Δ9-tetrahydrocannabinol negatively regulates neurite outgrowth and Akt signaling in hiPSC-derived cortical neurons.

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Abstract

Endocannabinoids regulate different aspects of neurodevelopment. *In utero* exposure to the exogenous psychoactive cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC), has been linked with abnormal cortical development in animal models. However, much less is known about the actions of endocannabinoids in human neurons. Here we investigated the effect of the endogenous endocannabinoid 2-arachidonoyl glycerol (2AG) and Δ^9-THC on the development of neuronal morphology and activation of signaling kinases, in cortical glutamatergic neurons derived from human induced pluripotent stem cells (hiPSCs). Our data indicate that the cannabinoid type 1 receptor (CB1R), but not the cannabinoid 2 receptor (CB2R), GPR55 or TRPV1 receptors, is highly expressed during the development of hiPSC-derived cortical neurons. Consistent with previous reports, 2AG and Δ^9-THC negatively regulated neurite outgrowth. Interestingly, acute exposure to both 2AG and Δ^9-THC inhibited phosphorylation of serine/threonine kinase extracellular signal-regulated protein kinases (ERK1/2), whereas Δ^9-THC also reduced phosphorylation of Akt (aka PKB). Taken together, our data suggest that hiPSC-derived cortical neurons highly express CB1Rs and are responsive to both endogenous and exogenous cannabinoids. Thus, hiPSC-neurons may represent a good cellular model for investigating the role of the endocannabinoid system in regulating cellular processes in human neurons.

Introduction

The endocannabinoid system is a neuromodulatory system with important roles in central nervous system (CNS) development, neuronal function and synaptic plasticity[1,2]. Perturbations in this system have been observed in psychiatric disorders such as schizophrenia[3], and there is a significant association between cannabis use and schizophrenia[3-6]. The endocannabinoid system is composed of endogenous cannabinoids, enzymes that synthesize and degrade endogenous cannabinoids and cannabinoid receptors. The Cannabinoid 1 receptor (CB1R) is a highly abundant receptor in the CNS, with strong expression in a number of brain regions, including the cortex[1,7]. Cannabinoid 2 receptors (CB2Rs) show much lower expression in the CNS, although recent work has shown high inducible expression of CB2Rs under pathological conditions[8]. Several other receptors, such as peroxisome proliferator activated receptors and transient receptor potential channels, are also engaged by cannabinoids[9].

CB1Rs are members of the superfamily of G protein-coupled receptors that inhibit adenylyl cyclase and activate mitogen-activated protein kinase by signaling through $G_{i/o}$ proteins. Stimulation of CB1R by cannabinoids has been shown to lead to the phosphorylation of serine/threonine kinase Akt and extracellular signal-regulated kinase ERK1/2[10]. These kinases may then activate or inhibit their substrates to influence cellular functions such as promoting survival, metabolism and differentiation. Both Akt and ERK1/2 activation have been shown to mediate neurite outgrowth[11,12], a key process in neuronal development and plasticity. Furthermore, the primary psychoactive component of cannabis (*Cannabis sativa*), Δ^9-tetrahydrocannabinol (Δ^9-THC) is also thought to act via the CB1R, resulting a wide

range of effects including a depression of the glutamatergic system[1]. This highlights the potential importance of the CB1R in relation to health and disease.

Much of our knowledge of the endocannabinoid system comes from work using *in vitro* and *in vivo* animal models. It remains unknown how well these data translate to the human neurons. Recent investigations into the role of endocannabinoids during cortical development have shown that cannabinoids regulate neuronal morphology, through small GTPase signaling pathways and rearrangement of the actin cytoskeleton, resulting in a negative impact on cortical development[13-15]. Recent advances in stem cell technology have enabled the reprogramming of human somatic cells into induced pluripotent stem cells (hiPSCs)[16,17], which can subsequently be differentiated in to specific neuronal subtypes[18]. Using this system Guennewig and colleagues demonstrated that exposure of hiPSC-neurons to Δ^9-THC resulted in significant alterations in genes involved with development, synaptic function, as well as those associated with psychiatric disorders[19]. This supports the use of hiPSCs as a cellular model to investigate the cellular actions of cannabinoids to understand their relevance for health and disease.

In this study, we first examined the expression of cannabinoid receptors during the differentiation of hiPSCs into cortical glutamatergic neurons. Subsequently, we investigated the impact of acute exposure to the endogenous cannabinoid 2-arachidonoyl glycerol (2AG), a full agonist for the CB1R, and Δ^9-THC, a partial agonist for CB1R, on the morphology of young iPSC-derived glutamatergic neurons. Furthermore, we assessed the ability of 2AG and Δ^9-THC to regulate the activity of ERk1/2, Akt and GSK3β signaling cascades. We report that CB1R is strongly expressed in iPSC-neurons and that 2AG and Δ^9-THC treatment negatively affect neuronal morphology and impact the Akt and ERK1/2 signaling pathways. These data

provide additional insight into the cellular roles of cannabinoids during the development of human cortical neurons.

Results

Generation of cortical neurons from hiPSCs

Within the developing CNS, the endocannabinoid system has been implicated in important neuronal processes such as synapse formation and neurogenesis[1,2]. CB1Rs are G-protein coupled receptors that are widely expressed in the brain[7], including the cortex[1,2]. Consistent with a role for the endocannabinoid system during the development of cortical neurons, acute (24 hour) exposure of hiPSC-neurons to Δ^9-THC results in the differential expression of multiple genes, including those involved in development[19]. Therefore, in order to further understand the role of cannabinoids during the development of human cortical neurons, we induced cortical differentiation from hiPSC. The cell lines were derived by reprogramming keratinocytes from neurotypic males (age ranging between 35 to 55 years). hiPSC lines were induce to neuroepithelial differentiation through dual SMAD inhibition (2i) for 8 days (**Figure 1A**). The small molecules inhibitors were removed during neuronal progenitor's differentiation from day 8 to day 20. Subsequently, the media was replaced with B27 supplemented with DAPT until day 30 to induce the generation of terminally differentiated neurons (**Figure 1A**). Immunocytochemical (ICC) analyses indicated that the derived hiPSC expressed high levels of the pluripotency markers OCT4, NANOG and SOX11, whereas early neural progenitor cell (NPC) markers PAX6, ZNF521 and NESTIN were up-regulated following 8 days of 2i induction (**Figure 1B and 1C**). After 26 days of neuronal differentiation young neurons

expressed high levels of FOXG1, TBR1, TBR2 and BRN2 factors, which are essential for cortical neurons differentiation (**Figure 1B and 1C**). Similarly, real time PCR (qPCR) analyses demonstrated that the neuroepithelial marker SOX2 was highly expressed 7 days after 2i induction and was gradually down-regulated during the 50 days of neuronal fate acquisition (**Figure 1D**). PAX6, a key regulator of cortical development, was found to be highly expressed from day 7 to day 50 during neuronal formation; peak expression was observed at day 22 (**Figure 1E**). The expression of CTIP2, a marker of deep layer cortical cell fate, rapidly increased from day 22 to day 28, peaking at day 26; subsequently, expression of CTIP2 was down-regulated until day 50 (**Figure 1F**). Conversely, expression of BRN2, a marker associated with upper cortical layer cell fate, showed expression peaks at days 23, 26 and once more at day 48 (**Figure 1G**). Collectively, these data demonstrate the generation of cortical glutamatergic neurons from hiPSCs.

CB1R expression during the cortical differentiation of iPSCs

Previous studies have suggested that the CB1R is highly expressed during neuronal development[1]. Therefore, we examined the expression of CB1R in hiPSC-derived NPCs and terminally differentiated hiPSC-derived cortical neurons. In all three hiPSC lines, CB1R showed similar gene expression patterns across the three iPSC lines. Specifically, CB1R mRNA levels significantly increased as hiPSCs differentiated from NPCs into neurons (**Figure 2A**). We also examined the expression of CB2R, GPR55 and TRPV1 receptors which are expressed in the brain and are engaged by cannabinoids in differentiated neurons[1,2]. The expression of these receptors was consistently mirrored between the three hiPSC lines and was significantly reduced compared with the expression of CB1R (**Figure 2B**). Western blotting of cell lysates

from hiPSC-neurons for CB1R, further validated the expression of this receptor at the protein level. A prominent band ~53 kDa consistent with the predicated molecular weight for the receptor, was readily observed (**Figure 2C**). Consistent with this, immunocytochemistry for CB1R in all three hiPSC-derived cortical neurons showed a could be readily observed. Immunoreactive puncta for CB1R was observed within the cell soma, and could be seen as punctate structures along MAP2-positive neurites in hiPSC-neurons (**Figure 2D**). Collectivity, these data demonstrate that CB1R is highly expressed at both mRNA and protein levels and is ideally localized to influence neuronal morphology as previously reported.

Effect of 2AG on neurite outgrowth in iPSC-neurons

There is growing appreciation that the endocannabinoid system is an important regulator of brain wiring during development through the modulation of several different processes including the specification of neuronal morphology[13,14,20-22]. As CB1R is localized along neurite, we reasoned that endocannabinoids may be involved in regulating the establishment of neuronal morphology in hiPSC-neurons. To test this prediction, we treated hiPSC-neurons at day 29 with 1 µM 2AG, a full agonist for the CB1R, for 24 hours before neurons were fixed and stained for MAP2 to outline neuronal morphology and subsequently imaged and quantified neurite development using a high-content screening platform (**Figure 3A**). We first assessed whether the treatment altered the number of MAP2-positive cells; this revealed no differences between conditions (**Figure 3B**; p=0.931). We next examined neuronal morphology and demonstrated that the average number of neurites (**Figure 3C**; p=0.713) and number of branch points (**Figure 3C**; p=0.780) was unaffected by treatment. However, 2AG treatment for 24 hours significantly reduced neurite length (**Figure 3D**; p<0.001)

in all three hiPSC-derived cortical neuron lines. Taken together, these data indicate that 2AG, potentially acting via the CB1R, results in an attenuation of neurite outgrowth in hiPSC-neurons.

2AG modulation of signaling kinases.

Stimulation of CB1R leads to the phosphorylation and activation of several signaling kinases, including Akt, ERk1/2 and GSK3β[2,23,24]. Akt and ERK1/2 pathways have been implicated in promoting neurite outgrowth[11,12], whereas GSK-3β, a well-defined substrate of Akt, has also been implicated in the regulation of neurite and axonal outgrowth and branching[25]. Interestingly, the negative effects of CB1R activation on neurite outgrowth have been linked with ERK1/2 signaling[19], although activation of CB1R has also been linked with Akt/GSK3β signaling *in vivo*[24]. Therefore, we examined whether CB1R activation by 2AG, generated changes in the activation state (phosphorylation) of Akt/GSK3β kinases in day 30 neurons. Interestingly, 2AG treatment resulted in a significant decrease in ERK1/2 phosphorylation after 30 minutes (**Figure 4A**; $p<0.05$). Conversely, no changes in the phosphorylation state of Akt or GSK3β were detected following 15 and 30 minutes of 2AG exposure (**Figure 4B and C**). These data demonstrated that 2AG. a full agonist for the CB1R, is capable of coupling the receptor to a negative regulation of ERK1/2.

Δ⁹-THC negatively regulates neurite outgrowth in hiPSC-neurons.

Δ⁹-THC has previously been shown to negatively regulate neurite outgrowth and growth cone dynamics, through the regulation of actin polymerization and microtubule stability in mouse cortical neurons[13,15,20]. In addition, exposure to Δ⁹-THC in hiPSC-neurons results in the alteration of multiple genes involved in development

and development of neuronal morphology[19]. Therefore, we were interested in determining whether acute exposure to Δ^9-THC could also alter neurite outgrowth. D29 hiPSC-neurons were treated with 3 µM Δ^9-THC for 24 hours before neuronal morphology was examined using a high-content screening platform (**Figure 5A**). Δ^9-THC treatment did not significantly affect cell number across the three hiPSC-lines (**Figure 5B**; p=0.647). Similar to 2AG, exposure to Δ^9-THC did not significantly alter neurite number (**Figure 5C**; p=0.426) or branch points (**Figure 5D**; p=0.859). However, Δ^9-THC 24 hour treatment significantly reduced the neurite length (**Figure 5E**; p=0.426). Taken together, these data indicate that Δ^9-THC negatively regulates neurite outgrowth in young hiPSC-neurons.

Effect of Δ^9-THC on the phosphorylation of serine/threonine kinases Akt, GSK-3β and mitogen-activated protein kinase ERK1/2.

Owing to our data demonstrating that 2AG negatively regulated ERK1/2 phosphorylation, we were next interested in understanding the effect of Δ^9-THC on the activation of signaling kinases involved in neurite outgrowth. D30 hiPSC-neurons were treated with vehicle or 3 µM Δ^9-THC for either 15 or 30 minutes. Similarly to 2AG treatment, Δ^9-THC induced a rapid decrease in ERK1/2 phosphorylation (**Figure 6A**; * $p < 0.05$). Interestingly, Δ^9-THC also caused a rapid reduction in levels of phosphorylated Akt, which was evident after 15 minutes (**Figure 6B**; * = $p < 0.05$). A non-significant trend towards a decrease in phosphorylated GSK3β was also evident following Δ^9-THC treatment, but this did not reach significance (**Figure 6C**; p=0.105). Collectively, these data indicate that Δ^9-THC negatively regulates both ERK1/2 and Akt phosphorylation, in contrast to 2AG, which regulates ERK1/2 exclusively.

Discussion

Recently it has been demonstrated that cannabinoid treatment affects neuronal function and glutamate receptor expression in hiPSC-derived dopaminergic neurons and excitatory neurons, respectively[26,27]. Moreover, treatment with Δ^9-THC induces a wide range of transcriptional changes in cortical hiPSC-neurons. This includes changes in gene involved in neurodevelopmental processes, as well as those associated with neurodevelopmental and psychiatric disorders[19]. Despite this, it is not clear what role the endocannabinoid system plays during neurodevelopment. In this study, we demonstrate that the CB1R is the predominate cannabinoid receptor expressed during early differentiation of cortical hiPSC-neurons. Furthermore, we demonstrate that treatment with both 2AG and Δ^9-THC result in a negative regulation of neurite outgrowth, as well as differential effects on the activation status of ERK1/2 and Akt signaling kinases. These data indicate that both 2AG and Δ^9-THC are likely signaling via the CB1R receptor, but are able to induce different signaling patterns, potentially owing to their pharmacological profile at these receptors.

Differentiated hiPSC-derived glutamatergic neurons showed significant upregulation of CB1R mRNA compared to undifferentiated NPCs. Moreover, hiPSC-derived glutamatergic-neurons also showed significantly higher levels of CB1R mRNA compared to CB2R, GPR55 and TRPV1. Stanslowsky et al. (2016) also observed upregulation of CB1R mRNA following terminal differentiation of hiPSC-derived dopaminergic neurons and the predominant expression of CB1R in hiPSC-derived neural precursors[27]. It has been reported that CB1R is highly expressed in newly differentiated glutamatergic neurons and is labelled in a punctate manner in the soma and neurites of these cells[20]. In agreement with previous reports, we observed that

CB1R protein is differentially up-regulated during neuronal differentiation in the three iPSC lines with different genetic background. For instance, CB1R was localized in the cell soma and along neurites in iPSC-derived glutamatergic neurons. These findings provide support for the idea that the CB1R may play an important role in early neurodevelopmental processes such as progenitor cell differentiation and neurite outgrowth, prior to the formation of synapses.

Studies on the effect of cannabinoids on neurite outgrowth have been attempted with various CB1R agonists and have produced conflicting results[2]. Whereas Δ^9-THC treatment in dopaminergic neurons had no effect on morphology[28], Δ^9-THC treatment reduced neurite length in primary neurons from E16.5 mouse cortices[15]. Similarly, activation of CB1R by HU210 has been shown to trigger neurite outgrowth in Neuro2A cells[29], while CB1R agonist WIN55,212-2 negatively regulates dendritic and axonal outgrowth in cultured rat hippocampal neurons[20]. We investigated the effects of the endogenous cannabinoid 2AG and the exogenous cannabinoid Δ^9-THC on neuronal morphology. In our study, we observed that both 2AG and Δ^9-THC decreased total neurite outgrowth in hiPSC-derived glutamatergic neurons. Vitalis et al. (2008) previously showed that basal activation of CB1R acts as a negative regulatory signal for neuritogenesis, and treatment with inverse agonist AM281 significantly increased total dendritic length[20]. These data and ours suggest that both endogenous and exogenous cannabinoids affect neuronal morphology in growing neurons. However, the specific downstream mechanisms underlying this process are not well-described, and further research is required to delineate how different cannabinoids affect neuritogenesis in growing and mature neurons, from humans and other animal species.

CB1R signaling is a complex process and cannabinoids can phosphorylate and activate members of all three families of multifunctional mitogen-activated protein kinases, as well as the PI3K/Akt pathway[30,31]. We examined the signal transduction pathways regulated by the activation of CB1Rs, focusing on the effect of 2AG and Δ^9-THC on the Akt and ERK1/2 pathways. Studies on the effect of cannabinoids on these pathways have been attempted with various CB1R agonists and have produced conflicting results. Several studies of non-neuronal cells and hippocampal slices and hippocampal neurons in living mice previously demonstrated that stimulation of CB1Rs by endocannabinoids anandamide, 2AG and exogenous Δ^9-THC activates ERK1/2[23,32], whereas stimulation of CB1R by selective CB1R agonist ACEA in primary cortical neurons did not alter the phosphorylation of ERK1/2 or Akt [33]. In contrast, an increase in the phosphorylation of Akt and of GSK3β by acute Δ^9-THC, anandamide and HU-210 administration has been demonstrated *in vivo*[24,34]. In this study, 2AG did not affect the phosphorylation and activation of Akt, whereas Δ^9-THC reduced the levels of phosphorylated Akt. Neither cannabinoid significantly altered the downstream effector GSK-3β, though both reduced the levels of phosphorylated ERK1/2. Overall, previous data and ours suggest that both endogenous and exogenous cannabinoids affect the ERK1/2 and Akt signaling pathways. It is possible that in different model systems, CB1R activation triggers different signaling pathways to regulate downstream gene expression and neuronal processes. The specific pharmacology of CB1R signaling is not well understood, and further research is necessary to describe how endogenous, exogenous and synthetic cannabinoids act on CB1R and how they may or may not interact to trigger or inhibit downstream signaling pathways.

Overall, the results of the present study suggest that young hiPSC-derived glutamatergic neurons are responsive to cannabinoids, and further support the notion

that this cellular system is a good model for investigating CB1R signaling[19]. CB1Rs were first identified as the main neuronal receptor for Δ^9-THC and are one of the most abundant G protein-coupled receptors in the brain[7]. They have previously been characterized as a retrograde synaptic regulator of inhibitory control of neuronal activity in mature neurons[35-37] and to affect both long-term potentiation (LTP) and depression (LTD) and to impair learning and memory[38]. CB1Rs and endocannabinoids are also highly expressed in the fetal brain and implicated in neuronal development processes such as neurite growth and axonal pathfinding[13,15,20-22,29]. As shown in this and other recent studies, both endogenous and exogenous cannabinoids alter neurite outgrowth, synaptic activity and synaptic protein expression in developing human hiPSC-derived neurons[26,27]. Moreover, as Δ^9-THC has recently been shown to alter expression of genes associated with neurodevelopmental and psychiatric disorders, there is evidence for perturbations of shared molecular pathways potentially exacerbated by Δ^9-THC[19]. Thus, these data collectively suggest that cannabinoids play a role in brain development and that overstimulation by exogenous cannabinoids or abnormal levels of endocannabinoids may perturb normal physiological processes, such as neuritogenesis, which may contribute to the pathophysiology of neurodevelopmental and psychiatric disorders.

Materials and Methods

Reagents

Δ^9-THC (ethanol solution, ab120447) were from Abcam. 2-Arachidonylglycerol (1298) was from Tocris. A list of antibodies used in this study can be found in **Table 1**.

Human induced pluripotent stem cells (hiPSCs)

hiPSC lines were generated from primary keratinocytes as described previously[39]. Participants were recruited and methods carried out in accordance to the 'Patient iPSCs for Neurodevelopmental Disorders (PiNDs) study' (REC No 13/LO/1218). Informed consent was obtained from all subjects for participation in the PiNDs study. Ethical approval for the PiNDs study was provided by the NHS Research Ethics Committee at the South London and Maudsley (SLaM) NHS R&D Office. Briefly, 1×10^5 primary hair root keratinocytes were reprogramed by introducing OCT4, SOX2, KLF4 and C-MYC factors with a CytoTune-iPS 2.0 Sendai expressing Reprogramming Kit (ThermoFisher, A16517). Transformed keratinocytes were plated onto an irradiated MEF feeder layer (Millipore) supplemented Epilife medium for ten days before switching to 'hES media', which consisted of KO-DMEM/F12 supplemented with 20% Knock-out serum replacement, Non-essential amino acids, Glutamax, β-mercaptoethanol (all from Life Technologies) and bFGF (10 ng/ml; Peprotech). After a further two weeks, reprogrammed colonies were selected and plated on Geltrex (Life technologies) coated Nunc treated multidishes (Thermo Scientific) into E8 media (Life Technologies). hiPSCs reprograming was validated by genome-wide expression profiling using Illumina Beadchip v4 and the bioinformatics tool 'Pluritest'. Additionally, the tri-lineage differentiation potential was stablished by embryoid body formation; ICCs to validate the expression of different pluripotency markers including Nanog, OCT4, SSEA4 and TRA1-81 and the alkaline phosphatase activity by Alkaline phosphatase expression kit (Milipore). The genomic stability was determined by G-banded karyotyping. hiPSCs were incubated in hypoxic conditions

at 37°C and maintained in E8 media replaced every 24 hours until the cells monolayer reach ~95% confluence.

Neuronal differentiation

Neuronal differentiation of hiPSCs was by achieved replacing E8 medium on confluent hiPSCs with neuralization medium: 1:1 mixture of N2- and B27 (Life Technologies) supplemented with 10 µM SB431542 (Sigma-Aldrich) and 1 µM Dorsomorpin (Calbiochem) for dual SMAD inhibition (2i). Cells were maintained at 37°C in normoxic conditions in 2i medium for 7 days with media was replacement every 24 hours. At day 7 the 2i induced neuroepithelial cells were then passaged using Accutase (Life Technologies) and re-plated at a 1:1 ratio in N2:B27 media without the small molecule inhibitors and supplemented with 10 µM Rock Inhibitor Y-27632 (Sigma Aldrich) until day 12. Cells were then passaged 2 more times following the same procedure described above at days 16 and 20. During neuronal induction, the formation of neural rosettes was evident from approximately day 10 until the differentiation of neural progenitors (NPC) around day 20. NPCs were maintained as mitotic progenitors in neuralization medium supplemented with 10 ng/ml bFGF, 5 µg/ml insulin, 1 mM l-glutamine, 100 µm non-essential amino acids, and 100 µM 2-mercaptoethanol. Dividing cells were routinely passaged to a 1:1 ratio to expand the NPC population as necessary. Moreover, NPC vials were frozen in a controlled freezing rate using Mr. Frosty containers in 10% DMSO in NPC media (Thermo Fisher). NPC cryovials were thaw by incubation for 3 minutes at 37°C and then transferring the cell suspension to a 15 ml tube containing 3 ml of NPC media. Subsequently, the tubes were centrifuged (1250RPM for 2 minutes) and the cells pellet was re-suspended with 3 ml of NPC + 10 µM Rock inhibitor media and plated as

above. NPC were terminally plated on 5 µg/ml poly-D-lysine and 2 µg/cm^2 laminin (Thermo Fisher) in coated Nunc treated multidishes, in B27 medium supplemented with 200 µM L-ascorbic acid and 10 µM DAPT (Calbiochem) for 7 days (day 27) to block NOTCH signaling. Subsequently, neurons were grown in B27 medium with 200 µM L-ascorbic acid until day 30 when they were used for experimentation.

Treatments

Acute pharmacological treatment (15 or 30 minutes) of cells were performed in ACSF solution (125 mM NaCl, 2.5 mM KCl, 26.2 mM NaHCO$_3$, 20 mM glucose, 5 mM Hepes, 2.5 mM CaCl$_2$ and 1.25 mM MgCl$_2$). Prolonged pharmacological treatment (24 hour) were performed in Neurobasal medium supplemented with B27 and 200 µM L-ascorbic acid.

qRT-PCR

Total RNA harvested and lysed with Trizol reagent (Life technologies) and isolated by centrifugation with 100% Chloroform, following by 100% isopropanol and lastly by 75% ethanol. The RNA was purified by precipitation with 100% ethanol and Sodium acetate (Life technologies) and quantify with the NanoDrop 1000 Spectrophotometer (Thermo scientific). Residual genomic DNA was removed by addition of TURBO DNA-free (Life technologies) and incubation at 37°C for 30 minutes. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA from each extraction using random hexamer primers and SuperScript III (Life Technologies) following the manufacturer's recommendations. qPCR was performed with HOT FIREPol EvaGreen qPCR Mix Plus ROX (Solis Biodyne) carried out according to the manufacturer's instructions in a total volume of 20 µl, containing 1:5 diluted cDNA,

qPCR mix and primers at to a final concentration of 0.3 µM. PCR reaction conditions: 95°C for 15 minutes for the initial denaturation followed by 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds during 33 cycles. The melting curve analyses was preformed from 60°C to 95°C with readings every 1°C. The 2^$\Delta\Delta$CT comparative method for relative quantification was used to quantify the genes expression. The data CT values were normalized to GAPDH, RPL27 and SDHA housekeeping genes.

Immunocytochemistry

Treated neurons were fixed with 4% formaldehyde plus 4% sucrose in PBS. Fixed neurons were permeabilized in 0.1% Triton-X-100 in PBS for 15 minutes and blocked in 4% normal goat serum in PBS for 1 hour at room temperature. Primary antibodies were added to the block solution in an antibody dependent concentration and incubated overnight at 4°C. Immunoreactivity was achieved by incubating the cells with 1:500 concentration of Alexa Fluor 594 conjugated anti-mouse IgG, Alexa Fluor 594 conjugated anti-goat IgG and Alexa Fluor 488 conjugated anti-rabbit IgG in block buffer. For nuclei staining a 1:2000 concentration of DAPI (Thermo Fisher) was used.

Imaging of immunofluorescence by high content image screening

NPCs were plated at a density of 1×10^4 cells/well on poly-D-lysine and laminin-coated optical-bottom 96 well plates with polymer base (ThermoScientific). Image acquisition was performed with a 20X objective for the genes OCT4, NANOG, SOX11, ZNF521, PAX6, NESTIN, FOXG1, TBR1, TBR2 and BRN1 by using CellInsight CX5 High Content Screen Platform (Thermo Fisher). The bioapplication Cell Health Profiling from the iDev software package (Thermo Fisher) using the nuclear staining

to assessed the viable cells. Intensity, shape and size features were used to determine positively labelled cells.

For neurite outgrowth assays, treated hiPSC-neurons were imaged using an Opera Phenix High Content screening platform (Perkin Elmer): images were acquired using a 20 x (NA 0.4) objective. The Harmony High Content Imaging and Analysis Software was used to determine the average neurite length and average branch number based on the MAP2 staining. For each hiPSC line, 3 biological replicates with 3 technical replicates per condition were imaged and analyzed: 15 randomly selected fields from each technical replicate was examined. Data from each technical replicate was averaged and used as a single data point and compared with each biological replicate and each hiPSC line. The means of percentages of positive cells and the average neurite length and branch number were compared by an ANOVA. Confocal images of hiPSC-neurons stained for MAP2 and CB1R were acquired using a Lecia SP5 confocal microscope using a 63x oil objective (NA 1.4) as a z-series. Post image acquisition, images were z-projected using ImageJ (rsb.info.nih.gov/ij/).

Western blotting

hiPSC-neurons cells were lysed in 20 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton-X-100, 5 mM EDTA pH 8 containing a cocktail of protease and phosphatase inhibitors. Detergent soluble lysate were resolved by SDS-PAGE, then immunoblotted with primary antibodies overnight at 4°C, followed by incubation with anti-mouse Alexa Fluor 680 and anti-rabbit Alexa Fluor 790 IgG (H+L) secondary antibodies for 1 hour at room temperature. Membranes were scanned with Odyssey imaging system (LI-COR). Intensities of bands were quantified by densitometry using Image J (rsb.info.nih.gov/ij/). Full length blots are shown in **Supplemental Figures 1-3.**

Statistical analysis

All statistical analysis was performed in GraphPad. Differences in $2^\wedge \Delta \Delta$ CT, relative expression, cell number and neurite parameters were identified by Student's unpaired t-tests, or for comparisons between multiple conditions the main effects and simple effects were probed by one-way-ANOVAs or two-way-ANOVAs with Tukey or Bonferroni correction for multiple comparisons. Differences were considered significant if *P* was lower than 0.05 (*P*<0.05). Error bars represent standard errors of the mean.

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Author contributions

C.S., L.D. and D.P.S. carried out experiments and data analysis; N.J.B., J.P. and S.B. provided reagents, assistance in experimental design and editing of manuscript; C.S., L.D. and D.P.S. wrote the manuscript; D.P.S. designed the project.

Competing Interests

Authors declare no conflict of interest

Figure Legends

Figure 1: Generation of glutamatergic cortical neurons from hIPSCs. (A) Schematic of hiPSC differentiation protocol used. **(B)** Representative images of hiPSCs, NPCs and neurons immunostained for markers of specific cell linages. **(C)** Number of DAPI positive cells positive for markers of hiPSC, NPC and neuronal cell

fate; error bars represent standard deviations (SD). **(D-G)** Expression profile of cell fate markers determined between day 7 and 50 following induction of neural differentiation; error bars represent SD.

Figure 2: Expression of cannabinoid receptors in hiPSC-neurons. (A) Expression of CB1R in NPCs and neurons in three independent hiPSC lines. In all three lines, CB1R expression increases as cells differentiate into neurons: ($F_{(2,12)}=6.49$, $p < 0.05$, Bonferroni Post Hoc, ***, $p < 0.001$, two-way ANOVA; n = 3 independent cultures for each line). **(B)** Comparison of the expression of CB1R, CB2R, GPR55 and TRPV1 receptors in day 30 hiPSC-neurons. Whereas CB1R is highly expressed, no expression of CB2R, GPR55 or TRPV1 receptors was detected in three hiPSC-lines: ($F_{(6,12)}=1.896$, $p < 0.05$, Bonferroni Post Hoc, ***, $p < 0.001$, two-way ANOVA; n = 2 independent cultures for each line). **(C)** Western blot demonstrating expression of CB1R in day 30 hiPSC-neurons derived from 2 independent hiPSC-lines. A prominent band is observed at ~53 kDa, predicted molecular weight of CB1R. **(D)** Representative confocal images of day 30 hiPSC-neurons immunostained for MAP2 (morphological marker) and CB1R. Immunoreactive puncta for CB1R can be found within the cell soma (white arrow heads) and along MAP2-psoitive neurites. Insets are of high magnification zooms of dendrite highlighted by white box in main image. Scale bar = 5 μm.

Figure 3: Negative regulation of neurite outgrowth in hiPSC-neurons by 2AG. (A) Representative images of MAP2 stained day 30 hiPSC-neurons as imaged using the Opera Phenix High Content Imager. **(B)** Assessing of the number of DAPI-positive
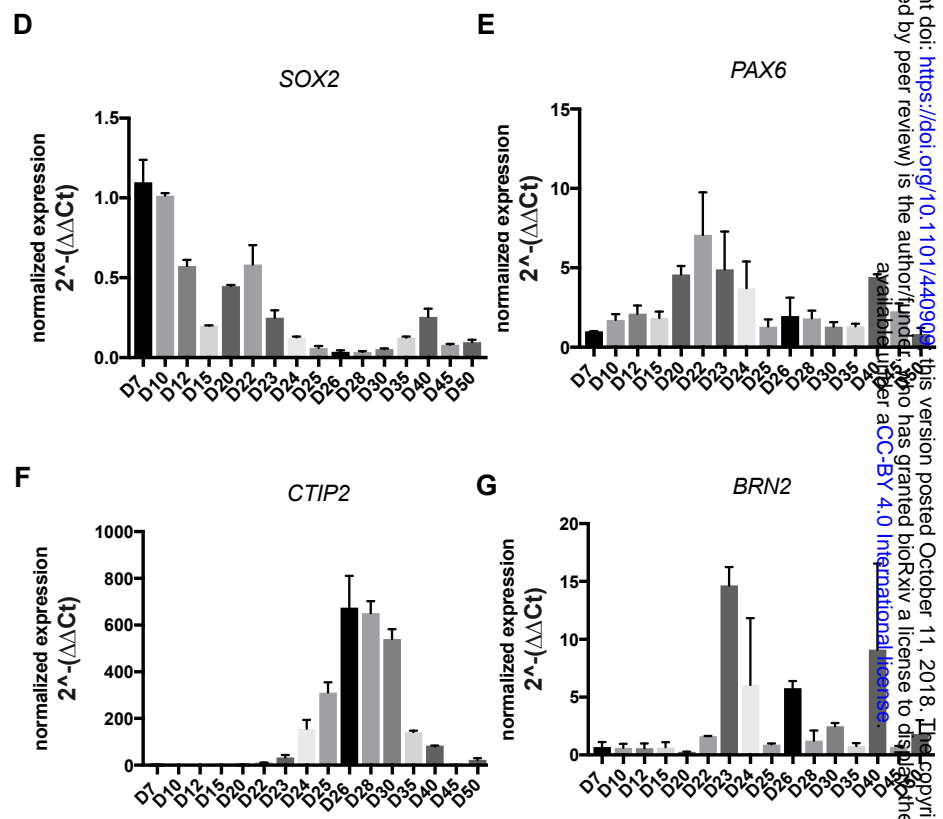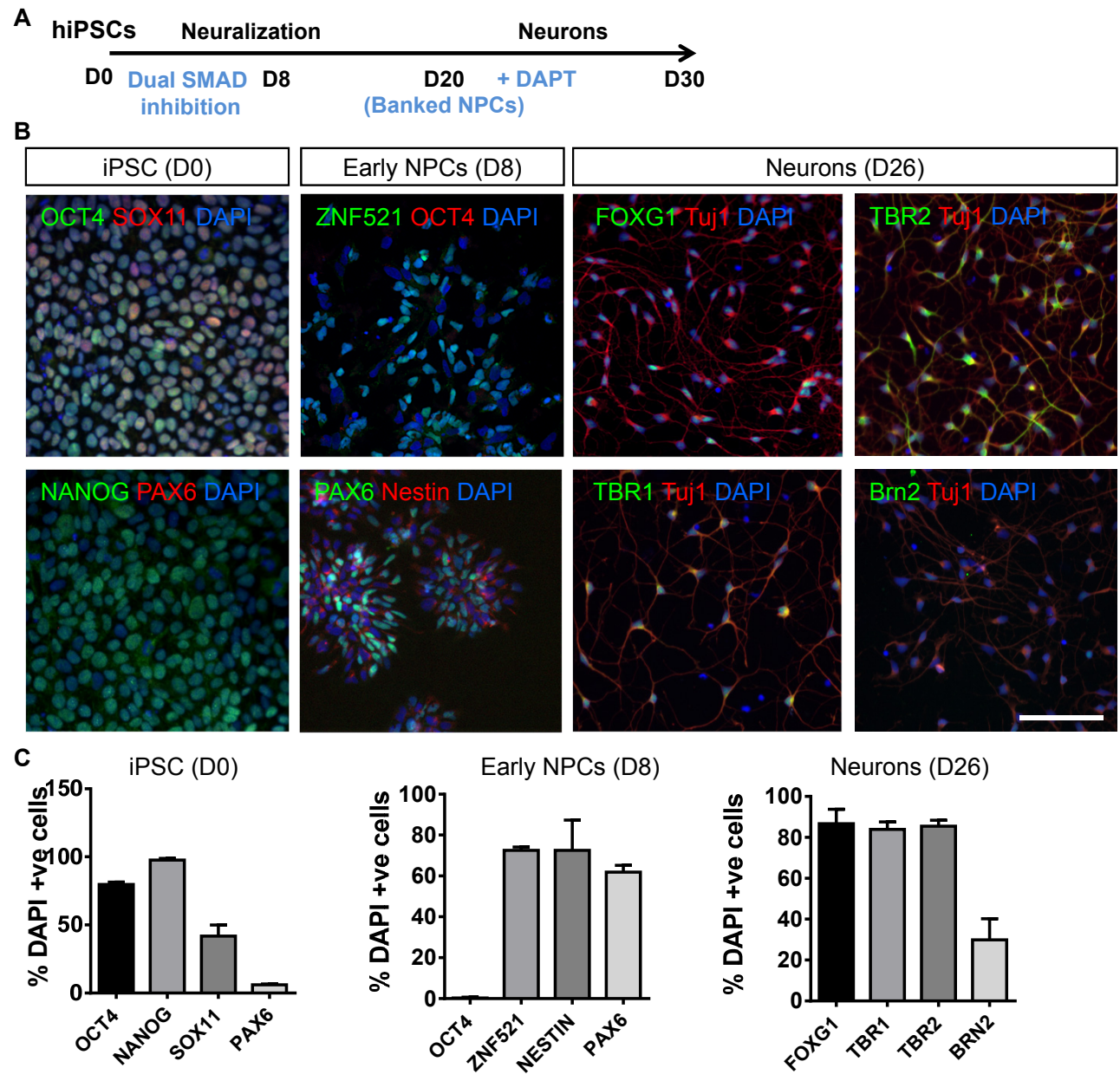
MAP2 neurons demonstrates no difference on overall neuronal number across conditions. Data are presented as Box plot showing min and max values (p = 0.931, Student t-test; n = 12; average data generated from 3 independent cultures for 3 independent hiPSC lines). **(C, D)** Assessment of average neurite number **(C)** and average branch number **(D)** revealed no difference between conditions (p = 0.713, and p = 0.78 respectively, Student t-test; n = 12; average data generated from 3 independent cultures for 3 independent hiPSC lines). **(E)** Treatment with 2AG for 24 hours significantly reduced total neurite length compared to vehicle control (***, p < 0.001, Student t-test; n = 12; average data generated from 3 independent cultures for 3 independent hiPSC lines). Scale bar = 50 µm.
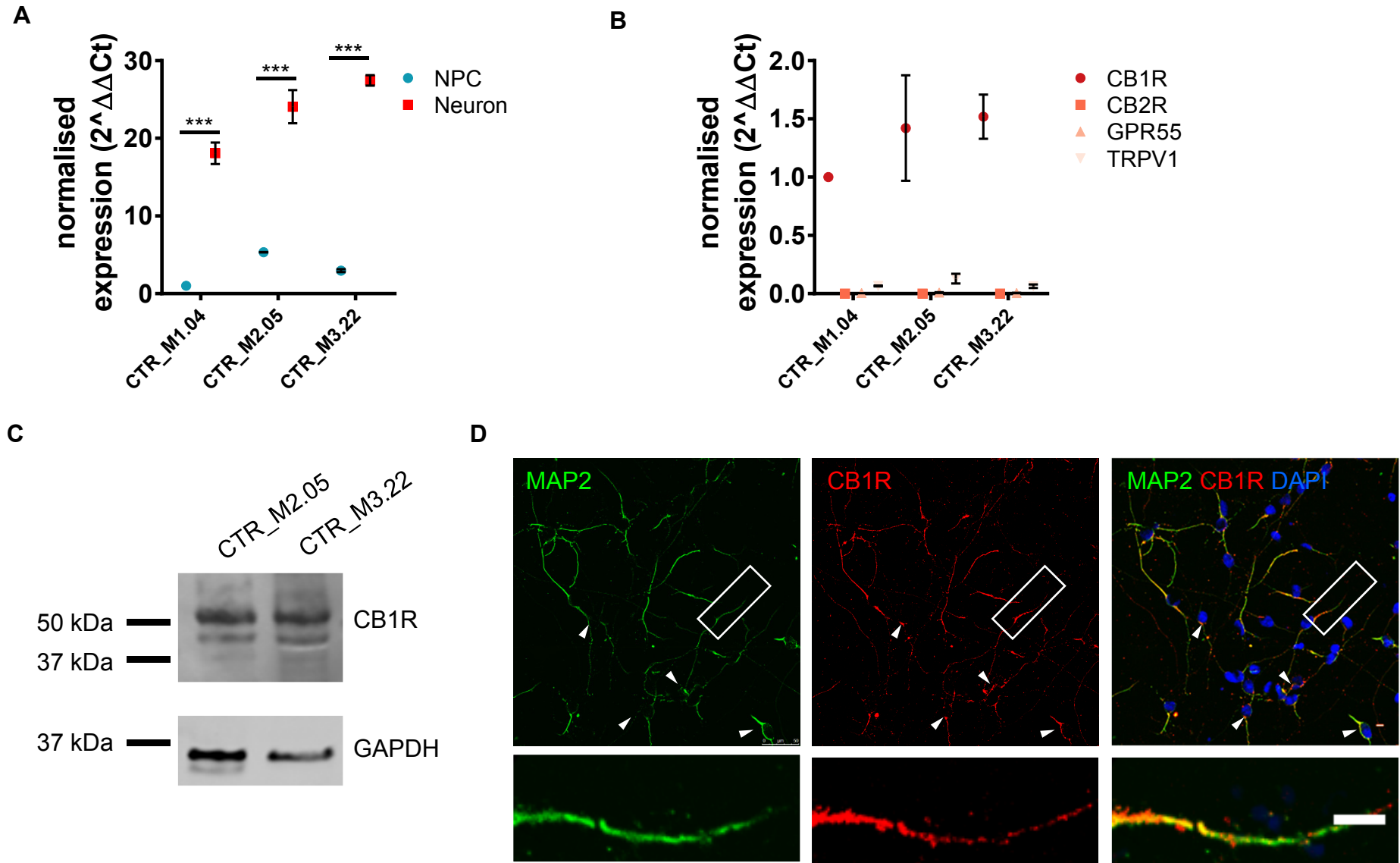
Figure 4: 2AG negative regulates ERK1/2 phosphorylation. (A) Western blot analysis of day 30 hiPSC-neurons treated with 2AG for 15 or 30 minutes compared to vehicle control conditions. Western blots probed for total and phosphor-ERK1/2, revealed a significant decrease in active (phosphorylated) ERK1/2 levels after 30 minutes of 2AG treatment ($F_{(2,6)}=6.593$, p < 0.05, Tukey Post Hoc, *, p < 0.05, one-way ANOVA; n = 3 per condition from 3 independent hiPSC-lines). **(B, C)** Cell lysates were also assessed for levels of active (phosphorylated) Akt and GSK3β; no difference was observed between conditions.

Figure 5: Δ[9]-THC negatively influences neurite outgrowth in hiPSC-neurons. (A) Representative images of MAP2 stained day 30 hiPSC-neurons following 24 hour treatment with vehicle or Δ[9]-THC, as imaged using the Opera Phenix High Content Imager. **(B)** Assessing of the number of DAPI-positive MAP2 neurons demonstrates
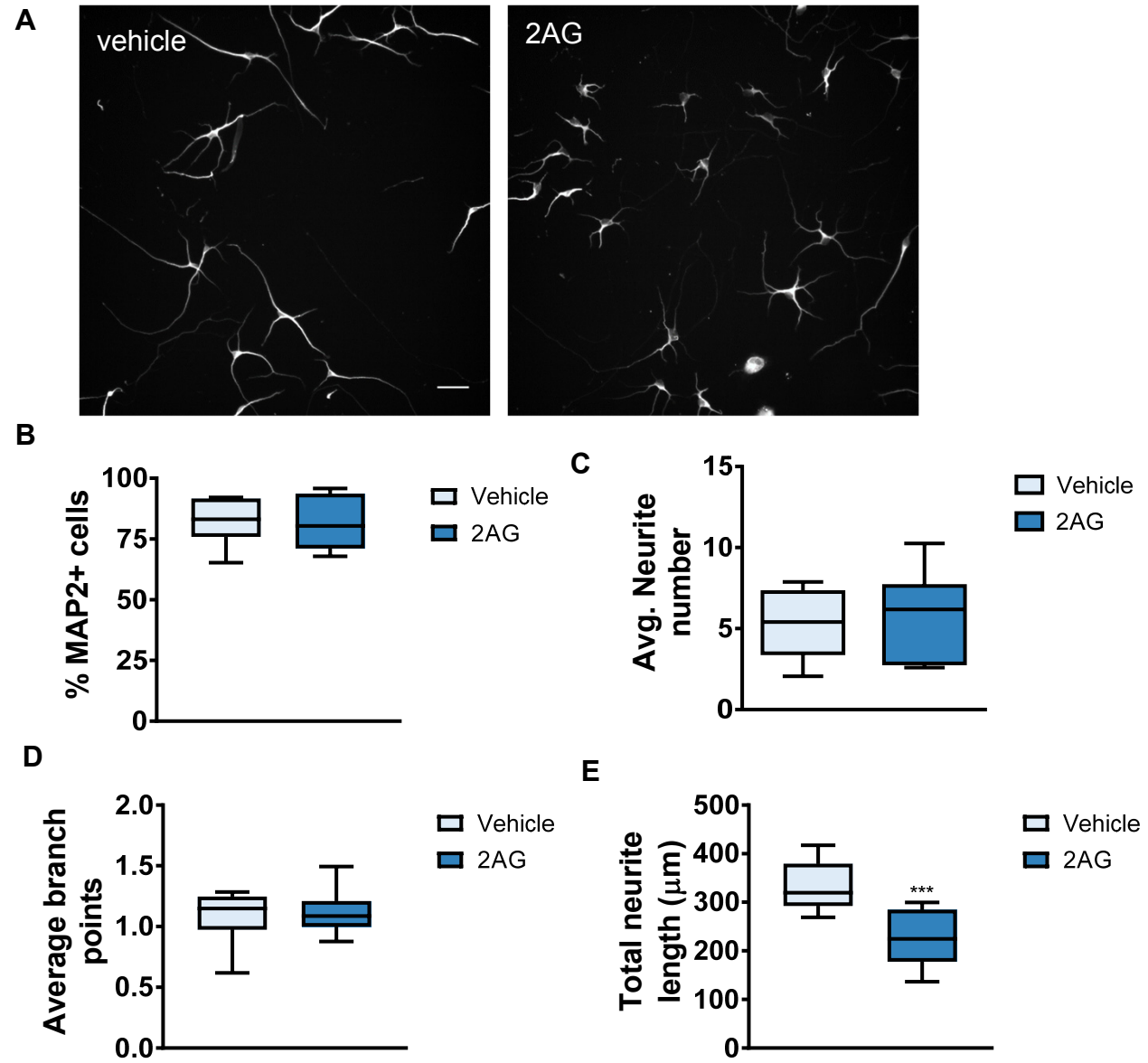
no difference on overall neuronal number across conditions. Data are presented as Box plot showing min and max values (p = 0.647, Student t-test; n = 12; average data generated from 3 independent cultures for 3 independent hiPSC lines). **(C, D)** Assessment of average neurite number **(C)** and average branch number **(D)** revealed no difference between conditions (p = 0.426, and p = 0.859 respectively, Student t-test; n = 12; average data generated from 3 independent cultures for 3 independent hiPSC lines). **(E)** Treatment with Δ^9-THC for 24 hours significantly reduced total neurite length compared to vehicle control (***, p < 0.001, Student t-test; n = 12; average data generated from 3 independent cultures for 3 independent hiPSC lines). Scale bar = 50 µm.

Figure 6: ERK1/2 and Akt phosphorylation is reduced following acute Δ^9-THC exposure. (A) Western blot analysis of day 30 hiPSC-neurons treated with Δ^9-THC for 15 or 30 minutes compared to vehicle control conditions. Western blots probed for total and phosphor-ERK1/2, revealed a significant decrease in active (phosphorylated) ERK1/2 levels after 30 minutes of 2AG treatment (F(2,6)=8.299, p < 0.05, Tukey Post Hoc, *, p < 0.05, one-way ANOVA; n = 3 per condition from 3 independent hiPSC-lines). **(B)** Assessment of phosphor Akt levels also revealed a reduction in active levels fo Akt following treatment with Δ^9-THC (F(2,6)=9.557, p < 0.05, Tukey Post Hoc, *, p < 0.05, one-way ANOVA; n = 3 per condition from 3 independent hiPSC-lines). **(C)** Cell lysates were also assessed for levels of active (phosphorylated) GSK3β; no difference was observed between conditions.
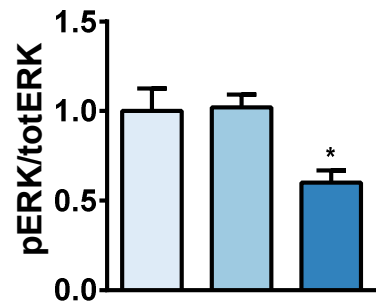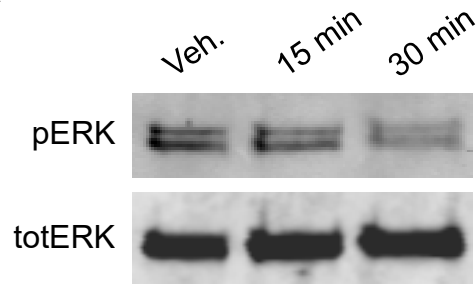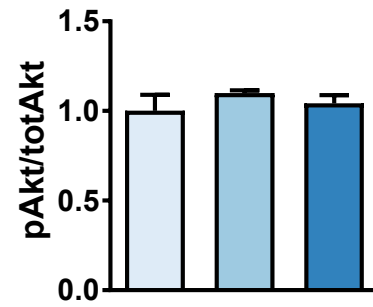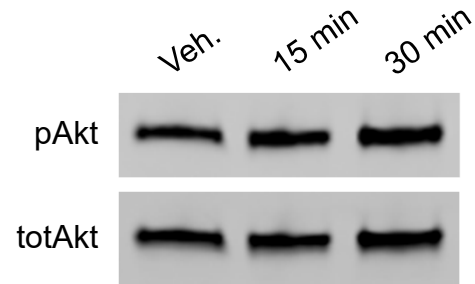
A hiPSCs → Neuralization → Neurons

D0 — Dual SMAD inhibition — D8 — D20 +DAPT (Banked NPCs) — D30

B

iPSC (D0): OCT4 SOX11 DAPI; NANOG PAX6 DAPI

Early NPCs (D8): ZNF521 OCT4 DAPI; PAX6 Nestin DAPI

Neurons (D26): FOXG1 Tuj1 DAPI; TBR2 Tuj1 DAPI; TBR1 Tuj1 DAPI; Brn2 Tuj1 DAPI

C iPSC (D0); Early NPCs (D8); Neurons (D26)

D SOX2

E PAX6

F CTIP2

G BRN2

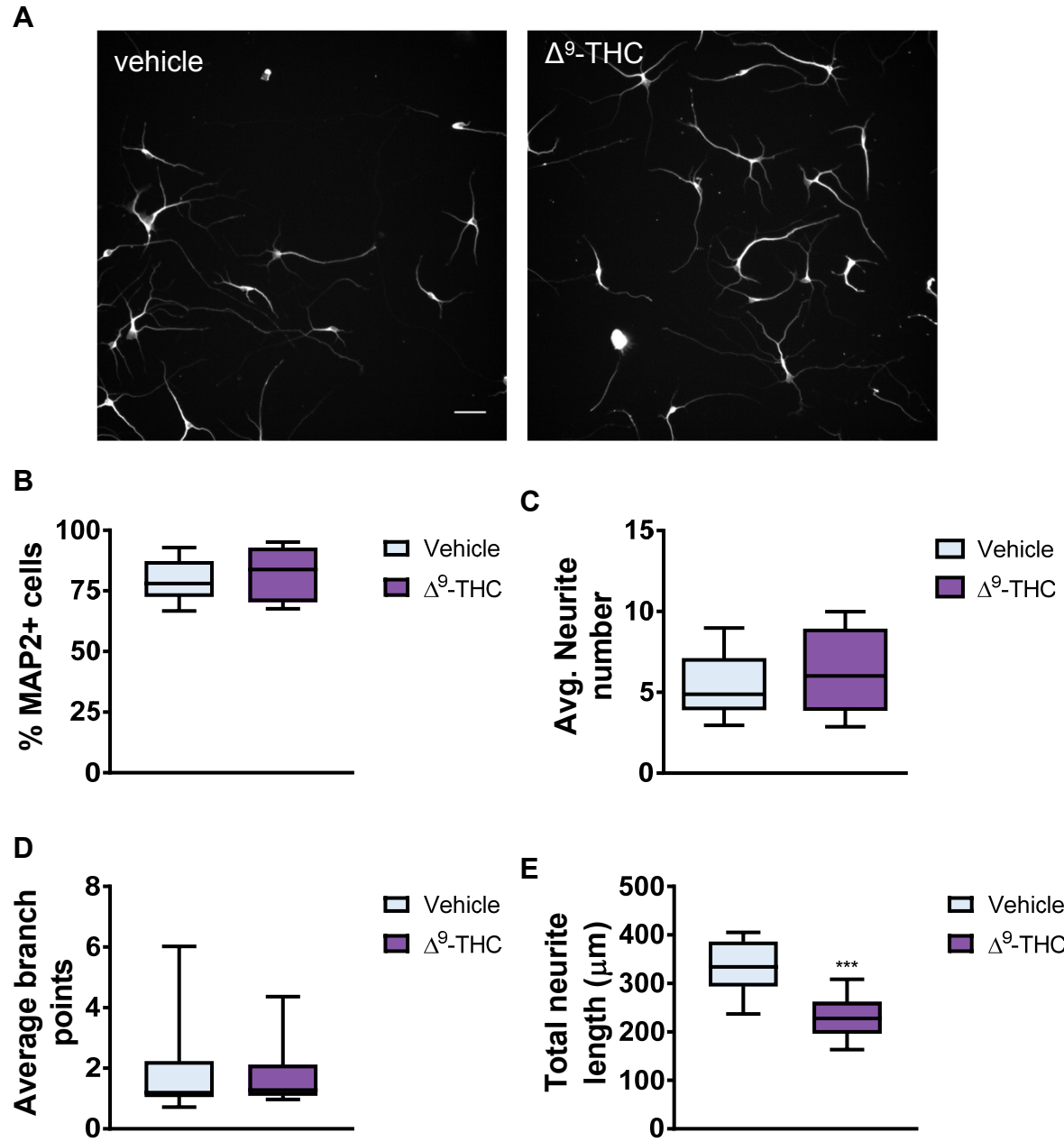Shum et al. Figure

Shum et al. Figure 2

Shum et al. Figure 3

Shum et al. Figure 4

A

vehicle

Δ⁹-THC

B

% MAP2+ cells

Vehicle

Δ⁹-THC

C

Avg. Neurite number

Vehicle

Δ⁹-THC

D

Average branch points

Vehicle

Δ⁹-THC

E

Total neurite length (μm)

Vehicle

Δ⁹-THC

***
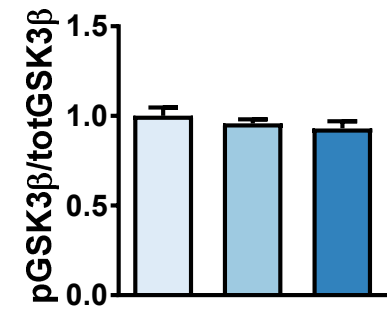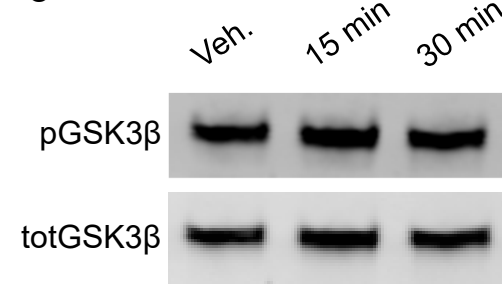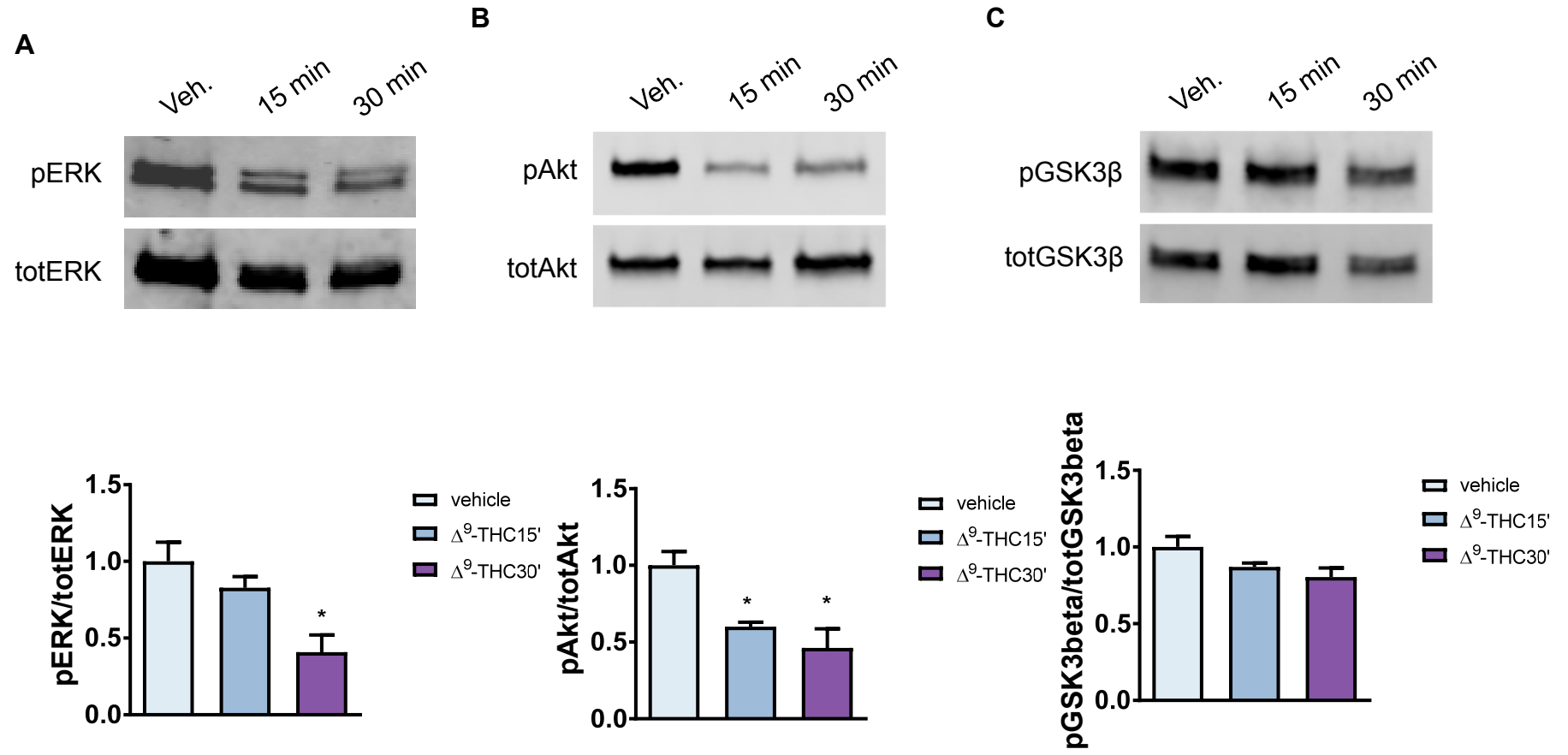
Shum et al. Figure 5

Shum et al. Figure 6

Table 1. List of antibodies used for immunohistochemistry

Antibody	Type	Host	Dilution	Supplier	Catalog Number
NESTIN	Monoclonal	Mouse	1:500	R&D Systems	MAB1259
OCT4	Monoclonal	Mouse	1:200	Santa cruz	Sc5279
PAX6	Polyclonal	Rabbit	1:200	Proteintech grPooup	12323-1-AP
SOX11	Polyclonal	Rabbit	1:50	Santa cruz	Sc20096
NANOG	Polyclonal	Goat	5ug/ml	R&D Systems	AF1997
ZNF521	Polyclonal	Rabbit	1:100	Atlas Antibodies	HPA023056
FOXG1	Polyclonal	Rabbit	1:500	Abcam	Ab18259
TBR1	Polyclonal	Rabbit	1:500	Abcam	Ab31940
TBR2	Polyclonal	Rabbit	1ug/ml	Abcam	Ab23345
BRN2	Polyclonal	Rabbit	1:500	Santa cruz	Sc28594
MAP2	Polyclonal	Chicken	1:1000	Abcam	Ab92434
CB1R	Polyclonal	Rabbit	1:50	Abcam	ab23703
Erk 1/2	Monoclonal	Mouse	1:2000	Cell signaling	L34F12
p-Erk	Polyclonal	Rabbit	1:1000	Cell signaling	T202 Y204
GSK3-beta	Monoclonal	Mouse	1:1000	Cell signaling	3D10
p- GSK3-beta	Polyclonal	Rabbit	1:1000	Cell signaling	D85E12
Akt	Monoclonal	Mouse	1:2000	Cell signaling	40D4
p-Akt	Polyclonal	Rabbit	1:1000	Cell signaling	5473