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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

of these compounds on the viability of cultured NSPCs derived from the adult mouse brain, using the MTT assay. Cells were treated with cannabinoids at 1 μM, the maximum non-toxic concentration, in normal growth medium (complete medium) or in the media without growth factors (B27 medium) for 2 days. None of the compounds had significant effect on the viability in complete medium (Fig. 1a); however, CBC raised cell viability in the B27 medium, in which NSPCs stop proliferating and start differentiating (Fig. 1b).

Next, to study which cell types are affected by CBC treatment during differentiation, we performed Q-PCR analysis for nestin as a stem cell marker, beta III tubulin (Tubb3) as an early neuronal marker, glial fibrillary acidic protein (GFAP) as astroglial marker, in the early phase of differentiation. In the absence of growth factors, NSPCs stop proliferating and differentiate into neuronal and glial lineages. There was no change in nestin mRNA levels in complete medium with growth factors (Fig. 1c), suggesting that CBC has no significant effect on the proliferation, which is consistent with the cell viability assay (Fig. 1a). However, in the absence of growth factors (B27 medium), CBC up-regulated nestin (Fig. 1d). CBD also showed positive effect on nestin, in line with a previous study (Wolf et al., 2010), although the effect of this compound was smaller than that of CBC.

To further clarify the effect of CBC on the maturation into neuronal and astroglial lineages, immunocytochemistry was performed using anti-MAP2ab (neurons) and -GFAP (astrocytes) antibodies in cells after 3 days of differentiation in B27 differentiation medium, which show distinct neuronal and astroglial morphology (Fig. 2a). As shown in (Fig. 2a), fewer GFAP-positive astroglial cells were observed, whereas MAP2ab-positive cells were unaffected (Fig. 2a and b). Western blotting confirmed significantly lower GFAP protein levels in the cells in the presence of CBC (Fig. 2b). As GFAP is known to be expressed also in stem cells (Robel et al., 2011), we performed Nestin/GFAP double staining to better identify the population affected by CBC (Fig. 2c). Indeed, a fraction of GFAP-positive cells turned out to be also Nestin-positive. However, the double GFAP/Nestin-stained population was not significantly altered after the treatment, and, therefore, the reduction of GFAP levels was likely to be attributed only to the GFAP-positive/Nestin-negative population (Fig. 2c, bottom left). Accordingly, the CBC-treated group showed more Nestin-positive/GFAP-negative cells (Fig. 2c, bottom right), consistent with the Q-PCR analysis (Fig. 1d). Taken together, these data suggest that CBC significantly potentiates the preservation of Nestin-positive cells while inhibiting the differentiation into the astroglial lineage during differentiation of NSPCs.

3.2. The effect of CBC on intracellular signalling

To elucidate the underlying mechanism for the effects of CBC, ERK1/2 phosphorylation was examined as an essential intracellular signalling event determining stem cell fate (Wang et al., 2009). NSPCs were pre-treated in the absence of growth factors for 16 h, and then exposed to CBC (1 μM) or vehicle. The results show that CBC induces ERK phosphorylation (Fig. 3a). Further, the ERK signalling inhibitor U0126 counteracted the CBC-induced up-regulation of nestin mRNA (Fig. 3b), whereas CBC still reduced GFAP expression levels in the presence of U0126 (Fig. 3c), suggesting the involvement ERK signalling in CBC effect on the Nestin-positive stem cell population during the initial phase of differentiation but not on astroglial differentiation. Normally, ERK phosphorylation occurs within 5–10 min (Migita et al., 2008; Shinjyo et al., 2009), which is in contrast to the slower action of CBC observed here (Fig. 3a). This may suggest an atypical mechanism for the effect of CBC on phospho-ERK levels.

3.3. The potential action of CBC through purinergic signalling

Our previous pharmacological study of the antinociceptive effect of CBC suggested the involvement, among others, of transient

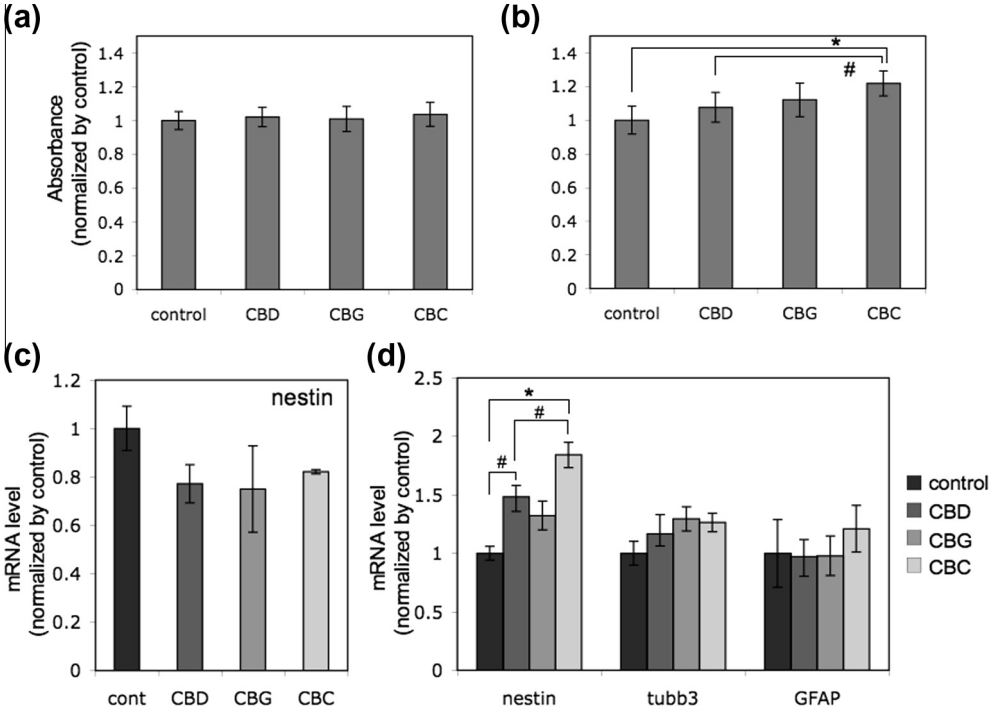

Fig. 1. The effect of cannabinoids on cell viability and mRNA levels of neural markers. Cells were treated with phytocannabinoids (1 μM) in complete media (a) or in B27 media without growth factors (b) for 2 days. The viability was measured by the MTT assay. Data are normalized by control condition and shown as means ± SEM, $N = 6$, $^{*}P < 0.01$, $^{\#}P < 0.05$ (ANOVA). (c and d) Cells were treated with phytocannabinoids (1 μM) in complete media (c) or in B27 media without growth factors (d) for 2 days. mRNA levels of neural markers were measured using Q-PCR. Means ± SEM, $N = 4$, $^{*}P < 0.001$, $^{\#}P < 0.05$ (ANOVA).

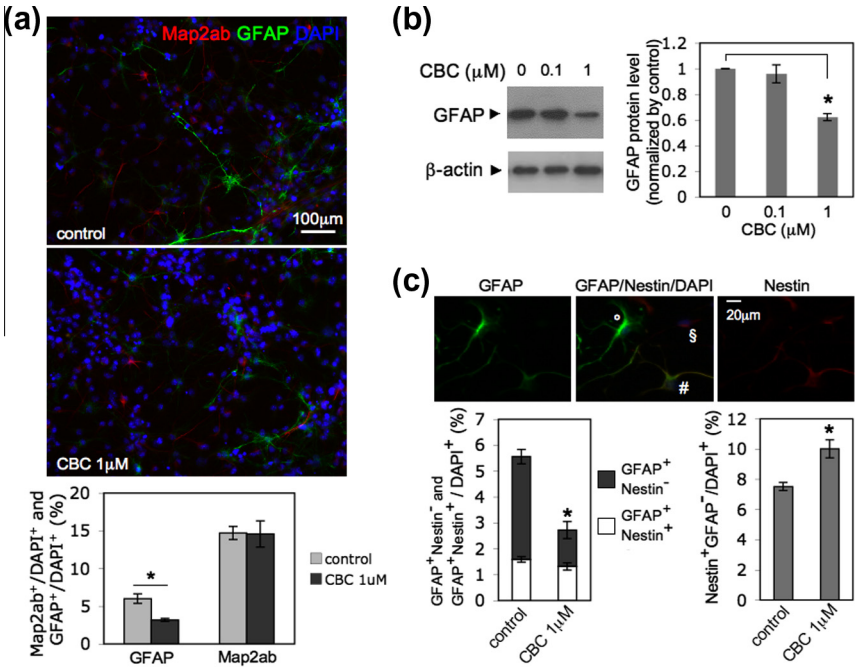

Fig. 2. The effect of CBC on neuronal and astroglial differentiation. Cells were grown in complete media for 2 days, then further incubated for 3 days in differentiation media without growth factors with CBC 1 μM or ethanol (control). (a) Neurons and astrocytes were detected by immunocytochemistry using specific antibodies. Red: Map2ab (neurons), Green: GFAP (astrocytes). Map2ab and GFAP positive cells were counted by a technician blinded to the experiment in three representative fields of each experiment, and the fractions of Map2ab and GFAP positive cells per total cells (DAPI) were presented. Means ± SEM, $N = 4$, $^{*}P < 0.01$ (Student's t-test vs control). (b) GFAP and β-actin in total cell lysates were detected by western blotting. Signal intensity was normalized against β-actin. Means ± SEM, $N = 3$, $^{*}P < 0.001$ (ANOVA). (c) Nestin (§), GFAP (°), and Nestin/GFAP (#) double positive cells were quantified by immunocytochemistry. The graphs show the % of total cells (DAPI) of each population. Means ± SEM, $N = 4$, $^{*}P < 0.01$ (Student's t-test vs control). (For interpretation of color in this Figure, the reader is referred to the web version of this article.)

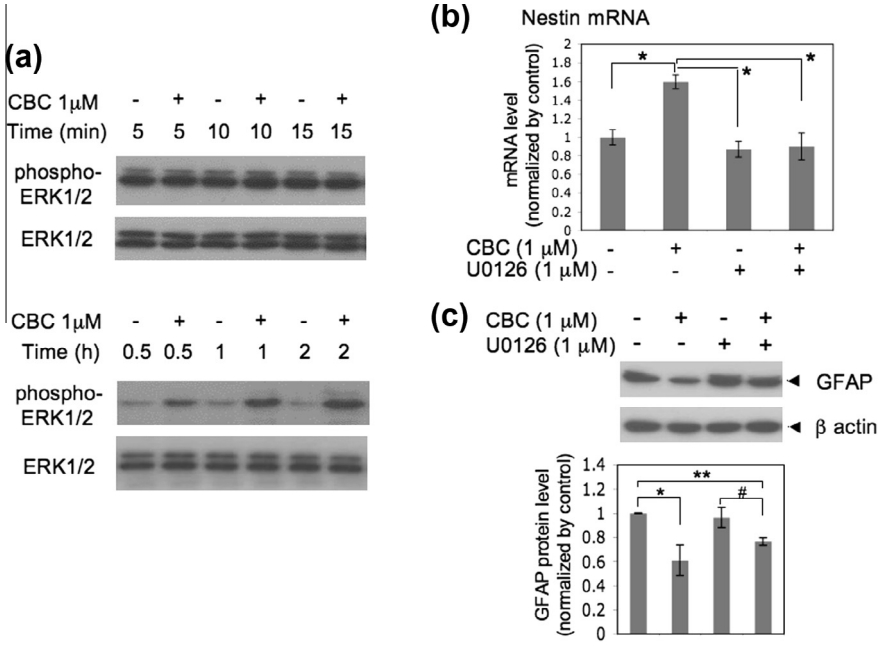

Fig. 3. The effect of CBC on ERK phosphorylation. (a) Cells were exposed to CBC for 5, 10, 15, 30 min, 1 and 2 h. Phospho-ERK1/2 and total ERK1/2 in total lysate were detected by western blotting using specific antibodies, respectively. (b and c) The effect of CBC on nestin and GFAP level in the presence or absence of U0126 was tested. Nestin mRNA: Means ± SEM, $N = 4$, $^{*}P < 0.001$ (ANOVA). GFAP protein: Means ± SEM, $N = 3$, $^{*}P < 0.001$, $^{**}P < 0.01$, $^{\#}P < 0.05$ (ANOVA).

receptor potential (TRP) channels of ankyrin type-1 (TRPA1) and adenosine A1 receptors in its actions on the electrical activity of some brainstem neurons (Maione et al., 2011). Thus, to find if either of these two proteins could be involved in the effect of CBC on NSPCs, the mRNA levels of TRPA1 and adenosine receptors in NSPCs before the induction of differentiation were measured by Q-PCR (Fig. 4a). Adenosine receptors A1 mRNA levels were significantly higher than those of other adenosine receptor subtypes

(A2a, A2b, and A3), whereas TRPA1 expression was not detected, suggesting the possible involvement of adenosine receptor A1 in the action of CBC. In fact, the selective A1 inhibitor DPCPX significantly reduced CBC-induced ERK phosphorylation (Fig. 4b). However, the slow response of ERK phosphorylation to CBC and the fact that U0126 only partially interfered with CBC effects (Fig. 3), suggest that CBC does not activate directly the adenosine A1 receptors, but may instead increase adenosine levels as discussed in the aforementioned report (Maione et al., 2011). Since adenosine levels in the brain under physiological conditions are largely reflected by adenosine triphosphate (ATP) levels at equilibrium (Daré et al., 2007), we hypothesized that these purinergic molecules are up-regulated in response to CBC. NSPCs were, therefore, treated with CBC for 24 h in the medium without growth factors (B27 medium), and ATP levels in the collected cell lysates were measured (Fig. 4c). ATP levels in the extracellular media were below detection, and hence we considered only the levels in the lysates. At 24 h after growth factor removal, ATP levels in the cells were significantly lower compared with the cells at 0 h (3.45 ± 0.26 nmol/10^6 cells). However, this significant reduction in ATP levels was not observed in the presence of 1 μM CBC. This suggests that CBC interferes with the reduction of ATP levels in NSPCs during differentiation, thereby activating purinergic signalling in these cells.

4. Discussion

CBC is one of the major non-psychotropic cannabinoids found in the cannabis plants. Anti-inflammatory effects have been shown for this compound in previous studies (Izzo et al., 2012), although its action in the brain was suggested to be only partly dependent on indirect activation of cannabinoid CB1 receptors (via inhibition of endocannabinoid inactivation and endocannabinoid level elevation), and due also to activation of TRPA1 channels and indirect activation of adenosine 1 receptors (via inhibition of the equilibrative nucleoside transporter [ENT] and adenosine level elevation) (Maione et al., 2011). The present study suggests that CBC can positively influence the viability of the Nestin-positive stem cell population in differentiating NSPCs through the up-regulation of ERK phosphorylation mediated by the adenosine A1 receptor. In fact, our Q-PCR analysis showed significant expression of adenosine receptor A1 in NSPCs and this receptor was previously reported to stimulate stem cell proliferation through ERK signalling (Migita et al., 2008). However, the required relatively high concentration of DPCPX (5 μM) and the expression of A1 and A3 receptors in these cells may suggest the involvement of A3 as well as A1 in the effect of CBC. Usually, ERK phosphorylation is a fast event that occurs within 5–15 min from stimulation (Migita et al., 2008), which is in contrast to the slow action of CBC found in this study. This may suggests that CBC activates adenosine receptors indirectly (and, hence, with a slower onset), i.e. after elevation of adenosine levels (via inhibition of adenosine reuptake by the ENT) (Maione et al., 2011). However, the effect of CBC was observed in the initial phase after the removal of growth factors, during which cells stop proliferating and start differentiating. Therefore, it is not clear whether CBC is acting directly on NSPCs or indirectly, by affecting differentiating cell populations, and further investigation at the cellular and molecular levels needs to be done to clarify this issue. Indeed, our results suggest that ERK phosphorylation is not responsible for the effect of CBC on astrocyte differentiation, which may indicate that different pathways are involved in CBC effects on Nestin-positive stem cells and GFAP-positive differentiating astrocytes.

Growing evidence suggests the significance of neurogenesis in the brain under various physiological and pathological conditions (Gould, 2007). For the benefit of brain health, it is therefore important to find factors that stimulate neurogenic potential either through direct action on NSPCs or through other cells that regulate neurogenesis. In fact, NSPCs are in intimate contact with, and under the control of, surrounding glial cells, namely microglia, brain resident immune cells, and astrocytes, which produce pro-/anti-inflammatory and neurotrophic factors (Seth and Koul, 2008). Importantly, astrocytes from neurogenic and non-neurogenic regions show different properties: permissive or inhibitory for neurogenesis, respectively (Barkho et al., 2006; Colangelo et al., 2012; Robel et al., 2011), indicating their crucial regulatory role in this phenomenon. Given the previously reported anti-inflammatory actions of CBC (Izzo et al., 2012; Wirth et al., 1980), it is tempt-

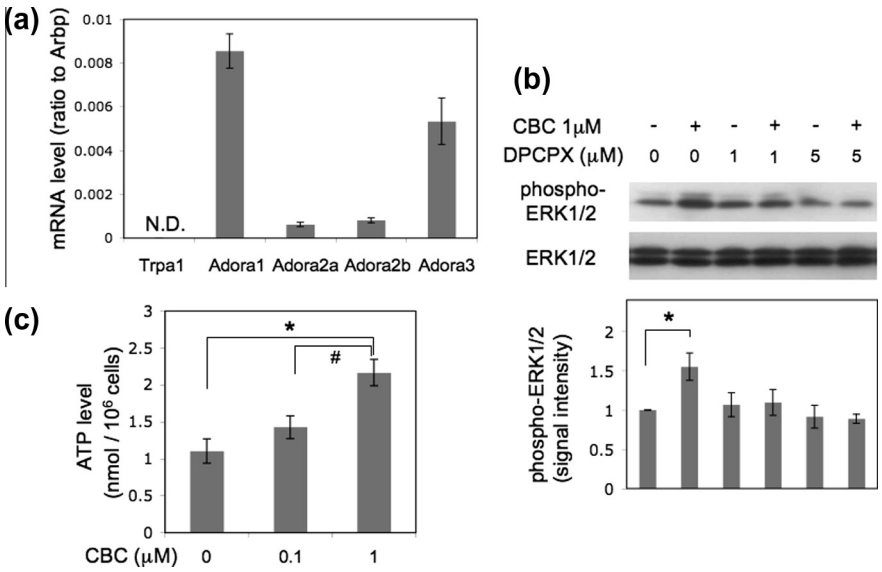

Fig. 4. The involvement of purinergic signalling in the effect of CBC. (a) mRNA expression of TRPA1 and adenosine receptors (A1, A2a, A2b, A3) in NPSCs. Relative expression to ribosomal protein P0 is shown (means ± SEM, $N = 4$). (b) The effect of DPCPX on CBC-induced ERK phosphorylation. The cells were treated with CBC in the presence or absence of DPCPX for 1 h. The signal intensity was measured, and the values normalized to control (without CBC and DPCPX) are shown below. The intensity of the control is shown as 1. Means ± SEM, $N = 3$, $^{*}P < 0.01$ vs control (ANOVA). (c) Intracellular ATP levels after differentiation induction in the presence or absence of CBC. Cells were treated with B27 medium for 24 h and ATP levels in the cell lysate was measured. Data are presented as nmol per 10^6 cells. Means ± SEM, $N = 3$, $^{*}P < 0.001$, $^{\#}P < 0.01$ (ANOVA).

ing to suggest that the potential benefit of CBC on neurogenesis occurs also through its inhibitory action on glial cell populations. In fact, in the present study, CBC inhibited astroglial differentiation of NSPCs, suggesting a possible pro-neurogenic effect of CBC through the suppression of reactive astrocytes that can be detrimental to neurogenesis (Colangelo et al., 2012; Robel et al., 2011).

Importantly, we found that CBC elevates ATP levels in differentiating NSPCs. Purinergic signalling is an essential regulator of neurogenesis during development and in adult brain after injury (Ulrich et al., 2012), as well as of astroglial activation (Daré et al., 2007), both through the activation of P1 receptors (adenosine receptor A1, A2a/b, and A3) and P2 receptors for ATP (P2X/P2Y). Considering that extracellular adenosine levels are regulated by the ENT, previously suggested to be inhibited by CBC (Maione et al., 2011), and by degradation of intracellular ATP to adenosine (Vlajkovic et al., 2009), it is plausible to speculate that the observed up-regulation of ATP is partly responsible for the effect of CBC on both the preservation of Nestin-positive stem cells, via adenosine elevation and indirect A1 activation, and astroglial differentiation, through ATP signalling at P2 receptors. This could explain why we found an inhibitory effect of the A1 receptor antagonist DPCPX only for the effect of CBC on Nestin-positive stem cells but not on GFAP-positive astrocytes. However, further studies are needed to clarify the underlying mechanism for the effect of CBC on intracellular ATP levels.

It is unlikely that CBC exerts the effects observed here via activation of cannabinoid receptors. First, CBC has moderate affinity ($K_i \sim 100$ nM) only for CB2 receptors, which are not expressed in the NPSC preparation used here (data not shown), and binds to CB1 receptors only at concentrations higher than 1 μM (V. Di Marzo, unpublished observations), whereas it inhibits endocannabinoid cellular reuptake only at concentrations higher than 10 μM (De Petrocellis et al., 2011). Secondly, CBC has no CB1 or CB2 agonist or antagonist activity in an *in vitro* functional assay in macrophages (Romano et al., 2013). Finally, while the stimulation of CB2 receptors stimulation promotes proliferation of NPSCs obtained through protocols different from the one used here (Molina-Holgado et al., 2007; Palazuelos et al., 2006), CB1 receptor activation promotes astroglial differentiation in NSPCs (Aguado et al., 2006), an effect opposite to that observed here.

In conclusion, CBC may exert potential actions on brain health through effects on adult neural stem cells. However the majority of stem cells *in vivo* reside in two different neurogenic niches, and these two populations have different properties and functional significance (Ming and Song, 2011), whereas our study was performed using whole brain-derived NSPCs. Therefore, although our study suggests a potential effect of CBC on neural stem cells in the adult brain, before considering the possible clinical implications of the present results, the action of this compound on each stem cell population *in vivo*, in the context of various physiological and pathological circumstances, needs to be investigated.

Acknowledgements and conflict of interest

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