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Regulation of neural progenitor cell fate by anandamide

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ABSTRACT

Exogenous application of neural progenitor cells (NPCs) has successful implications in treating brain disorders, and research is beginning to identify ways to mimic this exogenous application by activating endogenous stem cell compartments. The recent discovery of a functional endocannabinoid system in murine NPCs (mNPCs) represents one potential therapeutic means to influence endogenous stem cell compartments. High levels of the endogenous cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) persist during CNS inflammation and infection. The goal of this study was to assess the influence of AEA on mNPCs to identify how the endocannabinoid system influences mNPCs in vitro, a potential model to investigate effects of endocannabinoids on endogenous stem cell compartments. Our results show that AEA affects mNPC cell fate determination. Initial glial differentiation was observed, followed by induction of neuronal differentiation with AEA treatment. Cell survival and apoptosis was not affected by AEA. These effects were coupled by an increased phosphorylation of cAMP-responsive element (CRE) binding protein (CREB).

1. Introduction

The endocannabinoid system has been shown to modulate inflammatory and immune responses [1], inhibit pain [2], and reduce neuronal damage in models of excitotoxicity [3], ischemia [4], and traumatic brain injury [5]. The two primary endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [6,7], exert these effects through binding with the cannabinoid-1 and 2 receptors (CB-1R, CB-2R) [8,9]. The CB-1R is abundant in the CNS [8] while the CB-2R is present mostly on immune cells [9].

The therapeutic potential of neural progenitor cells (NPCs) has been well documented. For example, exogenous application of NPCs in an animal model of Multiple Sclerosis completely ameliorates the condition [10]. These successes rely on the exogenous administration of healthy NPCs, a problematic approach as there are difficulties including immune rejection and significant problems maintaining a pure, ample supply of cells. An alternative therapeutic approach would be to induce the endogenous NPC compartments to proliferate and expand, mimicking the results of exogenous application. However, little is known about the factors that regulate NPC growth and differentiation in endogenous stem cell compartments.

High levels of AEA and 2-AG persist in the brain during periods of traumatic brain injury [5,11–14] accompanied by no changes in AEA binding capacity with the CB-1R [15]. The discovery of CB-1 receptors on mNPCs coupled with an observed upregulation of the receptor during traumatic stress [16] suggests the possibility that AEA and 2-AG may act as signaling molecules that influence NPC maturation, thus providing a potential mechanism that explains the apparent neuroprotective role of the endocannabinoid system. However, there is also evidence suggesting that AEA can be a neurotoxic agent in the CNS [17], demanding further studies into cannabinoid signaling. Others have begun to explore the developmental signaling roles and influence of the endocannabinoid system on NPCs in healthy organisms using selective CB-1 and CB-2 agonists [18–21], however, the influence of AEA – a non-selective endogenous CB-1 agonist – is not well studied. The goal of this study is to mimic the traumatic environment with AEA to investigate the therapeutic potential of endocannabinoid signaling on mNPCs in vitro.

2. Materials and methods

2.1. Isolation of murine neural progenitor cells (mNPCs)

Dr. Jeffrey Spees at the University of Vermont College of Medicine Stem Cell Core Facility isolated mNPC's from post-natal day four C57/BL6 mouse brains. Brain tissue was mechanically disrupted and enzymatically dissociated with NeuroCult Enzymatic Dissociation Kit (StemCell Technologies Inc., Canada). Progenitor

Abbreviations: NPC, neural progenitor cell; AEA, anandamide; CNS, central nervous system; CB-1R, cannabinoid-1 receptor; CB-2R, cannabinoid-2 receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; BrdU, bromodeoxyuridine; CREB, cAMP-responsive element binding.

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cells and neurospheres were maintained in Neurobasal-A media (Invitrogen, Carlsbad, CA) with B27, 10 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast growth factor (FGF), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells (passages 2, 4, and 5) were cultured on poly D-lysine and laminin coated plates. When the cells achieved appropriate confluency, they were rinsed and maintained in similar media except without growth factors unless noted.

2.2. Reagents

Anandamide (AEA) was purchased from Sigma (St. Louis, Missouri). It was dissolved in molecular biology grade ethanol. All experiments contained an ethanol-only vehicle control, equivalent to the largest volume of ethanol present in the AEA treatments.

2.3. Cell viability and apoptosis

Cell apoptosis and survival was assessed using three methods: terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), calcein acetomethoxy ester (calcein AM), and UV–Annexin. Cells were treated with AEA at 0, .5, 5, and 10 μM AEA concentrations in the absence of growth factors.

2.3.1. Calcein AM assay

Calcein AM (2 μg/mL) was added to each well and incubated for 30 minutes. The fluorescence emitted from live cells was read on a Fluostar Galaxy machine (BMG Lab Technologies, Offenberg, Germany). Data is expressed as a percentage of live cells.

2.3.2. TUNEL

TUNEL detected apoptotic cells after 3 days of AEA treatment. Data is expressed as the percentage of non-apoptotic (TUNEL negative) cells. Two TUNEL methods were used. First, cells were manually counted after biotinylated dUTP was incorporated into late stage fragmented DNA using terminal deoxynucleotidyl transferase (Promega). Secondly, cells were incubated with streptavidin fluorescein isothiocyanate (Jackson Immunoresearch), and analyzed using flow cytometry.

2.3.3. UV–Annexin

Annexin stain (Molecular Probes) along with a live/dead dye (Invitrogen, Carlsbad, CA) – which enters compromised cell membranes and reacts with free amines in the cell interior – was used to assess early apoptosis, viable cells, and dead cells. Results were obtained using a flow cytometer.

2.4. Cell proliferation

Bromodeoxyuridine (BrdU), an S phase cell cycle marker, was used to assess cellular proliferation. AEA treatment was added for 1 or 2 days. Cells were pulsed with BrdU overnight, the analyzed on a flow cytometer. Secondly, Ki67 was used. A monoclonal antibody to Ki67 (BD Pharmingen) was applied, and cells were analyzed using flow cytometry. Manufacturer's recommended protocols were followed.

2.5. PCR and RT-qPCR

RNA was isolated with the RNeasy Kit (Qiagen). cDNA was prepared using Superscript III (Invitrogen, Carlsbad, CA). All cDNA samples were treated with RNase H to remove residual RNA. PCR was used to detect CB-1R expression while RT-qPCR was used to assess the expression levels of differentiation markers. For the CB-1R PCR reactions were performed under the following conditions: (1) 92 °C for 2 min, (2) 35 cycles of 92 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and (3) 72 °C for 2 min. PCR products were run on a 1.5% agarose gel and stained with ethidium bromide. The CB-1R primer set used was 5′ CCACTTCTTTTCCGCCTCCG 3′ with 5′ CTCCTGCCGTCATCTTTTCTTG 3′, and the CB-2R primer set was 5′ GGCTGACAAATGACACCCAGTC 3′ with 5′ CAGGAAGATA GCGTTGGAGTCG 3′. A NCBI BLAST search showed that the primers were specific to the CB-1R and CB-2R. Mouse spinal tissue, tissue known to express high levels of the CB-1R, was used as a control.

Taqman Master Mix with Taqman primers (Applied Biosystems) and probes were used for all RT-qPCR reactions. An ABI 7500 Fast Real time PCR System was used for amplification under the following settings: (1) 95 °C for 10 min (2) 95 °C for 15 s and 60 °C for 60 s for a total of 50 cycles. The delta CT method was used to analyze results with actin as the endogenous control. The following lineage specific markers were chosen: GFAP and GLAST for astrocytes, NG2, PDGFRα and MBP for oligodendrocytes, and enolase and β-tubulin III as neuronal markers. Experiments were replicated for $N = 3$. Differentiation analysis was done for a four-day treatment and a 7-day treatment.

2.6. Western blot

A western blot of the cannabinoid receptor was performed with an anti-cannabinoid receptor 1 antibody (Sigma) according to manufacturer's protocol. The primary antibody was used at 1:250.

2.7. Immunohistochemistry

Cells were stained for differentiation markers and the presence of the CB-1R. Cells were plated on coated (as described above) coverslips in 24-well plates. After a 1 week treatment period, being retreated at 4 days, coverslips were fixed in Zamboni's fixative (4% paraformaldehyde; 15% picric acid). Cells were incubated in block (10% horse serum, .3% Triton) in the following antibodies at 4 °C: β-tubulin III (Sigma; 1:200), GFAP (DAKO; 1:250), Nestin (Novus Biologicals; 1:250), PDGFRα (Santa Cruz; 1:50), CB-1R (Cayman Chemical Company; 1:10, a gift from Dr. Yong-Ho Lee). Following 2 washes, anti-chicken Cy2 and anti-rabbit Cy3 (both Jackson Immunoresearch) were applied as secondary antibodies for their respective primaries for 1 h at 4 °C.

2.8. CREB phosphorylation

After achieving 70–80% confluence, cells were incubated at 37 °C for either 15 min, 30 min, or 3 h with media containing AEA both with and without EGF/FGF. Cells were lysed in 100 μL of RIPA buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1% SDS) containing 0.3 mg/ml phenylmethylsulfonyl fluoride and protease inhibitor mix (16 μg/ml benzamidine, 2 μg/ml leupeptin, 50 μg/ml lima bean trypsin inhibitor, 2 μg/ml pepstatin A). Cell lysis was confirmed by visual inspection. Forskolin (5 μM) was dissolved in DMSO and used as a positive control.

Cells lysates were separated by SDS–PAGE using a 4–12%, 1.5 mm gel (Invitrogen, Carlsbad, CA). The proteins were transferred to a PVDF membrane. The membranes were blocked for 1 h in 5% nonfat dry milk in TBS with 0.1% Tween-20 (TBST) on a rocker. Pan CREB antibody was diluted 1:1000 with 1.5% BSA in TBST. PhosphoCREB antibody was used at 1:1000 with 1.5% BSA in TBST. After a 2 h incubation the membranes were rinsed in TBST. A horseradish peroxidase labeled secondary antibody was diluted 1:5000 in 5% nonfat dry milk in TBST. The blot was developed using enhanced chemiluminescence following the manufacturer's protocol. The relative intensity of the bands was analyzed by computer software.

2.9. Statistical analysis

A one-way ANOVA was performed to determine statistical significance, followed by Tukey's test to compare individual samples. Graphpad Prism 5 software was used for all analyses.

3. Results

3.1. mNPCs express the CB-1R, a key component of the endocannabinoid system

The cannabinoids AEA and 2-AG are known to act through two cannabinoid receptors, CB-1R and CB-2R. PCR was performed on cDNA isolated from mNPCs, grown in the presence of growth factors with and without 10 μM AEA. Our results show that mNPCs express the CB-1R, but not the CB-2R (Fig. 1E). The presence of the CB-1R was confirmed with a western blot (Fig. 1E) and confocal microscopy (Fig. 1C). Nestin, an NPC marker, confirms that the receptor is present on cells in an undifferentiated, pluripotent state.

3.2. Cell viability, apoptosis, and proliferation

AEA treatment appears to have no overall effect on cell viability. Results from the Calcein AM assay showed no statistically significant change in mNPC survival at AEA concentrations of 5 and 10 μM (Fig. 2A). To complement this, data from both the UV/Annexin and TUNEL assays – assays marking apoptotic cells – shows that AEA did not influence apoptosis on mNPCs (data not shown).

However, a statistically significant decrease in mNPC proliferation was observed at a 10 μM AEA treatment in the BrdU assay (Fig. 2B). This was confirmed with a KI67 stain (data not shown).

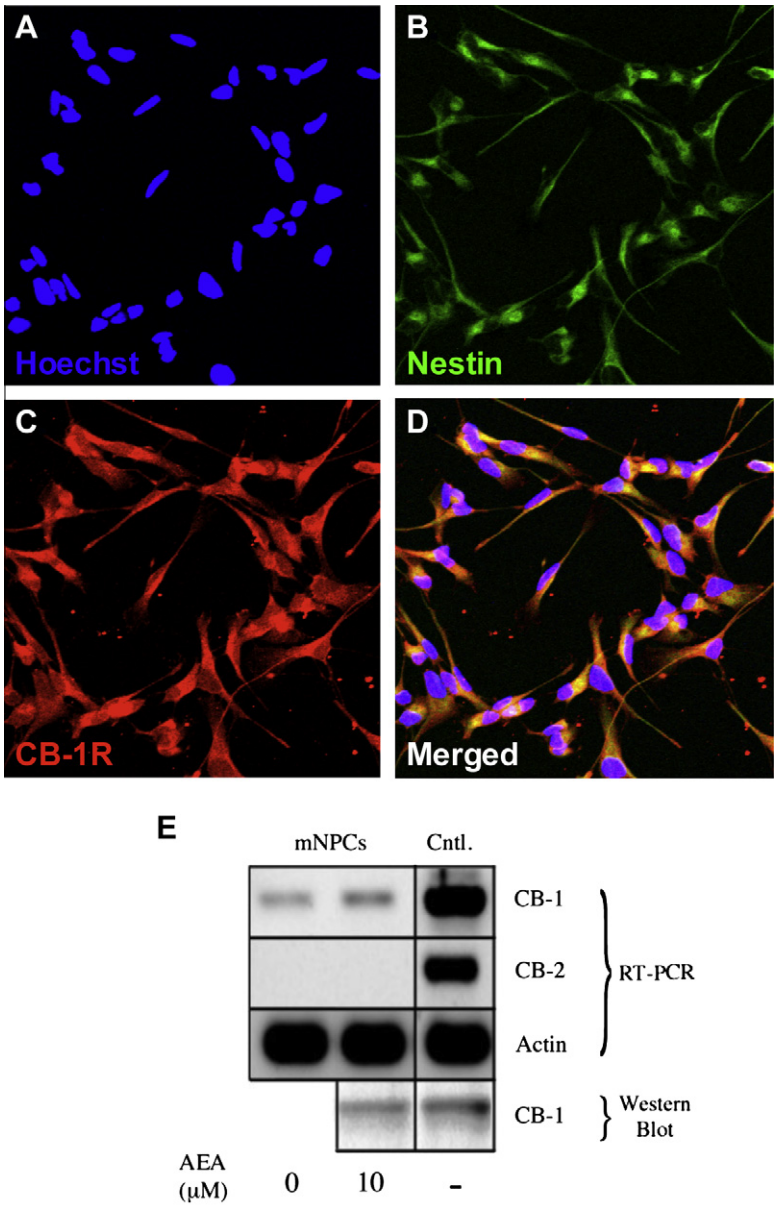

Fig. 1. Postnatal Neural Progenitor Cells Express the CB-1 Receptor. (A–D) Immunohistochemistry staining demonstrates that murine neural progenitor cells (identified as Nestin positive, (B)) express the CB-1R (C). Cell nuclei are stained with Hoechst (A). (E) RT-PCR confirms mRNA expression of the CB-1R but not the CB-2 receptor in cells grown in media treated and untreated with AEA. A western blot also confirms the presence of the CB-1 receptor. Mouse spinal tissue was used as a positive control for both the RT-PCR and western blot, and actin was used as a positive PCR control.

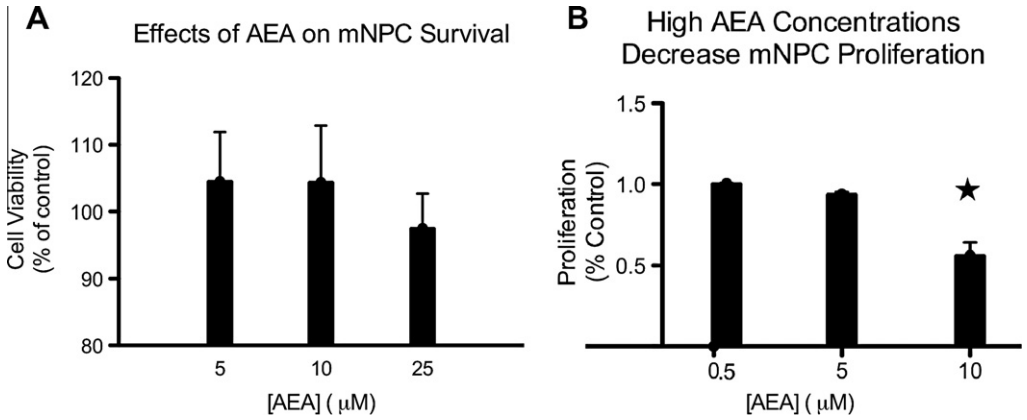

Fig. 2. Influence of AEA on mNPC survival and proliferation. mNPCs were allowed to grow either with or without AEA treatment. mNPC survival was assessed using a Calcein AM assay (A), and proliferation via a BrdU assay (B). There is a statistically significant ($p < .05$) decrease in survival at a 10 μM AEA treatment.

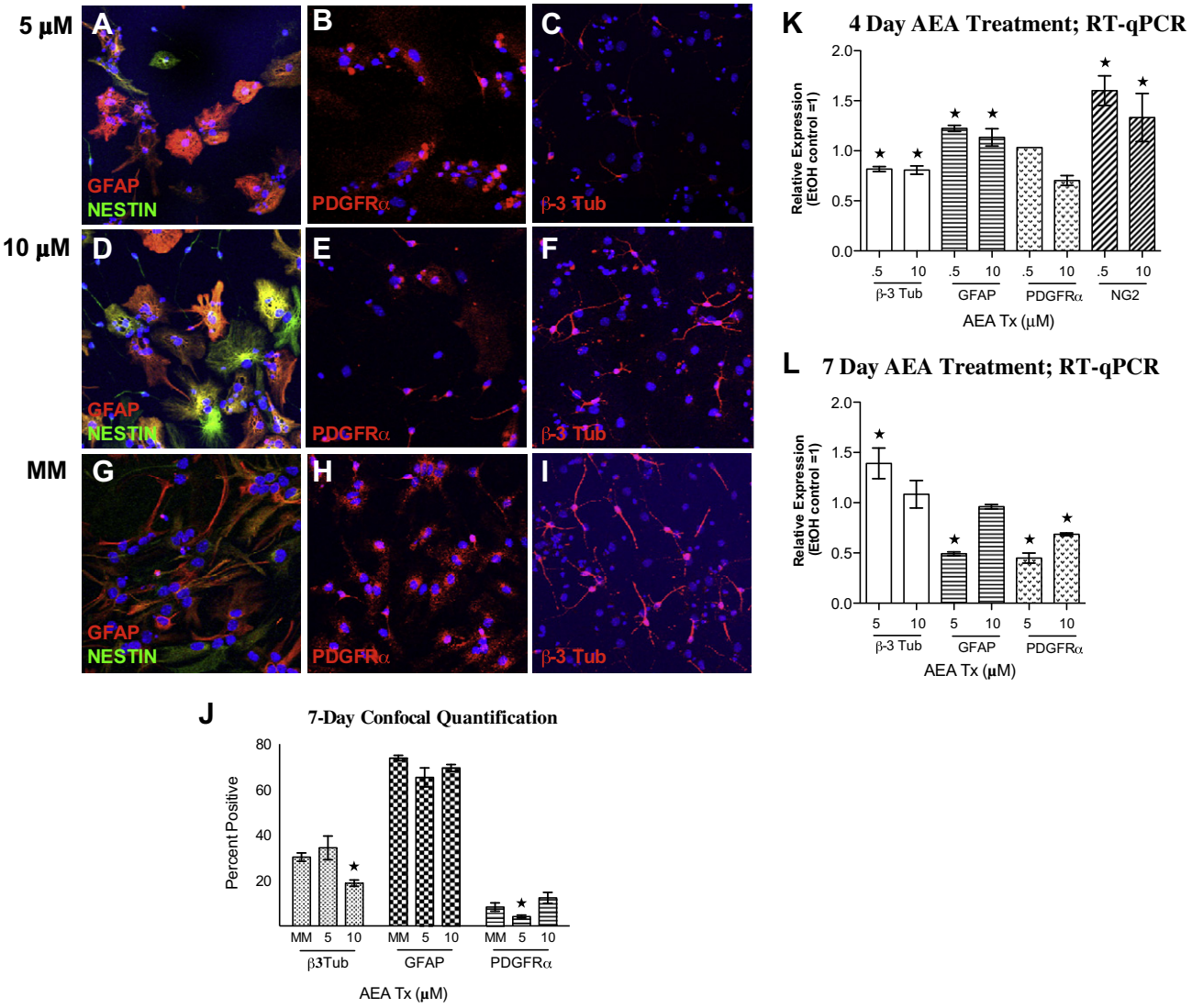

Fig. 3. AEA activates mNPC differentiation. mRNA was extracted from mNPCs treated and untreated with AEA to perform RT-qPCR with primers specific to the neuronal, astrocyte, and oligodendrocyte lineages. An acute, 4-day treatment promotes glial differentiation (K) while a more chronic 7-day treatment promotes neuronal differentiation (L). Induction into the neuronal lineage is confirmed at the protein level (A–J) via immunohistochemistry. * = statistically significant, $P<.05$.

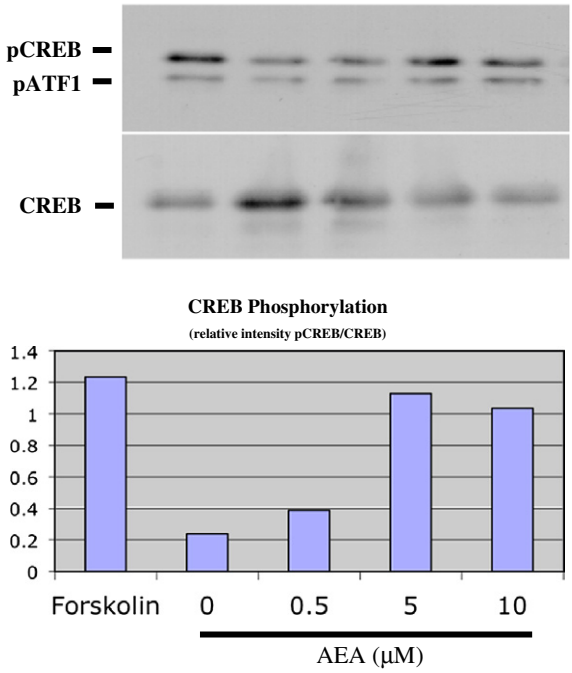

Fig. 4. AEA stimulates CREB phosphorylation. A western blot was performed on mNPCs treated with AEA, staining for both phosphorylated CREB (active protein) and non-phosphorylated CREB (inactive protein). The antibody is non-specific for pCREB and also binds pATF1. Our data demonstrates a statistically significant ($P < .05$) increase in the ratio of pCREB/CREB following AEA treatment.

3.3. AEA-induced glial differentiation evolves into a neuronal phenotype

RT-qPCR was performed with the astrocyte markers GFAP and GLAST, the oligodendrocyte precursor cell (OPC) markers NG2, MBP, and PDGFR-α, and the neuronal markers β-tubulin III and enolase. At a 4-day treatment, our results show a statistically significant increased expression of the early astrocyte marker GFAP and the early glial marker NG2, with a corresponding decrease in the neuronal marker β-tubulin III (Fig. 3K). No statistical change was found in mature lineage markers. When treated for 7 days, the differentiation pattern favored a neuronal phenotype as we demonstrate a statistically significant increase in β-tubulin III with a marked decrease in GFAP and PDGFR-α (Fig. 3L). These changes at the mRNA level are consistent with the protein expression of these differentiation markers (Fig. 3A–J) after differentiation in various AEA treatments for 7 days.

3.4. Mechanism of cannabinoid action

Cannabinoids are known to act through G-protein coupled receptors, suggesting that their effects may be modulated through either a G_i or G_s signaling pathway. To help elucidate which may be active, we performed a western blot against the cAMP-responsive element (CRE) binding protein (CREB) in its phosphorylated (active) and de-phosphorylated (inactive) state. The ratio of phosphorylated to de-phosphorylated CREB was calculated to show a statistically significant increase in the phosphorylation of CREB with AEA treatment; maximum phosphorylation was achieved at 5 μM (Fig. 4). The antibody also non-specifically binds to ATF-1, a CRE gene activator, further confirming an increased level of cAMP.

4. Discussion

Our major finding is that AEA application in vitro leads to no change in mNPC cell viability with a concurrent initial induction of glial differentiation via a cAMP-dependent pathway (Fig. 4). Between day 4 and day 7 of treatment, a significant switch from the glial lineage to the neuronal is observed (Fig. 3). At treatments of .5 and 5 μM AEA, no change in proliferation was observed while at 10 μM AEA, mNPC proliferation is decreased (Fig. 2B). In contrast, others have shown that the endocannabinoid system promotes mNPC proliferation [18,20]. However, these results do not necessarily conflict as they used specific CB-1R agonists/antagonists and various endocannabinoid-KO mice to look at CBR-dependent embryonic stem cell proliferation, while we used the non-specific agonist AEA [20,22], and studied its global effects on post-natal neural progenitor cells. We build off of their initial findings and show that AEA does not promote further mNPC proliferation post-natally, and instead AEA plays a role on cell lineage differentiation. As AEA possess non-specific binding, we hypothesize that these effects may be the result of receptor activation and cross-talk between a variety of pathways, a mechanism consistent with others [20,22,23]. Our data also showed that AEA treatment results in no change in cell viability or apoptosis despite the observation that proliferation is decreased at high concentration 10 μM.

AEA may provide neuroprotection during brain insult in two ways – first by inducing early mNPC differentiation into the glial lineage and secondly by increasing the available pool of neurons by inducing neuronal differentiation. Glial cells are thought to provide neuroprotective effects [24], suggestive that the endocannabinoid system is playing a neuroprotective role by inducing endogenous repair systems rather than inducing mNPC proliferation. Consistent with previous studies [19,25], our RT-qPCR analysis (Fig. 3) demonstrated an endocannabinoid upregulation of both the early glial markers GFAP and NG2 at 5 μM AEA treatment, a down-regulation of the early neuronal marker β-tubulin III, accompanied by no change in mature proliferation markers. This strategy may be done to target your body's limited energy at directly combating the damage, rather than at the costly process of cell division. However, we did observe a marked increase in the neuronal lineage marker β-tubulin III after 7 days of treatment. Whereas an acute effect of AEA may be to promote repair, a more chronic effect may be to induce NPCs to differentiate into neurons in order to replenish those that may be severely damaged during the toxic assault.

Our study focus is on the overall effect of AEA on mNPCs in order to help elucidate the function of AEA during neuronal stress. Our study confirmed the prescence of the CB-1R on mNPCs (Fig. 1) and demonstrated an increased ratio of pCREB/CREB in cells treated with AEA (Fig. 4). To our knowledge, this is the first study that demonstrates increased phosphorylation of CREB in mNPCs following AEA treatment. Thus, this protein may play a key role in the cellular changes following AEA treatment. This finding demands further investigation into the intracellular signaling cascades influenced by AEA.

In conclusion, this study demonstrates that acute AEA exposure induces mNPC glial differentiation with no change in cell viability, suggestive of a neuroprotective role of the endocannabinoid system on mNPC. A more chronic role may be to induce neuronal differentiation of NPCs. Our observation of increased pCREB expression demands further investigation into endocannabinoid signaling pathways involved in mNPC differentiation. Our study introduces many challenges in using the endocannabinoid signaling pathways to induce mNPC cell proliferation in vitro, however we provide evidence that cannabinoid activation of mNPCs offers insight into designing neuroprotective therapies targeting differentiation via a cAMP-dependent mechanism.

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