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Oleamide is a selective endogenous agonist of rat and human CB_1 cannabinoid receptors

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1 The ability of the endogenous fatty acid amide, *cis*-oleamide (ODA), to bind to and activate cannabinoid CB_1 and CB_2 receptors was investigated.

2 ODA competitively inhibited binding of the nonselective cannabinoid agonist [^{3}H]CP55,940 and the selective CB_1 antagonist [^{3}H]SR141716A to rat whole-brain membranes with K_i values of 1.14 μM (0.52–2.53 μM, Hill slope = 0.80, $n = 6$) and 2.63 μM (0.62–11.20 μM, Hill slope = 0.92, $n = 4$), respectively. AEA inhibited [^{3}H]CP55,940 binding in rat whole-brain membranes with a K_i of 428 nM (346–510 nM, Hill slope = −1.33, $n = 3$).

3 ODA competitively inhibited [^{3}H]CP55,940 binding in human CB_1 (hCB_1) cell membranes with a K_i value of 8.13 μM (4.97–13.32 μM, $n = 2$). In human CB_2 transfected (hCB_2) HEK-293T cell membranes, 100 μM ODA produced only a partial (42.5 ± 7%) inhibition of [^{3}H]CP55,940 binding.

4 ODA stimulated [^{35}S]GTPγS binding in a concentration-dependent manner ($EC_{50} = 1.64$ μM (0.29–9.32 μM), $R^2 = 0.99$, $n = 4$–9), with maximal stimulation of 188 ± 9% of basal at 100 μM. AEA stimulated [^{35}S]GTPγS binding with an EC_{50} of 10.43 μM (4.45–24.42 μM, $R^2 = 1.00$, $n = 3$, 195 ± 4% of basal at 300 μM). *Trans*-oleamide (*trans*-ODA) failed to significantly stimulate [^{35}S]GTPγS binding at concentrations up to 100 μM.

5 ODA (10 μM)-stimulated [^{35}S]GTPγS binding was reversed by the selective CB_1 antagonist SR141716A ($IC_{50} = 2.11$ nM (0.32–13.77 nM), $R^2 = 1.00$, $n = 6$).

6 The anatomical distribution of ODA-stimulated [^{35}S]GTPγS binding in rat brain sections was indistinguishable from that of HU210. Increases of similar magnitude were observed due to both agonists in the striatum, cortex, hippocampus and cerebellum.

7 ODA (10 μM) significantly inhibited forskolin-stimulated cyclic AMP (cAMP) accumulation in mouse neuroblastoma N1E 115 cells ($P = 0.02$, $n = 11$). ODA-mediated inhibition was completely reversed by 1 μM SR141716A ($P < 0.001$, $n = 11$) and was also reversed by pretreatment with 300 ng ml^{-1} pertussis toxin ($P < 0.001$, $n = 6$).

8 These data demonstrate that ODA is a full cannabinoid CB_1 receptor agonist. Therefore, in addition to allosteric modulation of other receptors and possible entourage effects due to fatty acid amide hydrolase inhibition, the effects of ODA may be mediated directly *via* the CB_1 receptor.
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Keywords: *Cis*-9,10-octadecanoamide, oleamide (ODA); HU210; 11-hydroxy-dimethylheptyl-Δ^8-tetrahydrocannabinol; cannabinoid agonist; endocannabinoid; CB_1 receptor; CB_2 receptor; rat; human; radioligand binding; [^{35}S]GTPγS binding

Abbreviations: AEA, Anandamide; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; CP55,940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]*trans*-4-(3-hydroxypropyl)-cyclohexanol; HU210, 11-hydroxy-dimethylheptyl-Δ^8-tetrahydrocannabinol; ODA or oleamide, *cis*-9,10-octadecanoamide; SR141716A, *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide; [^{35}S]GTPγS, guanosine 5′-O-[γ-[^{35}S]thio]triphosphate

Introduction

Cis-9,10-octadecanoamide (oleamide, ODA) is an endogenous sleep-inducing substance, first isolated from the cerebro-spinal fluid of sleep-deprived cats (Cravatt *et al*., 1995). The primary amide of oleic acid, ODA is an endogenous fatty acid amide of the same family of chemical messengers that includes the endocannabinoid anandamide (AEA). Although its synthetic pathway is unclear, ODA has been shown to be produced in mouse brain microsomes (Sugiura *et al*., 1996) and mouse neuroblastoma cells where ODA was found to be 78 times more abundant than AEA (Bisogno *et al*., 1997). ODA is a preferred substrate for the serine hydrolase FAAH, the enzyme responsible for the hydrolysis of all fatty acid amides including AEA (Boger *et al*., 2000a).

ODA has been shown to have various effects *in vitro* including inhibition of gap junction-mediated cell–cell

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communication (Boger *et al*., 1999), modulation of 5-HT_1, 5-$HT_{2A,C}$ and 5-HT_7 receptors (Thomas *et al*., 1997; 1999; Hedlund *et al*., 1999) and modulation of inhibitory ionotropic receptors such as the $GABA_A$ receptor (Coyne *et al*., 2002).

When administered *in vivo*, ODA produces similar effects to AEA. Both induce sleep, ODA increasing the slow wave sleep 2 (SWS2) phase (Cravatt *et al*., 1995; Boger *et al*., 1998; Yang *et al*., 1999; Huitron-Resendiz *et al*., 2001) and AEA increasing SWS2 and rapid eye movement (REM) sleep (Murillo-Rodriguez *et al*., 1998). ODA induces the classic tetrad of behaviours, used to identify cannabinergic activity, with a similar activity profile to AEA (Mechoulam *et al*., 1997). ODA produces a dose-dependent hypothermia and a decrease in locomotor activity in both mice and rats (Mechoulam *et al*., 1997, Huitron-Resendiz *et al*., 2001). ODA also induces catalepsy in mice (Mechoulam *et al*., 1997) but not in rats (Fedorova *et al*., 2001) and produces antinociception in both species (Mechoulam *et al*., 1997; Fedorova *et al*., 2001; Murillo-Rodriguez *et al*., 2001). Studies using the selective CB_1 receptor antagonist SR141716A have yielded conflicting results. SR141716A has been shown to reverse the effects of ODA on sleep (Mendelson & Basile, 1999), locomotor activity, ODA-potentiated 5-HT-mediated behaviours (Cheer *et al*., 1999) and antinociception (Fedorova *et al*., 2001) yet in a recent report failed to reverse locomotor activity and ptosis (Lichtman *et al*., 2002). SR141716A failed to reverse ODA-induced hypothermia in both studies that examined the effect (Fedorova *et al*., 2001; Lichtman *et al*., 2002). Further, Lichtman *et al*. (2002) investigated the ability of ODA to induce cannabinoid-like effects in CB_1 knockout mice. All three of the effects studied (hypolocomotion, hypothermia and ptosis) were induced by ODA in CB_1 knockout mice. However, the ability of ODA to induce sleep, analgesia or catalepsy in CB_1 knockout mice was not reported.

In vitro binding studies have also provided conflicting results. Boring *et al*. (1996) reported that ODA competitively inhibited [^{3}H]CP55,940 binding in rat brain membranes with a relatively low affinity (44 μM). However, ODA apparently failed to activate cannabinoid receptors (as demonstrated by the lack of [^{35}S]GTPγS binding) at concentrations up to 100 μM. Other groups found that ODA negligibly bound (Lichtman *et al*., 2002) or was unable to bind (Mechoulam *et al*., 1997; Sheskin *et al*., 1997) to either the CB_1 or CB_2 receptor at concentrations up to 10 μM. In order to explain the cannabinergic effects of ODA, with no apparent binding to the CB_1 receptor, an 'entourage' effect was suggested (Lambert & Di Marzo, 1999). ODA may potentiate or prolong the effects of endocannabinoids such as AEA by competitively inhibiting the enzyme FAAH (Mechoulam *et al*., 1997). A more recent study by Cheer *et al*. (1999) found that ODA inhibited [^{3}H]CP55,940 binding with an IC_{50} of 10 μM (with an increase of affinity in the presence of the FAAH inhibitor phenylmethylsulphonyl fluoride) suggesting that ODA might indeed exert some effects directly *via* the CB_1 receptor.

Owing to the inconsistent nature of both the *in vitro* and *in vivo* findings, the involvement of cannabinoid receptors in the actions of ODA is disputed. In the present study, radioligand binding was used to determine the ability of ODA and AEA to bind to the CB_1 receptor in rat whole-brain membranes and ODA to human-CB_1 (hCB_1) transfected human embryonic kidney (HEK)-293T cells. The ability of ODA to bind to the human-CB_2 (hCB_2) receptor in transfected HEK-293T cells was also tested. The [^{35}S]GTPγS membrane binding assay was used to determine the ability of ODA to activate rat brain CB_1 receptors and was compared with two positive controls, HU210 and AEA. Receptor specificity was determined by performing the assay in the presence of selective CB_1 receptor antagonists. [^{35}S]GTPγS autoradiography in rat brain sections was used to investigate the distribution of activated CB_1 receptors after exposure to ODA compared to the cannabinoid agonist HU210. Cyclic AMP (cAMP) accumulation assays were performed in N1E 115 cells, which endogenously express CB_1 receptors, to determine whether ODA inhibits adenylyl cyclase *via* the CB_1 receptor.

The data presented here indicate that ODA is a full endogenous cannabinoid receptor agonist with selectivity for the CB_1 receptor.

Some of the data presented have been communicated in preliminary form to the British Pharmacological Society (Ralevic *et al*., 2000; Leggett *et al*., 2002).

Methods

Materials

HU210, AEA and ODA were obtained from Tocris Cookson, U.K. SR141716A was supplied *via* the NIMH synthesis programme (contract NOIMH30003) and LY320135 was a gift from Eli Lilly (U.S.A.). Pertussis toxin was obtained from Sigma (U.K.) and stored at −20°C until required. *Trans*-ODA was a kind gift from Dr G. Lees (University of Sunderland). ODA and *trans*-ODA were made as stock solutions in ethanol or DMSO (10 mM) and stored at −20°C until required. HU210, SR141716A and LY320135 were made as stock solutions in ethanol (10 mM) and stored at −20°C until required.

Rat brain membrane preparation

Rats were killed by decapitation and brains (minus brainstem) were removed and rapidly homogenised in ice-cold Tris buffer (50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4). with a glass/teflon homogeniser. The homogenate was centrifuged at 20,000 × *g* at 4°C for 10 min and the pellet resuspended in Tris buffer. Homogenisation and centrifugation were repeated twice and the final pellet was resuspended in storage buffer (50 mM Tris, 1 mM EDTA, pH 7.4) to a protein concentration of 2 mg ml^{-1} for [^{35}S]GTPγS binding or 5 mg ml^{-1} for radioligand binding (Bradford, 1976).

hCB_1 and hCB_2 transient transfection and membrane preparation

HEK-293T cells were grown in Dubelcco's modified Eagle's medium (DMEM) with 10% foetal bovine serum and high glucose. Cells were transiently transfected with pcDNA3 encoding CB_1 or CB_2 using calcium phosphate precipitation and used for membrane preparation as described previously (Abadji *et al*., 1999). Briefly, at 24 h post-transfection, cells were harvested, washed twice in phosphate-buffered saline (PBS) and resuspended in PBS with 0.1% v v^{-1} protease inhibitor cocktail (Sigma Chemical Co., St Louis, MO, U.S.A.). Cell disruption was performed with nitrogen cavitation at 750 psi for 5 min. Following removal of cell debris and

nuclei by differential centrifugation, membranes were resuspended in TME buffer (25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA) + 7% sucrose. The membrane preparation was diluted to 0.6 μg ml^{-1} total protein concentration (Bradford, 1976) and stored at −70°C.

Mouse neuroblastoma N1E 115 cell culture

Mouse neuroblastoma N1E 115 cells were grown to confluence in culture flasks and maintained in DMEM with 10% foetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. Cells were then diluted and distributed into 24-well plates to a final volume of 1 ml per well 72 h prior to incubation with [^{3}H]adenine.

Radioligand binding in rat whole-brain membranes

Assay tubes were prepared in triplicate to a final volume of 1 ml containing 0.5 nM [^{3}H]CP55,940 (Perkin-Elmer Life Sciences Inc., U.S.A.) or [^{3}H]SR141716A (Amersham, U.K.) and 250 μg of protein diluted in Tris buffer (50 mM Tris · HCl, 2 mM EDTA, 5 mM $MgCl_2$ also containing 0.2 mg ml^{-1} bovine serum albumin (BSA), pH7.4). Stock drugs were diluted in the same buffer containing 5 mg ml^{-1} BSA. Tubes were incubated for 90 min at 30°C. Specific binding was calculated by subtracting nonspecific binding (NSB, determined in the presence of 1 μM HU210). Nonlinear regression analysis was carried out using Graphpad Prism3. K_i values for ODA and AEA were determined from IC_{50} values using a K_D of 550 pM for [^{3}H]CP55940 and 600 pM for [^{3}H]SR141716A, both as described in a recent review (Pertwee, 1997). Results are presented as mean values, with 95% confidence intervals.

Radioligand binding assays with HEK-293T membrane preparations containing hCB_1 and hCB_2 receptors

Radioligand binding assays were performed as previously described (Abadji *et al*., 1999). Membrane preparations were incubated with 4 nM [^{3}H]CP55,940 with varying concentrations of ODA. NSB was determined with 1 μM CP55,940. All conditions were prepared in triplicate. A Brandel cell harvester (Brandel Inc., Gaitherburg, MD, U.S.A.) was used to separate bound from unbound ligand and the former quantified by liquid scintillation counting. Results were analysed with Prism GraphPAD Software (GraphPAD Software, San Diego, CA, U.S.A.) as previously described (Chin *et al*., 1999) using the Cheng–Prusoff equation to determine K_i values with the K_D value of 4.7 nM for CP55,940 binding to CB_1 HEK 293T membrane preparations. Data are given as the mean from two independent experiments.

[^{35}S]GTPγS membrane binding

Membranes were preincubated with 100 μM GDP (ICN Biomedicals Inc., U.K.) and 1 mM theophylline (Sigma, U.K.) in assay buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$ containing 5 mg ml^{-1} BSA, pH 7.4) for 20 min at 30°C. Assay tubes were prepared in triplicate using the same buffer containing 500 μl preincubated membranes, 0.4 nM [^{35}S]GTPγS (Amersham, U.K.) and the appropriate concentrations of drug. The NSB was determined using 100 μM nonradiolabelled GTPγS and 1 μM HU210 was included as a positive control. Tubes were incubated for 45 min at 30°C. Free and bound [^{35}S]GTPγS were separated by rapid filtration (using filter mats presoaked in Tris buffer (pH 7.4) containing 5% BSA) with ice-cold Tris buffer (pH 7.4). Data are presented as mean values, with 95% confidence intervals for experiments performed in triplicate. EC_{50} and IC_{50} values were determined using nonlinear regression analysis of concentration response curves using Graphpad Prism3.

[^{35}S]GTPγS autoradiography

Rats were killed by decapitation and brains were rapidly removed and frozen on dry ice. Coronal brain sections (20 μM) were cut on a cryostat and thaw mounted onto glass microscope slides. Sections were preincubated with 100 mM GDP in buffer A (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) for 30 min at 25°C. Sections were then incubated for 90 min at 25°C with 0.2 nM [^{35}S]GTPγS in the presence or absence of 10 μM ODA or 1 μM HU210 in buffer B (50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH7.4 containing 0.2 mg ml^{-1} BSA). The NSB was determined by incubating sections with 0.2 nM [^{35}S]GTPγS in the presence of 100 μM nonlabelled GTPγS. After incubation, sections were washed, dried and exposed to β-max hyperfilm (Amersham, U.K.) for 72 h at −80°C. A [^{14}C] calibration strip was included to enable quantification of the autoradiograms after development. Autoradiographic images were analysed using NIH image (v1.62) for Macintosh computer. Quantification of images was achieved by densitometric analysis using calibrated ^{14}C standards to convert the data to disintegrations per minute. Data were then normalised and presented as % basal binding ± s.e.m. The Wilcoxon signed rank statistical tests were carried out using Graphpad Prism3 with $P<0.05$ considered significant.

Inhibition of forskolin-stimulated cAMP generation in mouse neuroblastoma N1E 115 cells with and without pertussis toxin pretreatment

Mouse neuroblastoma N1E 115 cells that express CB_1 but not CB_2 receptors (Zhou & Song, 2001) were grown on 24-well plates in DMEM with 10% foetal calf serum at 37°C in an atmosphere of 5% CO_2 and air. Cells were maintained at 37°C throughout the experiment. After 48 h, [^{3}H]adenine was added to a final concentration of 74 kBq ml^{-1} and 300ng ml^{-1} pertussis toxin was added when appropriate. Cells were incubated for a further 24 h before being washed twice with Hank's HEPES buffer (HHB) pH7.4 and left to equilibrate for 15 min in 1 ml of HHB. Rolipram (10 μM) (phosphodiesterase inhibitor) was added to each well to reduce enzymatic breakdown of cAMP followed immediately by antagonist (if required). Cannabinoids or vehicle (HHB) were added after 5 min and allowed to incubate for a further 5 min before the addition of 10 μl forskolin to a final concentration of 10 μM or 10 μl vehicle (ethanol). Cells were incubated for 15 min before the reaction was terminated by the addition of 50 μl (1 M) HCl. Samples (50 μl) were removed to determine total [^{3}H]adenine incorporation and 100 μl (25–35Bq) [^{14}C]cAMP was added to each well. [^{3}H]cAMP was resolved by single column chromatography according to Alvarez & Daniels (1992) using [^{14}C]cAMP as a recovery marker. Total [^{3}H]cAMP generated was estimated as the percentage conversion from total [^{3}H]adenine nucleotides. Results were expressed as % basal cAMP accumulated. Statistical analysis was performed by analysis of variance

followed by Bonferroni/Dunn *post hoc* tests using super-ANOVA for Macintosh computer or Graphpad Prism3.

Results

Effect of ODA and AEA on [^{3}H]CP55,940 binding and ODA on [^{3}H]SR141716A binding

ODA inhibited specific binding of [^{3}H]CP55,940 to rat whole-brain membranes in a concentration-dependent manner ($K_i = 1.14\,\mu$M (0.52–2.53 μM), Hill slope = 0.80, $n = 6$; Figure 1a).

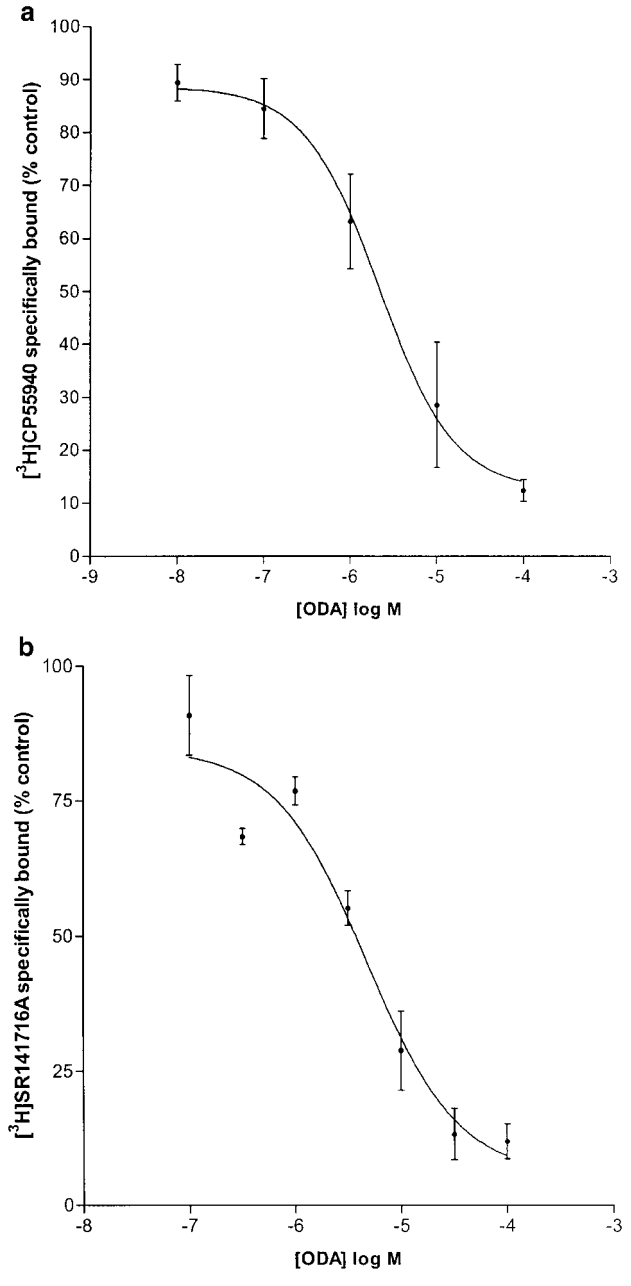

Figure 1 (a) Inhibition of specific [^{3}H]CP55,940 binding by *cis*-ODA (1 nM–100 μM), $K_i = 1.14\,\mu$m (0.52–2.53 μM, Hill slope = 0.80, $n = 6$). (b) Inhibition of specific [^{3}H]SR141716A binding by ODA (0.1 μM–100 μM), $K_i = 2.63\,\mu$M (0.62–11.20 μM, Hill slope = 0.92, $n = 4$).

Similarly, ODA inhibited binding of the selective CB_1 receptor antagonist [^{3}H]SR141716A to rat whole-brain membranes ($K_i = 2.63\,\mu$M (0.62–11.20 μM), Hill slope = 0.92, $n = 4$; Figure 1b). Analysis showed that the Hill slopes were consistent with single site binding. AEA concentration-dependently displaced 100% of specifically bound [^{3}H]CP55,940 in rat whole-brain membranes ($K_i = 428 \pm 82$ nM, Hill slope = -1.33 ± 0.15, $n = 3$). The positive cannabinoid agonist control, HU210 (used to determine NSB), inhibited 60% ($\pm 5\%$) of total bound [^{3}H]CP55,940 and 59% ($\pm 3\%$) of total bound [^{3}H]SR141716. In further experiments, ODA inhibited specific binding of [^{3}H]CP55,940 to hCB_1 transfected HEK-293T cell membranes in a concentration-dependent manner ($K_i = 8.14\,\mu$M (range 4.97–13.32 μM) $n = 2$; Figure 2). However, in hCB_2 transfected HEK-293T cell membranes ODA exhibited only a partial inhibition of [^{3}H]CP55,940 binding ($42.5 \pm 7\%$) at the maximal concentration (100 μM) employed.

[^{35}S]GTPγS membrane binding

Cis-ODA stimulated [^{35}S]GTPγS binding in rat whole-brain membranes in a concentration-dependent manner ($EC_{50} = 1.64\,\mu$M (0.29–9.32 μM), $R^2 = 0.99$, $n = 4$–9; Figure 3a). At a maximally effective concentration of 100 μM, ODA enhanced [^{35}S]GTPγS binding to $188 \pm 9\%$ of basal. *Trans*-ODA failed to stimulate [^{35}S]GTPγS binding at concentrations up to 100 μM ($n = 4$).

The selective CB_1 receptor antagonist SR141716A inhibited [^{35}S]GTPγS binding stimulated by 10 μM ODA ($IC_{50} = 2.11$ nM (0.32–13.77 nM), $R^2 = 1.00$, $n = 6$; Figure 3b), completely reversing enhanced [^{35}S]GTPγS binding at a concentration of 1 μM. Another selective CB_1 receptor antagonist, LY320135 (Felder *et al*., 1998) also inhibited ODA-enhanced [^{35}S]GTPγS binding ($IC_{50} = 12.44$ nM (1.63–95.16 nM), $R^2 = 1.00$, $n = 9$).

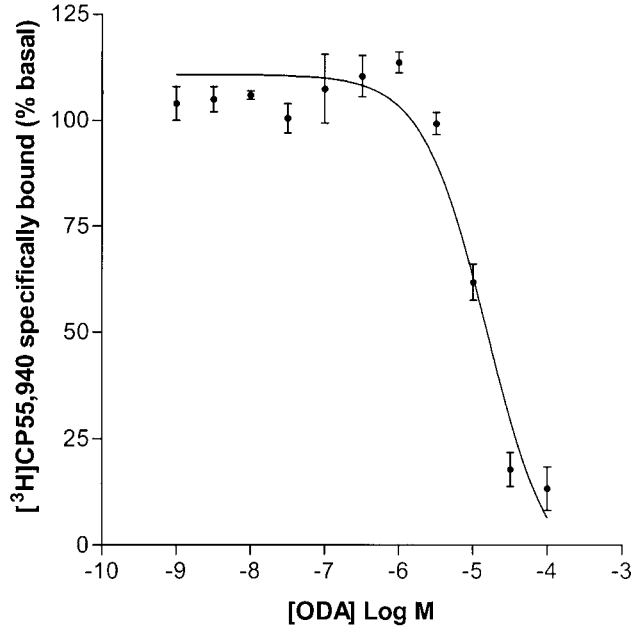

Figure 2 Inhibition of [^{3}H]CP55,940 binding in hCB_1 (transiently transfected HEK-293T) cell membranes by *cis*-ODA, $K_i = 8.13\,\mu$M (range 4.97–13.32 μM, $n = 2$). The figure represents one of two experiments conducted in triplicate.

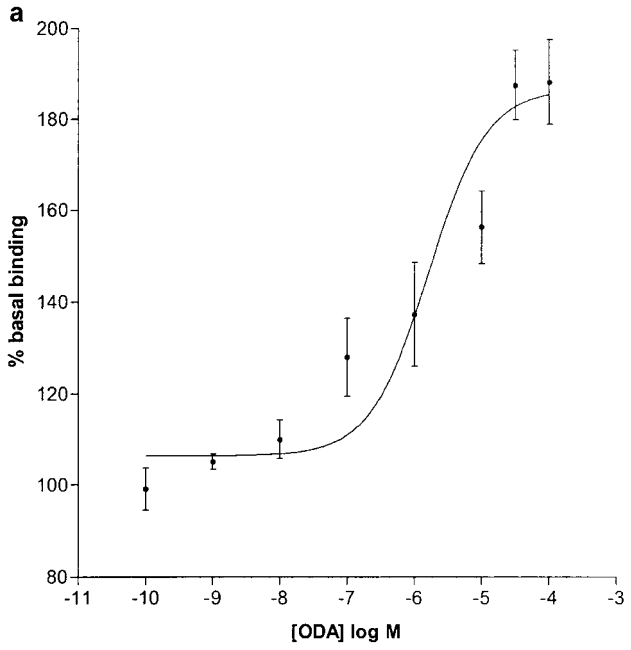

Figure 3 (a) ODA-stimulated [^{35}S]GTPγS binding in rat brain membranes ($EC_{50} = 1.64\,\mu$M, $n = 4$–9, $R^2 = 0.99$). (b) Inhibition of 10 μM ODA-stimulated [^{35}S]GTPγS binding by SR141716A ($IC_{50} = 2.11$ nM, $n = 6$, $R^2 = 1.00$).

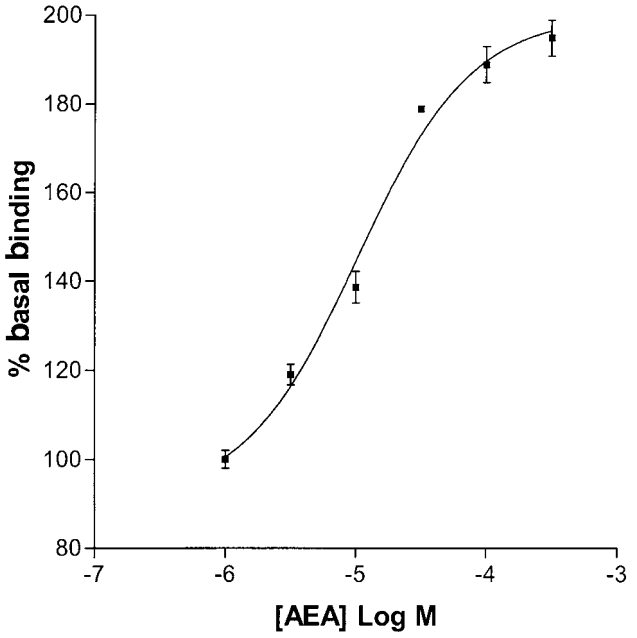

Figure 4 AEA-stimulated [^{35}S]GTPγS binding in rat brain membranes ($EC_{50} = 10.43\,\mu$M (4.45–24.42 μM), $n = 3$, best fit curve: $R^2 = 0.98$).

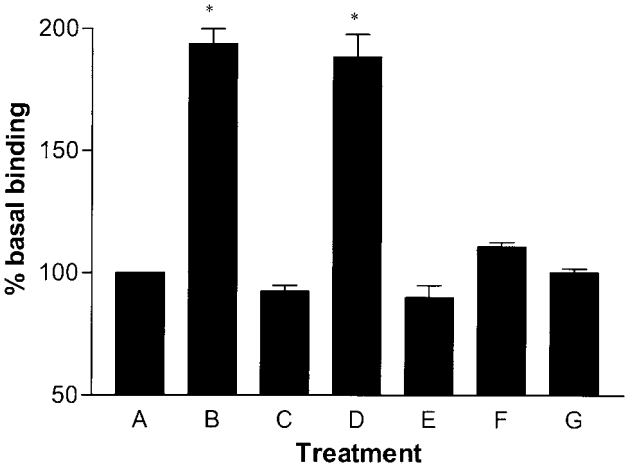

Figure 5 Antagonism of agonist-potentiated [^{35}S]GTPγS binding to rat brain membranes and effects of stereo isomers of ODA. (a) Basal binding, (b) 1 μM HU210, (c) 0.1 μM HU210 plus 1 μM SR141716A, (d) 100 μM *cis*-ODA, (e) 10 μM *cis*-ODA plus 1 μM SR141716A, (f) 10 μM *cis*-ODA plus 10 μM LY320135, (g) 100 μM *trans*-ODA (significance levels: $^*P < 0.01$ compared to basal binding).

AEA concentration-dependently increased [^{35}S]GTPγS binding ($EC_{50} = 10.43\,\mu$M (4.45–24.42 μM), $R^2 = 0.98$, $n = 3$; Figure 4). At the maximum concentration used (300 μM), AEA stimulated [^{35}S]GTPγS binding to 195$\pm$4% of basal. The positive control, HU210, concentration-dependently increased [^{35}S]GTPγS binding. At a maximally effective concentration, HU210 stimulated binding to 194$\pm$6% of basal ($EC_{50} = 1.46$ nM (0.45–4.78 nM), $R^2 = 0.98$, $n = 8$). SR141716A completely reversed HU210-stimulated [^{35}S]GTPγS binding at a maximally effective concentration of 1 μM ($n = 9$; Figure 5).

[^{35}S]GTPγS autoradiography

Autoradiography was used to investigate the regional distribution of specific [^{35}S]GTPγS binding in the presence of ODA or the high potency cannabinoid agonist HU210. Four brain regions were examined quantitatively: the striatum, cortex, hippocampus and cerebellum. HU210 (1 μM) significantly increased [^{35}S]GTPγS binding to 360$\pm$89% ($P < 0.05$, $n = 6$) of basal in the striatum, 244$\pm$39% ($P < 0.05$, $n = 6$) in the cortex, 225$\pm$31% ($P < 0.05$, $n = 6$) in the hippocampus and

287±67% ($P<0.05$, $n=5$) in the cerebellum. ODA (10 μM) significantly increased [^{35}S]GTPγS binding to 294±89% ($P<0.05$, $n=6$) in the striatum, 226±41% ($P<0.05$, $n=6$) in the cortex, 196±29% ($P<0.05$, $n=6$) in the hippocampus and 254±73% ($P<0.05$, $n=5$) in the cerebellum (Figures 6 and 7).

Effect of ODA on forskolin-stimulated cAMP generation

[^{3}H]cAMP accumulation after stimulation with forskolin (10 μM) was 192±17% of basal ($P=0.002$, $n=17$). HU210 (1 μM) significantly reduced forskolin-stimulated [^{3}H]cAMP accumulation reducing levels to 132±19% of basal ($P=0.05$, $n=11$). ODA (10 μM) significantly inhibited cAMP generation to 122±17% of basal levels ($P=0.02$, $n=11$). The selective CB_1 receptor antagonist SR141716A completely reversed the inhibitory effect of 10 μM ODA ($P=0.0003$, $n=11$) while having no significant effect when administered alone (Figure 8). Pretreatment of cells with pertussis toxin (300 ng ml^{-1}) completely abolished the inhibitory effect of ODA on forskolin-stimulated cAMP generation ($P<0.001$, $n=6$). In addition, pretreatment with pertussis toxin significantly elevated cAMP above non-pretreated levels in forskolin-treated ($P<0.05$, $n=6$) and ODA-treated cells ($P<0.05$, $n=6$, Figure 9).

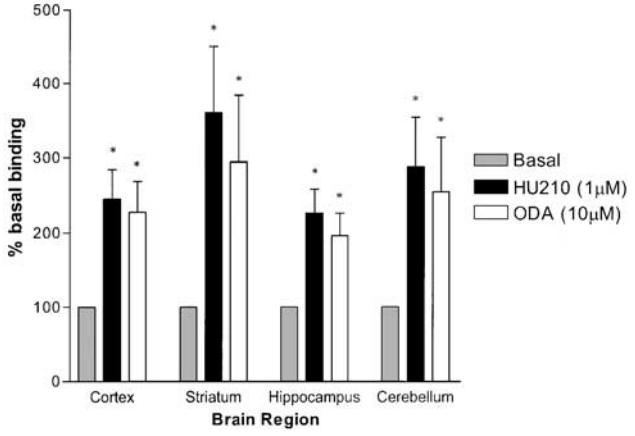

Figure 6 Regional increases in [^{35}S]GTPγS (autoradiographic) binding after stimulation with HU210 or ODA (bars indicate s.e.m.) compared to [^{35}S]GTPγS binding under basal conditions. Significance levels: *$P<0.01$ compared to basal.

Discussion

ODA shares many characteristics with the endocannabinoid AEA. Both ODA and AEA are fatty acid amides produced endogenously in the brain (Di Marzo, 1998) and both are hydrolysed by FAAH (Boger *et al*., 2000a, b). *In vivo* administration of ODA or AEA induces sleep that can be

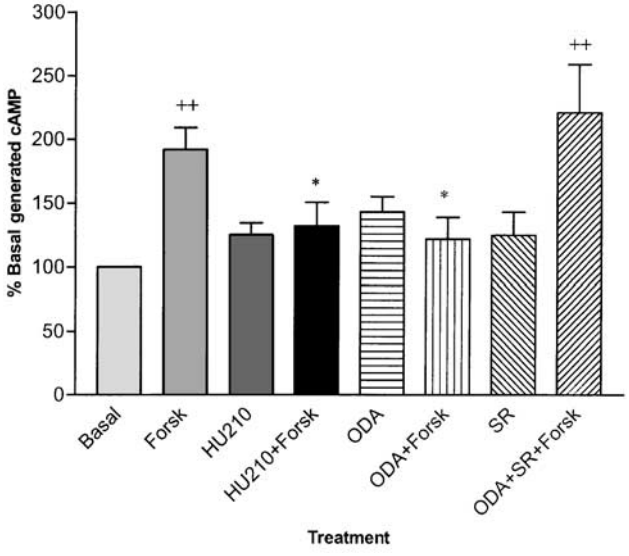

Figure 8 Inhibition of forskolin (Forsk)-stimulated cAMP generation in mouse neuroblastoma N1E 115 cells by 1 μM HU210 and 10 μM ODA and attenuation of ODA-mediated inhibition by the selective CB1 receptor antagonist SR141716A (1 μM). ODA, HU210 and SR141716A had no effect alone. ++ = $P<0.01$ compared to basal cAMP generation ($n=11$), * = $P<0.05$ compared to forskolin-stimulated cAMP generation ($n=11$).

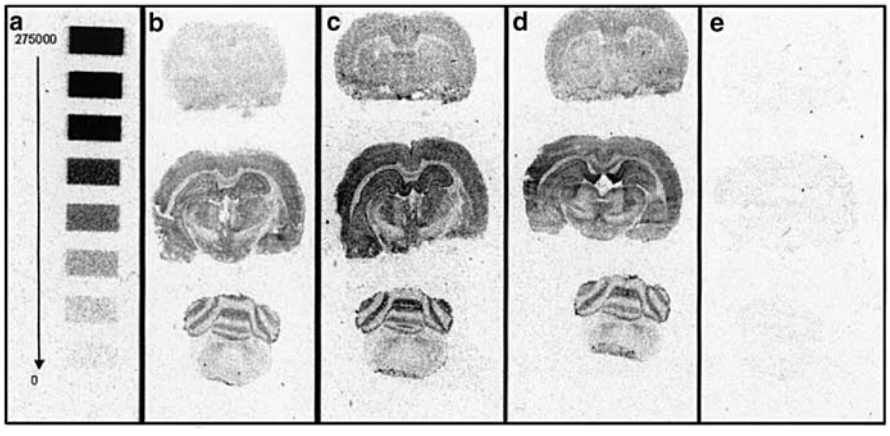

Figure 7 Example autoradiograms showing (a) ^{14}C calibration strip calibrated with disintegrations per minute (DPM) values from 275,000 DPM to 0 DPM (b) basal, (c) 1 μM HU210-treated slices, (d) 10 μM ODA-treated slices and (e) NSB. All examples are from the same experiment and show significantly enhanced [^{35}S]GTPγS binding after treatment with HU210 or ODA.

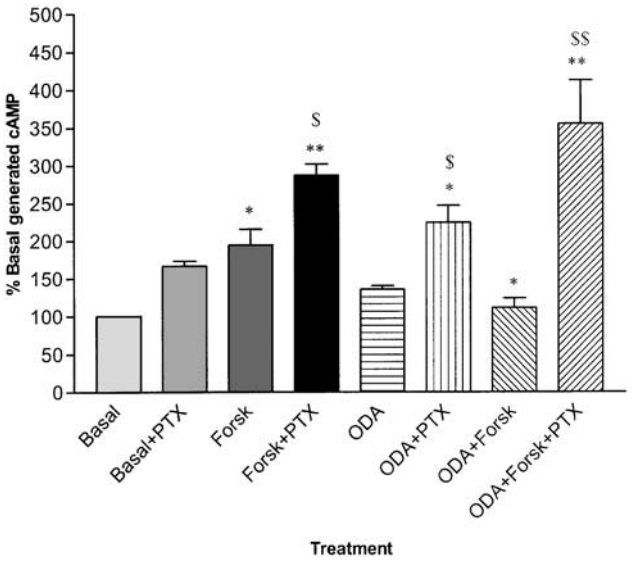

Figure 9 Inhibition of forskolin (Forsk)-stimulated cAMP generation in mouse neuroblastoma N1E 115 cells by 10 μM ODA and blockade of ODA-mediated inhibition by pretreatment with 300 ng ml^{-1} pertussis toxin (PTX). Pretreatment with PTX significantly increased cAMP accumulation in Forsk only and ODA only treated cells compared to respective non-pretreated cells. * = $P<0.05$ compared to basal cAMP levels, ** = $P<0.001$ compared to basal cAMP levels, + = $P<$ 0.05 compared to Forsk-stimulated cAMP levels, $ = $P<0.05$ compared to respective non-pretreated condition, $$ = <0.001 compared to non-pretreated condition.

reversed by SR141716A. ODA also produces the classical tetrad of behaviours associated with cannabinoid agonists with a similar profile to AEA (Mechoulam *et al*., 1997). SR141716A has been shown to reverse the effects of ODA on catalepsy, hypolocomotion and analgesia but not hypothermia (Fedorova *et al*., 2001). Lichtman *et al*. (2002) were, however, unable to reverse ODA-induced hypolocomotion in their study with the CB_1 antagonist. Similarly, SR141716A also fails to completely reverse elements of AEA pharmacology (Breivogel *et al*., 2001). A recent study has also demonstrated the ability of ODA to induce certain cannabinoid-like effects (hypolocomotion, hypothermia and ptosis) in CB_1 receptor knockout mice. The endogenous cannabinoid AEA has also been shown to have residual behavioural effects and stimulates [^{35}S]GTPγS binding in CB_1 knockout mice (Di Marzo *et al*., 2000; Wiley & Martin, 2002). This may be due to AEA activating multiple types of receptor: both CB_1 and CB_2 cannabinoid receptors, the VR_1 vanilloid receptor (Pertwee & Ross, 2002) and possibly a non-$CB_{1/2}$ cannabinoid receptor subtype (Mang *et al*., 2001). Despite sharing functional similarities with endocannabinoids, radioligand binding studies indicated that ODA had a low affinity for the cannabinoid CB_1 receptor. ODA was found to bind with a K_i of 44 μM in rat brain membranes (Boring *et al*., 1996), negligibly (Lichtman *et al*., 2002), not to bind at all (Mechoulam *et al*., 1997), or more recently, to bind with an IC_{50} of 10 μM in rat cerebellar membranes (Cheer *et al*., 1999). Additionally, Boring *et al*. (1996) reported that ODA, at concentrations up to 1 mM, was unable to enhance GTPγS binding to rat brain membranes, indicating an inability to activate G-protein-coupled receptors (GPCRs). Owing to the reported low affinity of ODA for the CB_1 receptor and the apparent inability of ODA to activate the CB_1 GPCR, an alternative explanation for its cannabinergic effects was required. One theory presented was that the effects of endocannabinoids are potentiated *via* the competitive inhibition, by ODA, of the enzyme FAAH; known as the entourage effect (Lambert & Di Marzo, 1999). However, ODA concentrations of $\geqslant 5\,\mu$M are required to inhibit FAAH-mediated hydrolysis of AEA (Mechoulam *et al*., 1997) and a non-hydrolysable analogue of ODA proved to be no more effective at inhibiting AEA hydrolysis (Fedorova *et al*., 2001). Recently, in a study using FAAH knockout mice, it was concluded that FAAH regulates but does not mediate the effects of fatty acid amides (such as ODA and AEA) *in vivo* (Lichtman *et al*., 2002).

Studies have indicated that ODA affects other neurotransmitter systems. ODA has been reported to modulate 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors (Huidobro-Toro & Harris, 1996; Thomas *et al*., 1997) and has been shown to act *via* an allosteric site on the 5-HT_7 receptor, thereby modulating receptor affinity (Hedlund *et al*., 1999). Recently, several cannabinoid agonists (tetrahydrocannabinol (THC), WIN55,212-2, AEA, JWH-015 and CP55,940) have been shown to inhibit human 5-HT_{3A} receptors *via* an allosteric site (Barann *et al*., 2002) highlighting another similarity in the actions of ODA and endogenous (and classical) cannabinoids. However, a recent report suggests that ODA does not modulate serotonergic transmission in the CA1 pyramidal neurons of the hippocampus (Dougalis *et al*., 2004) and it has also been shown that 5-HT_{1A} and 5-HT_{2C} antagonists have no effect on ODA-induced hypothermia, locomotor activity, analgesia or anxiety in rats (Fedorova *et al*., 2001). Cheer *et al*. (1999) reported a modulation of 5-HT_2 receptor affinity but with no effect on associated second messengers. ODA is also a nonselective modulator of inhibitory ionotropic receptors and has been shown to act indirectly *via* an allosteric site on the $GABA_A$ receptor (Coyne *et al*., 2002). Recently, it has been shown that the $GABA_A$ receptor antagonist bicuculline reverses ODA-induced hypothermia and analgesia (Fedorova *et al*., 2001) and elimination of the β subunit of the $GABA_A$ receptor prevents ODA-induced sleep (Laposky *et al*., 2001).

The radioligand binding results from the present study are similar to those found by Cheer *et al*. (1999) but contrast sharply with those of Boring *et al*. (1996), Lichtman *et al*. (2002) and Mechoulam *et al*. (1997). Here ODA inhibited [^{3}H]CP55,940 binding in rat whole-brain membranes with a K_i value of 1.14 μM (0.52–2.53 μM). This represents an affinity for ODA much closer to those previously calculated for AEA binding to various rat CB_1-containing membranes (52 nM, Devane *et al*., 1992; 143 nM, Hillard *et al*., 1995; 44 nM, Petitet *et al*., 1996; 266 nM, Rinaldi-Carmona *et al*., 1996) rather than the approximate 800 fold difference between ODA and AEA reported by Boring *et al*. (1996). Indeed, in the present study under the same experimental conditions, AEA was found to have an affinity of 428 nM (346–510 nM) a less than three-fold difference in affinity. ODA also inhibited binding of the selective CB_1 receptor antagonist [^{3}H]SR141716A ($K_i = 2.63\,\mu$M, 0.62–11.20 μM) to rat brain membranes, again demonstrating a relatively high affinity for the CB_1 receptor. ODA

also successfully inhibited [^{3}H]CP55,940 binding in hCB_1 HEK-293T cell membranes, although with marginally less affinity ($K_i = 8.14\,\mu$M). The affinity of AEA binding to transfected hCB_1 receptors was reported as 115 nM in HEK-293 cell membranes (Song & Bonner, 1996), although lower binding affinities have been quoted for AEA binding to hCB_1 in other model cells (252 nM, Mechoulam and Pride, 1995; 543 nM Felder *et al*., 1995).

ODA at concentrations up to 100 μM only partially displaced [^{3}H]CP55,940 binding to hCB_2 receptors, suggesting little affinity for the hCB_2 receptor. It should be noted, however, that selectivity of various agonists for cannabinoid receptors has been reported to vary by more than 2000-fold depending upon the species and assay conditions used (Pertwee, 1997; Iwamura *et al*., 2001).

The ability of ODA to bind to the CB_1 receptor does not necessarily infer agonist activity. Preliminary experiments using [^{35}S]GTPγS autoradiography resulted in significantly increased binding in only the paraventricular nucleus of the hypothalamus, although this was reversed by SR141716A (Beckett *et al*., 1999) and Boring *et al*. (1996) failed to observe any ODA-stimulated GTPγS binding in rat brain membranes at concentrations up to 1 mM. However, in the present study ODA enhanced [^{35}S]GTPγS binding in rat whole-brain membranes in a stereoselective manner with an EC_{50} of 1.64 μM, a 10-fold lower EC_{50} than AEA under the same conditions. SR141716A concentration-dependently inhibited the increase in ODA-stimulated [^{35}S]GTPγS binding, further indicating the effects of ODA to be mediated *via* the CB_1 receptor.

Although the distribution of ODA-induced increases in Fos protein and c-fos mRNA have been studied (Thomas *et al*., 1999), the distribution of ODA-stimulated GTPγS binding (and thus the distribution of ODA-activated GPCRs) has not. Having established that ODA stimulates significant increases in [^{35}S]GTPγS binding in rat brain membranes, the distribution of [^{35}S]GTPγS binding was examined in rat brain sections. The distribution of ODA-stimulated [^{35}S]GTPγS binding was indistinguishable from that stimulated by HU210, and the magnitude of [^{35}S]GTPγS binding stimulated by 10 μM ODA was comparable to that produced by 1 μM HU210 (Figure 7). This is consistent with ODA activating the same set of receptors as the cannabinoid HU210.

Since the evidence for ODA binding to and activating the CB_1 receptor may be considered controversial, its ability to inhibit cAMP generation *via* the CB_1 receptor would help to confirm its status as an endogenous cannabinoid agonist. Consistent with a previous report (Zhou & Song, 2001), HU210 (1 μM) inhibited forskolin-stimulated [^{3}H]cAMP accumulation by 65% in N1E 115 cells. ODA (10 μM) inhibited 76% of forskolin-stimulated [^{3}H]cAMP accumulation. ODA-mediated attenuation of cAMP accumulation was completely reversed by 1 μM SR141716A and inactivation of $G_{i/o}$ GPCRs by pretreatment with pertussis toxin (PTX) also completely reversed the inhibitory effect of ODA. These results indicate strongly that the cAMP attenuation is mediated *via* a $G_{i/o}$-linked GPCR likely to be a CB_1 receptor. Interestingly, PTX treatment had the effect of unmasking a lesser, stimulatory effect of ODA on cAMP generation. Similar cryptic positive coupling of CB_1 receptors to adenylyl cyclase has been reported in striatal neurons and CB_1-transfected Chinese hamster ovary cells after $G_{i/o}$ inactivation with PTX (Glass & Felder, 1997; Felder *et al*., 1998).

It is difficult to reconcile previous reports of a lack of effect of ODA on CB_1 receptors with the present data, although methodological factors may be responsible for some of the differences. The most likely of these is the difficulty in dissolving ODA into saline-based buffers at concentrations higher than 300 μM. Indeed, we were unable to dissolve ODA at any final assay concentration higher than this without an unacceptable increase in the concentration of nonaqueous solvent (DMSO or ethanol). Other factors such as buffer composition, temperature, rat strain and analytical methods may also have contributed to a difference in results. Similar methodological factors may account for the difference in agonist activity, as measured by GTPγS binding, in the present study compared to that reported by Boring *et al*. (1996). It should be noted that even well-recognised cannabinoid agonists such as THC and AEA have been reported not to enhance [^{35}S]GTPγS binding in membrane preparations (Griffin *et al*., 1998), a finding that has been negated by the weight of subsequent positive findings.

The apparent ability of ODA to induce certain behaviours in CB_1 knockout mice (Lichtman *et al*., 2002) may be due to interactions with other non-cannabinoid receptors, similar to those produced by a range of cannabinoid receptor agonists, or possibly with as yet uncharacterised cannabinoid receptor subtypes. ODA allosterically modulates $GABA_A$ receptors (Coyne *et al*., 2002) and bicuculline, a $GABA_A$ antagonist, reverses ODA-induced hypothermia (Fedorova *et al*., 2001). In addition, ODA allosterically modulates 5-HT receptors (Thomas *et al*., 1997) and established cannabinoid agonists have also been shown to act in this manner (Barann *et al*., 2002). Therefore, in addition to allosteric and possibly, direct modulation of other receptor types and inhibition of FAAH, the effects of ODA may actually be mediated directly *via* the CB_1 receptor. Although the affinity of ODA for the CB_1 receptor is somewhat lower than that reported for AEA, it should be noted that the basal concentration of ODA in the brain has not yet been reliably measured. On the basis of the concentration of ODA in neuroblastoma cells being much higher than that of AEA (Bisogno *et al*., 1997), it is conceivable that endogenous levels of ODA might be sufficient to occupy a significant proportion of CB_1 receptors *in situ* and ODA may, therefore, act as a low-affinity agonist.

In summary, the data presented clearly show that ODA occupies rat and human CB_1 (but not CB_2) receptors with micromolar affinity and that it has agonist activity comparable to that of HU210 and AEA, revealed by its ability to enhance GTPγS binding to membranes. Furthermore, ODA, at least in mouse neuroblastoma cells, can inhibit or stimulate (after $G_{i/o}$ inactivation) formation of the intracellular messenger cAMP in a CB_1 antagonist-sensitive fashion.

Thus, the evidence strongly supports the contention that ODA is a directly acting endogenous cannabinoid with selectivity for the CB_1 receptor.

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