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# Tetrahydrocannabinol-induced apoptosis of cultured cortical neurones is associated with cytochrome *c* release and caspase-3 activation

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**Abstract**

$\Delta^9$-Tetrahydrocannabinol (THC), the principal psychoactive component of marijuana, is associated with impaired cognition and altered cortical function. THC transduces its central effects via activation of the G-protein linked cannabinoid receptor CB1. In this study we report that THC induces morphological degenerative changes in cultured cortical neurones, such as membrane blebbing and formation of apoptotic bodies, that are consistent with the apoptotic pathway of cell death. The THC-induced apoptosis was blocked by the CB1 receptor antagonist AM251 and pertussis toxin (PTX), suggesting that this effect of THC involves receptor-mediated activation of the G-protein subtypes $G_i$ or $G_o$. THC also promoted translocation of mitochondrial cytochrome *c* to the cytosol and increased the activity of the cysteine protease caspase-3, in a PTX-sensitive manner. The results from this study suggest that coupling of THC to a PTX-sensitive G-protein promotes cytochrome *c* release, caspase-3 activation and subsequent degeneration of cultured cortical neurones. This apoptotic pathway may underlie the compromised neuronal function that is associated with marijuana usage.

*Keywords:* Tetrahydrocannabinol; Apoptosis; Caspase-3; Cytochrome c; Pertussis toxin; Cortical neurones

## 1. Introduction

$\Delta^9$-tetrahydrocannabinol (THC), the major psychoactive component of marijuana, has been shown to induce behavioural changes such as compromised cognition (Reisine and Brownstein, 1994) and impaired verbal and memory retrieval skills (Block and Ghoneim, 1993). The behavioural manifestations of THC have been proposed to be due to altered functional activity of the frontal cortex in humans (Mathew et al., 1997) and rats (Jentsch et al., 1997), possibly by altering neurotransmitter release in this brain region (Jentsch et al., 1998; Gessa et al., 1998).

To date, two subytpes of cannabinoid receptor, CB1 and CB2, have been identified (Howlett, 1990) with the CB1 receptor being responsible for mediating the central effects of THC (Matsuda et al., 1990). The CB1 has been localised in several brain regions including the cortex, hippocampus and cerebellum (Berrendero et al., 1998) and the amino acid sequence of this receptor is consistent with a tertiary structure typical of G-protein coupled receptors (Matsuda et al., 1990). The CB1 receptor has been shown to couple to a variety of signalling pathways including the cAMP pathway (Howlett, 1995), mitogen-activated protein (MAP) kinase (Bouaboula et al., 1999) and the regulation of ion channels (Mackie and Hille, 1992) in a pertussis toxin (PTX)-sensitive manner, providing evidence for the CB1 receptor coupling to the G-protein subtypes $G_i$ or $G_o$.

While THC modulates a variety of neuronal functions including ion channel activity (Mackie and Hille, 1992) and neurotransmitter release (Jentsch et al., 1998), recent studies have demonstrated that THC has a toxic effect on cultured hippocampal neurones (Chan et al., 1998), C6 glioma cells (Sanchez et al., 1998) and non-neuronal cells such as macrophages and lymphocytes (Zhu et al., 1998). The proposed mechanisms underlying the apoptotic effects of THC include the generation of reactive oxygen species (Chan et al., 1998), sphingomyelin hydrolysis (Sanchez et al., 1998) and activation of the

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cysteine protease, caspase-1 (Zhu et al., 1998). Apoptotic cell death may be characterised by several morphological and biochemical parameters, including reduced cell volume, blebbing of the plasma membrane, condensation of nuclear chromatin and DNA fragmentation (Raffray and Cohen, 1997). These apoptotic events are initiated by members of the caspase superfamily and activation of caspase-3 has been demonstrated to be of particular significance in the induction of apoptosis, by virtue of its ability to cleave structural and nuclear proteins (Janicke et al., 1998; Hampton et al., 1998). The trigger for caspase-3 activation involves translocation of mitochondrial cytochrome *c* to the cytoplasm (Rosse et al., 1998), a process which is regulated by the Bcl family of mitochondria-associated proteins (Korsmeyer, 1995).

In this study we demonstrate that exposure of cultured cortical neurones to THC induces the morphological and biochemical features of apoptosis including membrane blebbing, DNA fragmentation, release of mitochondrial cytochrome *c* and activation of caspase-3. These apoptotic events proceed in a PTX-sensitive manner suggesting that THC-induced apoptosis is dependent upon activation of PTX-sensitive G-proteins in this preparation.

## 2. Methods

### *2.1. Culture of cortical neurones*

Primary cortical neurones were established from postnatal one day old Wistar rats and maintained in neurobasal medium. Rats were decapitated and cerebral cortices removed. The dissected cortices were incubated in phosphate-buffered saline (PBS) containing trypsin (0.25%) for 20 min at 37°C. The tissue was then triturated (×5) in PBS containing soyabean trypsin inhibitor (0.1%) and DNAse (0.2 mg/ml) and gently filtered through a sterile mesh filter. Following centrifugation, 2000×***g*** for 3 min at 20°C, the pellet was resuspended in neurobasal medium, supplemented with heat inactivated horse serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml) and glutamax (2 mM). Suspended cells were plated out at a density of $0.25\times10^6$ cells on circular 10 mm diameter coverslips, coated with poly-L-lysine (60 μg/ml), and incubated in a humidified atmosphere containing 5% $CO_2$:95% air at 37°C. After 48 h 5 ng/ml cytosine-arabino-furanoside was included in the culture medium to prevent proliferation of non-neuronal cells. Culture media were exchanged every 3 days and cells were grown in culture for up to 14 days.

### *2.2. Drug treatment*

PTX was reconstituted in phosphate-buffered saline and cells were incubated with 200 ng/ml PTX for 24 h at 37°C. THC was obtained from Sigma–Aldrich Company Ltd. (Dorset, UK) and was diluted to the required concentration with warmed culture media. Absolute alcohol was used as carrier or vehicle for THC. Cells were incubated with the CB1 receptor antagonist AM251 (10 μM) for 30 min prior to THC treatment.

### *2.3. Evaluation of cell degeneration*

Following treatment of cultured cortical neurones with THC the cells were fixed in 4% paraformaldehyde for 30 min at room temperature, stained using the Rapi-diff II staining procedure (DiaCheM International Ltd., Lancs, UK) and viewed under ×100 magnification. Cells displaying degenerative features (e.g shrinkage, membrane blebbing, formation of apoptotic bodies) were counted and expressed as a percentage of the total number of cells examined (400–500 cells/coverslip). At least four coverslips were counted for each treatment in a single culture preparation, and the average value was taken. Pooled data was obtained from a total of 4–5 primary cultures prepared on different days.

### *2.4. TdT-mediated-UTP-end nick labelling (TUNEL)*

Apoptotic cell death was assessed using the DeadEnd colorimetric apoptosis detection system (Promega Corporation, Madison, USA). Briefly, cells were fixed with paraformaldehyde (4%), permeabilised with Triton-X100 (0.1%), and biotinylated nucleotide was incorporated at 3′-OH DNA ends using the enzyme Terminal deoxynucleotidyl Transferase (TdT). Horseradish-peroxidase-labelled streptavidin then bound to the biotinylated nucleotide and this was detected using the peroxidase substrate $H_2O_2$ and the chromogen diaminobenzidine. Cells were then viewed under light microscopy at ×100 magnification, where the nuclei of TUNEL positive cells stained brown. Apoptotic cells (TUNEL positive), were counted and expressed as a percentage of the total number of cells examined (400–500 cells/coverslip).

### *2.5. Cytochrome c western immunoblot*

Following incubation with THC (5 μM) cortical neurones were harvested in lysis buffer (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 5 μg/ml pepstatin A, 2 μg/ml leupeptin, 2 μg/ml aprotinin, pH 7.4) and left on ice for 20 min. Cells were then centrifuged (10 000×***g*** for 10 min at 4°C) and the supernatant containing the cytosolic fraction was prepared for SDS-polyacrylamide gel electrophoresis. The cytosolic fraction was diluted to 50 μg protein/ml with sodium dodecyl sulphate (SDS) sample buffer (150 mM Tris-HCl pH 6.8, 10% v/v glycerol, 4% w/v SDS, 5% v/v β-mercaptoethanol, 0.002% w/v Bromophenol Blue). Samples were

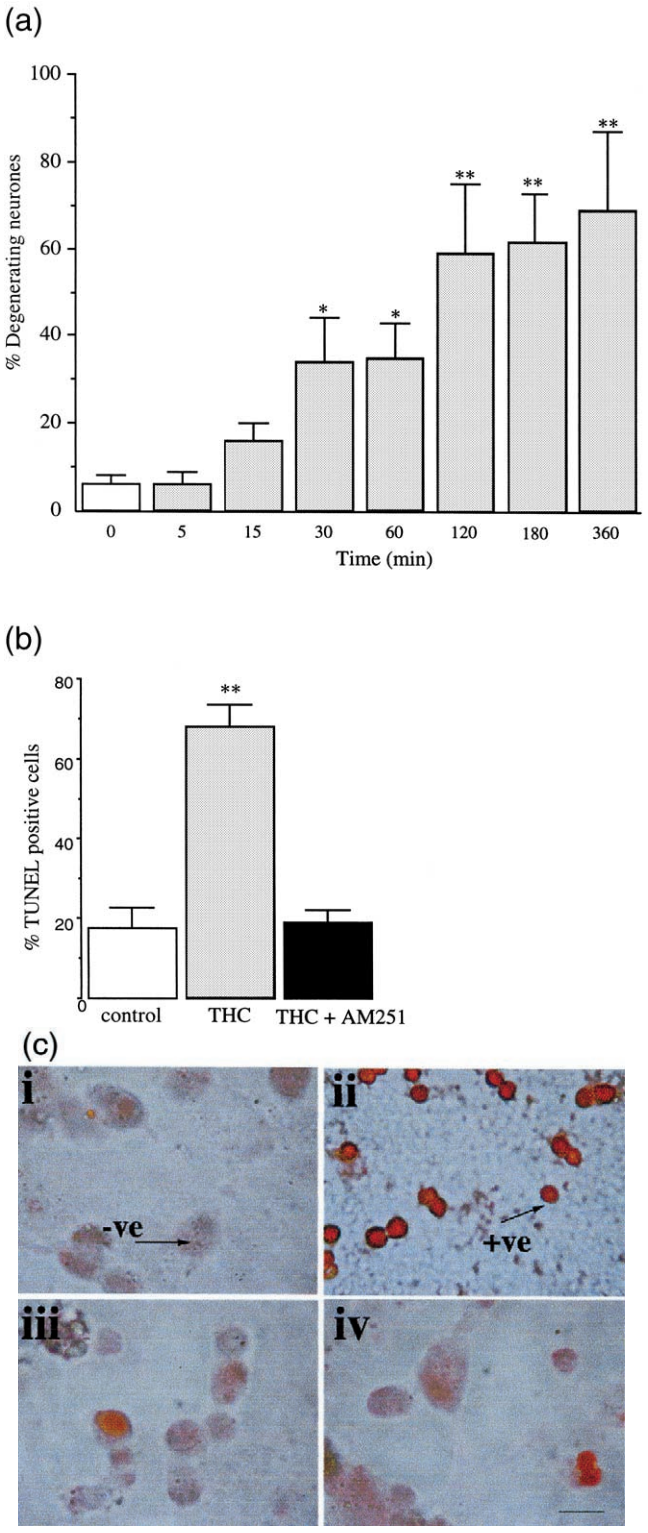


Fig. 1. THC promotes degeneration of cultured cortical neurones. (a) Cortical neurones were exposed to THC (5 μM) for 30–360 min. A significant increase in neuronal degeneration was observed following exposure to THC for 30, 60, 120, 180 and 360 min. Results are expressed as mean±s.e.m. for 5 observations, * $p<0.05$, ** $p<0.01$. (b) Preincubation of the cortical neurones with the CB1 receptor antagonist AM251 (10 μM) for 30 min abolished the THC-induced increase in apoptotic cells (as assessed by TUNEL) observed at 180 min. Results are expressed as mean±s.e.m. for 7 observations, ** $p<0.01$. (c) Representative image of cortical neurones stained with TUNEL. (i) Under control conditions few cells stained positive for DNA fragmentation using TUNEL. (ii) THC-treated neurones exhibited a significant increase in TUNEL-positive cells. (iii) Treatment with AM251 alone had no effect on the number of TUNEL-positive neurones. (iv) Treatment with AM251 in the presence of THC significantly reduced the number of TUNEL-positive cells (−ve, TUNEL-negative neurones; +ve, TUNEL-positive neurones; scale bar is 25 μm).

then heated to 100°C for 3 min. Cytosolic proteins (1 μg per lane) were separated by electrophoresis on a 10% polyacrylamide minigel, transferred to nitrocellulose membrane (Sartorius, Germany) and immunoblotted with a polyclonal anti-cytochrome *c* antibody (Santa Cruz Biotechnology Inc., California, USA) purified from rabbit serum. Immunoreactive bands were detected using horseradish peroxidase conjugated anti-rabbit IgG (Sigma, UK) and an enhanced chemiluminescence (ECL) system (Amersham, UK). Molecular weight markers were used to verify the molecular weight of protein bands and purified cytochrome *c* protein was used for positive identification of protein bands corresponding to cytochrome *c*. Band widths were quantified using densitometric analysis (GelWorks PC software).

### 2.6. Measurement of caspase-3 activity

Cleavage of the fluorogenic caspase-3 substrate (DEVD-aminofluorocoumarin (AFC); Alexis Corporation, USA) to its fluorescent product was used as a measure of caspase-3 activity. Cortical neurones were harvested in lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 5 mM DTT, 5 mM EDTA, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin; pH 7.4), subjected to 3 freeze–thaw cycles and centrifuged at 10 000 rpm for 10 min at 4°C. Samples of supernatant (90 μl) were incubated with the DEVD peptide (500 μM; 10 μl) for 1 h at 30°C. Incubation buffer (900 μl; 100 mM HEPES containing 10 mM DTT; pH 7.4) was added and fluorescence was assessed (excitation, 400 nm; emission, 505 nm).

### 2.7. Measurement of caspase-1 activity

Cleavage of the fluorogenic caspase-1 substrate (YVAD-AFC; Alexis Corporation, USA) to its fluorescent product was used as a measure of caspase-1 activity. Cortical neurones were harvested in lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 5 mM DTT, 5 mM EDTA, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin; pH 7.4), subjected to 3 freeze–thaw cycles and centrifuged at 10 000 rpm for 10 min at 4°C. Samples of supernatant (90 μl) were incubated with the YVAD peptide (500 μM; 10 μl) for 1 h at 30°C. Incubation buffer (900 μl; 100 mM HEPES containing 10 mM

DTT; pH 7.4) was added and fluorescence was assessed (excitation, 400 nm; emission, 505 nm) by spectrofluorimetry.

## 3. Results

### 3.1. Time course for THC-induced cell death

Exposure of cultured cortical neurones to THC (5 μM) induced cell death, as assessed by analysis of morphological features of degeneration, in a time dependent manner with maximal cell death occurring 120–360 min following THC treatment (Fig. 1). In control cells, 7% of cells displayed degenerative features and this was significantly increased to 34% ($p<0.05$, ANOVA, $n=5$), 36% ($p<0.05$, ANOVA, $n=5$), 57% ($p<0.01$, ANOVA, $n=5$), 62% ($p<0.01$, ANOVA, $n=5$) and 69% ($p<0.01$, ANOVA, $n=5$) in cells treated with THC for 30, 60, 120, 180 and 360 minutes, respectively. The proclivity of THC to induce neurodegeneration was concentration-dependent since exposure of neurones to a lower THC concentration (0.5 μM) for 180 min also significantly increased the number of degenerating neurones from 8% to 43% ($p<0.05$, ANOVA, $n=5$), while THC at a concentration of 5 nM failed to induce neurodegeneration (6% degenerating neurones).

The TUNEL technique was used to demonstrate that THC-induced DNA fragmentation was blocked by the CB1 receptor antagonist AM251 (10 μM: Fig. 1(b)). In control cells 17% of cells were TUNEL positive and this was significantly increased to 68% TUNEL-positive cells following treatment with THC (5 μM) for 180 minutes ($p<0.01$, Student's paired $t$-test, $n=7$). Exposure of cells to THC in the presence of AM251 failed to produce any significant increase in TUNEL staining (19% TUNEL-positive cells). Representative TUNEL staining of cortical neurones treated with THC±AM251 is shown in Fig. 1(c).

### 3.2. PTX pretreatment prevents THC-induced cell death

Treatment of cortical neurones with PTX (200 ng/ml) for 24 h prevented the THC-induced neurodegeneration (Fig. 2). Since a maximal THC-induced cell death was found to occur at 180 min the effect of PTX on cell death at this timepoint was evaluated. In cells treated with THC for 3 h the percentage of cells displaying morphological features of degeneration was significantly increased from 10% to 59% ($p<0.01$, Student's $t$-test, $n=5$). While pretreatment of cells with PTX alone had no effect on neuronal viability (12% neurones degenerating), it prevented the THC-induced increase in cell degeneration (20% neurones degenerating).

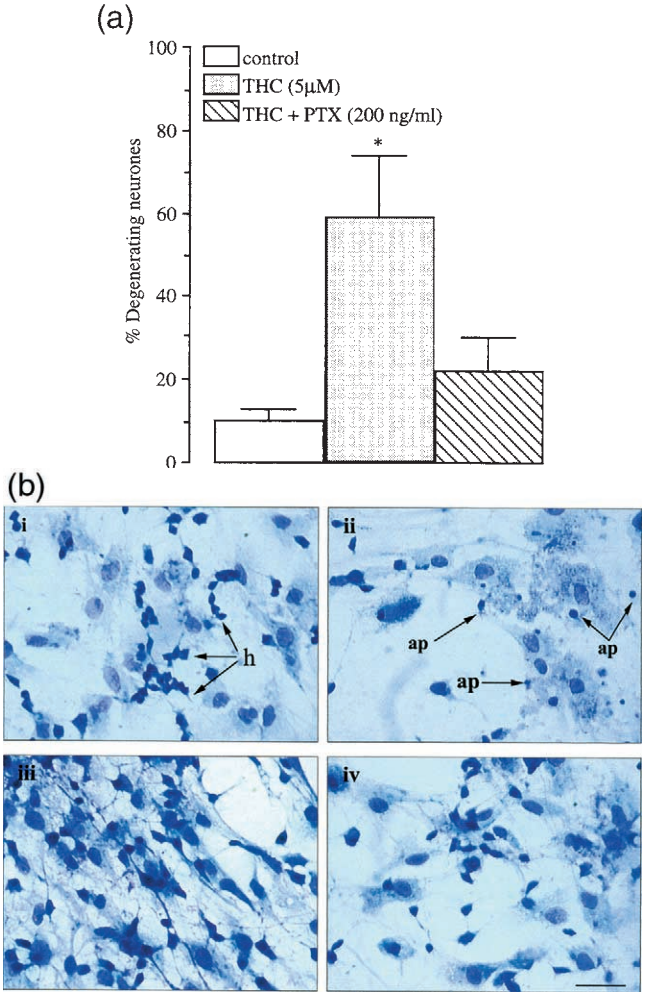


Fig. 2. PTX prevents THC-induced neurodegeneration. (a) Cortical neurones were pretreated with PTX (200 ng/ml) for 24 h and exposed to THC (5 μM) for 3 h. The number of cells displaying morphological features of degeneration was assessed. THC significantly increased neuronal degeneration and this was reduced by PTX. Results are expressed as mean±s.e.m. for 5 observations, * $p<0.01$. (b) Sample photos of (i) control cortical neurones, (ii) THC-treated cells, (iii) PTX-treated cells and (iv) cells treated with THC in the presence of PTX, which were stained with the RapiDiff kit. The arrows indicate examples of healthy (h) and apoptotic (ap) neurones. Scale bar is 50 μm.

### 3.3. THC-induced release of mitochondrial cytochrome c is PTX-sensitive

The ability of THC to promote translocation of mitochondrial cytochrome *c* into the cytoplasm was assessed by western immunoblot on cytoplasmic samples using an anti-cytochrome *c* antibody, in conjunction with densitometric analysis (Fig. 3(a)). In control cells the mean band width was 2.49±0.15 (arbitrary units; mean±s.e.m., $n=4$) and this was significantly increased by THC to 3.48±0.27 ($p<0.05$, Student's paired $t$-test, $n=4$). PTX treatment had no effect on band width (2.11±0.21) and it prevented the THC-induced increase in band width (2.29±0.43). A sample immunoblot dem-

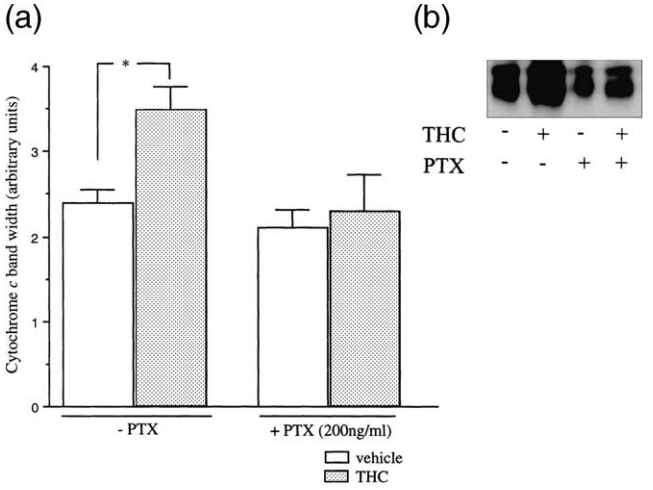


Fig. 3. THC promotes translocation of cytochrome *c* from the mitochondria to the cytosol. Cortical neurones were incubated with THC (5 μM) for 3 h, cells were harvested and cytosolic fractions were analysed for cytochrome *c* expression using western immunoblot. (a) THC significantly increased cytochrome *c* concentration in the cytosol. While PTX treatment had no effect on cytosolic cytochrome *c* expression, PTX inhibited the THC-induced release of mitochondrial cytochrome *c*. Results are expressed as mean±s.e.m. of 5 observations, * $p<0.01$. (b) A sample western immunoblot which demonstrates the increase in cytosolic cytochrome *c* expression in THC-treated cortical neurones (lane 2) compared to control cells (lane 1). While PTX had no effect on cytosolic cytochrome *c* expression (lane 3) it abolished the ability of THC to promote translocation of mitochondrial cytochrome *c* to the cytoplasm (lane 4).

onstrating the translocation of cytochrome *c* following THC treatment, and the abolition of this effect in PTX-treated samples is shown in Fig. 3(b).

### *3.4. THC has no effect on caspase-1 activity in cultured cortical neurones*

Exposure of cortical neurones to THC (5 μm) for 3 h had no effect on activity of caspase-1 (Fig. 4(a)). Caspase-1 activity was 0.23±0.06 pmoles AFC produced/mg protein/min (mean±s.e.m., $n=4$) in control cells, 0.17±0.03 pmoles AFC produced/mg protein/min in THC-treated cells and 0.15±0.01 pmoles AFC produced/mg protein/min in cells treated with THC in the presence of PTX (200 ng/ml).

### *3.5. THC increases caspase-3 activity in a PTX-sensitive manner*

Treatment of cortical neurones with THC (5 μm) for 3 h significantly increased the activity of caspase-3 (Fig. 4(b)). Caspase-3 activity in control cells was 0.11±0.09 pmoles AFC produced/mg protein/min (mean±s.e.m.) and this was significantly increased to 0.26±0.05 pmoles AFC produced/mg protein/min in THC-treated cells ($p<0.05$, Student's paired *t*-test, $n=5$). In the presence of PTX (200 ng/ml), THC had no effect on caspase-3

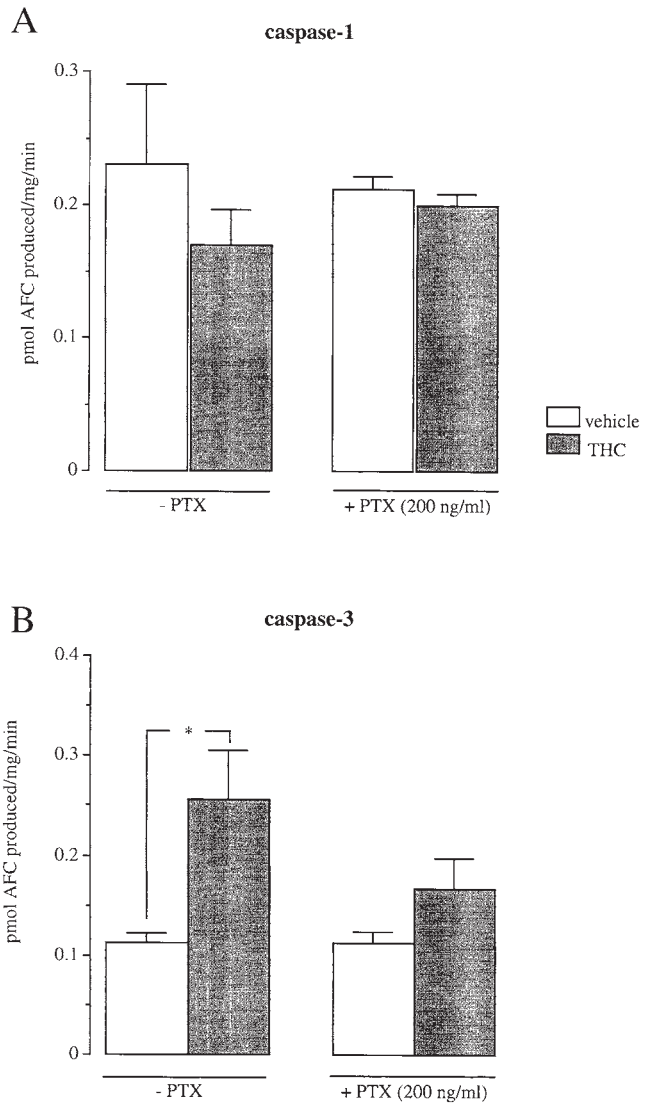


Fig. 4. Effect on THC on caspase activity in cultured cortical neurones. (a) Caspase-1 activity was assessed by cleavage of the fluorogenic peptide YVAD. Exposure of cells to THC (5 μM) for 3 h had no effect on caspase-1 activity. Results are expressed as mean±s.e.m. for 5 observations. (b) Caspase-3 activity was assessed by cleavage of the fluorogenic peptide DEVD. Exposure of cells to THC (5 μM) for 3 h significantly increased caspase-3 activity. Pretreatment of the cells with PTX abolished the THC-induced increase in caspase-3 activity. Results are expressed as mean±s.e.m. for 5 observations, * $p<0.01$.

activity (0.17±0.03 pmoles AFC produced/mg protein/min, $n=5$). In cortical neurones exposed to PTX alone, caspase-3 activity was comparable to that measured in control cells (0.11±0.06 pmoles AFC produced/mg protein/min).

Since activation of caspase-3 is likely to precede cell death the time-course of THC-induced activation of caspase-3 was examined. Caspase-3 activity was 0.09±0.03 pmoles AFC produced/mg protein/min in control cells and was not significantly affected by exposure to THC (5 μM) for 5 min (0.15±0.09 pmoles AFC produced/mg protein/min) or 15 min (0.19±0.05 pmoles AFC

produced/mg protein/min). However, exposure of the neurones to THC for 30 min significantly increased caspase-3 activity to 0.23±0.08 pmoles AFC produced/mg protein/min ($p<0.05$, Student's paired *t*-test, $n=5$) and a 120 min exposure to THC significantly increased caspase-3 activity to 0.25±0.04 pmoles AFC produced/mg protein/min ($p<0.05$, Student's paired *t*-test, $n=5$). Thus, THC-induced activation of caspase-3 occurs prior to the morphological features of degeneration, reaching a maximal activation at 120 min.

## 4. Discussion

The aim of this study was to examine the effects of THC on neuronal viability and to characterise the mechanisms underlying THC-induced cell death. THC was found to promote degeneration of cultured cortical neurones in a time-dependent manner, with a maximal effect occurring 3 hours post-treatment. The proclivity of THC to induce neurodegeneration was blocked by the CB1 antagonist AM251 and PTX, implicating a role for the CB1 receptor and subsequent activation of the G-protein subtypes $G_o/G_i$ in this cell death cascade. While THC had no effect on caspase-1 activity, it significantly increased cytochrome *c* release from the mitochondria and caspase-3 activity, in a PTX-sensitive manner. These results suggest that G-protein coupling of the cannabinoid receptor to effectors involved in the regulation of cytochrome *c* release and caspase-3 activation may be pertinent in mediating the neurotoxic properties of THC.

The finding that THC promotes degeneration of cultured cortical neurones is consistent with the effects of THC on hippocampal neurones (Chan et al., 1998) which has been shown to be due to CB1 receptor activation. The results presented here demonstrate that the toxic effects of THC involve a PTX-sensitive G-protein, which is consistent with the evidence that links the CB1 receptor to activation of the G-protein subtypes $G_i/G_o$ (Howlett, 1995). The role of G-proteins in neuronal apoptosis is not clear since PTX-sensitive G-proteins have been found to exert both pro-apoptotic (Farkas et al., 1998) and anti-apoptotic effects (Jakob and Kreiglsten, 1997). This study implicates PTX-sensitive G-proteins as facilitators of THC-induced apoptosis, with potential effectors being members of the Bcl family of mitochondria-associated proteins. This suggestion stems from the finding that THC caused the release of cytochrome *c* from the mitochondria in a PTX-sensitive manner. Cytochrome *c* release is regulated by the mitochondria-associated proteins Bcl and Bax, which are anti- and pro-apoptotic, respectively, by virtue of their ability to inhibit or promote cytochrome *c* release (Rosse et al., 1998). In macrophages, THC has been found to decrease expression of Bcl-2 (Zhu et al., 1998) which would be expected to promote cytochrome *c* release. The accumulation of cytochrome *c* in the cytosol plays a pivotal role in the activation of caspase-3, a key executor of cell death which has been shown to cleave structural and nuclear proteins as part of the death cascade (Janicke et al., 1998). In this study THC was found to activate caspase-3 in cultured cortical neurones, presumably as a downstream consequence of the THC-induced liberation of cytochrome *c* from the mitochondria. The ability of THC to impact on these components of the cell death pathway was blocked by PTX, suggesting that the neurotoxic effects of THC are mediated by coupling of a PTX-sensitive G-protein to cytochrome *c* release and caspase-3 activation. The nature of the link between PTX-sensitive G-protein activation and release of mitochondrial cytochrome *c* was not addressed in this study, however, PTX-sensitive G-proteins have been demonstrated to couple to the monomeric GTP-binding protein Ras (Crespo et al., 1994) and Ras has the proclivity to regulate expression of members of the Bcl-2 family (Yu et al., 1997). Therefore, an interaction between Ras and Bcl-2 may underlie the release of cytochrome *c*, and subsequent caspase-3 activation, which is stimulated by a PTX-sensitive G-protein following exposure of cells to THC. The CB1 receptor is also linked to induction of c-jun-N-terminal kinase and p38 mitogen-activation protein kinase activity in a PTX-sensitive manner (Rueda et al., 2000), and since these kinases have been linked to neuronal apoptosis by increasing mitochondrial bax expression and promoting caspase activation (Ghatan et al., 2000), it is possible that they play a role in THC-induced neuronal cell death. The CB1 receptor is also linked to inhibition of adenylyl cyclase activity (Childers and Deadwyler, 1996) and ceramide accumulation (Galve-Roperh et al., 2000) both of which may play a role in linking the CB1 receptor-associated G-protein to cytochrome *c* release and caspase-3 activation.

In macrophages and lymphocytes the toxic effects of THC have been shown to involve caspase-1 (Zhu et al., 1998), a cysteine protease which is responsible for converting the proinflammatory cytokine interleukin-1ß into its biologically active form. While caspase-1 has been shown to play a role in apoptosis in other neuronal systems (Vereker et al., 2000), THC did not alter caspase-1 activity in cortical neurones, suggesting that this caspase is not pertinent in THC-induced neuronal cell death.

Plasma concentrations of THC are in the low μM range following consumption of a single marijuana cigarette (Chiang and Barnett, 1984), and THC concentrations in the brain are likely to be comparable given that THC is a lipophilic molecule and will readily cross the blood–brain barrier. The dose of THC used in this study, which was found to be neurotoxic to cultured cortical neurones, was within this concentration range. Our finding that neonatal cultured neurones responded to THC by undergoing apoptosis within a few hours may reflect a developmental response profile to THC, since

studies have shown that adult animals exposed to THC for longer periods of time do not undergo neuronal apoptosis (Galve-Roperh et al., 2000). Our results are more likely to reflect responses of the neonatal nervous system to THC, such as would occur when THC crosses the fetoplacental barrier. The proclivity of THC to induce apoptosis in neonatal neurones may underlie the central nervous system abnormalities (Freid, 1980) and impaired neurobehavioral development (Freid and Watkinson, 1990) that occurs in infants exposed to marijuana in utero.

It is of interest note that in vivo administration of THC fails to induce neurotoxicity in rats (Galve-Roperh et al., 2000). Similarly, THC has neuroprotective properties against glutamate-induced toxicity and ischemia, which are due to its antioxidant effects (Hampson et al., 2000). The ability of THC to protect against oxidative cell death is thought to occur via a receptor-independent mechanism (Chen and Buck, 2000). In contrast, THC is toxic to cultured hippocampal neurones (Chan et al., 1998) and subcutaneous administration of cannabinoids induces morphological changes in the hippocampus that are consistent with ischemic or toxic damage (Lawston et al., 2000). Thus, the data regarding the effects of THC on neuronal viability is conflicting and this may reflect the influence of developmental factors and differential responsiveness of specific brain regions to THC (Louw et al., 2000).

In summary, the finding that THC activates the caspase-3 cell death pathway to cause degeneration of cortical neurones may contribute to the impaired cognitive function and neurodevelopmental deficits that are associated with exposure to marijuana.

## References


Berrendero, F., Garcia, L., Hernandez, M.L., Romero, J., Cebeira, M., De Miguel, R., Ramos, J.A., Fernandez-Ruiz, J.J., 1998. Localization of mRNA expression and activation of signal transduction mechanisms for cannabinoid receptor in rat brain during fetal development. Development 125 (16), 3179–3188.

Block, R.I., Ghoneim, M.M., 1993. Effects of chronic marijuana use on human cognition. Psychopharmacology 110 (1–2), 219–228.

Bouaboula, M., Bianchini, L., McKenzie, F.R., Pouyssegur, J., Casellas, P., 1999. Cannabinoid receptor CB1 activates the Na/H exchanger NHE-1 isoform via Gi-mediated mitogen-activated protein kinase signalling transduction pathways. FEBS Letters 16 (449), 61–65.

Chan, G.C., Hinds, T.R., Impey, S., Storm, D.R., 1998. Hippocampal neurotoxicity of $\Delta^9$-tetrahydrocannabinol. Journal of Neuroscience 18 (14), 5322–5332.

Chen, Y., Buck, J., 2000. Cannabinoids protect cells from oxidative cell death: a receptor-independent mechanism. Journal of Pharmacology and Experimental Therapeutics 293 (3), 807–812.

Chiang, C.W., Barnett, G., 1984. Marijuana effect and delta-9-tetrahydrocannibinol plasma level. Clinical Pharmacology and Therapeutics 36 (2), 234–238.

Childers, S.R., Deadwyler, S.A., 1996. Role of cyclic AMP in the actions of cannabinoid receptors. Biochemical Pharmacology 52 (6), 819–827.

Crespo, P., Xu, N., Simonds, W.F., Gutkind, J.S., 1994. Ras-dependent activation of MAP kinase pathway mediated by G-protein ßγ subunits. Nature 369, 418–420.

Farkas, I., Baranyi, L., Takahashi, M., Fukuda, A., Liposits, Z., Yamamoto, T., Okada, H., 1998. A neuronal C5a receptor and its associated apoptotic signal transduction pathway. Journal of Physiology 15 (507), 679–687.

Freid, P.A., 1980. Marijuana use by pregnant women: neurobehavioral effects on neonates. Drug and Alcohol Dependency 6 (6), 415–424.

Freid, P.A., Watkinson, B., 1990. 36- and 48-month neurobehavioral follow-up of children pre-exposed to marijuana, cigarettes and alcohol. Journal of Development and Behavioral Pediatrics 11 (2), 49–58.

Galve-Roperh, I., Sanchez, C., Cortes, M.L., Pulgar, T.G., Izquierdo, M., Guzman, M., 2000. Anti-tumoral action of cannabinoids: Involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nature Medicine 6 (3), 313–319.

Gessa, G.L., Casu, M.A., Carta, G., Mascia, M.S., 1998. Cannabinoids decrease acetylcholine release in the medial-prefrontal cortex and hippocampus, reversal by SR141716A. European Journal of Pharmacology 355 (2–3), 119–124.

Ghatan, S., Larner, S., Kinoshita, Y., Hetman, M., Patel, L., Xia, Z., Youle, R.J., Morrison, R.J., 2000. p38 MAP kinase mediates bax translocation in nitric-oxide-induced apoptosis in neurones. Journal of Cell Biology 150 (2), 335–347.

Hampson, A.J., Grimaldi, M., Lolic, M., Wink, D., Rosenthal, R., Axelrod, J., 2000. Neuroprotective antioxidants from marijuana. Annals of the New York Academy of Sciences 899, 274–282.

Hampton, M.B., Fadeel, B., Orrenius, S., 1998. Redox regulation of the caspases during apoptosis. Annals of the New York Academy of Sciences 20 (854), 328–335.

Howlett, A.C., 1990. Reverse pharmacology applied to the cannabinoid receptor. Trends in Pharmacological Science 11 (10), 395–397.

Howlett, A.C., 1995. Pharmacology of cannabinoid receptors. Annual Review of Pharmacology and Toxicology 35, 607–634.

Jakob, R., Kreiglsten, J., 1997. Influence of flupirtine on a G-protein coupled inwardly rectifying potassium current in hippocampal neurones. British Journal of Pharmacology 122 (7), 1333–1338.

Janicke, R.U., Sprengart, M.L., Wati, M.R., Porter, A.G., 1998. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. Journal of Biological Chemistry 273 (16), 9357–9360.

Jentsch, J.D., Andrusiak, E., Tran, A., Bowers, M.B., Roth, R.H., 1997. Delta 9-tetrahydrocannabinol increases prefrontal cortical catecholaminergic utilization and impairs spatial working memory in the rat: blockade of dopaminergic effects with HA966. Neuropsychopharmacology 16 (6), 426–432.

Jentsch, J.D., Verrico, C.D., Le, D., Roth, R.H., 1998. Repeated exposure to delta-9-tetrahydrocannibinol reduces prefrontal cortical dopamine metabolism in the rat. Neuroscience Letters 246 (3), 169–172.

Korsmeyer, S.J., 1995. Regulators of cell death. Trends in Genetics 11 (3), 101–105.

Lawston, J., Borella, A., Robinson, J.K., Whitaker-Azmitta, P.M., 2000. Changes in hippocampal morphology following chronic treatment with the synthetic cannabinoid WIN 55, 212-2. Brain Research 877 (2), 407–410.

Louw, D.F., Yang, F.W., Sutherland, G.R., 2000. The effect of delta-9-tetrahydrocannabinol on forebrain ischemia in rat. Brain Research 857 (1–2), 183–187.

Mackie, K., Hille, B., 1992. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proceedings of the National Academy of Sciences of the USA 89, 3825.

Mathew, R.J., Wilson, W.H., Coleman, R.E., Turkington, T.G.,

DeGrado, T.R., 1997. Marijuana intoxication and brain activation in marijuana smokers. Life Sciences 60 (23), 2075–2089.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Raffray, M., Cohen, G.M., 1997. Apoptosis and necrosis in toxicology; A continuum or distinct modes of cell death? Pharmacological Therapeutics 75 (3), 153–177.

Reisine, T., Brownstein, M.J., 1994. Opiod and cannabinoid receptors. Current Opinion in Neurobiology 4 (3), 406–412.

Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B., Borner, C., 1998. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome *c*. Nature 391, 496–499.

Rueda, D., Galve-Roperh, I., Haro, A., Guzman, M., 2000. The CB1 cannabinoid receptor is coupled to activation of C-jun N-terminal kinase. Molecular Pharmacology 58 (4), 814–820.

Sanchez, C., Galve-Roperh, I., Canova, C., Brachet, P., Guzman, M., 1998. $\Delta^9$-tetrahydrocannabinol induces apoptosis in C6 glioma cells. FEBS Letters 25 (436), 6–10.

Yu, K., Ying-Nan, P.C., Ravera, C., Bayonna, W., Nalin, C.M., Mallon, R., 1997. Ras-dependent apoptosis correlates with persistent activation of stress-activated protein kinases and induction of isoforms of Bcl. Cell Death Differentiation 4, 745–755.

Vereker, E., Campbell, V., Roche, E., McEntee, E., Lynch, M.A., 2000. Lipopolysaccharide inhibits long-term potentiation in the rat dendate gyrus by activating caspase-1. Journal of Biological Chemistry 275, 26252–26258.

Zhu, W., Freidman, H., Klein, T.W., 1998. $\Delta^9$-tetrahydrocannabinol induces apoptosis in macrophages and lymphocytes: involvement of Bcl-2 and caspase-1. Journal of Pharmacology and Experimental Therapeutics 286, 1103–1109.