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Rapid communication

The effect of cannabichromene on adult neural stem/progenitor cells

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ABSTRACT

Apart from the psychotropic compound Δ^9-tetrahydrocannabinol (THC), evidence suggests that other non-psychotropic phytocannabinoids are also of potential clinical use. This study aimed at elucidating the effect of major non-THC phytocannabinoids on the fate of adult neural stem progenitor cells (NSPCs), which are an essential component of brain function in health as well as in pathology. We tested three compounds: cannabidiol, cannabigerol, and cannabichromene (CBC), and found that CBC has a positive effect on the viability of mouse NSPCs during differentiation *in vitro*. The expression of NSPC and astrocyte markers nestin and Glial fibrillary acidic protein (GFAP), respectively, was up- and down-regulated, respectively. CBC stimulated ERK1/2 phosphorylation; however, this effect had a slower onset in comparison to typical MAPK stimulation. A MEK inhibitor, U0126, antagonized the up-regulation of nestin but not the down-regulation of GFAP. Based on a previous report, we studied the potential involvement of the adenosine A1 receptor in the effect of CBC on these cells and found that the selective adenosine A1 receptor antagonist, DPCPX, counteracted both ERK1/2 phosphorylation and up-regulation of nestin by CBC, indicating that also adenosine is involved in these effects of CBC, but possibly not in CBC inhibitory effect on GFAP expression. Next, we measured ATP levels as an equilibrium marker of adenosine and found higher ATP levels during differentiation of NSPCs in the presence of CBC. Taken together, our results suggest that CBC raises the viability of NSPCs while inhibiting their differentiation into astroglia, possibly through up-regulation of ATP and adenosine signalling.

1. Introduction

The neural stem/progenitor cell (NSPC) population in the adult brain is essential for brain plasticity under normal physiological conditions as well as during the recovery from brain injuries. In the adult brain, these cells reside predominantly in two stem cell niches, namely the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. NSPCs can differentiate into three phenotypes: neurons, astrocytes and oligodendrocytes. The role of adult neurogenesis is established under physiological and pathological conditions (Ming and Song, 2011). It includes the contribution of SGZ neurogenesis to the major hippocampal functions of learning/memory and affective behaviours, and of SVZ neurogenesis to olfaction in healthy subjects and the replenishment of lost cellular constituents following injury (Saha et al., 2012). On the other hand, the significance of glial differentiation is less defined. Astrocytes are essential components in the brain as physical and metabolic support and also as integral players in nervous system networks (Araque and Navarrete, 2010). However, reactive astrocytes can suppress the recovery from brain injury through negative regulation of neuroregeneration (Wilhelmsson et al., 2004), whereas subpopulations of astrocytes were suggested to be quiescent neural stem cells (Robel et al., 2011), indicating a double-edged role of astroglial differentiation depending on the context.

Phytocannabinoids are found in significant quantities in cannabis. The plant *Cannabis sativa* produces about 80 such compounds, including Δ^9-tetrahydrocannabinol (THC), which is the major psychotropic component and specifically binds to G-protein-coupled receptors named cannabinoid (CB1 and CB2) receptors (Bisogno and Di Marzo, 2010). Although most attention has been paid to THC and its psychotropic actions, the therapeutic properties of other phytocannabinoids with lesser or no psychotropic effects are now emerging (Izzo et al., 2012). These include anti-inflammatory effects under neuropathological situations (Kozela et al., 2011; Martín-Moreno et al., 2011), as well as pro-neurogenic activity in the adult hippocampus (Wolf et al., 2010).

Based on this background, we examined the potential effects of three major phytocannabinoids, i.e. cannabidiol (CBD), cannabigerol (CBG), and cannabichromene (CBC), on the fate of adult NSPCs *in vitro*, for the potential benefit of the future clinical use of these herbal products in the treatment of neurological diseases or injuries through the regulation of adult neurogenesis.

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2. Experimental procedures

2.1. Cell culture

Adhesive NSPCs were prepared from the whole brains of 8-week old mice as previously described (Ray and Gage, 2006) except for the culture medium. Isolated NSPCs were passaged 12 times before the experiment. For Cell viability assay, cells were plated at 1×10^4/mL density in complete medium: neurobasal medium (Life Technologies) supplemented with B27 supplement (Life Technologies) and growth factors, 20 ng/ml bFGF (Life Technologies), 5–10 μg/ml Heparin and 20 ng/ml EGF (Sigma), and cultured at 5% CO_2 37 °C. For studying differentiating cells, cells were plated onto poly-L-ornithine (50 μg/ml)/laminin (5 μg/ml)-coated plastic or glass coverslips (immunocytochemistry) and treated with B27 medium: neurobasal medium supplemented with B27 but without growth factors.

2.2. Cell viability assay

Cell viability was assessed by the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were cultured in 48-well plates and then incubated with MTT (5 mg/mL) at 5% 37 °C for 4 h before cell lysis by SDS buffer (50% (v/v) N,N_-dimethyl formamide, 20% (w/v), SDS pH 4.5). The formazan product was measured using 620 nm absorbance (GENios Pro, TECAN).

2.3. Quantitative PCR (Q-PCR)

Total RNA was extracted with TRIZOL reagent (Life Technologies). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Life Technologies) according to the manufacturer's instruction, and mRNA levels were analyzed using IQ SYBR Green supermix (Bio-Rad) in a 10 uL scale by CFX384 Real Time System (Bio-Rad). Following primers were used for amplification; Nestin: CCCTGAAGTCGAGGAGCTG, CTGCTGCACCTCTAAGCGA; GFAP: CGGAGACGCATCACCTCTG, AGGGAGTGGAGGAGTCA TTCG; Tubb3: TAGACCCCAGCGGCAACTAT, GTTCCAGGTTCCAAGTCCACC; Adora1: TGTGCCCGGAAATGTACTGG, TCTGTGGCCCAATGTTGATAAG; Adora2a: GCCATCCCATTCGCCATCA, GCAATAGCCA AGAGGCTGAAGA; Adora2b: AGCTAGAGACGCAAGACGC, GTGG GGGCTGTAATGCACT; Adora3: ACGGACTGGCTGAACATCAC, AGACA ATGAAATAGACGGTGGTG. All data were normalized against Acidic ribosomal phosphoprotein P0 (Arbp): AGATTCGGGATATGCT GTTGGC, TCGGGTCCTAGACCAGTGTTC.

2.4. Immunocytochemistry

Cells were plated in complete medium on poly-L-ornithine/laminin-coated glass coverslips. Following 24 h incubation, the medium was replaced with fresh medium without growth factors and incubated for 3 days. The cells were fixed with 4% (w/v) paraformaldehyde. Nonspecific binding was blocked with 10% (v/v) fetal bovine serum and 0.1% (v/v) Triton X-100, and incubated with primary antibodies: mouse anti-Map2ab (Sigma–Aldrich; 1:1000), mouse anti-Nestin (abcam; 1:1000), rabbit anti-GFAP (abcam; 1:2000), overnight at 4 °C. Secondary antibodies anti-mouse Alexa594 (Life Technologies; 1:1000) and anti-rabbit Alexa488 (Life Technologies; 1:2000) were used for immunofluorescence). The immunofluorescence was studied with an epifluorescence microscope (Leica AF6000) equipped with the appropriate filter and, then, acquired using a digital camera (Leica, DFC 320) connected to the microscope and image analysis software (Leica, LAS AF). For the counting of $GFAP^+DAPI^+$ and $Map2ab^+DAPI^+$, three fields of 1250 × 900 μm, which contained 300–500 cells on average, were counted for each experiment ($N = 4$).

2.5. Western blot analysis

Cells were plated at 2×10^4/well and cultured on poly-L-ornithine/laminin-coated 6-well plastic plates for 2 days and then treated with the compound in B27 media for another 3 days. For ERK1/2 phosphorylation, NSPCs were pre-treated in the absence of growth factors for 16 h, which brings ERK phosphorylation to baseline levels, and then exposed to CBC or vehicle. After media removal, plates were rinsed with ice-cold PBS, incubated in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% (v/v) Triton X-100, protease and phosphatase inhibitor cocktails; Sigma–Aldrich) for 5 min on ice. Cells were scraped off the plates, centrifuged for 10 min at 14,000g at 4 °C, and supernatants were collected as total cell lysates. Protein concentration was determined using Bradford protein assay kit (Bio-Rad). For phospho- and total ERK1/2 analysis, cells were directly dissolved in SDS–PAGE sample loading buffer (below). Proteins (0.5 μg/well) were dissolved in SDS–PAGE sample loading buffer (62.5 mM Tris–HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol, 2% SDS, 5% (w/v) sucrose, 0.005% (w/v) bromophenol blue), heated for 5 min, and separated by 10% SDS–polyacrylamide gel. The proteins were transferred onto polyvinylidene fluoride membrane (Bio-Rad) in transfer buffer (192 mM glycine, 25 mM Tris base, 20% (v/v) methanol). Non-specific binding was blocked by 5% skim milk in TBST (50 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl and 0.05% Tween 20) for 1 h at room temperature. Primary antibodies: rabbit anti-GFAP (Dako, 1:2000), mouse anti-beta actin (Sigma-Aldrich, 1:5000), rabbit anti-phospho ERK1/2 (Cell Signaling Tech, 1:1000), and rabbit anti-ERK1/2 (Cell Signaling Tech, 1:2000) were applied in TBST overnight at 4 °C, followed by secondary antibodies: anti-rabbit horseradish peroxidase (HRP) conjugates (Bio-Rad) for GFAP, phospho- and total-ERK1/2, or anti-mouse HRP conjugates (Bio-Rad) for beta-actin. Detection was performed using chemiluminescent reagent (Bio-Rad, HRP Substrate Kit) and X-ray films (Thermo Scientific, CL-XPosure Film). The signal intensity of GFAP was analyzed using Adobe Photoshop 7.0 (histogram tool was applied to the inverted image of Western blot; Adobe Systems).

2.6. ATP assay

Cells were plated onto 6 well plates in complete medium. After 24 h, cells treated with CBC or control in B27 medium for 24 h, collected, and rinsed by PBS. ATP level in the cells were measured using fluorometric ATP assay kit (abcam) according to the manufacturer's instruction. Fluorescent intensity was measured using fluorescence reader 'GENios Pro' (TECAN), the values were normalized against protein concentration of the samples.

2.7. Statistical analysis

Statistical analysis was performed with one-way ANOVA followed by Tukey HSD test or Student's *T* test using BrightStat online statistics (Stricker, 2008).

3. Results

3.1. The effect of phytocannabinoids on the viability and differentiation of NSPCs

To test the effect of CBD, CBG, and CBC on adult neurogenesis directly through the action on NSPCs, we have tested the effects