined by their continuous presence for several neurosphere passages using wild-type and $CB_1^{-/-}$ neurospheres. The exponential growth of NPs was significantly enhanced by WIN-55,212-2 and URB597 (Fig. 3*C*). This effect was prevented by SR141716 treatment and impaired in CB_1 knockout-derived NPs.

The functional relevance of the eCB system in vivo was determined using FAAH knockout mice that posses increased brain levels of eCBs (6). As shown in Fig. 4*A*, FAAH knockout embryos showed a marked increase in hippocampal BrdU-positive cells, a finding that correlates with higher number of $nestin^+$ cells (cell number per section; $FAAH^{+/+}$: 88±25, n=5; $FAAH^{-/-}$: 171±34, n=4; P<0.01).

The endocannabinoid system remains functionally active in the regulation of adult brain progenitor cell proliferation

Our observations that the eCB system promotes progenitor cell proliferation prompted us to study whether eCBs were also active in adult NPs. As it was the case for embryonic progenitors, adult brain-derived NPs express the CB_1 receptor and FAAH (Fig. 5*A* and *B*). These elements of the eCB system were expressed in $nestin^+$ cells and BrdU-labeled cells. Moreover, adult NPs proliferate in response to cannabinoids, leading to increased $BrdU^+$ and $nestin^+$ cells after cannabinoid challenge (Fig. 5*C*). In agreement, $BrdU^+$ cell counting in the adult dentate gyrus showed that hippocampal proliferation is increased in FAAH knockout mice as compared with wild-type littermates (Fig. 4*B*).

DISCUSSION

Our results evidence the existence of a functional eCB system, constituted by the endogenous ligands AEA and 2AG, the CB_1 receptor, and the eCB-inactivating FAAH enzyme that actively participates in the control of NP cell proliferation. Progenitor-derived eCBs are biologically active and promote cell proliferation and neurosphere generation in a CB_1-dependent manner. The relevance of these actions of the eCB system was proved by the observation that neurosphere generation is impaired in CB_1-deficient NPs. Similarly, $CB_1^{-/-}$ progenitors fail to respond to cannabinoid-induced neurosphere generation. Finally, cannabinoid action on NP self-renewal ability indicates that eCB regulation of NP proliferation occurs, at least in part, in early progenitor cells, although it does not preclude concomitant actions on intermediate-amplifying progenitors. eCB-induced activation of proliferative signal transduction pathways involved in the regulation of neural cell fate include the phosphatidylinositol 3-kinase/Akt pathway and the extracellular signal-regulated kinase pathway (8). Alternatively, the eCB system may interact with growth factors that are essential for the expansion and regulation of progenitor cells. In particular, activated CB_1 receptor can transactivate tyrosine kinase receptors of the EGF receptor family (24). Similarly, FGF-2-induced neural cell growth has been proposed to involve 2AG production (25).

CB_1 expression is developmentally regulated (17), and it has been proposed to include also progenitor cells (22, 26). In the present report, we obtained evidence that the eCB system remains expressed in adult brain progenitors and enhanced eCB levels increase hippocampal progenitor proliferation. Our results are in agreement with the observations that the eCBs AEA and 2AG participate in the control of neural cell fate by modulating the balance between cell death and survival (7, 8). AEA may reduce brain damage in excitotoxicity models (27), and neuronal degeneration due to traumatic brain injury is attenuated by 2AG (7). Thus, eCBs generated on demand on brain injury may result in CB_1-induced neuroprotection (9, 10) and NP proliferation (the present study). In agreement, brain injury results in progenitor proliferation, a process that may represent a compensatory mechanism by which the injured brain minimizes damage intensity (15, 28). Nonetheless, it is worth noting that other studies have shown the existence of eCB neurotoxic actions (29, 30), an effect that probably reflects the ability of AEA to modulate vanilloid receptor TRPV1 (31).

Numerous studies have recently addressed the regulatory mechanisms of progenitor cell proliferation in the adult brain (14, 15) that contribute to the generation of new functional neurons with the ability to integrate properly in hippocampal circuits (16, 32, 33). This process is considered to constitute a new form of brain plasticity (14, 16), in which the eCB-induced NP proliferation reported here may play an important role. These findings, together with the neuroprotective role of the eCB system (7, 9, 10) and the developmentally regulated pattern of CB_1 receptor expression (17) and eCB production (1), support the concept that this endogenous system should be considered as novel signaling cues that participate in the regulation of NP cell proliferation.

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Fig. 1

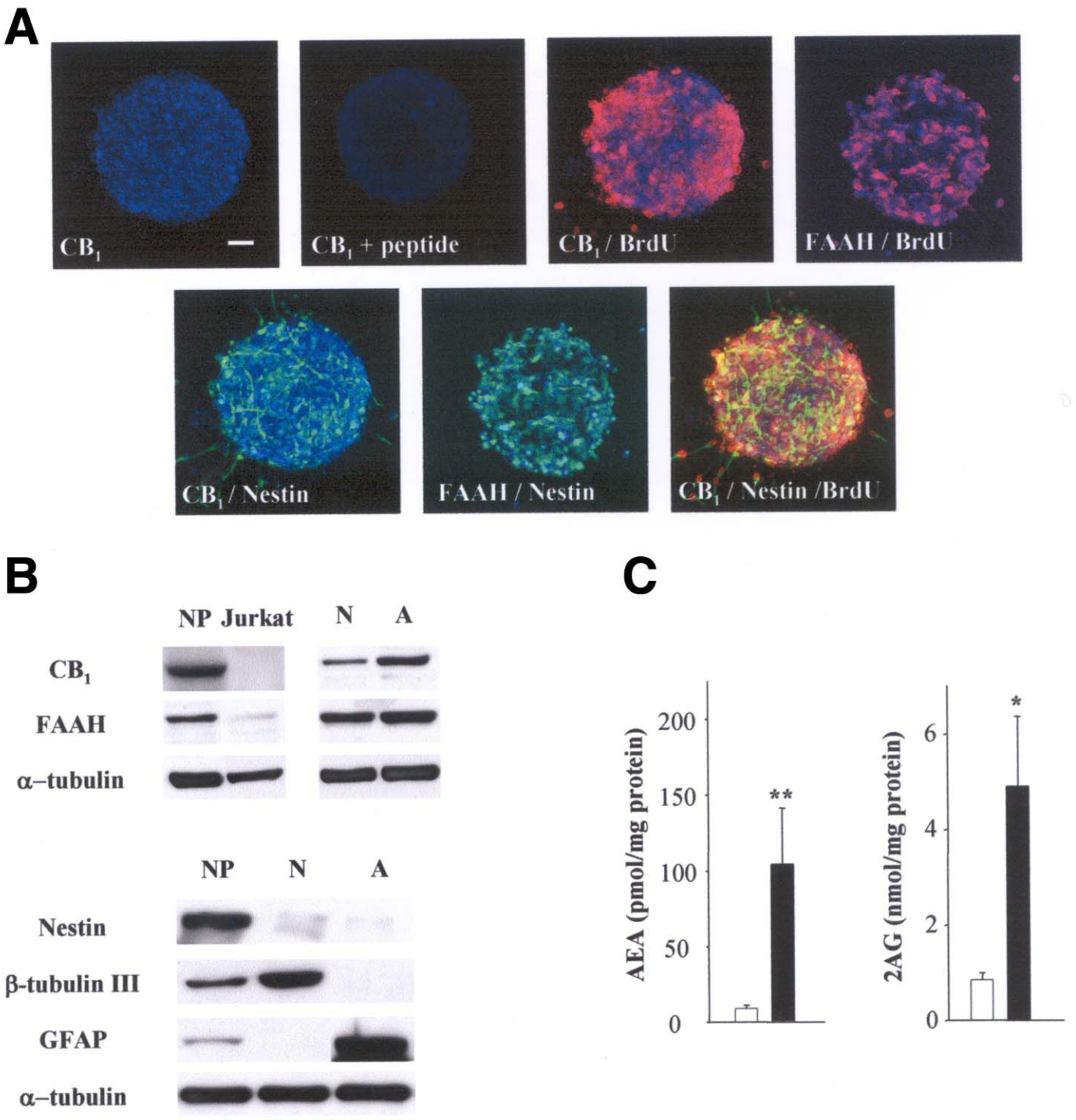

Figure 1. The eCB system is expressed in NPs. ***A***) Neurospheres were immunostained for BrdU (red) and nestin (green) and CB_1 receptor and FAAH (blue). Colocalization is shown in merged images. Control for specificity of CB_1 staining was performed by preincubating the antibody with antigenic peptide. Scale bars = 25 μm. ***B*, *upper panels***) Western blot analysis of CB_1 and FAAH expression in NPs. N, neurons; A, astrocytes; Jurkat cells were employed as CB_1-negative control, and α-tubulin levels as loading control. ***B*, *lower panels***) Characterization of the neural cell cultures employed with indicated neural markers. ***C***) NPs produce eCBs. AEA and 2AG levels in neurospheres treated with vehicle (white bars) and 5 μM A23187 for 2 min (black bars). Significantly different from controls: $*P < 0.05$, $**P < 0.01$. Results correspond to 4 (***A*** and ***C***) or 5 (***B***) independent experiments. Representative immunofluorescence images (***A***) and luminograms (***B***) are shown.

Fig. 2

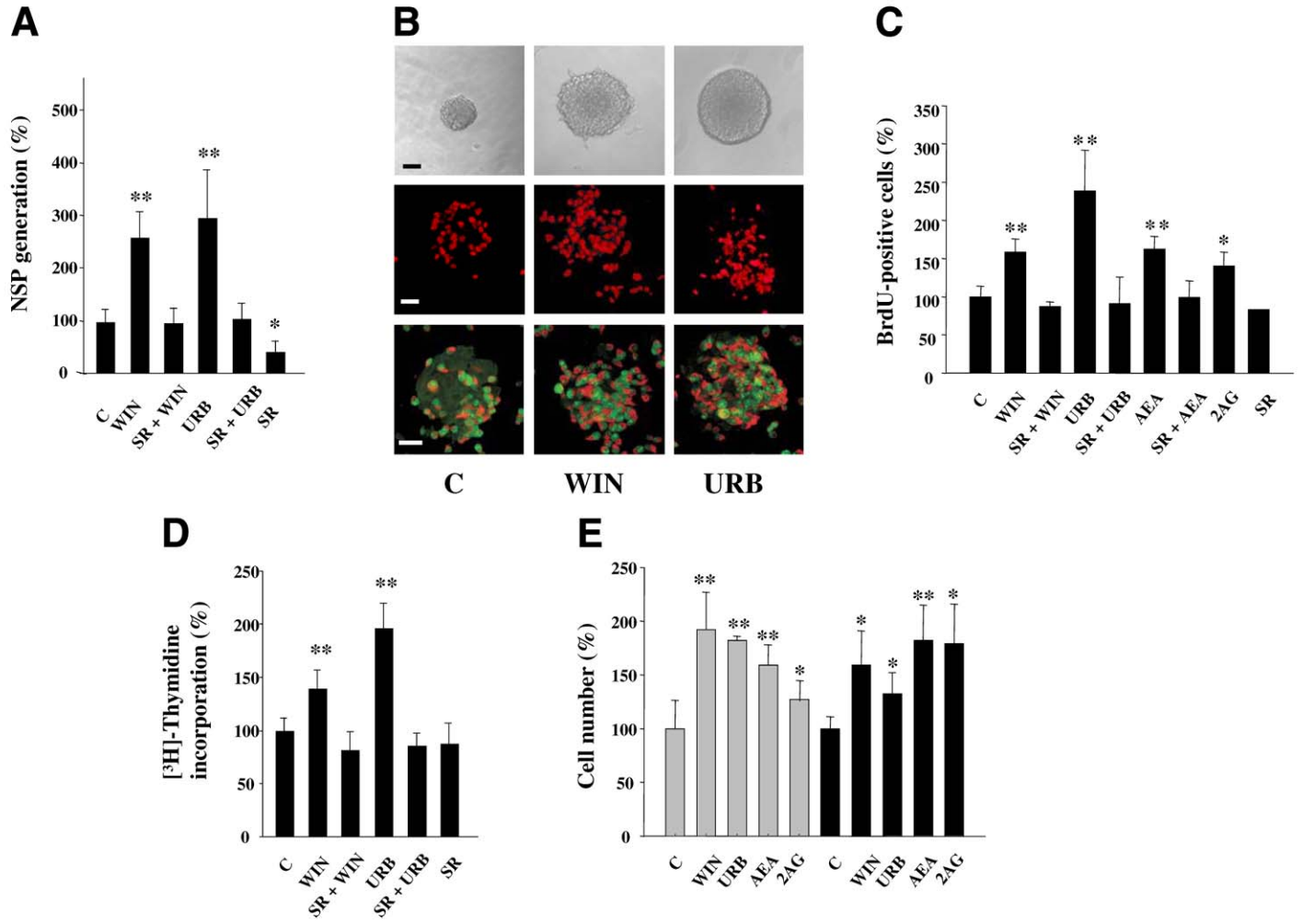

Figure 2. Cannabinoids stimulate neurosphere generation and NP proliferation. ***A*)** Percentage of wells containing neurospheres was quantified after 7 days of incubation of NPs in neurosphere-growing medium with vehicle (C), 30 nM WIN-55,212-2 alone or together with 2 μM SR141716, 30 nM URB597 alone or together with SR141716, and SR141716 alone. ***B*)** Representative phase contrast microscopy images (***upper panels***) of vehicle-, WIN-, or URB-treated neurospheres (from ***left*** to ***right***); immunofluorescence images are shown for BrdU (***middle panels***), and for K_i-67 (green) and nestin (red) (***lower panels***). ***C*)** Quantification of $BrdU^+$ NP cells from dissociated neurospheres stimulated for 16 h as above or in the presence of 10 μM AEA and 10 μM 2AG. ***D*)** [^{3}H]thymidine incorporation in neurospheres grown in the presence of the indicated stimuli. ***E*)** Quantification of K_i-67^+ (gray bars) and $nestin^+$ (black bars) NP cells from dissociated neurospheres after incubation with indicated stimuli. Scale bar = 25 μm. Results correspond to 4 (***A***, ***C***) or 3 (***D***, ***E***) independent experiments. Significantly different from controls: $^*P < 0.05$, $^{**}P < 0.01$.

Fig. 3

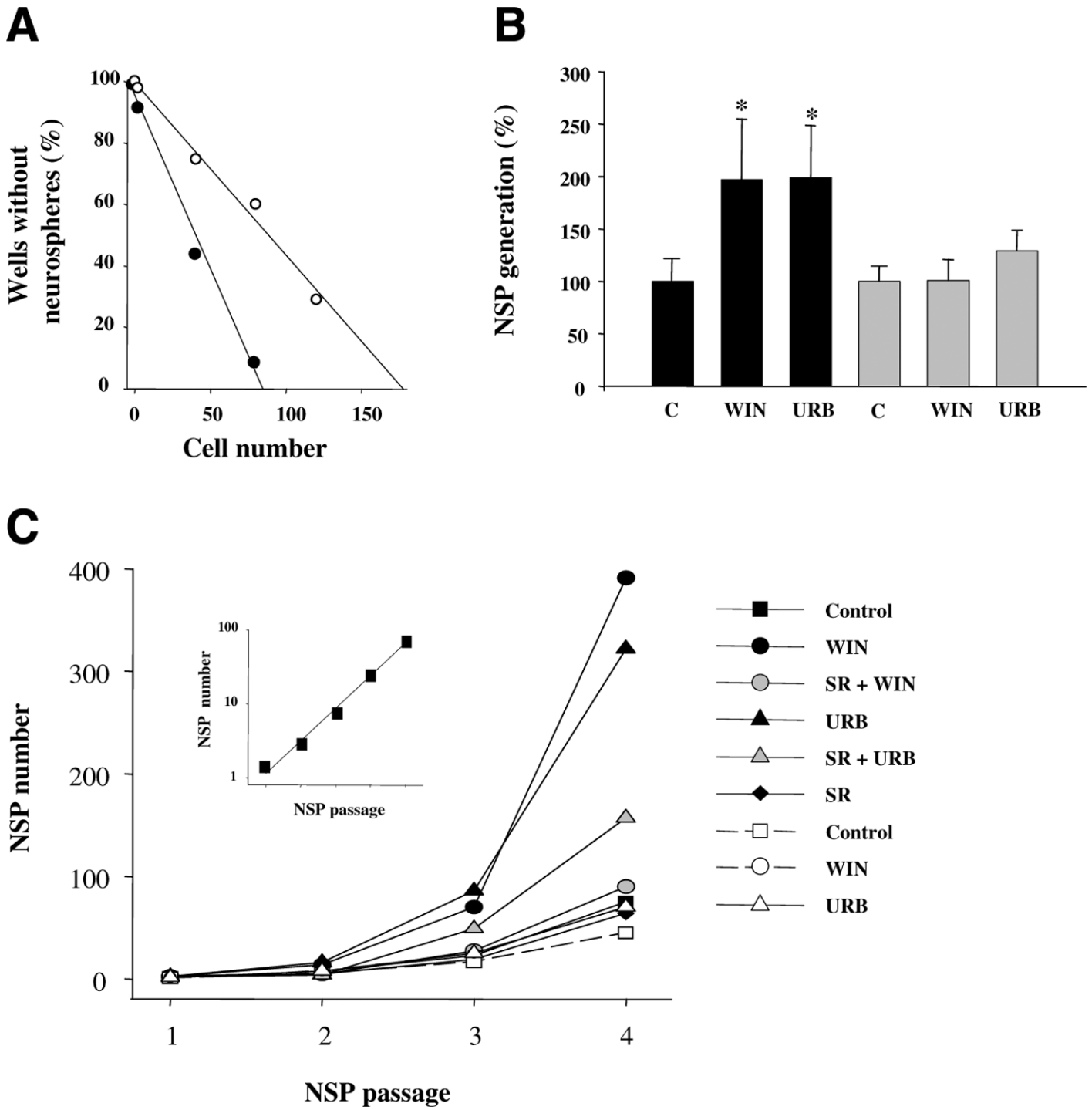

Figure 3. Impaired neurosphere generation in CB_1 knockout mice. ***A*)** Limit dilution analysis of neurosphere generation activity in CB_1-deficient mice (○) and their wild-type littermates (●). ***B*)** Effect of WIN-55,212-2 and URB597 on neurosphere-forming cells derived from wild-type (black bars) and CB_1-knockout mice (gray bars). ***C*)** Self renewal of NPs derived from wild-type (solid lines) and CB_1-knockout mice (dashed lines). Number of neurospheres was quantified after 4 consecutive neurosphere passages in the continuous presence of the indicated stimuli. ***Inset*)** Representative logarithmic plot of neurosphere generation. Results correspond to 4 (***A***, ***B***) or 3 (***C***) independent experiments. Significantly different from controls: $*P < 0.05$.

Fig. 4

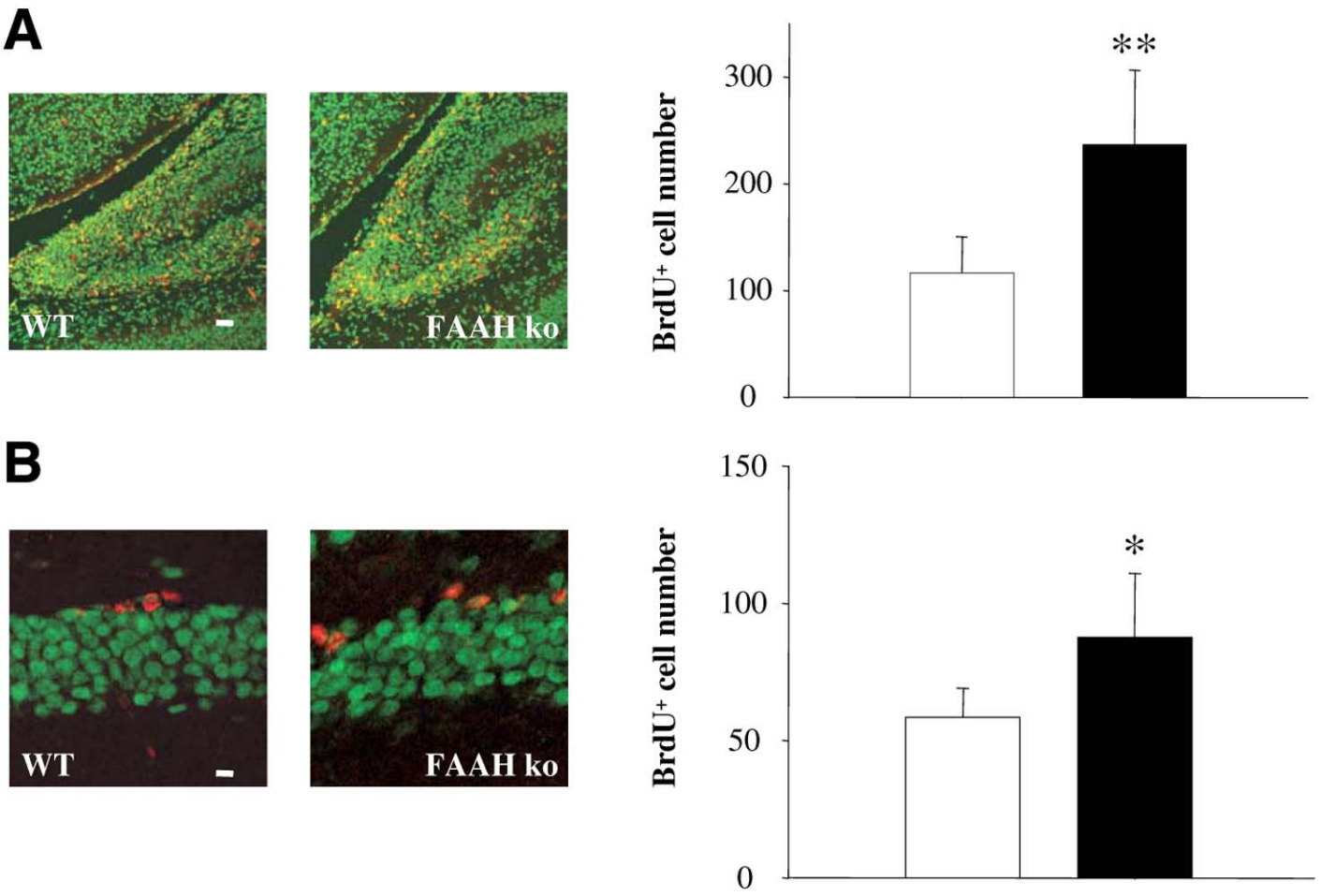

Figure 4. Endocannabinoids promote NP proliferation in vivo. ***A*)** Number of BrdU-positive cells per section in the dentate gyrus of wild-type (white bars; n=5) and FAAH$^{-/-}$ (black bars; n=4) mouse embryos. Representative immunostainings (***left panels***) of BrdU^{+} cells (red) counterstained with Yoyo-1 (green). Scale bar = 30 μm. ***B*)** Number of BrdU^{+} cells *per* section in wild-type (white bars, n=4) and FAAH$^{-/-}$ (black bars; n=4) adult mice. Representative immunostainings (***left panels***) of BrdU^{+} cells (red) costained with NeuN (green). Scale bar = 8 μm. Significantly different from controls: *$P < 0.05$; **$P < 0.01$.

Fig. 5

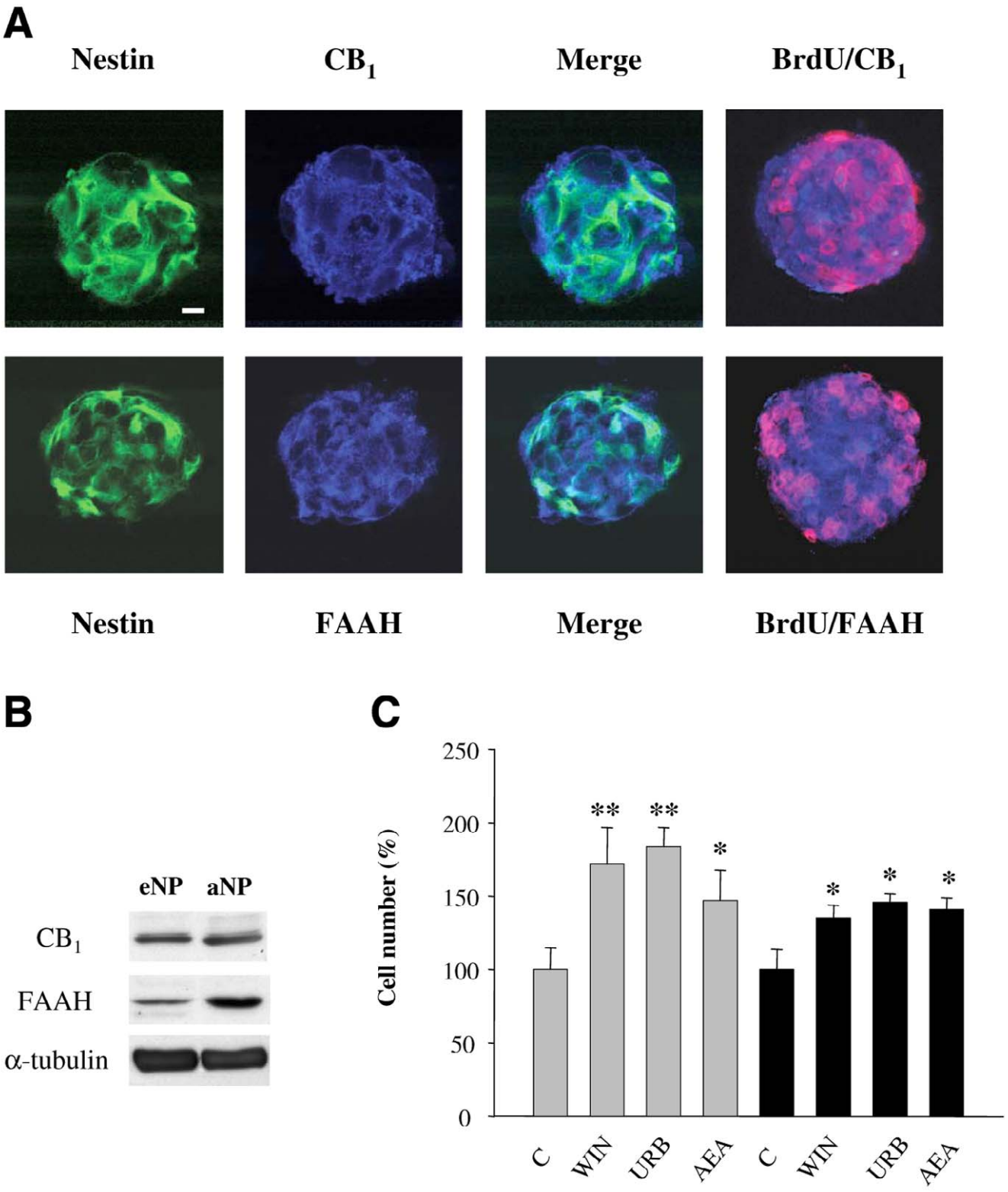

Figure 5. The eCB system is functionally active in adult mouse hippocampal NPs. ***A*)** Immunofluorescence of CB_1 and FAAH (blue) together with nestin (green) or BrdU (red). Scale bar = 25 μm. ***B*)** Western blot of CB_1 and FAAH in embryonic NPs (eNP) and adult NPs (aNP). ***C*)** Quantification of $BrdU^+$ (gray bars) and $nestin^+$ (black bars) NP cells after cannabinoid treatment (n=3). Significantly different from controls: $^*P < 0.05$, $^{**}P < 0.01$.