Characterisation of Differentiated NSC-34 Motor-Neuronal Cells as a Model of Oxidative Stress Induced Neurodegeneration and Cannabinoid Neuroprotection

A thesis submitted in partial fulfilment of the

HONOURS DEGREE of BACHELOR OF HEALTH AND MEDICAL SCIENCES

In

The Discipline of Pharmacology

Adelaide Medical School

The University of Adelaide

by Jonathan Harry James

November 2020

Abstract

Phytocannabinoids, bioactive products of the *Cannabis Sativa* plant, are increasingly being considered as potential treatments for Motor Neuron Disease (MND). One common *in vitro* model to study the mechanisms of MND, such as oxidative stress, is the motor-neuron like NSC-34 cell line differentiated with *all-trans* retinoic acid (atRA).

The aims of the present study were threefold: (1) To characterise the response of differentiated NSC-34 to; the three phytocannabinoids Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabichromene (CBC); Bediol® whole medicinal cannabis extract; and the synthetic CB_1 agonist arachidonyl-2′-chloroethylamide (ACEA) and antagonist AM 251. (2) To investigate the potential neuroprotective properties of THC, CBD, CBC and Bediol® against H_2O_2-induced toxicity, and (3) to investigate the morphological outcomes of CB_1 modulation during NSC-34 differentiation.

Differentiated NSC-34s displayed increased viability when treated with subtoxic concentrations of THC and Bediol® ($p<0.05$). Differentiation also resulted in increased viability of cells exposed to high concentrations of CBC (20μM, $p<0.01$) and ACEA (100μM, $p<0.0005$). Only Bediol® showed potential antioxidant effects, reducing toxicity induced by 250 and 350μM H_2O_2 ($p<0.05$). Both atRA and ACEA promoted NSC-34 differentiation ($p<0.0005$, $p<0.05$ respectively) as well as neurite length and number. This effect, for ACEA only, was negated by the addition of AM 251 ($p<0.01$).

Taken together, these results support the key role of atRA-induced differentiation of NSC-34 cells when studying cannabinoid neuroprotection and suggest the therapeutic antioxidant potential of whole medicinal cannabis extract. They also support the potential role of CB_1 activation in NSC-34 differentiation, a result previously undocumented in this cell line.

249 words

Introduction

The Epidemiology and Cellular Mechanisms of Motor Neuron Disease

Motor Neuron Disease (MND) is a fatal neurodegenerative disease characterised by the progressive functional decline of both upper and lower motor neurons.[1] Onset of symptoms generally occurs between the ages of 50 - 65 with a mean survival time of 3-5 years.[1-3] In Australia, MND carries an estimated prevalence of 8.7 per 100,000, significantly higher than 2.7 per 100,000 estimated throughout Europe and North America, although this may be due to global under reporting.[2, 4] The economic cost of MND in Australia is estimated to exceed $430 million annually, particularly due to associated morbidity and early mortality.[4]

MND cases can be classified into two distinct categories. The familial form of the disease (fMND) accounts for only 5-10% of MND cases diagnosed, with the remaining 90-95% of cases classified as sporadic (sMND). Mutations in over 30 genes have been associated with fMND, including those encoding the key antioxidant superoxide dismutase-1 (SOD1), TAR-DNA binding protein-43 (TDP-43), and *C9orf72*.[5, 6]. The aetiology of sMND, on the other hand, remains elusive. Several environmental exposures have been implicated as sMND risk factors including agricultural pesticides, herbicides and certain industrial chemicals.[7] Regular cyanobacterial blooms have also been linked to temporo-spatial clusters of sMND around rural waterways, such as Lake Wyangan near Griffith, NSW.[8]

Both forms of the disease are characterised by similar mechanisms on the cellular level. These include oxidative stress, neuroinflammation, mitochondrial dysfunction, dysregulation of autophagosomal flux, glutamate excitotoxicity, disruption of cytoskeletal structure and protein aggregation.[2] Many of these mechanisms are self-perpetuating though and much like the aetiology of sporadic cases, the true pathogenesis of MND remains a mystery.

To date, therapeutic approaches to MND have only achieved moderate success in symptom management, as opposed to the modification of disease progression [9]. The only currently approved treatment for MND is riluzole, which acts to reduce excitotoxicity through the inhibition of presynaptic glutamate release.[10] While riluzole has been demonstrated to increase mean survival time by 2-3 months under specific treatment criteria, its clinical use remains somewhat limited.[11] As such, there remains an urgent need to elucidate the pathogenesis of MND in order to develop effective therapeutics effective in slowing disease progression.

The Endocannabinoid System and Three Key Phytocannabinoids

Over recent years, the endocannabinoid system has emerged as a target of particular therapeutic potential in the treatment of many chronic diseases.[12] This system consists of the G-protein coupled endocannabinoid receptors, their endogenous ligands, and associated pathways for synthesis, transport and degradation.[13] Two main endocannabinoid receptors, CB_1 and CB_2, have been successfully cloned and studied to date, although evidence strongly suggests the existence of more.[14] CB_1 receptors are generally expressed presynaptically on neuronal cells, and are one of the most highly expressed excitatory receptors in the nervous system.[15, 16] CB_2 receptors, on the other hand, are generally expressed by glial and immune cells.[17] CB_1 receptors act to increase mitogen activated protein kinase (MAPK) signalling and inhibit adenylyl cyclase,. They play a key role in neurodevelopment, pain modulation, short and long term plasticity, and cellular response to insult and injury.[16,18] In neurodegeneration, endocannabinoid signalling is involved in cellular responses against neuroinflammation, oxidative stress and excitotoxicity.[19]

Several key endogenous CB_1 agonists have been identified to date, the main two being *N*-arachidonoylethanolamine (anandamide) and 2-arachidonyl glycerol (2-AG). Anandamide acts a partial agonist at CB_1 and CB_2 receptors, while 2AG instead acts as full agonist at both.[20, 21]

Uniquely, both compounds are continuously synthesised by presynaptic neurons and as such CB_1 receptors exhibit relatively high endogenous occupancy rates, although the relative expression of endocannabinoids varies strongly by location.[18]

Phytocannabinoids, bioactive products of the *Cannabis Sativa L.* plant, have long been used by humans for therapeutic benefit, with anecdotal evidence stretching back over 5000 years.[20,22] To date, over 120 unique compounds have been identified from the *Cannabis Sativa L*, including the many derivatives of cannabigerol, terpenes, flavonoids and other polyphenols, many of which interact with the endocannabinoid system with varying efficacies.[23, 24] Phytocannabinoids have also exhibited potential interactions with other key receptors and signalling pathways in the nervous system. These including the G-protein coupled receptors GPR55 and GPR18, transient receptor-potential channels (TRPVs), 5-HT serotonin receptors, opioid receptors and peroxisome proliferator-activated receptors (PPARs).[25, 26] A growing body of literature supports the therapeutic potential of phytocannabinoid treatment options for a wide range of conditions including neurodegeneration, chronic pain, inflammation, muscle spasticity and epilepsy.[13, 20, 27-29]

First isolated in 1964, Δ^9-tetrahydrocannabinol (THC) is one of the most widely studied phytocannabinoids to date.[30] The psychoactive effects of THC are largely modulated by CB_1 receptors, to which it is a partial agonist with similar affinity to anandamide. Interestingly, relatively high levels of endogenous CB_1 occupancy heavily affects the pharmacological effects of THC[18] While the addition of THC at synapses where anandamide predominates may have an excitatory effect, it may have an antagonist effect in synapses predominated by 2AG.[18] As such, THC modulation of CB_1 receptors is much more nuanced than a simple agonist relationship, and activity heavily depends on the synaptic microenvironment. Indeed, treatment with high doses of the CB_1 antagonist rimonabant only mildly ameliorated the psychoactive changes brought on THC.[31]

Cannabidiol (CBD) is another widely studied phytocannabinoid, exhibiting antioxidant, anti-inflammatory properties and a wide safety profile for therapeutic use.[32, 33] While void of psychoactive properties, recent evidence suggests that it may act as a weak allosteric antagonist of both CB_1 and CB_2 receptors.[34] Another phytocannabinoid of therapeutic interest is the CB_2 receptor agonist cannabichromene (CBC).[35] While relatedly understudied compared to CBD and THC, it has been demonstrated to increase viability of adult neural progenitor stem cells via increased phosphorylation of extra-cellular signal related kinase (ERK).[36] CBC has also been demonstrated to inhibit cellular uptake of anandamide, and play an anti-inflammatory role, potentially due to its role as a TRPV agonist. [37, 38]

THC, CBD and CBC all display promising therapeutic potential against certain mechanisms associated with motor neuron disease. Sativex, an oral spray containing THC and CBD, is currently approved for the treatment of muscle spasticity in multiple sclerosis, a neurodegenerative disease sharing many of the same toxicity mechanisms as MND.[39] In a transgenic mouse model of MND, treatment with Δ^9-tetrahydrocannabinol was found to increase both motor function and mean survival time. [40] Indeed, a phase III clinical trial investigating cannabidiol (CBD) oil as a novel therapeutic for MND is currently recruiting patients in Queensland, Australia.[41]

One of the major critiques of both *in vitro* and *in vivo* MND research to date is the heavy reliance on fMND, particularly the murine SOD 1^{G93A} mutation.[40] Despite accounting for the vast majority of cases, sMND by comparison is relatively understudied. As such, there is a need for a robust, non-transgenic *in vitro* model to study cellular mechanisms of MND in more depth. [40, 42]

The NSC-34 cell line

The NSC-34 cell line is a hybrid between murine neuroblastoma and motor neuron-enriched embryonic spinal cord cells.[43] While undifferentiated in their proliferative state, the combination of serum-starvation and *all-trans* retinoic acid (atRA) and has been demonstrated to induce a motor neuron-like state. These cells exhibit morphological changes such neuritic outgrowths and develop the ability to generate action potentials.[11] Differentiated NSC-34s also express choline acetyl transferase (ChAT), neuronal growth markers such as the axonal protein β-Tubulin III, and are generally more resistant to neurotoxic events such as oxidative stress and glutamate excitotoxicity.[42, 44] In addition, differentiation has been demonstrated cause a 5-fold increase NSC-34 expression of CB_1 receptors, suggesting that endocannabinoid signalling plays a key role in the survival of these cells post differentiation.[19] This being said, there are several inconsistencies in the literature that still need to be addressed. [45]

Despite NSC-34 cells being regularly employed in the study of cholinergic neurodegeneration, a standardised differentiation protocol in yet to be developed and fully characterised. Reported differentiation times range from 48 hours to over 4 weeks, with atRA concentrations varying between 1μM and 10μM.[19, 45, 46] By comparison, a small body of evidence suggests that differentiation may be independent of atRA treatment.[45] In addition, several studies bypassed differentiation altogether.[45] This highlights the need for a robust, standardised and well characterised protocol for NSC-34 differentiaton.[45]

It has also been suggested that differentiated cells do not express all NMDA receptor subunits at sufficient quantities to provide an appropriate model for glutamate excitotoxicity. [45] While this cell line may not exhibit sustained calcium influx in response to treatment with glutamate, this does not necessarily invalidate their use in the study of neurodegenerative mechanisms. Indeed, treatment

with the anti-excitotoxic treatment riluzole was unable to reduce cell death induced by the reactive oxygen species H_2O_2 or the protein kinase C inhibitor staurosporine, suggesting these toxicity pathways may be independent of glutamate signalling.[44] As such, differentiated NSC-34s may still provide a suitable model for the study of other toxicity mechanisms associated with MND, such as oxidative stress.

Interestingly, the addition of the potent and specific CB_1 agonist arachidonyl-2′-chloroethylamide (ACEA) to serum-reduced differentiation media promoted differentiation of primary murine neural stem cells into motor neuronal cells, but not astrocytes or oligodenrocytes.[47] This treatment caused a 2.5-fold increase in the percentage of cells expressing the axonal protein B-tubulin III, an effect that was negated in the presence of the specific CB_1 antagonist AM 251. ACEA also increased the number of neurites per cell and the length of these neurites, two other morphometric indicators of neuronal differentiation.[47] In light of this, CB_1 activation may also play an important role in promoting morphological changes in differentiating NSC-34 cells.

Aims and Hypotheses

Based on the preceding literature, the author hypothesises that differentiation will alter NSC-34 response to cannabinoid treatment, and hence any neuroprotective effects conferred against H_2O_2-induced oxidative stress. Furthermore, CB_1 receptor excitation during differentiation will promote the formation of motor-neuron like morphological changes seen in NSC-34s. To this end, the present study aims to; (1) characterise the effect of differentiation with *all*-trans retinoic acid on NSC-34 response to the phytocannabinoids Δ^9-tetrahydrocannabinol, cannabidiol, cannabichromene and the whole medicinal cannabis extract Bediol®; (2) investigate the effect of differentiation on NSC-34 response to H_2O_2-induced oxidate stress and any neuroprotective properties conferred by

these cannabinoids; and (3) investigate the effects of CB_1 receptor modulation during NSC-34 differentiation on the morphological outcomes of the process.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's Modified Essential Medium (DMEM, D6429), Penicillin/Streptomycin (P/S), Non-essential amino acids (NEAA), *all-trans* retinoic acid (atRA), 30% hydrogen peroxide (H_2O_2), arachidonyl-2′-chloroethylamide (ACEA), phosphate buffered saline (PBS, pH=7.4), 10x Trypsin EDTA, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Dimethyl Sulfoxide (DMSO) and Ethanol were obtained from Chem-Supply (Port Adelaide, SA, Australia) and Trypan Blue from Bio-Rad Laboratories (Gladesville, NSW, Australia).

Δ^9-Tetrahydrocannabinol was obtained from the National Measurement Institute (Department of Industry, Science, Energy and Resources, ACT, Australia), cannabidiol from Cerlliant Analytical Reference Standards (Round Rock, TX, USA) and cannabichromene from Sapphire Bioscience (Redfern, NSW, Australia). Bediol® whole medicinal cannabis extract, containing 8% CBD and 6.3% THC, was sourced from Novachem (Heidelberg West, VIC, Australia). Bediol® was extracted at 100mg/ml in 100% ethanol and all dilutions calculated by primary constituent (CBD). All chemicals and reagents used were of an analytical grade.

NSC-34 Cell Culture and Differentiation

NSC-34 were kindly donated by Dr Hakan Muyderman (Flinders University, SA, Australia), and maintained in DMEM supplemented with 5% FBS, 1% penicillin/streptomycin and 1% NEAA.[42, 48] Cells were split every three days and maintained at 37ºC under 5% CO_2.

For cannabinoid-response and oxidative-stress induced toxicity experiments NSC-34 cells were trypsinised and seeded into 96-well plates at a density of 1 x 10^4 cells per well ($3x10^4$ cells per cm^2) in 100µl DMEM supplemented with 5% FBS. After 24 hours seeding, media was replaced with differentiation media consisting of DMEM supplemented with 10µM all-trans retinoic acid, 1% FBS, 1% P/S and 1% NEAA. After 48 hours differentiation, approximately half of the cells exhibited motor-neuron like morphology, with long neuritic projections visible under light microscopy (Figure 1)

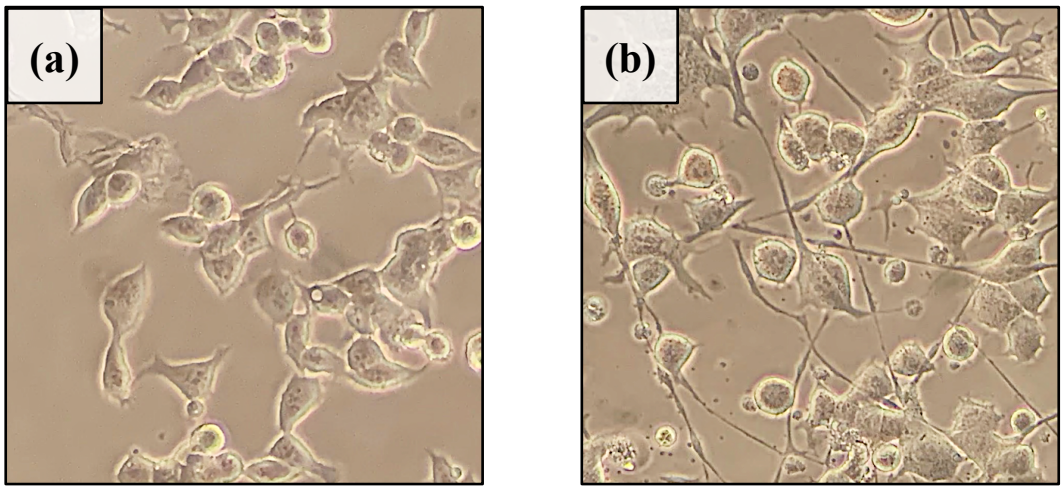

Figure 1: *Light microscopy of (a) undifferentiated NSC-34 cells, and (b) NSC-34 cells after 48 hour differentiation in the presence of 10µM all-trans Retinoic Acid and 1% FBS (x200 magnification)*

Cannabinoid Concentration-Response and Toxicity

After differentiation, media was removed and replaced with serum-free DMEM containing 10nM-100µM of CBD, THC and ACEA for 24 hours. Due to solubility constraints, CBC and AM 251 were instead treated at 10nM-20µM. For a more therapeutically aligned comparison, Bediol® whole cannabis extract containing 8% CBD and 6.3% THC was also tested. Undifferentiated NSC-34s

were treated under the same conditions. Vehicle controls were included for 1% DMSO, 0.1% DMSO, 1.36% ethanol and 3.14% methanol. All treatment conditions were performed in triplicate, and experiments repeated three times over consecutive passages.

H_2O_2-Induced Oxidative Stress and Cannabinoid Neuroprotection

Differentiated NSC-34 cells were treated with serum-free DMEM containing 1µM THC, CBD, CBC or Bediol® standardised to 1µM CBD equivalent. After 1 hour of cannabinoid pre-treatment, serial dilutions of 50, 150, 250, 350 and 450µM H_2O_2 were added, and cells incubated for a further 24 hours. All treatment conditions were performed in triplicate, and experiments repeated four times over consecutive passages.

MTT Cytotoxicity Assay

Following treatment, cell viability was assessed using the thiazolyl blue tetrazolium bromide (MTT) colorimetric cytotoxicity assay.[49] Briefly, treatment media was replaced with serum-free DMEM containing 0.25mg/ml MTT. After two hours, active mitochondria had converted MTT to an insoluble blue formazan salt, which was dissolved by replacing media with 100µl DMSO per well. Plates were agitated for 30 seconds to ensure homogeneity and absorbance at 570nm determined using a Synergy MX microplate reader (Biotek Instruments Inc., Winooski, VT, USA).

Morphometric Analysis of NSC-34 Differentiation

For morphometric analysis, Ibidi 8-slide live cell imaging slide wells were seeded with 3 x 10^4 cells per cm^2 in 300µl proliferation medium. Cells were then differentiated for 48 hours in 1% DMEM supplemented with 10µM atRA, 1µM ACEA or a combination of the two. These conditions were then repeated with the addition of the synthetic CB_1 antagonist AM 251 (1µM). After differentiation, cells were imaged using a Ti-E Live Cell Imaging System (Nikon Corporation, Tokyo, Japan). Four images were analysed per well using a variation of the Scholl method for

quantification of dendritic branching, a method previously used to asses differentiation outcomes both in NSC-34s and other neuronal cell lines.[45, 50, 51]. Briefly, 20 cells were randomly selected for tracing from a 0.16cm^2 area using the Mersenne-Twister random number generator. Neurites were traced from the centre of the soma using the NeuronJ plugin for ImageJ (Version 1.53) and cells considered differentiated if they possessed at least one neurite longer that 50µM.[45, 52] From this tracing data the percentage of cells differentiated, average neuritic length per differentiated cell and the number of neurites per differentiated cell were calculated. Experiments were repeated four times, resulting in the tracing of over 300 cells per treatment condition.

Statistical Analysis

All data was processed and analysed using Prism 9.0.0 for Mac (GraphPad Software, CA, USA). MTT absorbance data was averaged between technical replicates and standardised. Results were expressed as a percentage of cannabinoid-free control for concentration-response experiments, or H_2O_2-free control for oxidative stress toxicity experiments. Two-way ANOVA tests with Bonferroni Post-Hoc tests were performed on all results. $p>0.05$ was considered statistically significant for all tests.

RESULTS

Cannabinoid Toxicity

To determine the appropriate cannabinoid treatment concentrations for further study, concentration response data was determined for; the three phytocannabinoids, CBD, THC and CBC; Bediol® whole medicinal cannabis extract; and the synthetic CB_1 ligands ACEA and AM 251 (Figure 2).

CBD elicited a similar response under both conditions, with a significant decrease in cell viability detected at 10µM ($p<0.0005$). CBC resulted in toxicity at 20µM ($p<0.01$) in undifferentiated cells, but increased cell viability in the differentiated cells (+18.9 ± 9.0%, $p<0.01$). Treatment with 1µM

THC also increased cell viability by 23.1 ± 4.8% ($p<0.01$) in differentiated cells only although this was followed by a sharp decrease in viability of both cell types at 10μM (differentiated $p<0.05$, undifferentiated $p<0.0005$). Bediol® elicited a similar response curve to THC, with toxicity seen at 10μM CBD equivalent under both differentiation conditions ($p<0.0005$) but increased viability at 1μM CBD equivalent in differentiated cells only (+31.6 ± 7.0%, $p<0.005$).

The synthetic CB_1 agonist ACEA caused a minor reduction in cell viability at 100μM in the undifferentiated NSCs ($p<0.01$), although this effect was reversed with a 55.5 ± 4.4% ($p<0.0005$) increase in differentiated cells. 10μM AM 251, on the other hand caused significant toxicity to NSC-34s that were both differentiated ($p<0.0005$) and undifferentiated ($p<0.01$).

Based on the results above, 1μM was chosen as the most appropriate concentration of cannabinoid treatment for further studies.

Significant vehicle toxicity (not displayed) was not detected for the highest concentrations of methanol (3.14%) and ethanol (1.36%) in both differentiated and undifferentiated cells. 1% DMSO was well tolerated by undifferentiated cells but resulted in a 26.5 ± 2.4% increase in differentiated cell viability ($p<0.05$). This effect was not seen from the 0.1% DMSO vehicle present at 10μM concentrations of CBD and CBC. All further concentrations used were <0.1% DMSO.

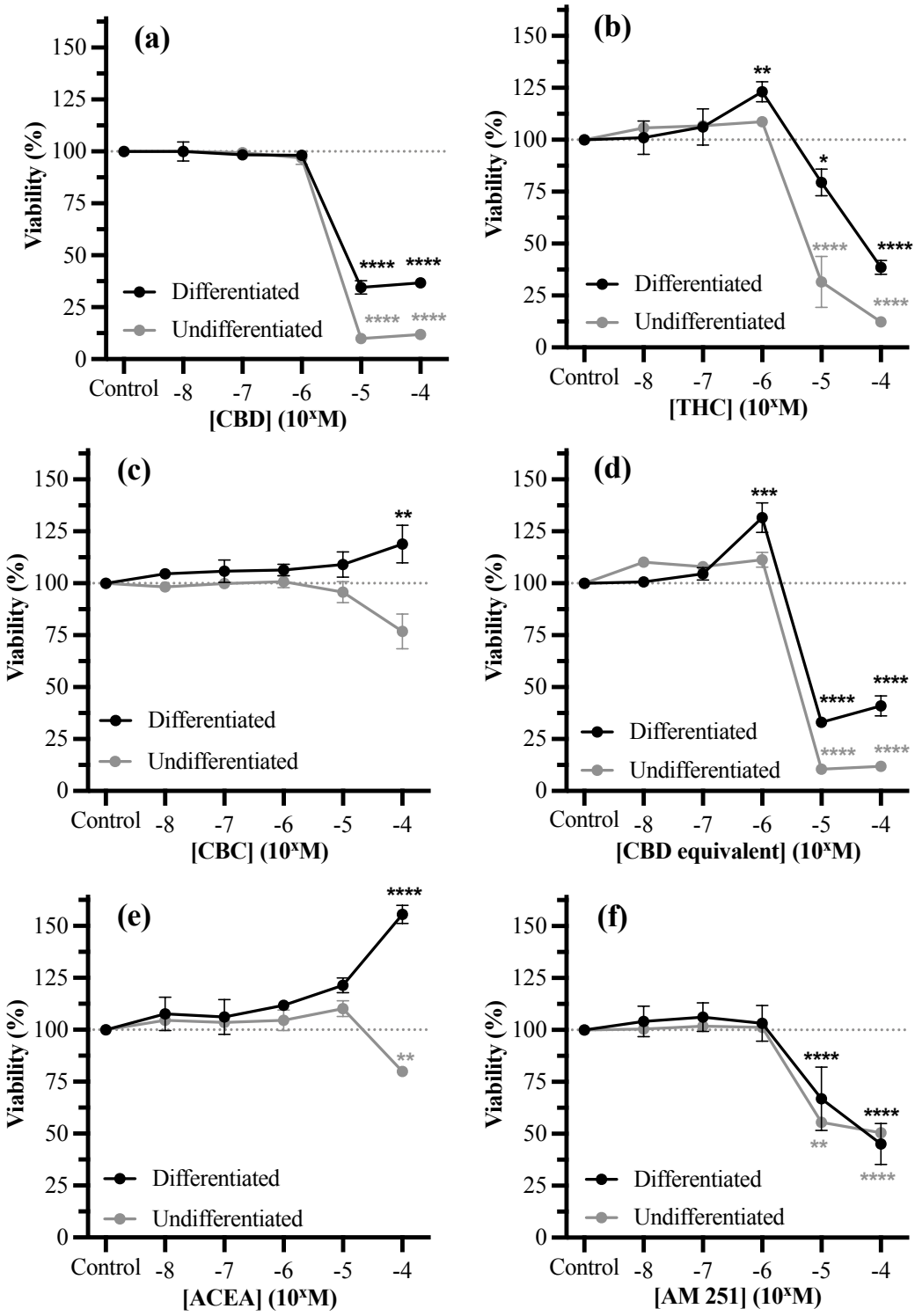

Figure 2: 48 hour differentiation with 10µM *all-trans* retinoic acid altered NSC-34 response to (a) cannabidiol (CBD), (b) Δ^9-tetrahydrocannabinol (THC) (c) cannabichromene (CBC); (d) Bediol® whole medicinal cannabis extract; (e) arachidonyl-2′-chloroethylamide (ACEA), and (f) AM 251. Data is displayed as mean ± SEM, where n=3. *p<0.05, **p<0.01, ***p<0.005, ****p<0.0005 vs. cannabinoid-free control.

H_2O_2-Induced Oxidative Stress

To investigate potential antioxidant effects of cannabinoids under investigation, differentiated and undifferentiated NSCs were co-treated for 24 hours with H_2O_2 and 1µM CBD, THC, CBC or Bediol® standardised to 1µM CBD equivalent (Figure 3).

Both undifferentiated and differentiated NSCs displayed a concentration-dependent toxicity response to H_2O_2-induced oxidative-stress, with a large reduction in viability at 450µM H_2O_2 (32.6 ± 6.6% vs. 31.3 ± 1.3% respectively)

None of the cannabinoids under review displayed any significant neuroprotective effects on undifferentiated NSC-34 cells over any concentrations of H_2O_2, as determined via MTT assay. This result was mirrored in the differentiated cells co-treated with 1µM CBD or CBC. 1µM THC treatment on the other hand increased differentiated cell viability in the presence of 0 and 50µM H_2O_2 ($p<0.05$), although this effect was absent at higher H_20_2 concentrations. Bediol®, on the other hand displayed no significant effects at low concentrations of H_2O_2 but reduced toxicity induced by treatment with 250 and 350µM H_2O_2 ($p<0.05$).

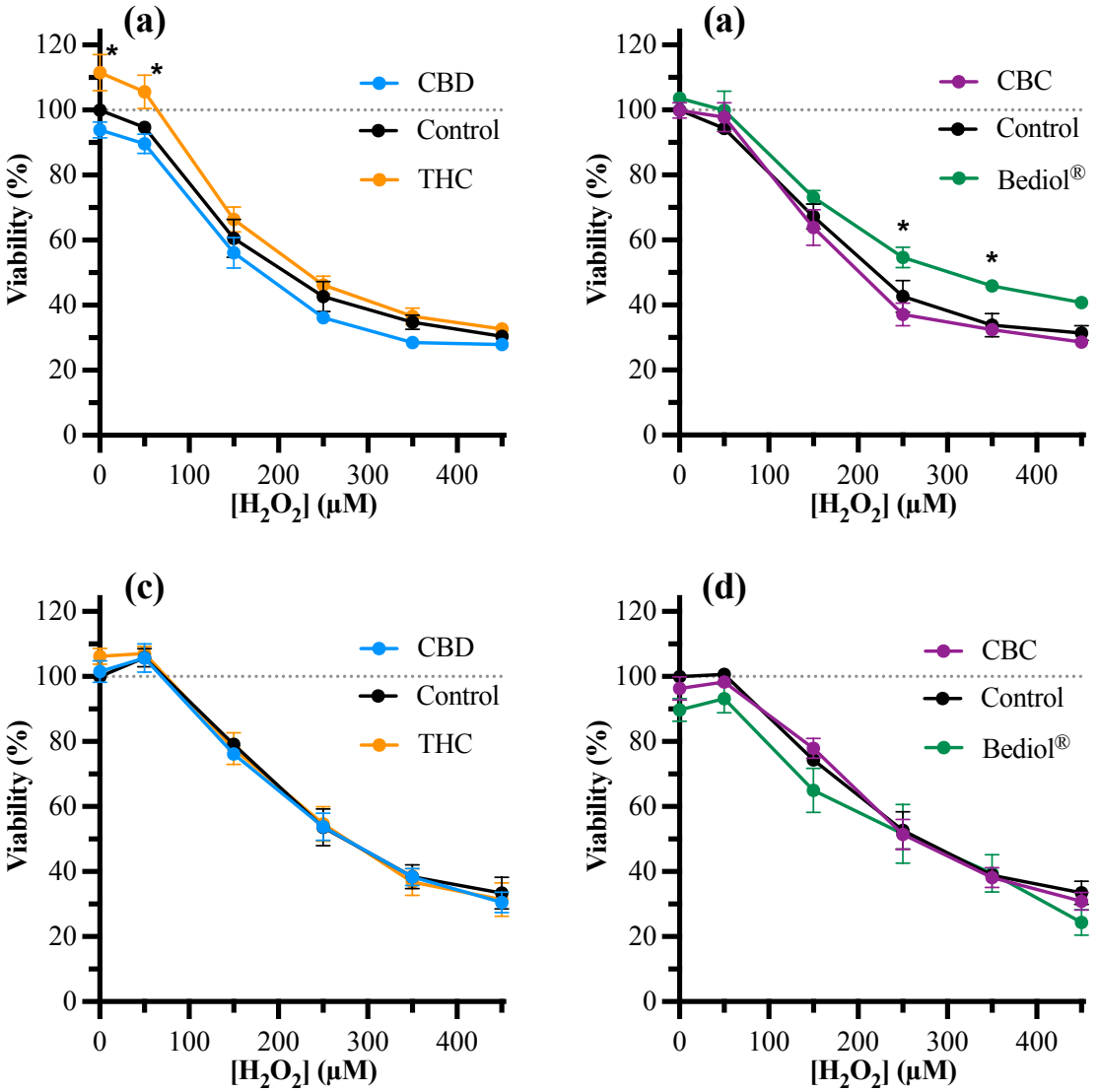

Figure 3: Comparison of the effects of 1-hour pre-treatment of (a) Δ^9-tetrahydrocannabinol (THC, 1μM) and cannabidiol (CBD 1μM), or (b) cannabichromene (CBC 1μM) and Bediol® whole medicinal cannabis extract (CBD equivalent, 1μM) on H_2O_2 induced neurotoxicity in differentiated NSC-34 cells. Data is displayed from undifferentiated cells for comparison; (c) and (d) respectively. Data displayed as mean ± SEM, where n=4. *$p<0.05$ vs. untreated control.

Morphometric Analysis of NSC-34 Differentiation

Minimal differentiation was observed when NSC-34 were treated for 48 hours in DMEM containing only 1% FBS. Only 7.6 ± 3.3% of cells exhibiting at least one neurite longer than 50μm (Figure 4.a). This percentage increased when differentiation media was supplemented with 10μM atRA (38.1 ± 6.1%, $p<0.0005$), 1μM ACEA (27.1 ± 5.0%, $p<0.05$) or a combination of the two (51.3 ± 2.7%, $p<0.0005$). Indeed, the combination of atRA and ACEA increased the number of cells differentiated beyond that of ACEA alone ($p<0.01$) but not atRA.

All three conditions also increased the average length of neurites in cells that did undergo differentiation (atRA: 160.3 ± 23.0 μm, ACEA: 135.7 ± 10.9 μm, combined: 167.3 ± 12.3 μm vs. control: 59.9 ± 6.0μm) (Figure 4.b) as well as the average number of neurites longer than 50μm per differentiated cell (atRA: 1.59 ± 0.18, ACEA: 1.50 ± 0.12, combined: 1.73 ± 0.09 vs. control: 1.00 ± 0.0) (Figure 4.c).

To investigate the role that CB_1 receptors play in NSC-34 differentiation, experiments were repeated in the presence of the competitive CB_1 antagonist AM 251 (1μM). AM 251 significantly negated all three morphological changes under investigation (7.8 ± 2.9% differentiated, 69.7 ± 11.2 μm average neurite length and 1.13 ± 0.09 neurites per differentiated cell) induced by coincubation with 1μM ACEA alone, but not that induced by atRA or the combination of the two (Figure 5). It should also be noted that 1μM AM 251 alone did not significantly alter any morphological characteristics when compared with 1% FBS differentiation media alone (not shown).

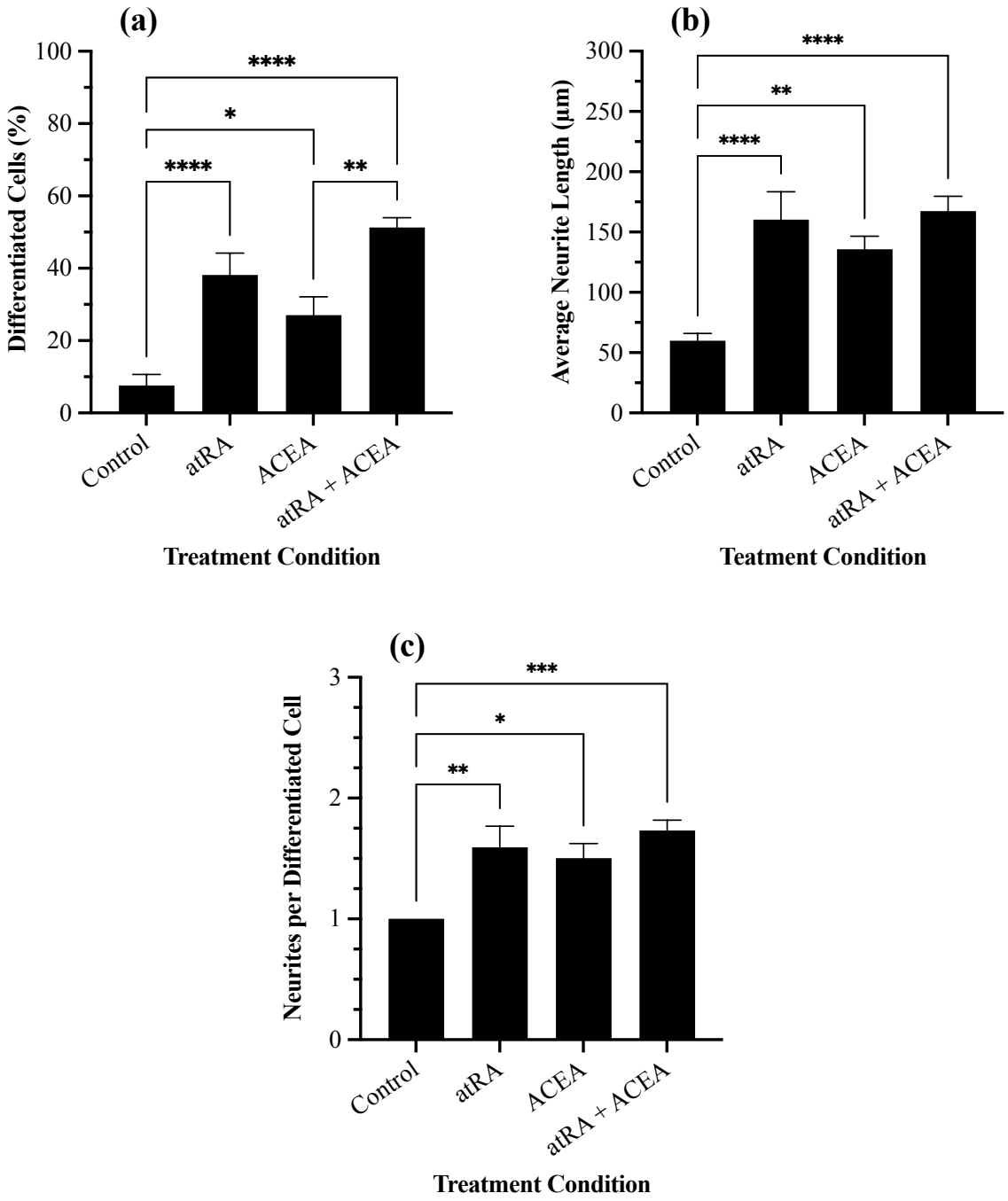

Figure 4: Morphological changes were increased in NSC-34 cells after 48 hour differentiation in serum-reduced (1% FBS) DMEM supplemented with *all-trans* Retinoic Acid (atRA, 10µM), arachidonyl-2′-chloroethylamide (ACEA, 1µM) or the combination of the two when compared to differentiation media alone. (a) The percentage of cells displaying neuritic growths longer than 50µm, (b) the average neuritic length of differentiated cells, and (c) average number of neurites per differentiated cell all significantly rose under all three conditions. Data is displayed as mean ± SEM where n=4. *p<0.05, **p<0.01, ****p<0.0005 vs. untreated control.

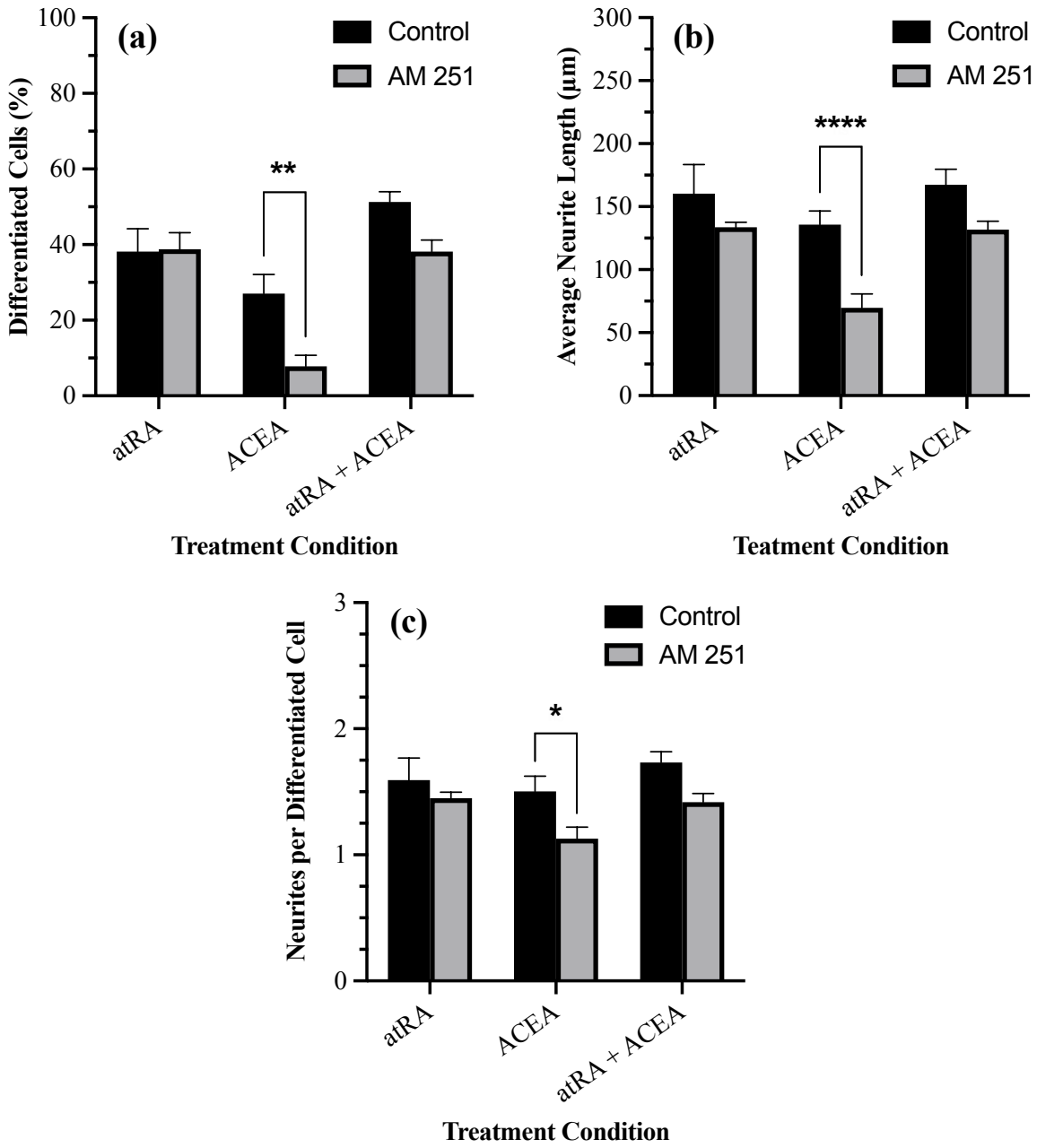

Figure 5: Motor-neuron like morphological changes induced by 48 hour coincubation with arachidonyl-2′-chloroethylamide (ACEA, 1µM), but not *all-trans* Retinoic Acid (atRA, 10µM), were reduced by the addition of the synthetic CB_1 antagonist AM 251 (1µM). These changes include (a) the percentage of cells displaying neuritic growths longer than 50µm, (b) the average neuritic length of differentiated cells, and (c) the average number of neurites per differentiated cell. Data is displayed as mean ± SEM where n=4. *p<0.05, **p<0.01, ****p<0.0005 vs. untreated control.

Discussion

In order to characterise how differentiation affects NSC-34 cellular responses to cannabinoid treatments and determine appropriate treatment concentrations for further study, concentration-response curves were developed for; the phytocannabinoids CBD, THC, CBC; and the whole medicinal cannabis extract Bediol®. The potent, specific CB_1 agonist ACEA and antagonist AM 251 were also investigated for a more thorough understanding of the role of CB1 receptors.

Interestingly, differentiated NSC-34 viability increased at 1μM THC and then dropping away at higher concentrations. This effect suggests that THC plays a neuroprotective role in these cells at lower concentrations prior to reaching toxicity. The CB_1 agonist ACEA elicited a similar response, albeit cell viability increased only at a much higher concentration (100μM), implying that this neuroprotective effect seen may be mediated by the CB_1 receptors expressed in higher quantities by these cells post-differentiation.[47] Differentiated NSC-34s also displayed a hormetic increase in cell viability to Bediol® whole medicinal cannabis extract standardised to 1μM by CBD concentration.

In terms of oxidative stress, differentiation did not appear to alter NSC-34 response to H_2O_2 toxicity alone. Indeed, while THC did play a neuroprotective role at low and absent H_2O_2 concentrations, none of the phytocannabinoids studied displayed individual antioxidant properties against H_2O_2 in either differentiated or differentiated cells.

Bediol® on the other hand did display an antioxidant effect, reducing toxicity induced by 250 and 350μM H_2O_2 in differentiated NSC-34 cells only. It should be noted that a 1μM CBD equivalent treatment of Bediol® also included 787nM THC, along with unknown quantities of unspecified phytocannabinoids. Hence, the neuroprotective and anti-inflammatory properties seen may also be due to over 120 phytocannabinoids potentially present in the extract.[23] Furthermore, it has

previously been suggested that the combination of multiple cannabinoids may bestow neuroprotective effects greater than the sum of the individual phytocannabinoids. [53] [54]

Not only did atRA-induced differentiation modulate cellular responses to THC, CBC, ACEA and Bediol®, it also potentiated the antioxidant effects of Bediol®. These results suggest that differentiation of NSC-34s is an essential step when investigating cannabinoids as novel therapeutics.

Differentiation of NSC-34s for 48 hours in media supplemented with 10µM atRA and 1% FBS significantly increased the percentage of cells presenting neuritic growths when compared to the serum-reduced media alone. This result was consistent across the two other morphological indicators of differentiation assessed, average length and number of these growths per differentiated cell. Together these three morphological changes provide further support the essential role of differentiation in determining NSC-34 cellular response to treatment conditions. These results also confirm the suitability of the differentiation protocol (10µM atRA, 1% FBS, 48h) used in prior studies.

The CB_1 agonist ACEA also induced motor-neuron like morphological changes in NSC-34s, a novel effect previously undocumented in this cell line. The addition of AM 251 negated these morphological changes due to ACEA, but not atRA, further supporting the notion that these changes were due to interaction with CB_1 receptors. The combination of these two pro-differentiation compounds did not increase morphological changes beyond that of the two individually, suggesting that these effects may not be cumulative. Further study is required on this front to fully elucidate the mechanisms behind CB_1 induced differentiation of NSC-34s.

One of the main limitations of assessing differentiation solely on cellular morphology is the relatively low percentage of cells that take on these morphological changes. In previous studies, only 40% of cells were considered differentiated after a 4 day differentiation with 1μM atRA, and only 20% of primary neural stem cells differentiated with ACEA expressed similar morphology[42, 44]. This being said, differentiation is a complex, multifactorial process and basic morphology does not fully reflect how a cell will act physiologically. To this end, the author proposes further study of ACEA-induced NCS-34 differentiation focusing on expression of key motor-neuronal features, such as choline acetyl transferase and glutamate receptors.

The results presented in the present study open up many exciting avenues for further investigation. While atRA differentiation caused significant morphological changes to NSC-34s, and heavily modified cellular responses to cannabinoid treatments, further work is required to test the consistency and physiological extent of these changes. CB_1 activation, such as via the synthetic agonist ACEA, also provides a novel avenue for NSC-34 differentiation and requires a further understanding.

Conclusion

MND is a complex, multifactorial, poorly understood disease and hence poses many challenges to the development of novel therapeutics. Based on the findings of the present study, treatment of NSC-34 cells with 10μM *all-trans* retinoic acid for 48 hours successfully promotes differentiation. Interestingly, the synthetic CB_1 agonist ACEA promoted similar morphological changes, a novel effect previously undocumented in this cell line. Differentiation modulates NSC-34 cells response to cannabinoid treatment. It also potentiates the antioxidant effects of Bediol® whole medicinal cannabis extract against H_2O_2-induced toxicity, adding to the growing body of literature supporting the investigation of phytocannabinoids as novel treatments for MND.

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Acknowledgements

The author would like to thank Dr Jane Sibbons (Adelaide Microscopy) for her assistance with live cell imaging and slide preparation, and Dr Hakan Muyderman (Flinders University) for donating the NSC-34 cell line used for all experiments. No external funding sources was used in the completion of this project.

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