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RESEARCH PAPER

Interactions between CB_1 receptors and TRPV1 channels mediated by 12-HPETE are cytotoxic to mesencephalic dopaminergic neurons

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Background and purposes: We recently proposed the existence of neurotoxic interactions between the cannabinoid type 1 (CB_1) receptor and transient receptor potential vanilloid 1 (TRPV1) channels in rat mesencephalic cultures. This study seeks evidence for the mediator(s) and mechanisms underlying the neurotoxic interactions between CB_1 receptors and TRPV1 *in vitro* and *in vivo*.
Experimental approach: The mediator(s) and mechanism(s) for the interactions between CB_1 receptors and TRPV1 were evaluated by cell viability assays, immunocytochemistry, Fura-2 calcium imaging, mitochondrial morphology assay, ELISA and Western blot assay *in vitro* in neuron-enriched mesencephalic cultures. Injections into the substantia nigra and subsequent cell counts were also used to confirm these interactions *in vivo*.
Key results: The neurotoxic interactions were mediated by 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HPETE), an endogenous TRPV1 agonist. CB_1 receptor agonists (HU210 and WIN55,212-2) increased the level of 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), a downstream metabolite of 12(S)-HPETE, which stimulates TRPV1-mediated death of mesencephalic neurons, both *in vitro* and *in vivo*. The neurotoxicity was mediated by increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through TRPV1, consequently leading to mitochondrial damage and was attenuated by baicalein, a 12-lipoxygenase inhibitor.
Conclusion and implications: Activation of CB_1 receptors in rat mesencephalic neurons was associated with biosynthesis of 12(S)-HPETE, which in turn stimulated TRPV1 activity, leading to increased $[Ca^{2+}]_i$, mitochondrial damage and neuronal death.
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Keywords: cannabinoid type 1 receptor; transient receptor potential vanilloid 1; 12-hydroperoxyeicosatetraenoic acid; COX-2

Abbreviations: AA, arachidonic acid; CB_1, cannabinoid type 1; Eth-1, ethidium homodimer-1; TRPV1, transient receptor potential vanilloid 1; 12(S)-HPETE, 12(S)-hydroperoxyeicosatetraenoic acid

Introduction

The cannabinoid type 1 (CB_1) receptor belongs to the seven-transmembrane domain family of G-protein-coupled receptors (Matsuda *et al*., 1990; Munro *et al*., 1993). Endogenous ligands of the CB_1 receptor are mainly derivatives of polyunsaturated fatty acids, including the ethanolamide of arachidonic acid (AA) termed 'anandamide' (AEA, arachidonylethanolamide) (Devane *et al*., 1992; Mechoulam *et al*., 1998) and 2-arachidonoylglycerol (Sugiura *et al*., 1995; Mechoulam *et al*., 1998). Several synthetic cannabinoids, such as HU210 (De Petrocellis *et al*., 1998; Pop, 1999) and WIN55,212-2 (Sarfarez *et al*., 2005), have also been characterized. However, the effects of endogenous and synthetic cannabinoids are not always identical as shown in presynaptic effects of AEA and WIN55,212-2 on neurotransmitter release from glutamatergic nerve endings in the hippocampus (Cannizzaro *et al*., 2006).

The widespread distribution of the CB_1 receptor in rat brain, confirmed from autoradiographic (Herkenham *et al*.,

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1991), immunohistochemical (Tsou *et al*., 1998), and *in situ* hybridization (Mailleux and Vanderhaeghen, 1992) studies, suggest that this protein plays a significant role in the CNS. This hypothesis is supported by recent data showing CB_1 receptor-mediated activity in several regions of the rat brain, including basal ganglia (Romero *et al*., 2002). There is accumulating experimental evidence that cannabinoids protect neurons from toxic insults. For instance, AEA and HU210 protect neurons against excitotoxicity *in vivo* (van der Stelt *et al*., 2001) and *in vitro* (Molina-Holgado *et al*., 2005). These neuroprotective effects are blocked by the CB_1 receptor antagonists, SR141716A and AM281. Recent experiments on CB_1 receptor gene-deleted mice demonstrate that the endocannabinoid system is involved in neuroprotection against kainic acid-induced seizure (Marsicano *et al*., 2003; Monory *et al*., 2006). The CB_1 receptor mediates the neuroprotective effects of the agonists, WIN55,212-2 and Δ^9-tetrahydrocannabinol (THC) (the principal psychoactive component of marijuana) against quinolinic acid-induced striatal damage (Pintor *et al*., 2006) and NMDA-induced retinal neurotoxicity (El-Remessy *et al*., 2003).

In contrast, several investigators have reported neurotoxic effects of cannabinoids. For instance, AEA induces apoptosis by activation of CB_1 receptor in bone marrow-derived dendritic cells (Do *et al*., 2004). Additionally, THC triggers the degeneration of cultured hippocampal (Chan *et al*., 1998) and cortical (Campbell, 2001) neurons, accompanied by cytochrome *c* release. This neurotoxicity is blocked by the CB_1 receptor antagonist, AM251. WIN55,212-2 induces apoptosis in cerebellar granule cells by activation of the CB_1 receptor, which is completely reversed in the presence of SR141716A (Pozzoli *et al*., 2006). Consistent with these reports, recent experiments by our group show that AEA induces degeneration of mesencephalic dopaminergic neurons through mitochondrial disruption and cytochrome *c* release, which is blocked by AM251 (Kim *et al*., 2005). Similarly, HU210 elicits dopaminergic neuronal death, which is again antagonized by AM251.

Transient receptor potential vanilloid subtype 1 (TRPV1) is a non-selective cation channel activated by vanilloids, such as capsaicin (Caterina and Julius, 2001; Kim *et al*., 2005), endogenous ligands, including AEA (Di Marzo *et al*., 2001; Kim *et al*., 2005) and N-arachidonoyl-dopamine (Huang *et al*., 2002), and products of lipoxygenases, such as 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HPETE) (Hwang *et al*., 2000). Among the lipoxygenase products, 12(S)-HPETE biosynthesized from AA by 12-lipoxygenase (Hwang *et al*., 2000; Shin *et al*., 2002) is the most potent TRPV1 agonist (Hwang *et al*., 2000). 12(S)-HPETE induces long-term depression in the hippocampus (Kim *et al*., 2005), and neurodegeneration in mesencephalic cultures (Canals *et al*., 2003). We recently showed that 12(S)-HPETE triggers TRPV1-mediated microglial cell death in the substantia nigra *in vivo* (Kim *et al*., 2006). Interestingly, bradykinin, a potent inflammatory mediator, interacts with bradykinin B_2 receptors in sensory neurons, and activates TRPV1 by production of 12(S)-HPETE from AA. This process is effectively blocked by the TRPV1 antagonist, capsazepine (Shin *et al*., 2002).

In view of our findings that capsazepine and AM251 reverse HU210- and capsaicin-induced neurotoxicity, by inhibiting Ca^{2+} influx in cultured mesencephalic dopaminergic neurons co-expressing the CB_1 receptor and TRPV1 (Kim *et al*., 2005), we examined the possibility of functional interactions between the two receptors. Our results indicated that stimulation of the CB_1 receptor with its agonists, HU210 and WIN55,212-2, produced 12(S)-HPETE, which in turn activated TRPV1 and caused neurodegeneration by Ca^{2+} influx in neuron-enriched mesencephalic cultures. Additional experiments demonstrated that HU210- and WIN55,212-2-induced expression of COX-2 also contributed to neurotoxicity, but this was independent of Ca^{2+} influx by TRPV1.

Materials and methods

Neuron-enriched mesencephalic cell cultures

Sprague-Dawley rat ventral mesencephalon were isolated from embryonic day 14 (E14) foetal brain and dissected as described previously (Choi *et al*., 2003b; Kim *et al*., 2005; Lee *et al*., 2005). Tissues were cut into small segments and incubated in Ca^{2+}-, Mg^{2+}-free Hanks' balanced salt solution (CMF-HBSS) for 10 min at 37 °C. Cultures were replaced with a 0.01% trypsin solution in CMF-HBSS, incubated for an additional 9 min, rinsed twice in RF (Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD, USA) supplemented with 10% foetal bovine serum, 6 mg mL^{-1} glucose, 204 μg mL^{-1} L-glutamine, 100 U mL^{-1} penicillin-streptomycin) and mechanically triturated. Dissociated cells were manually counted with a haemocytometre, and plated on 12-mm round aclar plastic coverslips precoated with poly-D-lysine (0.1 mg mL^{-1}) and laminin (2 μg mL^{-1}) housed in 24-well culture plates (1×10^5 cells per coverslip) for immunohistochemistry and cell viability assay, or on 25 mm round glass coverslips precoated with poly-D-lysine and laminin in 35 mm culture dishes (4×10^5 cells per coverslip) for measurement of cytosolic Ca^{2+} and observation of mitochondria. Cells were incubated in a humidified incubator at 37 °C, 5% CO_2 for 24 h. To suppress the proliferation of glial cells, at 2 days *in vitro* (DIV), the medium was replaced with a chemically defined serum-free medium (DM) composed of Ham's nutrient mixture (F12-Dulbecco's modified Eagle's medium) and supplemented with insulin, transferrin, selenium, glucose, L-glutamine and penicillin-streptomycin. At the fourth day *in vitro*, cultures were transferred to DM without insulin, transferrin, selenium and used for each experiment. When immunostained with cell-type specific antibodies, cultures contained 5% astrocytes and less than 1% microglia (Choi *et al*., 2003b; Lee *et al*., 2005). As described previously, the remaining cells were presumed to be neurons, of which 4.5–6% were TH-immunopositive (TH-ip) (Lee *et al*., 2005).

Cell viability (live and dead) assays

Mesencephalic neurons seeded on 25 mm coverslips were treated with CB_1 receptor agonists, and the cultures were stained 24 h later with 2 μM calcein-acetoxymethyl ester and 4 μM ethidium homodimer-1 (Eth-1). The calcein-positive live cells (green) and ethidium-positive dead cells (red) were

visualized using a confocal laser scanning microscope (Olympus) (Kim *et al*., 2005, 2006).

Immunocytochemistry
Cultures and brain tissue were prepared for immunostaining as described (Kim *et al*., 2005). The primary antibodies included those directed against neuron-specific nuclear protein (Chemicon, Temecula, USA), TH (Pel-freez, Biologicals, Rogers, AR, USA) and cytochrome *c* (Promega, Madison, WZ, USA). For Nissl staining, some of the substantia nigra tissue samples were stained in 0.5% cresyl violet. Stained cells were viewed and analysed under a bright-field microscope (Nikon) or viewed with a confocal laser scanning microscope (Olympus).

Measurement of intracellular Ca^{2+}
Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were assayed as described (Kim *et al*., 2005, 2006). Briefly, mesencephalic neurons seeded on 25 mm coverslips were preloaded with 5 μM Fura-2 dye plus 2% Pluronic F-127. After incubation for 30 min at 37 °C, the cells were washed three times in HBSS (containing no $CaCl_2$), supplemented with 145 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 20 mM HEPES (pH 7.4), to remove excess dye. The cells were selected by fluorescence microphotometry in HBSS containing 1.8 mM $CaCl_2$. $[Ca^{2+}]_i$ was determined on the basis of the ratio of Fura-2 fluorescence (R) at 340 and 380 nm, which were measured at 10 s intervals with a Zeiss inverted microscope and CCD camera, and analysed using an Ion Application (Empix, Cheektowaga, NY, USA).

12(S)-hydroxyeicosatetraenoic acid immunoassay
As described (Shin *et al*., 2002), the level of 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), a downstream metabolite of 12(S)-HPETE, in neuron-enriched mesencephalic cultures was determined by using a 12(S)-HETE enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA). The 12(S)-HETE antibody showed low crossreactivity (<0.3%) with 5(S)- or 15(S)-HETE, or prostaglandins, and with 12(R)-HETE (2.5%). Briefly, standards and samples were loaded in 96-well plates coated with goat anti-rabbit IgG. Alkaline phosphatase conjugated with 12(S)-HETE and rabbit polyclonal antibody to 12(S)-HETE were added to each well and incubated for 2 h. Colorimetric density of the reaction was read at 405 nm immediately after incubation with *p*-nitrophenyl phosphate for 3 h.

Mitochondrial morphology assay
As described (Kim *et al*., 2005, 2006), cultured mesencephalic neurons seeded on 25 mm coverslips were incubated with 250 nM Mito-Tracker dye for 30 min and washed three times in HBSS. The cells were finally examined under a confocal laser scanning microscope at 10 s intervals.

Western immunoblot analysis
As described (Kim *et al*., 2005, 2006), mitochondrial fractions from cultured mesencephalic neurons treated with HU210 or WIN55,212-2 were prepared. Mesencephalic neurons were homogenized with buffer containing 20 mM HEPES, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride and a protease inhibitor cocktail (Sigma, St Louis, MO, USA). The homogenates were centrifuged at 500 *g* for 10 min at 4 °C, and the supernatant was centrifuged at 100 000 *g* for 1 h at 4 °C in an ultracentrifuge (Beckman, Fullerton, CA, USA). The supernatant from this centrifugation was considered as the cytosolic fraction and the pellet was considered as the membrane and mitochondria-rich fraction. The protein concentration was determined using a BCA kit. Equal amounts of protein were separated by 12% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) using an electrophoretic transfer system (Bio Rad, Hercules, CA, USA). The membranes were immunoblotted with anti-cytochrome *c* (BD Pharmingen, San Jose, CA, USA) and proteins were visualized using the ECL kit (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). To confirm whether CB_1 receptor agonists increased COX-1 and COX-2, whole proteins from cultured mesencephalic neurons treated with HU210 or WIN55,212-2 were also prepared. Mesencephalic neurons were homogenized in ice-cold lysis buffer containing 20 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride plus protease inhibitor cocktail. The homogenates were centrifuged for 20 min at 14 000 *g* at 4 °C and each of the supernatants was transferred to a fresh tube. The protein concentration was determined using a BCA kit and samples were boiled at 100 °C for 5 min before gel loading. Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-COX-1 or anti-COX-2 (Santa Cruz, Santa Cruz, CA, USA), and visualized using the ECL kit. For semiquantitative analyses, the density of immunoblot bands was measured with the Computer Imaging Device and accompanying software (Fuji Film).

Stereotaxic injection of 12(S)-HPETE in vivo
All experiments were performed in accordance with approved animal protocols and guidelines established by Ajou University. Anaesthesia was induced with chloral hydrate (i.p. injection with 360 mg kg^{-1} as described by Choi *et al*. (2003a, 2005)), in female Sprague-Dawley rats (approximately 230–250 g). They then received a unilateral injection of 3 μL 12(S)-HPETE, capsazepine, or mixture of 12(S)-HPETE and capsazepine, at a rate of 0.5 μL min^{-1}, into the right substantia nigra (AP −5.3, ML −2.3, DV −7.6 mm from bregma), according to the atlas of Paxinos and Watson (1998), using a 30-gauge Hamilton syringe attached to an automated microinjector (De Petrocellis *et al*., 1998). After injection, the needle was left in place for an additional 5 min before slow retraction. Animals were humanely killed by an overdose of chloral hydrate at 7 days post-lesion and brains were removed, sectioned and stained with cresyl violet, or immunostained with antibodies to neuron-specific nuclear protein or TH.

Stereological estimation

As described (Choi *et al*., 2003a; Kim *et al*., 2005), the total number of TH-positive neurons in the substantia nigra was counted in the various groups of animals at 7 days after vehicle, 12(S)-HPETE or capsazepine injection using the optical fractionator method using the Olympus CAST (Computer Assisted Stereological Toolbox) system version 2.1.4. This is an unbiased stereological method of cell counting that is not affected by either the volume of reference (substantia nigra pars compacta) or the size of the counted elements (neurons).

Statistical analysis

All values are expressed as mean ± s.e.mean. Statistical significance was assessed by ANOVA using Instat 3.05 (GraphPad Software), followed by Student–Newman–Keuls analyses.

Materials

Materials were purchased from the following companies: cell viability assay (Live and Dead kit), Fura-2-AM, Mito-Tracker (Molecular Probes, Eugene, OR, USA), capsazepine, AM251, HU210, WIN55,212-2 (Tocris, Ellisville, MO, USA), anti-cytochrome *c* (Promega or Pharmingen), 5(S)-, 12(S)- and 15(S)-HPETE, NS398, DuP-697 (Cayman, Ann Arbor, MI, USA), anti-COX-1, anti-COX-2 (Santa Cruz), baicalein, REV-5901, indomethacin (Biomol, Plymouth Meeting, PA, USA). HU210, WIN55,212-2 and NS398 were dissolved in dimethyl sulphoxide (DMSO), DuP-697 was dissolved in dimethylformamide and then diluted with sterile phosphate-buffered saline (1:1 solution of dimethylformamide/phosphate-buffered saline), and others were dissolved in ethanol. The final concentration of all vehicles for treatment on cultures was 0.5% and there was no neurotoxicity, compared with vehicle-untreated controls. The vehicle used to dissolve 12(S)-HPETE for stereotaxic injection in the substantia nigra was sterile phosphate-buffered saline containing 14% ethanol (De Petrocellis *et al*., 1998; Kim *et al*., 2005).

Results

Evidence for neurotoxic interactions between CB_1 receptors and TRPV1

We had proposed that functional interactions between CB_1 receptors and TRPV1 contributed to the death of cultured mesencephalic neurons (including dopaminergic neurons), as in neuron-enriched mesencephalic cultures co-expressing both receptors, neurotoxicity induced by the CB_1 receptor agonist, HU210, was inhibited by the TRPV1 antagonist, capsazepine (Kim *et al*., 2005). To confirm these findings, we initially examined whether another CB_1 receptor agonist, WIN55,212-2 (Nagayama *et al*., 1999; Ramer *et al*., 2003; Pintor *et al*., 2006), induced neurotoxicity in our cultures. Treatment of neuron-enriched mesencephalic cultures with 6 μM WIN55,212-2 resulted in a marked increase in the number of dead Eth-1-positive cells (Figure 1e), accompanied by a decline in the number of calcein-acetoxymethyl ester-positive live cells (Figure 1d), compared with vehicle (0.5% DMSO)-treated controls (Figures 1a and b). This toxicity was attenuated by pretreatment with 5 μM capsazepine for 5 min (Figures 1g and h). Similar results were obtained with 3 μM HU210 (data not shown). Cells were quantified as a percentage of untreated control values. Treatment with 1 μM WIN55,212-2 or 1 μM HU210 had no effects (data not shown), whereas 6 μM WIN55,212-2 and 3 μM HU210 produced a 66% ($P<0.001$) and 76% ($P<0.001$) increase in Eth-1-positive cells, respectively, compared to non-treated control cultures (Figure 1j). Pretreatment with AM251, a CB_1

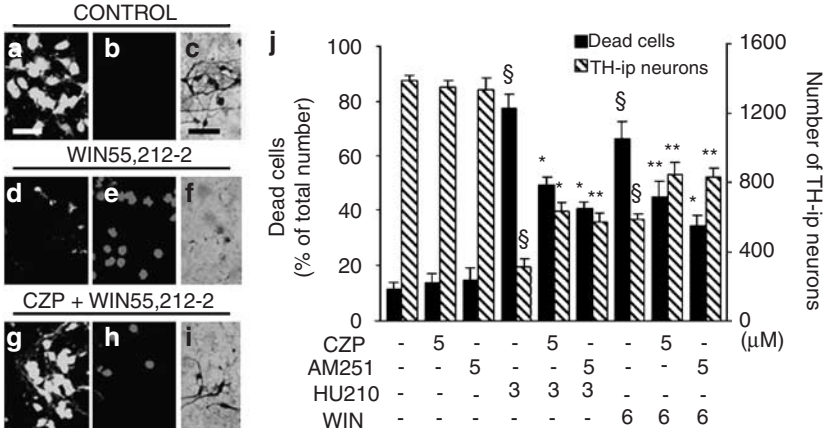

Figure 1 Neurotoxicity induced by WIN55,212-2 or HU210 in neuron-enriched mesencephalic cultures. Cultures were treated with 0.5% dimethyl sulphoxide (DMSO) as a control (**a**–**c**), 6 μM WIN55,212-2 (WIN) (**d**–**i**) or 3 μM HU210 for 24 h. Where indicated, cells were pretreated with 5 μM transient receptor potential vanilloid subtype 1 (TRPV1) antagonist capsazepine (CZP; **g**–**i**) or 5 μM CB_1 receptor antagonist AM251 before 5 min, and stained with calcein-acetoxymethyl ester (calcein-AM, green for live; **a**, **d**, **g**) and ethidium homodimer-1 (Eth-1, red for dead; **b**, **e**, **h**), or immunostained with TH antibody (**c**, **f**, **i**), respectively. In all cultures, cell death was assessed by counting the number of Eth-1-positive dead cells or TH-immunopositive (ip) cells (**j**). All values represent the mean ± s.e.mean of triplicate cultures in four separate platings. §$P<0.001$, significant from control; *$P<0.01$ and **$P<0.05$, significant from treatment with HU210 or WIN55,212-2. Scale bars: **a**–**b**, **d**–**e**, **g**–**h**, 40 μm; **c**, **f**, **i**, 100 μm. A colour version of this figure is available online.

receptor antagonist, partially attenuated the number of Eth-1-positive cells induced by WIN55,212-2 (31%; $P<0.01$) and HU210 (36%; $P<0.001$). Moreover, pretreatment with 5 μM capsazepine, a TRPV1 antagonist, reduced the number of Eth-1-positive cells observed with 6 μM WIN55,212-2 (21%; $P<0.01$) and 3 μM HU210 (25%; $P<0.001$). Tyrosine hydroxylase immunostaining data confirmed that the addition of WIN55,212-2 to cultures led to the death of dopaminergic neurones (Figure 1f), compared to non-treated control cultures (Figure 1c). Similarly, this neurotoxicity was attenuated upon pretreatment with capsazepine for 5 min (Figure 1i). Quantification of cells as a percentage of non-treated control values disclosed that WIN55,212-2 and HU210 reduced the number of TH-ip neurons by 58% ($P<0.001$) and 77% ($P<0.001$), respectively (Figure 1J). Moreover, pretreatment with AM251 partially prevented the reduction in TH-ip neurons by WIN55,212-2 (18%; $P<0.05$) and HU210 (19%; $P<0.05$). Similarly, pretreatment with 5 μM capsazepine attenuated the effects of 6 μM WIN55,212-2 (19%; $P<0.05$) and 3 μM HU210 (21%; $P<0.01$).

We recently demonstrated that HU210 stimulates influx of extracellular Ca^{2+} in cultured mesencephalic neurons, resulting in cell death (Kim *et al*., 2005). Accordingly, we examined the effects of WIN55,212-2 on Ca^{2+} influx, in our cultures. Administration of 3 μM HU210 (Figure 2a) or 6 μM WIN55,212-2 (Figure 2b) led to an increase in Fura-2 fluorescence intensity in cultured mesencephalic neurons in the presence of extracellular Ca^{2+}, signifying elevation of $[Ca^{2+}]_i$. HU210- and WIN55,212-2-induced increases in $[Ca^{2+}]_i$ were abolished in Ca^{2+}-free extracellular solution (Figures 2c and d) and the presence of 5 μM capsazepine (Figures 2e and f), respectively. Moreover, $[Ca^{2+}]_i$ elevation was blocked by pretreatment with 5 μM AM251 (data not shown), consistent with our earlier report (Kim *et al*., 2005).

Next, we determined whether the HU210- or WIN55,212-2-induced increase in $[Ca^{2+}]_i$ contributes to mitochondrial disruption in live cells, and consequent cytochrome *c* release. In vehicle-treated controls, cells displayed intact mitochondrial structure, as determined with Mito-Tracker fluorescence (Figures 2g and h). In contrast, mitochondrial disruption was evident after 30 min in cultures treated with 3 μM HU210 (Figures 2i and j). Pretreatment of cells with 5 μM capsazepine for 5 min prevented HU210-induced mitochondrial damage (Figures 2k and l). Analogous to data obtained with HU210, the effects of 6 μM WIN55,212-2 on mitochondrial damage were inhibited by capsazepine (data not shown). Double immunofluorescence staining with Mito-Tracker and cytochrome *c* antibodies revealed that in untreated controls, cytochrome *c* was localized to mitochondria (Figure 2m), whereas in cells treated with 3 μM HU210 (Figure 2n) or 6 μM WIN55,212-2 (Figure 2o), cytochrome *c* was redistributed into the cytosol, indicative of release from mitochondria. This finding was further corroborated by Western blot analyses (Figure 2p).

12(S)-hydroperoxyeicosatetraenoic acid mediates neurotoxic interactions

Activation of CB_1 receptors stimulates the production of AA (Chan *et al*., 1998; Demuth *et al*., 2005), leading to the biosynthesis of 12(S)-HPETE by 12-lipoxygenase (Hwang *et al*.,

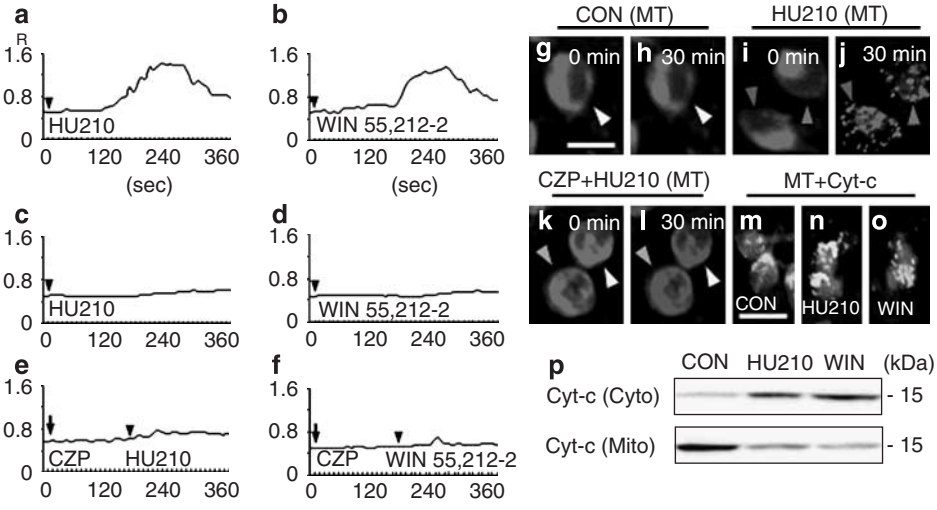

Figure 2 Fluorescence in Fura-2 loaded cultured mesencephalic neurons. Intracellular Ca^{2+} concentration was measured in neuron-enriched mesencephalic cultures treated with 3 μM HU210 (left panel, arrowhead) or 6 μM WIN55,212-2 (right panel, arrowhead) in the presence (**a**–**b**) or absence (**c**–**d**) of 1.8 mM extracellular Ca^{2+}. Response to 3 μM HU210 (**e**) or 6 μM WIN55,212-2 (**f**) of cultures pretreated with 5 μM capsazepine (CZP: arrow) in the presence of 1.8 mM extracellular Ca^{2+}. Data were averaged from 20 to 25 randomly selected cells for each condition and the results shown are representative of four independent experiments. (**g**–**j**) Mitochondrial disruption in neuron-enriched mesencephalic cultures treated with 3 μM HU210 (**i**, **j**) or 0.5% dimethyl sulphoxide (DMSO) as a control (**g**, **h**) in the presence of 1.8 mM extracellular Ca^{2+}. (**k**, **l**) Pretreatment with 5 μM CZP inhibited HU210-induced mitochondrial disruption. In live cells, mitochondrial structure was stained with Mito-Tracker fluorescence dye (MT). Each coloured arrow indicates the same cells. (**m**–**o**) Localization of cytochrome *c* (green) immunoreactivity and MT (red) in cells treated with vehicle (**m**), 3 μM HU210 (**n**) or 6 μM WIN55,212-2 (WIN; **o**) for 12 h. (**p**) Western blot analysis of cytochrome *c* levels after treatment of cells with 3 μM HU210 or 6 μM WIN55,212-2 for 12 h. The results are representative from three independent experiments. Cyt-*c*, cytochrome *c*; Con, control; Cyto, cytosolic fraction; Mito, mitochondrial fraction. Scale bars: **g**–**l**, 20 μm; **m**–**o**, 30 μm. A colour version of this figure is available online.

2000; Shin *et al*., 2002). 12(S)-hydroperoxyeicosatetraenoic acid, an endogenous TRPV1 agonist, is implicated in neurodegeneration in mesencephalic cultures (Canals *et al*., 2003). We suggested that stimulation of CB_1 receptors with HU210 or WIN55,212-2 generated 12(S)-HPETE, which in turn activated the ligand-gated calcium channel, TRPV1, leading to Ca^{2+} influx, mitochondrial damage and subsequent neuronal cell death. Thus, we assessed the effects of baicalein, a 12-lipoxygenase inhibitor, on HU210- or WIN55,212-2-induced neurotoxicity in mesencephalic cultures. Interestingly, co-treatment with 10 μM baicalein partially attenuated the number of Eth-1-positive cells induced by 3 μM HU210 (21%; $P<0.01$) or 6 μM WIN55,212-2 (20%; $P<0.01$), as well as TH-ip neurons in the presence of 3 μM HU210 (19%; $P<0.05$) or 6 μM WIN55,212-2 (17%; $P<0.05$) (Figure 3a). However, 10 μM REV-5901, a 5-lipoxygenase inhibitor, had no effect (Figure 3a).

Next, we analysed whether baicalein altered HU210- or WIN55,212-2-induced Ca^{2+} influx in mesencephalic cultures. Co-treatment with 10 μM baicalein suppressed both HU210- (Figure 3b) and WIN55,212-2-induced (Figure 3c) increase in $[Ca^{2+}]_i$, whereas REV-5901 (10 μM) had no effect (Figures 3d and e). Moreover, as shown in Figure 3f, application of 3 μM HU210 or 6 μM WIN55,212-2 elicited approximately three-fold increase in the level of 12(S)-HETE ($P<0.001$), a downstream metabolite of 12(S)-HPETE, in cultured mesencephalic neurons. These results indicate extensive biosynthesis of 12(S)-HPETE through activation of CB_1 receptors.

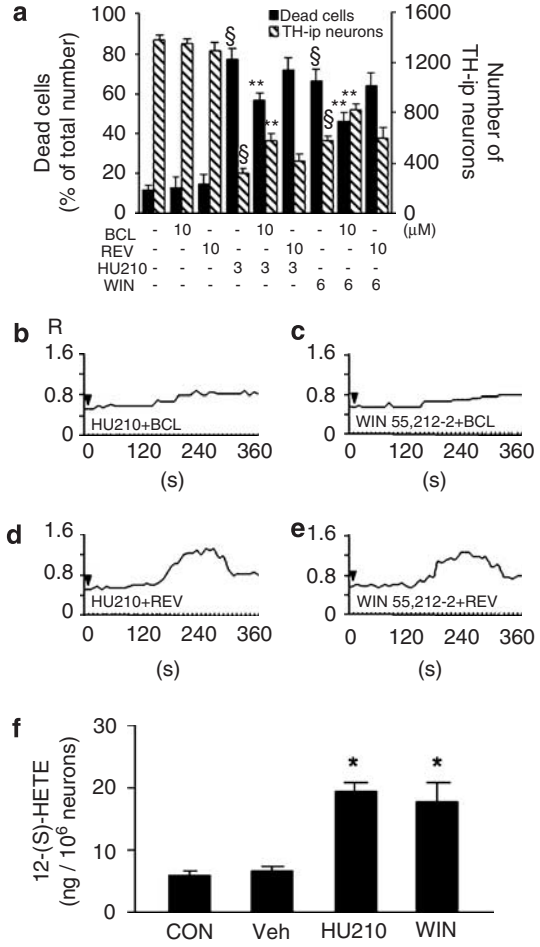

Figure 3 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HPETE) mediates the neurotoxic interactions between transient receptor potential vanilloid subtype 1 (TRPV1) and cannabinoid type 1 (CB_1) receptors. (**a**) Number of dead cells and TH-immunopositive (ip) neurons in neuron-enriched mesencephalic cultures treated with 0.5% dimethyl sulphoxide (DMSO), 3 μM HU210, or 6 μM WIN55,212-2, or co-treated with 10 μM 12-lipoxygenase inhibitor baicalein (BCL) or 10 μM 5-lipoxygenase inhibitor REV-5901 (REV), and stained with calcein-acetoxymethyl ester and ethidium homodimer-1 (Eth-1), or immunostained with TH antibody, respectively, at 24 h later. All values represent the mean ± s.e.mean of triplicate cultures in four separate platings. $^{\S}P<0.001$, significant from control; $^{**}P<0.05$, significant from treatment with HU210 or WIN55,212-2. (**b**–**e**) Response to 3 μM HU210 or 6 μM WIN55,212-2 of cultures co-treated with 10 μM baicalein (**b**–**c**, arrowhead) or REV (**d**–**e**, arrowhead) in the presence of 1.8 mM extracellular Ca^{2+}. Data were averaged from 20 to 25 randomly selected cells for each condition and the results shown are representative of four independent experiments. (**f**) 12(S)-HETE-specific enzyme immunoassay showing the increase of 12(S)-HETE at 30 min after treatment with 3 μM HU210 or 6 μM WIN55,212-2 (WIN) in cultured mesencephalic neurons. CON, non-treated control. Veh, 0.5% DMSO. The statistical significance of differences was assessed using one-way ANOVA, followed by Student–Newman–Keuls analyses. All values represent the mean ± s.e.mean of triplicate cultures in four separate platings. $^{*}P<0.001$, significant from control (CON or Veh). A colour version of this figure is available online.

12(S)-HPETE induces death of mesencephalic dopaminergic neurons through TRPV1 activation in vitro *and* in vivo

Further experiments were performed to confirm 12(S)-HPETE-induced neurotoxicity. In neuron-enriched mesencephalic cultures, treatment with 1–2 μM 12(S)-HPETE had no effect (data not shown). In contrast, application of 6 μM 12(S)-HPETE reduced the number of TH-ip neurons by 41% ($P<0.001$) (Figure 4a). Pretreatment of these cultures with 5 μM capsazepine partially attenuated the effects of 12(S)-HPETE (24%; $P<0.01$), signifying TRPV1-mediated neurotoxicity. Moreover, administration of 6 μM 12(S)-HPETE led to an increase in the intensity of Fura-2 fluorescence within a few seconds (Figure 4b), indicative of $[Ca^{2+}]_i$ elevation. This increase in $[Ca^{2+}]_i$ was abolished in the presence of 5 μM capsazepine (Figure 4c) and Ca^{2+}-free extracellular solution (data not shown). Similar to HU210 (Figures 2i and j) or WIN55,212-2 (data not shown), mitochondrial disruption was also noted within 5 min in 6 μM 12(S)-HPETE-treated cultures (Figures 4d and e), and pretreatment with 5 μM capsazepine for 5 min prevented the 12(S)-HPETE-induced mitochondrial damage (Figures 4f and g). Double immunofluorescence staining with Mito-Tracker and cytochrome *c* antibodies also revealed that cytochrome *c* was redistributed into the cytosol (data not shown), indicative of release from mitochondria. Treatment with 6 μM 15(S)-HPETE also led to a 21% reduction in the number of TH-ip neurons ($P<0.01$). However, this neurotoxicity was not blocked upon pretreatment with 5 μM capsazepine, implying that the effects of 15(S)-HPETE are independent of TRPV1 (Figure 4a). In contrast, application of 6 μM 5(S)-HPETE had no effects on cell viability, in our cultures.

At 7 days, after intranigral injection of 12(S)-HPETE (200 pmol 3 μL^{-1}) *in vivo*, a significant decrease was evident in the number of Nissl-stained (Figures 5c and d),

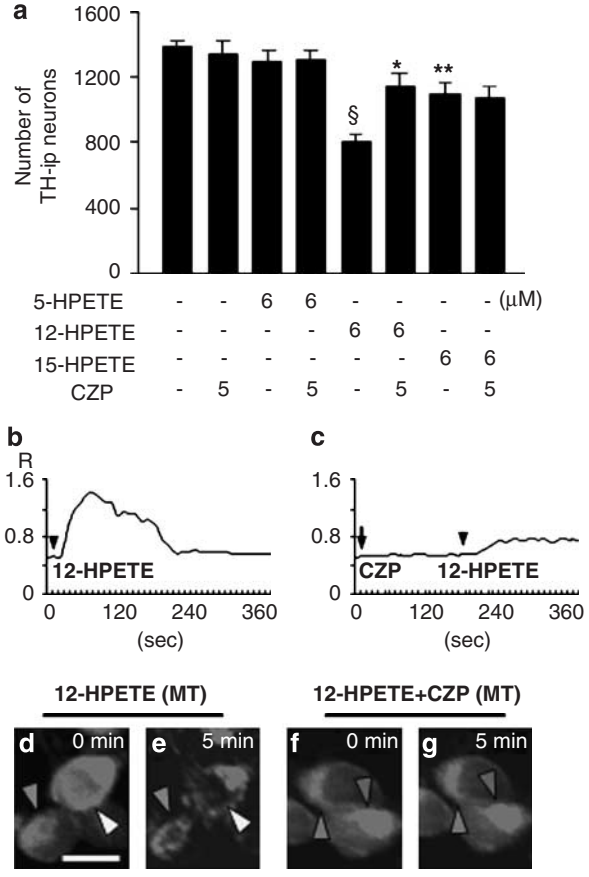

Figure 4 Neurotoxicity induced by 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HPETE) in neuron-enriched mesencephalic cultures. (**a**) Cultures were treated with 6 μM 5(S)-, 12(S)-, or 15(S)- HPETE for 24 h. Where indicated, cells were pretreated with 5 μM capsazepine (CZP) 5 min earlier than treatment with HPETEs and immunostained with TH antibody. Death of TH-immunopositive (ip) neurons was assessed by counting the number of TH-ip cells. All values represent the mean ± s.e.mean of triplicate cultures in four separate platings. $^{\S}P<0.001$ and $^{**}P<0.01$, significant from control; $^{*}P<0.01$, significant from treatment with 12-HPETE. (**b–c**) Changes of fluorescence were measured in cultures treated with 6 μM 12-HPETE (**b**, arrowhead), or pretreated with 5 μM CZP (arrow) before treatment with 12-HPETE (**c**, arrowhead) in the presence of 1.8 mM extracellular Ca^{2+}. Data were averaged from 20 to 25 randomly selected cells for each condition and the results are representative from three independent experiments. (**d**, **e**) Mitochondrial disruption in neuron-enriched mesencephalic cultures treated with 6 μM 12-HPETE in the presence of 1.8 mM extracellular calcium. (**f**, **g**) Inhibition of 12-HPETE-induced mitochondrial disruption by pretreatment with 5 μM CPZ. Scale bars: **d**–**g**, 20 μm. A colour version of this figure is available online.

neuron-specific nuclear protein-ip (Figures 5g and h) and TH-ip cells (Figures 5k and l) in the substantia nigra *in vivo*, compared with vehicle-treated controls (Figures 5a–b, e–f and i–j), respectively. Quantification of cells as a percentage of neurons on the ipsilateral side compared with the contralateral side revealed that 12(S)-HPETE suppresses the number of TH-ip neurons by 29% (Figure 4m; $P<0.001$). However, co-injection of 500 pmol capsazepine with 200 pmol 12(S)-HPETE induced a 20% increase in the number of TH-ip neurons ($P<0.05$), implying the involvement of TRPV1. The vehicle or capsazepine alone had no effects (data not shown) (Kim *et al*., 2005).

COX-2 contributes to cannabinoid-induced neurotoxicity independent of Ca^{2+} influx through TRPV1

The next step was to determine whether cannabinoids are involved in the expression of COX in neuron-enriched mesencephalic cultures. Western blot analysis disclosed that at 5 h post-treatment, 3 μM HU210 or 6 μM WIN55,212-2 markedly enhanced the expression of COX-2, but not COX-1 in our cultures (Figures 6a and b; $P<0.001$, significant compared to control). To determine whether COX expression contributes to cannabinoid-induced loss of dopaminergic neurons, we employed the non-selective COX inhibitor, indomethacin (Shin *et al*., 2002). Co-treatment with 5 μM indomethacin partially attenuated the loss of TH-ip neurons induced by 3 μM HU210 (30%; $P<0.001$) or 6 μM WIN55,212-2 (22%; $P<0.001$) (Figure 6c). Interestingly, indomethacin had no effect on $[Ca^{2+}]_i$ induced by HU210 (Figure 6d) or WIN55,212-2 (Figure 6e), suggesting that COX-induced neurotoxicity is independent of Ca^{2+} influx, which possibly occurs through TRPV1 (Hwang *et al*., 2000; Shin *et al*., 2002). Additional experiments showed that the relatively selective COX-2 inhibitors, NS398 (Lee *et al*., 2006) and DuP-697 (Choi *et al*., 2003a), at a concentration of 5 μM, attenuated death of TH-ip neurons induced by 3 μM HU210 (27%; $P<0.001$; 27%; $P<0.001$) and 6 μM WIN55,212-2 (21%; $P<0.01$; 19%; $P<0.01$), respectively (Figure 6c).

Discussion

In this study, we have shown that CB_1 receptor activation by its agonists (HU210 or WIN55,212-2) resulted in the biosynthesis of a well-characterized agonist of TRPV1, the endogenous lipid, 12(S)-HPETE (Hwang *et al*., 2000; Shin *et al*., 2002), by the 12-lipoxygenase pathway (Shin *et al*., 2002), which in turn activated the ligand-gated calcium channel, TRPV1, leading to an increase in $[Ca^{2+}]_i$, mitochondrial damage and death of dopaminergic neurons in neuron-enriched mesencephalic cultures, co-expressing both receptors (CB_1 receptors and TRPV1; Kim *et al*., 2005). To our knowledge, this is the first study to show that 12(S)-HPETE serves as a neurotoxic mediator of the two receptors.

We additionally show that treatment with 12(S)-HPETE causes degeneration of mesencephalic dopaminergic neurons in the substantia nigra *in vivo* and *in vitro* in a TRPV1-dependent manner. Finally, our *in vitro* experiments show that cannabinoid-induced expression of COX-2 contributes to neurodegeneration independently of Ca^{2+} influx through TRPV1 (Hwang *et al*., 2000; Shin *et al*., 2002).

In mesencephalic cultures, HU210- or WIN55,212-2-induced neurotoxicity and increase in $[Ca^{2+}]_i$ by TRPV1 were blocked by the 12-lipoxygenase inhibitor, baicalein, but not the 5-lipoxygenase inhibitor, REV5901. 12(S)-hydroperoxyeicosatetraenoic acid induced neurotoxicity in mesencephalic dopaminergic neurons *in vitro* and *in vivo*, as well as an increase in $[Ca^{2+}]_i$. 15(S)-hydroperoxyeicosatetraenoic acid, a product of 15-lipoxygenase, additionally produced neurodegeneration in mesencephalic cultures. Notably, capsazepine attenuated neurotoxicity induced by 12(S)-HPETE, but not 15(S)-HPETE, suggesting that 15(S)-HPETE-induced toxicity towards

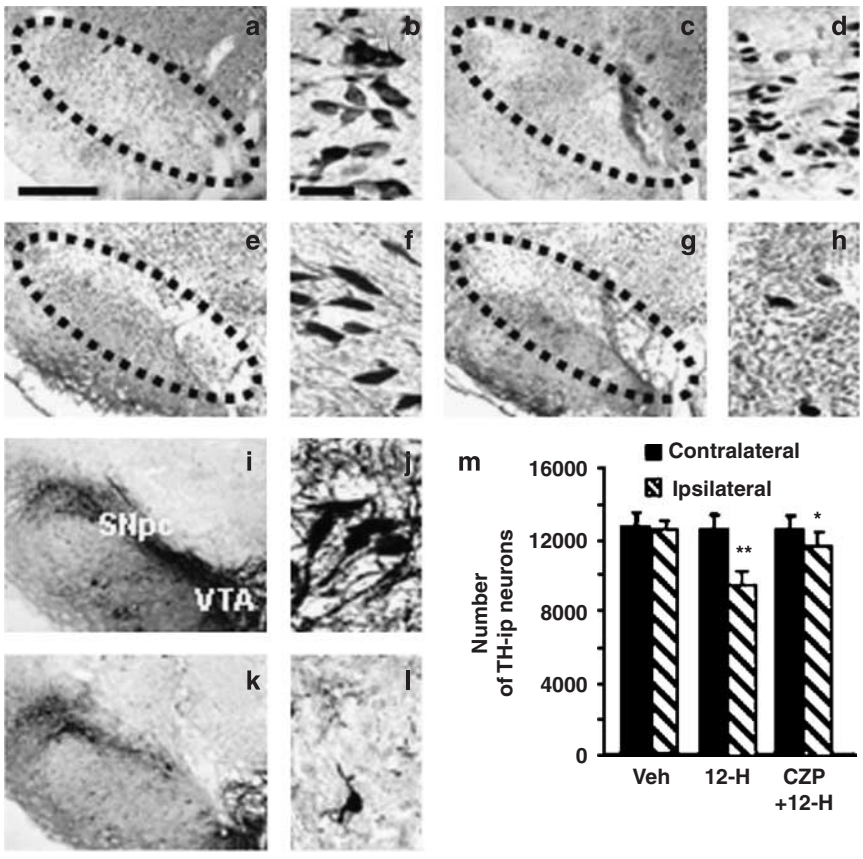

Figure 5 Neurotoxicity induced by 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HPETE) in the substantia nigra (SN) *in vivo*. (**a–l**) 12-HPETE-induced neurotoxicity in the substantia nigra of rat brains. Animals were administered a unilateral injection of 200 pmol 12-HPETE in 3 μL of Phosphate-buffered saline containing 14% ethanol (**c-d, g–h, k–l**) or vehicle (**a–b, e–f, i–j**) into the substantia nigra and killed 7 days later. Brain tissues were stained with cresyl violet (**a–d**) or immunostained with antibodies to neuron-specific nuclear protein (NeuN) (**e–h**) or TH (**i–l**). Dotted lines indicate substantia nigra pars compacta (where dopaminergic neurons were degenerated). Scale bars: **a, c, e, g, i, k,** 300 μm; **b, d, f, h, j, l,** 50 μm. SNpc, substantia nigra pars compacta; VTA, ventral tegmental area. (**m**) Number of TH-immunopositive (ip) neurons in the SN treated with 12-HPETE in the absence or presence of capsazepine. Animals receiving intranigral 12-HPETE (200 pmol) with or without administration of capsazepine (500 pmol) were killed 7 days after injection. Brain tissues were cut and immunostained with antibody to TH. TH-ip neurons were counted using a stereological technique in the whole SN. Six to eight animals were used for each experimental group. $^{**}P<0.001$, significant from vehicle; $^{*}P<0.05$, significant from 12-HPETE (12-H).

dopaminergic neurons occurred in a TRPV1-independent manner, probably induced by internucleosomal DNA fragmentation, caspase-3 activation or chromatin condensation related to oxidative stress (Kalyankrishna *et al*., 2002; Sordillo *et al*., 2005). A product of 5-lipoxygenase, 5(S)-HPETE, had no effect. The data suggest that among the lipoxygenase products, 12(S)-HPETE is an effective neurotoxic mediator of CB_1 receptors and TRPV1 channels and probably induces neurotoxicity in a TRPV1-dependent manner. We additionally observed HU210- or WIN55,212-2-induced production of 12(S)-HETE, a downstream metabolite of 12(S)-HPETE, which itself is an immediate product of 12-lipoxygenase (Hwang *et al*., 2000). Similarly, bradykinin, an algesic peptide acting on B_2 bradykinin receptors, excites sensory nerve endings by activating TRPV1 by production of 12(S)-HPETE, as evaluated by the production of 12(S)-HETE (Shin *et al*., 2002). However, 12(S)-HETE was unable to induce cell death in mesencephalic cultures (Canals *et al*., 2003), confirming that 12(S)-HPETE is the metabolite accounting for neurotoxicity. This finding is supported by current data that application of 12(S)-HPETE leads to loss of mesencephalic dopaminergic neurons in the substantia nigra *in vivo* and *in vitro*. Alternatively, Ca^{2+} influx by stimulation of CB_1 receptors may contribute to the formation of AEA (Di Marzo *et al*., 1994), an endogenous ligand of both CB_1 receptors and TRPV1 (Hermann *et al*., 2003; Kim *et al*., 2005). This would explain why HU210- or WIN55,212-2-induced neurotoxicity is blocked by capsazepine. However, we have not examined the effects of HU210 or WIN55,212-2 on AEA formation.

In mesencephalic cultures, HU210, WIN55,212-2 or 12(S)-HPETE do not enhance $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} or the presence of the TRPV1 receptor antagonist, capsazepine. In cultured hippocampal neurons (Chan *et al*., 1998) and resting T cells (Rao *et al*., 2004), THC-induced increase in $[Ca^{2+}]_i$ was not observed in the absence of extracellular Ca^{2+} or the presence of the CB_1 receptor antagonist, SR141716A. Recently, we showed that in cultured mesencephalic neurons, capsaicin, AEA or HU210 do not enhance $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} or in the presence of capsazepine or AM251 (Kim *et al*., 2005). These results collectively suggest that the increase in $[Ca^{2+}]_i$ mediated by TRPV1 or CB_1 receptors is dependent on extracellular Ca^{2+}. This is further supported by the finding

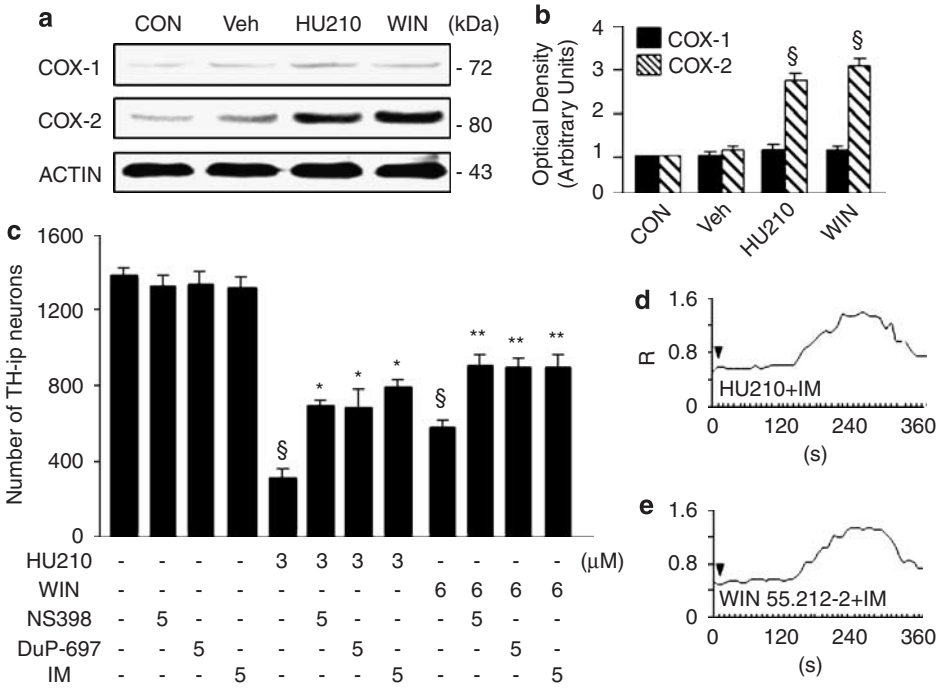

Figure 6 COX-2 mediates CB_1 receptor-induced neurotoxicity in neuron-enriched mesencephalic cultures. (**a**) Western blot analysis showing levels of COX-1 and COX-2 expression at 5 h after treatment with 3 μM HU210 or 6 μM WIN55,212-2 (WIN) in neuron-enriched mesencephalic cultures. The results are representative from three independent experiments. Non-treated- (CON) or 0.5% dimethyl sulphoxide (DMSO)- (Veh) treated cultures were used as controls. (**b**) Mean ± s.e.mean of four pooled samples per each treatment. $^{\S}P<0.001$, significant from control. (**c**) Number of TH-immunopositive (ip) cells in neuron-enriched mesencephalic cultures treated with 0.5% DMSO, 3 μM HU210, or 6 μM WIN55,212-2, or co-treated with 5 μM indomethacin (IM, COX inhibitor), 5 μM NS398 or 5 μM DuP-697 (COX-2 inhibitors), and immunostained with TH antibody at 24 h later. In all cultures, cell death was assessed by counting the number of TH-ip cells. All values represent the mean ± s.e.mean of triplicate cultures in four separate platings. $^{\S}P<0.001$, significant from control; $^{*}P<0.001$ and $^{**}P<0.01$, significant from HU210 or WIN55,212-2. (**d**–**e**) Changes of fluorescence were measured in cultures co-treated with 5 μM IM and 3 μM HU210 (**d**), or 6 μM WIN55,212-2 (**e**) in the presence of 1.8 mM extracellular Ca^{2+}. Data were averaged from 20 to 25 randomly selected cells for each condition and the results are representative from three independent experiments.

that an endoplasmic reticulum Ca^{2+} pump inhibitor, thapsigargin (Kim *et al*., 2005), has no effect on Ca^{2+} influx induced by HU210, WIN55,212-2 or 12(S)-HPETE in the presence of extracellular Ca^{2+}. However, the patterns of Ca^{2+} influx differ in cultures treated with HU210, WIN55,212-2 and 12(S)-HPETE. Application of HU210 or WIN55,212-2 causes a delayed increase in $[Ca^{2+}]_i$ that reaches a maximum at 220–240 s and returns to basal levels at 320–340 s, whereas 12(S)-HPETE evokes an initial rapid rise of $[Ca^{2+}]_i$, peaking at 30–40 s, followed by slow recovery of $[Ca^{2+}]_i$ over the next 2 min. This is comparable to our recent data (Kim *et al*., 2005) in that patterns of Ca^{2+} influx by 12(S)-HPETE are similar to those of capsaicin, whereas those induced by HU210 or WIN55,212-2 mimic the effects of AEA. This may explain our observation that the time required for HU210- or WIN55,212-2-induced mitochondrial damage was at least six times as long as that required for 12(S)-HPETE-induced damage. Moreover, this apparent discrepancy is also supported by the current finding that CB_1 receptor stimulation with specific agonists (HU210 or WIN55,212-2) leads to the biosynthesis of 12(S)-HPETE from AA by 12-lipoxygenase (Hwang *et al*., 2000; Shin *et al*., 2002), which activates TRPV1, thus enhancing Ca^{2+} influx.

It seems noteworthy that Ca^{2+} influx by stimulation of CB_1 receptors may contribute to the formation of endocannabinoids, such as AEA (Di Marzo *et al*., 1994, 2001; Hermann *et al*., 2003), that are metabolized by various lipoxygenases (Ueda *et al*., 1995; Kozak and Marnett, 2002) and these metabolites may activate TRPV1 (Hwang *et al*., 2000; Craib *et al*., 2001; Shin *et al*., 2002). We previously reported that HU210-induced neurotoxicity was attenuated by pretreatment with AM251 or capsazepine, suggesting functional cross-talk between TRPV1 and CB_1 receptors, and we discussed the possibility that the increase in $[Ca^{2+}]_i$-induced biosynthesis of AEA might be related to this interaction (Kim *et al*., 2005). In this study, we demonstrate that activation of CB_1 receptors results in elevation of $[Ca^{2+}]_i$ by TRPV1, leading to mitochondrial damage with cytochrome *c* release and eventual neurodegeneration in cultured mesencephalic dopaminergic neurons. In addition, CB_1 receptor agonists-induced Ca^{2+} influx was inhibited by treatment with capsazepine (Figures 2e and f) or baicalein (Figures 3b and c). Our results suggest that a mediator, which can activate TRPV1 after activation of CB_1 receptors, must be induced earlier than the increase in $[Ca^{2+}]_i$, consequently indicating that HPETE metabolites of endocannabinoids in response to the increase in $[Ca^{2+}]_i$ (Di Marzo *et al*., 1994; Kozak and Marnett, 2002) could be produced after activation of TRPV1 by induction of 12(S)-HPETE. These data also demonstrate that 12(S)-HETE induced by CB_1 receptor agonists, shown in Figure 3f, could be derived from 12(S)-HPETE biosynthesized from AA by 12-lipoxygenase (Hwang

et al., 2000; Shin *et al*., 2002), although the misidentification of 12-lipoxygenase-induced endocannabinoid metabolites as 12(S)-HETE is likely to occur. These metabolites would resemble the prostaglandin ethanolamides (prostamides; Woodward *et al*., 2008) which gate TRPV1 and evoke calcium signalling *in vitro* and *in vivo* (Matias *et al*. 2004; Glass *et al*., 2005; Spada *et al*. 2005). However, we cannot completely exclude the possibility that the observed effects are due to HPETE metabolites derived from AEA and 2-arachidonoylglycerol because AEA or 2-arachidonoylglycerol formed in response to Ca^{2+} influx could be metabolized to AA by fatty acid amide hydrolase or monoacylglycerol lipase (Sugiura *et al*., 1995).

Cannabinoids, such as AEA or THC, stimulate the release of AA from a variety of cells, including cultured astrocytes (Shivachar *et al*., 1996), cerebral cortical slices (Reichman *et al*., 1988) and hippocampal neurons (Chan *et al*., 1998). Arachidonylethanolamide, THC, HU210 or WIN55,212-2 induce COX-2 protein and/or mRNA expression in H4 human neuroglioma cells (Ramer *et al*., 2003), cerebral microvascular endothelium (Chen *et al*., 2005) or human non-pigmented ciliary epithelial cells (Rosch *et al*., 2006). In this context, there is growing evidence that increased levels of COX-2 and its product prostanoids or prostaglandins from AA, are implicated in cell death of cortical (Hewett *et al*., 2000; Kim *et al*., 2001), hippocampal (Chan *et al*., 1998; Kim *et al*., 2001), spinal motor neurons (Drachman and Rothstein, 2000) and nigral dopaminergic neurons (Choi *et al*., 2003a; Carrasco *et al*., 2005). These studies additionally show that COX-2-mediated neurotoxicity is prevented by specific inhibitors. In our experiments, cannabinoids (HU210 or WIN55,212-2) induced COX-2 expression, and indomethacin (a non-selective COX inhibitor) as well as NS398 and DuP-697 (selective COX-2 inhibitors) partially attenuated the loss of dopaminergic neurons in mesencephalic cultures. Interestingly, HU210- or WIN55,212-2-induced Ca^{2+} influx, possibly mediated by TRPV1, was not inhibited by indomethacin. Overall, our data suggest that COX-2 participates in cannabinoid-induced death of dopaminergic neurons in mesencephalic cultures in a Ca^{2+}- and TRPV1-independent manner. However, the possibility remains that TRPV1 may be involved in COX-2 neurotoxicity. This hypothesis is supported by the findings that COX-2 metabolites of AEA and 2-arachidonoylglycerol are prostaglandin ethanolamides (prostamides) which gate the TRPV1 and evoked calcium signalling *in vitro and in vivo* (Kozak and Marnett, 2002; Matias *et al*., 2004; Glass *et al*., 2005; Spada *et al*., 2005).

In contrast, a number of earlier studies show that CB_1 receptors have neuroprotective effects against ischaemia (Nagayama *et al*., 1999) and excitotoxicity (van der Stelt *et al*., 2001; Marsicano *et al*., 2003). As discussed previously (Marsicano *et al*., 2003; Kim *et al*., 2005), this inconsistency (neurotoxicity *vs* neuroprotection) is probably due to differences between the effects of endocannabinoids produced on demand and directly administered CB_1 agonists. The current data confirm direct neurotoxicity mediated by CB_1 receptors following the application of the agonists, HU210 and WIN55,212-2, whereas in studies showing CB_1 receptor-induced neuroprotective effects, the actions of CB_1 receptors were evaluated during ischaemic and excitotoxic injury, which may compromise their physiological functions due to recruitment in preventing secondary damage following insult. Alternatively, the biphasic effects of CB_1 receptors (protective or toxic) may depend on the specific concentrations of CB_1 agonists. At very low concentrations, WIN55,212-2 protects dopaminergic neurons in the ventral tegmental area, but displays detrimental effects at higher concentrations under ischaemic conditions (Melis *et al*., 2006).

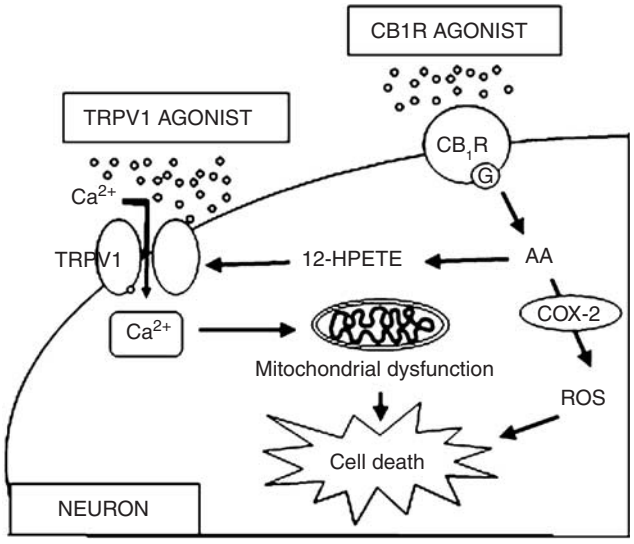

Figure 7 Diagram showing that 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HPETE) mediates functional interactions between cannabinoid type 1 (CB_1) receptors and transient receptor potential vanilloid subtype 1 (TRPV1). The CB_1 receptor couples to a G-protein activating arachidonic acid (AA), which can be metabolized by 12-lipoxygenase to (12(S)-HPETE). 12(S)-HPETE is an endogenous agonist of TRPV1 and activates TRPV1, leading to Ca^{2+} influx by TRPV1, mitochondrial disruption and eventual cell death. In addition, activation of CB_1 receptors is also able to enhance COX-2 expression, which can induce neuronal cell death regardless of Ca^{2+} influx by TRPV1. ROS, reactive oxygen species.

In conclusion, the present findings support a mechanism whereby the endogenous TRPV1 agonist, 12(S)-HPETE, produced by activation of CB_1 receptors with specific agonists mediates neurotoxic interactions between the two receptors by Ca^{2+} influx through TRPV1, although it is still unclear whether other interactions between the two receptors in response to production or release of endocannabinoids by activation of CB_1 receptors can contribute to neurodegeneration in the CNS. In addition to this mechanism, our results suggest that COX-2 produced by cannabinoids may serve as a neurotoxic signal, independently of Ca^{2+} influx by TRPV1 (Figure 7).

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Conflict of interest

The authors state no conflict of interest.

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