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Title Page

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5 **The endocannabinoid 2-arachidonylglycerol is a negative allosteric modulator of the**
6
7 **human A₃ adenosine receptor**
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Abstract

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5 Studies of endogenous cannabinoid agonists, such as 2-arachidonylglycerol (2-AG), have revealed
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7 their potential to exert modulatory actions on other receptor systems in addition to their ability to
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9 activate cannabinoid receptors. This study investigated the effect of cannabinoid ligands on the human
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11 adenosine A₃ (hA₃R) receptor. The endocannabinoid 2-AG was able to inhibit agonist ([¹²⁵I]N6- (4-
12
13 amino-3-iodobenzyl) adenosine-5'-(N-methyluronamide) – [¹²⁵I]AB MECA) binding at the hA₃R.
14
15 This inhibition occurred over a narrow range of ligand concentration and was characterized by high
16
17 Hill coefficients suggesting a non-competitive interaction. Furthermore, in the presence of 2-AG, the
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19 rate of [¹²⁵I] AB MECA dissociation was increased, consistent with an action as a negative allosteric
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21 modulator of the hA₃R. Moreover, by measuring intracellular cAMP levels, we demonstrate
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23 that 2-AG decreases both the potency of an agonist at the hA₃R and the basal signalling of
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25 this receptor. Since the hA₃R has been shown to be expressed in astrocytes and microglia, these
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27 findings may be particularly relevant in certain pathological states such as cerebral ischemia where
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29 levels of 2-AG and anandamide are raised.
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54 Keywords: G protein coupled receptor (GPCR), Adenosine A₃ receptor, endocannabinoid, 2-
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56 arachidonylglycerol, allosteric modulation
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1.1 Introduction

The cannabinoid and adenosine pathways have overlapping roles in both the modulation of neurotransmitter release in the central nervous system and modulation of the immune response [1, 2]. The endogenous nucleoside adenosine activates four distinct adenosine receptors, which all belong to the family of G protein coupled receptors (GPCR): the adenosine A₁, A_{2A}, A_{2B} and A₃ receptor [1]. Of these the adenosine A₃ receptor is the most recently discovered and has a wide distribution, with its mRNA being present in testis, lung, heart, placenta, brain, spleen, liver, uterus, proximal colon and bladder [3-5]. There is now extensive evidence for the involvement of the A₃ adenosine receptor in many disease pathways [6]. The A₃ receptor has been implicated in the modulation of inflammatory effects, for example stimulation of the A₃ receptor expressed in RBL-2H3 rat mast cells with adenosine leads to degranulation and may protect mast cells from apoptosis [7]. The A₃ receptor also plays an important role in both neuroprotective and neurodegenerative effects in the brain [6].

Of several endogenous agonists for cannabinoid receptors identified thus far the most notable are anandamide and 2-arachidonylglycerol (2-AG) [2]. Their physiological effects are mainly mediated via the cannabinoid receptors CB₁ and CB₂ [2]. These endocannabinoids have been implicated in both neuromodulatory roles and immunomodulatory functions (e.g. through the regulation of cytokine release and immune cell migration) [8]. CB₁ receptor expression levels are highest in the CNS, whereas CB₂ receptors have a close association with the immune system and mRNA has been detected in a wide variety of immune cells and tissues such as

1 mast cells and microglia [2]. Interestingly, the endocannabinoid 2-AG has been shown to
2 decrease the immunological activation of guinea pig mast cells in a mechanism mediated by
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4 CB₂ receptors and opposite to the effect mediated by adenosine described above [9].
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6 Consequently, it was of interest to investigate the potential for cannabinoid ligands to
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8 modulate the action of the adenosine A₃ receptor.
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12 It should be noted that a number of studies have implicated other sites of action of these
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14 endocannabinoids in addition to their ability to activate cannabinoid CB₁ and CB₂ receptors
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16 [10]. For example, neurobehavioural studies carried out in cannabinoid CB₁ knockout mice
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18 showed that regardless of the lack of CB₁ receptors, anandamide still exerted cannabimimetic-
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20 like activity, even though the CB₁ receptor is believed to be the only receptor for
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22 cannabinoids in the brain [11]. Interestingly, cannabinoids inhibit the production of cytokines
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24 such as tumour necrosis factor- α from activated microglial cells but this activity does not
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26 involve any known CB receptor subtype for it occurs at high concentrations of ligands [12,
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28 13]. Furthermore anandamide has been shown to inhibit ligand binding to central 5-HT
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30 receptors and muscarinic acetylcholine receptors [14-16]. In both cases this has been shown
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32 to be a direct effect, and not mediated by the interaction of the endocannabinoid with either
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34 CB₁ or CB₂ receptors. This is particularly relevant for disease states such as ischemia and
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36 schizophrenia where the local concentration of the endocannabinoid can be elevated [17-20].
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38 Given that cannabinoid receptors and adenosine A₃ receptors are co-localised, for example in
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40 glia and immune cells such as mast cells [2, 6, 21, 22], it is possible that elevated levels of
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42 endocannabinoids may exert a 'spill-over' effect on adenosine receptor binding and/or
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44 functional properties. In the present study we focus on the hA₃ receptor and demonstrate that
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46 the endocannabinoid ligands do indeed have a direct effect on the ligand binding of hA₃
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48 receptors. Furthermore, this study provides evidence that this interaction is non-competitive
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1 and independent of an interaction with either CB₁ or CB₂ receptors. The inhibition of both
2 agonist and antagonist binding is characterised by steep Hill slopes over a narrow
3 concentration range. This effect is specific to the A₃ receptor, with no effect of cannabinoid
4 ligands being observed on the ligand binding ability of A₁ or A_{2A} adenosine receptors.
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6 Moreover, measuring intracellular cAMP levels, we demonstrate that 2-AG decreases both the
7 potency of an agonist at the hA₃R and the basal signalling of this receptor. An investigation of
8 ligand dissociation kinetics demonstrated that eicosanoid ligands, including the endogenous
9 ligands anandamide and 2-AG, are negative allosteric modulators of ligand binding at the
10 adenosine A₃ receptor.
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29 **2 Materials and Methods**

30 **2.1 Materials**

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36 [125I] AB MECA was purchased from Perkin Elmer BV, Groningen, NL. The following
37 cannabinoid ligands were obtained from Tocris Ltd., Avonmouth, UK: ACEA, N-(2-
38 Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; ACPA, N-(Cyclopropyl)-5Z,8Z,11Z,14Z-
39 eicosatetraenamide; AM251, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-
40 methyl-1H-pyrazole-3-carboxamide; AM630, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-
41 1H-indol-3-yl](4-methoxyphenyl)methanone; CP55940, (-)-cis-3-[2-Hydroxy-4-(1,1-
42 dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; GW 405833, 1-(2,3-
43 Dichlorobenzoyl)-5-methoxy-2-methyl-3-[2-(4-morpholinyl)ethyl]-1H-indole; JWH 133,
44 (6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-
45 dibenzo[b,d]pyran; WIN55212-2; (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-
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1 morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone. All
2 other compounds and reagents were purchased from Sigma, NL.
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9 2.2 Cell culture

10 Chinese hamster ovary (CHO) cells stably expressing either the wild type human adenosine
11 A₁ receptor (hA₁) or the wild type human adenosine A₃ receptor (hA₃) were grown in a 1:1
12 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium
13 containing 10% newborn calf serum, streptomycin (50 µg.ml⁻¹), penicillin (50 IU.ml⁻¹) and
14 G418 (0.2 mg.ml⁻¹) at 37 °C in 5% CO₂. HEK 293 cells stably expressing the wild type
15 human A_{2A} adenosine receptor were grown in DMEM containing 10 % newborn calf serum,
16 streptomycin (50 µg.ml⁻¹), penicillin (50 IU.ml⁻¹) and G418 (0.5 mg.ml⁻¹) at 37 °C in 7% CO₂,
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The cells were subcultured twice weekly at a ratio of 1:20. For all membrane preparations the cells were transferred to large 14 cm diameter plates.

2.3 Membrane Preparation

For all cell lines described above the method of membrane preparation was as follows. Cells were detached from the plates by scraping them into 5 mL PBS, collected and centrifuged at 200 x g (1000 rpm) for 5 minutes. Cell pellets derived from 30 plates were pooled and resuspended in 20 mL of ice-cold 50 mM Tris-HCl buffer, pH 7.4. An Ultra-Turrax was used to homogenise the cell suspension. The cytosolic and membrane fractions were separated using a high speed centrifugation step of 100,000 x g, (31,000 rpm) in a Beckman Optima LE-80K ultracentrifuge) at 4 °C for 20 minutes. The pellet was resuspended in 10 mL of Tris

1 buffer and the homogenisation and centrifugation step repeated. The resulting pellet was
2 resuspended in 50 mM Tris-HCl buffer, pH 7.4. For hA₁-CHO and hA_{2A}-HEK293 membrane
3 preparations, adenosine deaminase (ADA) was added to a final concentration of 0.8 IU/ml.
4 For hA₃-CHO membrane preparations no ADA was added since the human A₃ receptor has a
5 low affinity for adenosine.
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11 2.4 Radioligand binding assays – [¹²⁵I] AB MECA equilibrium binding assays

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19 CHO membranes expressing either the human adenosine A₁ receptor (10 µg / reaction) or the
20 adenosine A₃ receptor (7.5 µg / reaction) were incubated with 0.1 nM of the radiolabelled
21 agonist [¹²⁵I] AB MECA in 50 mM Tris-HCl, 10 mM MgCl₂, pH 8.0 with a final volume of
22 100 µL. Reactions were incubated for 3 hours at 25 °C (hA₃-CHO) or 2 hours at 25 °C (hA₁-
23 CHO) and then terminated by rapid filtration through Whatman GF/B filters using a Brandel
24 cell harvester. Filters were washed three times with 4 mL of ice-cold 50 mM Tris-HCl pH 7.4
25 and dried before radioactivity was determined using a Beckman 5500B γ counter (Perkin
26 Elmer, Shelton, CT). Non specific binding was determined in the presence of 10 µM CPA
27 (hA₁-CHO) or 100 µM NECA adenosine-5'-N-ethylcarboxamide (hA₃-CHO). For saturation
28 binding of [¹²⁵I] AB MECA to CHO cell membranes expressing the hA₃ receptor, conditions
29 were as above apart from 5 µg of membrane protein was added per reaction and a range of
30 concentrations of [¹²⁵I] AB MECA was used between 0.025 nM and 5 nM.
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51 The ability of NECA, CPA, DPCPX (8-cyclopentyl-1,3-dipropylxanthine) or various
52 cannabinoid ligands to inhibit [¹²⁵I] AB MECA binding to both hA₁ and hA₃ was also tested.
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54 In these experiments stock solutions of the various adenosine receptor ligands or various
55 cannabinoids were prepared at a concentration of 40 mM in DMSO. Subsequent dilutions
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1 were made in the relevant buffers. Control incubations contained the same concentrations of
2 dimethyl sulfoxide (1.25 % max)
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4 5 6 7 2. 5 Radioligand binding assays – [³H] ZM 241385 equilibrium binding assays 8 9

10 For membranes of HEK 293 cells stably expressing the hA_{2A} receptor, [³H] ZM241385 (4-(2-
11 [7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5,}triazin-5-yl amino]ethyl)phenol) was used
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13 as the radioligand. Membranes containing 40 µg of protein were incubated in a total volume
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15 of 400 µL of 50 mM Tris/HCl (pH 7.4) and [³H] ZM 241385 (final concentration 2.0 nM) for
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17 2 h at 25 °C in a shaking water bath. Nonspecific binding was determined in the presence of
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19 100 µM CPA. The incubation was terminated by filtration as described above. Radioactivity
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21 was determined using a Tri-Carb 2900TR liquid scintillation analyzer (Perkin Elmer, Shelton,
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36 2.6 Radioligand binding assays – [³H] PSB-11 equilibrium binding assays 37 38 39 40

41 Binding experiments of 4 nM [³H] PSB-11 ((8*R*)-8-Ethyl-1,4,7,8-tetrahydro-4-5H-
42 imidazo[2,1-*i*]purin-5-one) to membranes of CHO cells expressing human A₃ adenosine
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44 receptors (50 µg of protein per reaction) were carried out in duplicate at 25 °C for 2 hours in
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46 100 µL of buffer containing 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM EDTA, pH 8.0. Non-
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48 specific binding was defined as that obtained in the presence of 100 µM NECA. Tested
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50 compounds were dissolved in dimethyl sulfoxide as described above. Reactions were
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52 terminated by filtration through GF/B filters on a cell harvester (Brandel, Gaithersburg, MD)
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1 and radioactivity was determined using a Tri-Carb 2900TR liquid scintillation analyzer
2 (Perkin Elmer, Shelton, CT).
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7 2.7 Dissociation of the radiolabelled, selective agonist [¹²⁵I] AB MECA from hA₃-CHO or
8 hA₁-CHO membranes
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14 [¹²⁵I] AB MECA (0.1 nM) was pre-incubated with hA₁-CHO or hA₃-CHO membranes for 2
15 or 3 hours respectively in the reaction buffers described above. Non-specific binding was
16 determined with the addition of 10 μM NECA before pre-incubation. Dissociation was
17 initiated by the addition of NECA (final concentration 10 μM) with or without cannabinoid
18 ligands and determined at various time points. Non-specific binding in the presence of tested
19 agents was also determined in parallel. After an additional incubation of 5 hours at 25 °C, the
20 reactions were terminated by filtration through GF/B filters and radioactivity determined as
21 described above.
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39 2.8 Dissociation of the radiolabelled, selective antagonist [³H] PSB-11 from hA₃-CHO
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46 Membranes (60 μg / reaction) were incubated with 4 nM [³H] PSB-11 in a total assay volume
47 of 100 μL for 120 minutes at 25 °C. Dissociation was initiated by the addition of 10 μM
48 NECA with or without cannabinoid ligands and determined at various time points after
49 equilibration had been reached. Non-specific binding was determined using 10 μM NECA.
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51 The reactions were terminated by filtration through GF/B filters and radioactivity determined
52 as described above.
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2.9 cAMP determination.

Intracellular cAMP levels were measured using a LANCE cAMP 384 kit (Perkin Elmer, The Netherlands) as described previously [23]. In short, to each well different concentrations of Cl-IB-MECA (2-Chloro-*N*⁶-(3-iodobenzyl)-5'-*N*-methylcarbamoyladenosine) in stimulation buffer (PBS with 5 mM Hepes, pH 7.4 supplemented with 0.1 % BSA, rolipram (50 μM) and cilostamide (50 μM)) was added in a volume of 5 μL in the absence (control) or presence of 2-AG or AM 251. Then 4.5 μL CHO_{hA3} cell suspension in stimulation buffer was seeded into a 384-well plate (approximately 5000 cells/well), which was followed by incubation for 15 min at room temperature. Subsequently, 2.5 μL forskolin (1 μM) was added and the mixture was incubated for 30 min at room temperature. Finally, 3 h after adding detection mix (6 μL) and cAMP antibody solution (6 μL), intracellular cAMP levels were measured using a TR-FRET assay on a Victor spectrometer (Perkin Elmer, The Netherlands) according to instructions of the supplier.

2.10 Data analysis

Binding parameters were estimated using Graphpad Prism version 5. Data sets of total and non-specific binding assay were analysed simultaneously via non-linear regression. Analysis was performed according to a hyperbolic, one-site mass action binding model to derive individual estimates of total receptor density (B_{\max}) and radioligand-receptor equilibrium dissociation constant (K_D) as shown in the following equation:

$$\text{Total binding} = \frac{B_{\max} x}{x + K_D} + NSx \quad (1)$$

Radioligand inhibition binding isotherms were analysed according to the following logistic function:

$$Y = \frac{(top - bottom)x^{nH}}{x^{nH} + IC_{50}^{nH}} \quad (2)$$

Where Y denotes the percent specific binding, top and bottom denote the maximal and minimal asymptotes respectively, x denotes the inhibitor potency (midpoint location) parameter and n_H denotes the Hill slope factor. Where appropriate, and assuming simple competition, IC_{50} values were converted to K_i values using the Cheng and Prusoff (1973) equation.

The dissociation of [125 I] AB MECA and [3 H] PSB-11 from the human A_3 receptor was best described by a mono-exponential decay according to the following equation for mono-exponential decay:

$$Y = (a - b)e^{-k_{off} \cdot x} + b \quad (3)$$

Where a represents the vertical span of the data, b is the plateau value, x is the time (min), and k_{off} is the dissociation rate constant (min^{-1}). In contrast the dissociation of [125 I] AB MECA from the human A_1 receptor was best described by a bi-phasic decay.

In the functional, cAMP accumulation assay, agonist concentration response curves were fitted to the following four-parameter Hill equation using prism 5:

$$response = \frac{(top - bottom)}{1 + (10^{\log EC_{50}} / x)^{nH}} \quad (4)$$

1 Where top represents the maximal asymptote of the concentration response curves, bottom
2 represents the lowest asymptote and the concentration-response curves, $\log EC_{50}$ represents the
3 logarithm of the agonist EC_{50} , x represents the concentration of the agonist and n_H represents
4 the Hill slope. Data shown are the mean \pm SEM. Comparisons between models were
5 performed by F-test using Prism. Unless otherwise stated values of $p < 0.05$ were taken as
6 statistically significant.
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3 Results

3.1 Saturation and inhibition binding assays in membranes of CHO cells stably expressing the human hA₃ adenosine receptor

Saturation binding experiments performed for [¹²⁵I] AB MECA at the hA₃R expressed in CHO membranes yielded a K_d of 1.86 ± 0.69 nM and a B_{max} of 1.16 ± 0.28 pmol/mg (**Table 2**). At the working concentration of 0.1nM [¹²⁵I] AB MECA, binding to the hA₃R was completely abolished with the addition of 100 μM GTP indicating that only the high affinity (G protein-coupled) state of the receptor was occupied at this concentration (data not shown). To examine the effects of various cannabinoid ligands (**figure 1**) on the binding of [¹²⁵I] AB MECA to hA₃-CHO membranes, equilibrium binding experiments were performed. Incubation of [¹²⁵I] AB MECA with increasing concentrations of either the endocannabinoids 2-AG or anandamide, the structurally related eicosanoid ACPA or the structurally distinct CB₁ antagonist AM 251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) revealed in each case that the cannabinoid ligand was able to compete in a concentration dependent manner with radioligand binding at the hA₃ adenosine receptor. A pIC₅₀ value of approximately 5 was obtained for all three cannabinoid ligands, with ACPA being the most potent (**see table 1, figure 2**). In every case high Hill coefficients were observed which were significantly larger than and different from unity (p < 0.05). Since a high Hill slope is not consistent with simple competition IC₅₀ values were not converted to K_i values. It should be noted that in the case of both AM 251 and ACPA a full inhibition of [¹²⁵I] AB MECA was observed. However, 2-AG produced a maximum displacement of 80% of specific [¹²⁵I] AB MECA binding. Hill coefficients larger than unity

1 are suggestive for a non-competitive interaction. We extended this study to include a wider
2 range of cannabinoid ligands. As shown in figure 1 this range included both subtype selective
3 and non-selective antagonists and agonists. All tested cannabinoid ligands, when used at a
4 concentration of 10 μ M, are able to displace [125 I] AB MECA, with ACPA (N-(Cyclopropyl)-
5 5Z,8Z,11Z,14Z-eicosatetraenamide) producing the most significant effect, displacing 90 % of
6 bound [125 I] AB MECA. To determine a rank order this data was analysed using a one-way
7 ANOVA followed by a Bonferroni's post-hoc test. ACPA (N-(Cyclopropyl)-5Z,8Z,11Z,14Z-
8 eicosatetraenamide) produced the most significant effect, displacing 90 % of bound [125 I] AB
9 MECA consistent with the data shown in Table 1. The remaining rank order in terms of
10 percentage bound [125 I] AB MECA displaced was as follows: ACPA>AM251, ACEA, AM
11 630, 2 AG, anandamide, GW 405833, WIN 55212-2 > JWH 133. Consequently no pattern in
12 terms of the receptor specificity or efficacy of the ligands was observed. Interestingly, the
13 lipid oleamide, an allosteric enhancer at serotonin receptors did not displace bound [125 I] AB
14 MECA suggesting that the above effect was not simply a non-specific effect due to high
15 concentrations of lipid (data not shown).
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39 **3.2 Inhibition binding assays in membranes of CHO cells stably expressing the human** 40 **hA₁ adenosine receptor or HEK 293 cells stably expressing the hA_{2A} adenosine receptor**

41 To investigate the specificity of this effect, this study was extended to a CHO cell line
42 expressing the hA₁ adenosine receptors or a HEK 293 cell line which stably expresses the
43 hA_{2A} adenosine receptor. For the human A₁ receptor, a 10 μ M concentration of each of the
44 various cannabinoid ligands was unable to inhibit [125 I] AB MECA binding. It is interesting
45 to note that cannabinoid ligands can displace [125 I] AB MECA at the hA_{3R} but not the human
46 A₁ receptor. In the case of the hA_{2A} receptor a 10 μ M concentration of either ACPA, 2-AG or
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1 AM 251 was unable to inhibit the binding of the radiolabeled antagonist [³H] ZM 241385.

2 This suggests that the effect of cannabinoids on the binding of ligands to the adenosine
3 receptors is specific to the human A₃ receptor.
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10 11 12 13 14 **3.3 Competition binding of [¹²⁵I] AB MECA versus the agonist NECA with and without** 15 **the presence of the endocannabinoid 2-AG** 16 17

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22 In order to gain further insight into the mechanism with which endocannabinoid 2-AG may
23 inhibit ligand binding at the hA₃ receptor, competition experiments of [¹²⁵I] AB MECA
24 binding in the presence of increasing concentrations of NECA were performed with and
25 without the addition of 10 μM 2-AG. Although the absolute specific binding of the
26 radioligand was reduced with the addition of 10 μM of 2-AG there was no observed effect on
27 the inhibitory potency of NECA (control: pK_i = 7.17 ± 0.21, + 2-AG pK_i = 7.02 ± 0.04,
28 Student's t-test, P > 0.05, Graphpad prism version 5). This again suggests that the
29 endocannabinoid does not compete for the orthosteric site of hA₃ receptor and must therefore
30 have an alternative allosteric site of action (**figure 3**).
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48 **3.4 Saturation binding of [¹²⁵I] AB MECA to membranes stably expressing the hA₃** 49 **receptor in the presence of increasing concentrations of the endocannabinoid 2-AG** 50 51

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56 To further investigate and confirm the non-competitive inhibition of ligand binding at the hA₃
57 adenosine receptor by the endocannabinoid 2-AG, [¹²⁵I] AB MECA saturation binding
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1 experiments were performed. These revealed a trend for 2-AG to reduce radioligand
2 maximal binding capacity in a concentration dependent manner. Non-linear regression
3 analysis of the data, followed by F-test demonstrated that the simplest model accommodating
4 all observations was a reduction in B_{\max} with no change in radioligand affinity with the
5 increasing presence of 2 AG ($p > 0.05$). Indeed a one way ANOVA analysis followed by a
6 bonferonni post-test revealed that whilst there was no significant difference observed in
7 affinity, there was a significant reduction in B_{\max} with the addition of 1, 3 or 10 μM 2 AG
8 compared to the control condition (**Table 2**). This is consistent with a non-competitive mode
9 of interaction.
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24 **3.5 Inhibition assays using the radiolabelled antagonist [^3H] PSB-11**

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29 Although unlikely given the specific nature of the interaction with the hA_3 receptor, it is
30 possible that inhibition of ligand binding by cannabinoid ligands is an artefact due to the
31 physicochemical properties of [^{125}I] AB MECA. It was therefore decided to use the
32 radiolabelled antagonist [^3H] PSB-11. In experiments analogous to those used with the
33 radiolabelled agonist [^{125}I] AB MECA, the ability of increasing concentrations of either
34 ACPA, 2-AG or AM 251 to displace [^3H] PSB-11 binding was assessed. In the case of both
35 ACPA and AM 251, a pIC_{50} of ~ 4.8 was gained, and steep curves with high Hill coefficients
36 were observed (**table 1, figure 4**). The endogenous ligands 2-AG and anandamide show a
37 lower potency for the inhibition of [^3H] PSB-11 with a pIC_{50} of 4.16 ± 0.11 and 4.25 ± 0.12
38 respectively. Again high Hill coefficients were observed for both 2-AG (4.1 ± 0.4) and
39 anandamide (2.6 ± 0.3) and these Hill coefficients were significantly different from unity ($p <$
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3.6 Dissociation kinetic assays in CHO hA₃ membranes

The inhibition binding assays described above indicated that the cannabinoid ligands interact with the hA₃ receptor at a site different from the orthosteric site and consequently in a manner that is non-competitive with both an agonist and an antagonist. Many compounds that allosterically affect the binding of orthosteric compounds such as [¹²⁵I] AB MECA at the hA₃ receptor do so by altering the dissociation rate of these compounds from the receptor. To determine whether the various cannabinoids possess this ability, [¹²⁵I] AB MECA dissociation kinetic assays were performed. Complete dissociation of [¹²⁵I] AB MECA measured using excess NECA (an agonist) was observed after 10 hours at 25 ° C (data not shown) and yielded a k_{off} of $0.007 \pm 0.0005 \text{ min}^{-1}$ (**table 3, figure 5**). Repeating this experiment in the presence of 10 μM 2-AG or 10 μM anandamide caused a significant increase in the rate of dissociation (**table 3**). Interestingly the above experiment performed in the presence of AM 251 or WIN 55212-2 ((*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de)-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone)) yielded no increase in dissociation rate. The endocannabinoids 2-AG and anandamide are eicosanoids, in contrast to AM 251 or WIN 55212-2 which as a diarylpyrazole or (aminoalkyl)indole respectively have a chemically distinct structures. To see if this pattern is observed across all of the cannabinoid ligands used in this study a 'kinetic screen' was performed. In this screen dissociation was initiated again using excess NECA with or without the presence of the various cannabinoid ligands (**figure 1**). The dissociation was stopped at 120 minutes, a time-point that allows a good window to observe both the effects of allosteric enhancers and inhibitors. Again in this one point screen it can be noticed that 2-AG causes a two fold decrease in the amount of [¹²⁵I] AB MECA bound as compared to excess NECA alone. This is consistent with the increased rate of

1 dissociation observed with the addition of 2-AG observed in the full dissociation curve
2 experiment. Similarly AM 251 showed a level of [125 I] AB MECA binding that was not
3 significantly different from the control experiment with the excess of unlabelled NECA alone
4 and again this is consistent with previous full curve experiments. The eicosanoid ligands,
5 which have a structure that is highly related to 2-AG (**figure 1**), produced a decrease in bound
6 [125 I] AB MECA as compared to that of the excess NECA alone condition (data not shown).
7 Conversely, cannabinoid ligands with a structure distinct from the eicosanoid structure such
8 as CP 55940, GW 405833 and WIN55212-2 did not significantly change the amount of [125 I]
9 AB MECA bound as compared to the NECA control condition. In addition, the non-
10 eicosanoid lipid oleamide, which has been shown to be an allosteric enhancer of ligand
11 binding at the serotonin receptors had no effect on the dissociation of [125 I] AB MECA. In
12 summary then cannabinoid ligands with an eicosanoid structure are negative allosteric
13 modulators of the hA₃ receptor, a property which is not extended to cannabinoids of other
14 chemical classes or indeed other lipids.
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39 **3.7 Dissociation kinetic assays in CHO-hA₁ membranes**

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Dissociation kinetics experiments revealed an interesting and novel action of eicosanoid
cannabinoid ligands as allosteric inhibitors at the human adenosine A₃ receptor. An obvious
next experiment was to see if this effect could be observed for another adenosine receptor.
The ability of the endocannabinoid 2-AG or the CB₁ antagonist AM 251 to affect the
dissociation rate of [125 I] AB MECA from hA₁-CHO membranes was tested. Dissociation of
[125 I] AB MECA from the hA₁ adenosine receptor was best described using a two phase decay
analysis (F test, P<0.05) with a k_{fast} of $0.26 \pm 0.1 \text{ min}^{-1}$ and a k_{slow} of 0.029 ± 0.02 (**table 4**).

1 The addition of 10 μM AM 251 or 10 μM 2-AG did not significantly affect either the rates of
2 the fast or slow phases of dissociation. This demonstrates that the action of eicosanoid
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3.8 Dissociation kinetic assays in CHO-hA₃ membranes using the radiolabelled antagonist [³H] PSB-11

Since both anandamide and 2-AG inhibit the binding of the antagonist [³H] PSB-11 to the hA₃ adenosine receptor, it was interesting to investigate the effect of 10 μM 2-AG on the dissociation of [³H] PSB-11 from CHO membranes expressing the hA₃ receptor. Furthermore since 2-AG displays a lower potency in its ability to displace [³H] PSB-11 binding as compared to [¹²⁵I] AB MECA binding the effect of 100 μM 2-AG was also tested.

Dissociation kinetics experiments demonstrate that the addition of 10 μM 2-AG or indeed 100 μM 2-AG has no effect on the rate of dissociation of [³H] PSB-11 (control $k_{\text{off}} = 0.31 \pm 0.02 \text{ min}^{-1}$, + 10 μM 2-AG $k_{\text{off}} = 0.29 \pm 0.01 \text{ min}^{-1}$, + 100 μM 2-AG $k_{\text{off}} = 0.32 \pm 0.02$). This suggests that 2-AG is a negative allosteric modulator of agonist but not antagonist binding.

3.9 The addition of 10 μM 2-AG causes both a decrease in basal hA₃ adenosine receptor signalling and a six fold decrease in the potency of the full agonist CI-IB-MECA.

The addition of 10 μM and 100 μM 2-AG to forskolin stimulated hA₃-CHO cells caused a concentration dependent and significant increase in cAMP levels (**figure 6Ai**). Interestingly when a parallel experiment was performed on CHO cells that did not express the hA₃

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adenosine receptor, no such increase occurred (**figure 6A ii**). This suggests that the increase in forskolin-stimulated cAMP levels caused by the addition of 2-AG is mediated by a direct interaction of 2-AG with the hA₃ receptor and not via an interaction with adenylate cyclase. We extended these studies to monitor the effect of increasing concentrations of the full agonist CI-IB-MECA on the intracellular cAMP levels of hA₃-CHO cells with and without the addition of 10 μM 2-AG or the non-eicosanoid inverse agonist AM 251 (**figure 6B**). Again, with the addition of 10 μM 2-AG a decrease in basal hA₃ receptor signalling was observed. Furthermore, 10 μM 2-AG caused a six fold decrease in the potency of CI-IB-MECA from 23 ± 13 nM to 135 ± 36 nM, (P <0.05, Student's t-test, Graphpad Prism version 5). Interestingly, this apparent antagonism could not be overcome with an increased concentration of CI-IB-MECA. This is consistent with the allosteric nature of the interaction of 2-AG with the hA₃ receptor shown by ligand binding studies. In contrast the addition of AM 251 had no significant effect on the potency of CI-IB-MECA (16 nM ± 5, Student's t-test, Graphpad prism version 5).

4 Discussion

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2 This study demonstrates a direct interaction of endocannabinoid ligands with the adenosine
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4 A₃ receptor which leads to the inhibition of ligand binding. Our study suggests that this is an
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6 allosteric effect rather than a direct competition with the orthosteric binding site of the A₃
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8 receptor. Accordingly kinetic studies and functional studies demonstrate that eicosanoids act
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10 as allosteric inhibitors at the A₃ receptor. These studies were extended to demonstrate that the
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12 endocannabinoid 2-AG exerts its affect via a distinct site to two previously characterised
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14 allosteric modulators of the adenosine A₃ receptor.
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22 The direct effects of endocannabinoid ligands tested at the A₃ receptor expressed in CHO cell
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24 membranes resulted in inhibition of both agonist ([¹²⁵I] AB MECA) and antagonist ([³H]
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26 PSB-11) binding that was characterised by very steep inhibition curves. This is not in accord
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28 with simple mass action kinetics, indicating a non-competitive mode of interaction.
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31 Furthermore in the case of 2-AG [¹²⁵I] AB MECA binding was not reduced to nonspecific
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33 binding levels. Again this is characteristic of an allosteric interaction, as opposed to that of a
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35 competitive antagonist [24]. To confirm this we performed competition assays of increasing
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37 concentrations of NECA with [¹²⁵I] AB MECA. This revealed no effect of the
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39 endocannabinoid 2-AG on the potency of NECA, but was characterised by a non-competitive
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41 reduction in the total binding. This is consistent with an effect on the maximal number of A₃
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43 binding sites but not affinity. Similar results were obtained for saturation binding of [125I]
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45 AB MECA to hA3-CHO membranes. In the presence of increasing concentrations of 2-AG
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47 no change in affinity was observed, simply a concentration dependent decrease in B_{max}.
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52 However, at the adenosine A₁ receptor the addition of 10 μM 2-AG showed no effect on
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54 ligand binding suggesting that the effect is specific for the adenosine A₃ receptor. This
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56 specificity allows us to discount such effects as micellar formation, or the coating of the
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receptor surface by the hydrophobic cannabinoid ligands. Furthermore, the lipid oleamide did not displace [125 I] AB MECA binding at the hA₃ adenosine receptor. This underlines the specificity of the observed effect both in terms of the hA₃R and the eicosanoid ligands described. Cannabinoid ligands have been shown to alter membrane fluidity [25, 26]. Our results could be reconciled with a mechanism of action for the cannabinoid ligands that occurs via membrane perturbation affecting the functioning of membrane-associated proteins such as GPCRs. To test this hypothesis we monitored the effect of benzyl alcohol, an agent which is frequently used to disrupt membranes[27], on equilibrium binding of [125 I] AB MECA to CHO cell membranes expressing either hA₁ or hA₃ adenosine receptors. In both cases radioligand binding was inhibited in a concentration-dependent manner by benzyl alcohol (data not shown). This demonstrates that although increasing membrane fluidity or indeed causing membrane breakdown does inhibit radioligand binding at GPCRs it does so in a non-receptor-subtype selective manner in contrast to 2 AG.

To further characterise the non-competitive action of the cannabinoid ligands in reducing [125 I] AB MECA binding, the ability of these ligands to influence the rate at which [125 I] AB MECA dissociates from the receptor was tested. Dissociation kinetic studies revealed that, whilst the eicosanoids increase the rate of ligand dissociation, other cannabinoids of unrelated structures do not. Ligands with an eicosanoid structure, including the endocannabinoids 2-AG and anandamide act as negative allosteric modulators and increase the rate of dissociation of the agonist [125 I] AB MECA. This is in contrast to the previous findings of Christopoulos et al. who demonstrated that anandamide, whilst inhibiting radioligand binding at the muscarinic M₁ and M₄ receptors with high Hill coefficients, did not affect the rate of ligand dissociation [15]. We extended the study to monitor the effect of 2-AG on the rate of dissociation of the selective hA₃ receptor antagonist [3 H] PSB-11. No effect was observed

1 using a concentration of 10 μM 2-AG, suggesting that the endocannabinoid at such a
2 concentration is a negative allosteric modulator of agonist but not antagonist binding at the
3 human A_3 receptor. Similarly the potency of the inhibition antagonist binding by 2-AG was
4 much lower than that observed for agonist binding. This suggests then that 2-AG modulates
5 agonist binding to a greater extent than antagonist binding.
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14 It was of importance to assess the effect of 2-AG on the function of the hA_3 adenosine
15 receptor. By monitoring intracellular cAMP levels in the presence of increasing
16 concentrations of the agonist Cl-IB-MECA the dose dependent inhibition of forskolin
17 stimulated cAMP mediated by the hA_3 receptor was demonstrated. With the addition of 10
18 μM 2-AG we observed both a decrease in basal hA_3R signalling and a six fold decrease in the
19 potency of Cl-IB-MECA. This finding is in complete agreement with the data from our
20 radioligand binding studies. Thus, 2-AG acts as an allosteric inhibitor of hA_3R ligand binding
21 and function. In contrast AM 251 a non-eicosanoid ligand, displays no effect on the potency
22 of Cl-IB-MECA. Interestingly this compound along with all other tested non-eicosanoid
23 ligands has no effect on ligand dissociation. Therefore both kinetic binding and functional
24 studies suggest that although all tested cannabinoid ligands did inhibit ligand binding at the
25 hA_3 receptor, a different mechanism and/or binding site may exist for the non-eicosanoid
26 ligands as compared to the eicosanoid ligands. It should be noted that both anandamide and
27 2-AG are metabolically unstable and may be broken down into the eicosanoid arachidonic
28 acid. However, it has been demonstrated that CHO cells (as used in this study) do not possess
29 a 2-AG removal mechanism and therefore the potential breakdown of this eicosanoids should
30 not influence the findings reported in this study [28]. The results of this study could be
31 reconciled with cannabinoid ligands influencing receptor binding via their interaction with a
32 cannabinoid receptor such as CB_1 , CB_2 or indeed GPR55 which has recently revealed to be a
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1 novel cannabinoid receptor [29, 30]. However, data from this study is not consistent with this
2 explanation. Firstly in the cAMP experiments on ‘empty’ CHO cells no significant effect of
3 2-AG addition on the basal or forskolin stimulated conditions was observed. If either CB₁ or
4 CB₂ cannabinoid receptors were expressed, a concentration dependent decrease in forskolin
5 stimulated cAMP production would be expected consistent with their ability to activate G_i G
6 proteins. In contrast, in the CHO cell line expressing the hA₃ receptor a significant decrease
7 in forskolin stimulated cAMP was observed even in the absence of the hA₃ adenosine receptor
8 ligand. This would suggest a direct interaction with the hA₃ receptor. Secondly, the
9 inhibitory effect on ligand binding was not restricted to ligands with selectivity for a
10 cannabinoid receptor subtype. In contrast a differential effect was observed dependant on
11 ligand structure, with eicosanoid ligands only increasing the rate of ligand dissociation.
12 Finally, AM 251, an antagonist at the CB₁ receptor, has recently been shown to activate
13 GPR55 [31]. In contrast, 2 AG showed no effect at this receptor. Therefore the data
14 presented in this study cannot be reconciled with an interaction with GPR55. The decrease in
15 basal adenosine A₃ receptor activity observed upon the addition of 2 AG suggests that the
16 eicosanoid could be acting as an allosteric inverse agonist. However, the presence of
17 adenosine causing this basal hA₃ adenosine receptor signal cannot be ruled out.
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46 The levels of the endocannabinoid anandamide in the brain can be raised in certain
47 pathological states such as cerebral ischemia or schizophrenia [17, 19, 20]. 2-AG has been
48 shown to have an affinity for cannabinoid receptors that is in the low micromolar range [2].
49 Accordingly this endocannabinoid is present in the brain at levels up to 800 times that of
50 anandamide and again this level can be raised in ischemic conditions [17, 32, 33].
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1 particularly relevant in these conditions. It is important to state that this interaction has yet to
2 be demonstrated in vivo. However, numerous studies have suggested other sites of action of
3 endocannabinoids in addition known cannabinoid receptors [10]. For example, cannabinoids
4 inhibit the production of cytokines such as tumour necrosis factor- α from activated microglial
5 cells but this activity does not involve any known cannabinoid receptor subtype for it occurs
6 at high concentrations of ligands [12, 13]. Interestingly, the expression of adenosine A₃
7 receptors has been demonstrated in microglia [34]. More recently the important role of the
8 adenosine A₃ receptor in modulating Toll-like receptor mediated responses in microglia has
9 been demonstrated [35]. This study would suggest that, by a direct interaction with the
10 adenosine A₃ receptor, the endocannabinoid 2-AG may modulate this role.
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27 It should be noted that other endogenous allosteric modulators have been identified, such as
28 endogenous cations, the peptide Leu-Ser-Ala-Leu acting on 5-HT_{1B} receptors and the
29 amidated lipid oleamide acting on 5-HT₂ and 5-HT₇ receptors [36, 37]. Finally, most relevant
30 to this work, the inhibition of ligand binding and function of muscarinic M₁ and M₄ receptors
31 by anandamide has been shown to be non-competitive and mediated by a direct interaction
32 with the muscarinic receptors [15, 16].
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44 The recently published crystal structures of the β_2 -adrenergic receptor, bound with the inverse
45 agonists carazolol and timolol respectively, reveal a specific binding site for cholesterol
46 molecules with the receptor. [38, 39]. In one study, incorporation of cholesterol induced an
47 increase in affinity for an inverse agonist but did not affect agonist binding [39]. This
48 illustrates that a change in the lipid environment of a receptor can modulate the ligand binding
49 and function of that receptor. Increases in the levels of lipids such as 2-AG, then, may
50 modulate receptor function via their specific interactions with that receptor. The modulation
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1 of the hA₃R but not the hA₁R demonstrates the selectivity of 2-AG, and suggests the presence
2 of a specific binding site on the hA₃R. It is interesting to note that Hanson et al. find that the
3 proposed cholesterol binding site is present in 44% of class A GPCRs [39]. Again this
4 demonstrates a degree of selectivity in the binding of cholesterol to certain GPCRs. As
5 mentioned in the introduction, actions of endocannabinoids which are independent of their
6 interaction with either CB₁ or CB₂ cannabinoid receptors are particularly well documented.
7 The allosteric modulation of G protein-coupled receptors, including the adenosine A₃
8 receptor, may thus represent yet another mechanism by which endocannabinoid ligands exert
9 their physiological effects independently of their interaction with CB₁ or CB₂ cannabinoid
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28
29 We thank Prof C.E. Müller (University of Bonn, Germany) for providing us with [³H] PSB-
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13 specific cholesterol binding site is established by the 2.8 Å structure of the human beta2-
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15 adrenergic receptor. *Structure* 2008;16:897-905.

16 17 18 **Legends for Figures**

19 20 21 22 **Figure 1**

23
24 **A summary of the structures of cannabinoid ligands used in this study, with both an**
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26 **icosanoid and non-icosanoid structure. Also included is the structure of oleamide, a**
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28 **non-icosanoid lipid.** The selectivity of the various ligands for either cannabinoid CB1 or CB2
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30 receptors is noted based on data from Pertwee (2006) *Cannabinoid receptor ligands*. Tocris
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37 38 39 40 **Figure 2**

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42 **Displacement of [¹²⁵I] AB MECA by various cannabinoid ligands is characterised by**
43
44 **high Hill coefficients.**

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46 The ability of the cannabinoid ligands 2-AG (▲), AM 251 (●), anandamide (▼) and ACPA
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48 (■) to inhibit 0.1 nM [¹²⁵I] AB MECA binding at the hA₃ expressed in CHO membranes was
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50 investigated. Incubation was for 3 hours at 25 °C in Tris-HCl buffer pH 8.0, 10 mM MgCl₂,
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55 56 57 **Figure 3**

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The addition of 10 μM 2-AG reduces maximal [^{125}I] AB MECA binding but does not change the affinity of an agonist at the hA_3 adenosine receptor. Inhibition binding of the agonist NECA against 0.1 nM [^{125}I] AB MECA in the absence or presence of 10 μM 2 AG at the hA_3 receptor expressed in CHO cell membranes. Incubation was for 3 hours at 25 °C in 50 mM Tris-HCl buffer pH 8.0, 10 mM MgCl_2 .

Figure 4

Displacement of the antagonist [^3H] PSB-11 by various cannabinoid ligands is characterised by high Hill coefficients. Inhibition binding of 2-AG (●), anandamide (■), AM 251 (▲) and ACPA (▼) against 4 nM [^3H] PSB-11 at the hA_3 receptor expressed in CHO membranes. Incubation was for 2 hours at 25 °C in 50 mM Tris-HCl buffer, 10 mM MgCl_2 , 1mM EDTA pH 8.0.

Figure 5

The addition of 10 μM 2-AG increases the rate of dissociation of [^{125}I] AB MECA from CHO membranes expressing the hA_3 receptor. Membranes were incubated with 0.1 nM [^{125}I] AB MECA at 25 °C for 3 hours in 50 mM Tris-HCl buffer pH 8.0, 10 mM MgCl_2 , , before dissociation was started by addition of 10 μM NECA alone (●), or in combination with 10 μM 2-AG (■), 10 μM anandamide (□), 10 μM WIN55212-2 (Δ) or AM 251 (▲). Normalised curves represent the best fit via non-linear regression analysis. Data points represent the mean \pm S.E.M. of three experiments conducted in duplicate.

Figure 6

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A) The addition of 10 μ M and 100 μ M 2-AG causes a concentration-dependent increase in forskolin stimulated cAMP production in CHO cells expressing the human adenosine A₃ receptor (ii), but not in CHO cells with no adenosine A₃ receptor expression (i). (Data was analysed using a one-way ANOVA with a bonferroni post-hoc test , significant differences are noted as follows: $p < 0.05$ - *, $P < 0.01$ - **, $P > 0.001$ - *)**

B) The modulatory effects of 10 μ M 2-AG or 10 μ M AM 251 on the dose-response curve of Cl-IB-MECA for the inhibition of the forskolin-stimulated cAMP production in CHO cells expressing the human adenosine A₃ receptor. The addition of 10 μ M 2-AG caused a significant, six-fold reduction in the potency of the full hA₃R agonist Cl-IB-MECA from 23 ± 13 nM to 135 ± 36 nM. The addition of 10 μ M AM 251 has no effect on the potency of CL-IB-MECA (16 ± 5 nM)., ($P < 0.05$, Student's t-test, Graphpad Prism version 5, representative graphs from three experiments performed in duplicate)

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Table 1

Displacement of [¹²⁵I] AB MECA or of [³H] PSB-11 from CHO membranes expressing the hA₃ receptor by the cannabinoid ligands ACPA, anandamide, 2-AG and AM 251.

Data are means (±SEM) of 3 experiments performed in duplicate

	Displacement of [¹²⁵ I] AB MECA		Displacement of [³ H] PSB-11	
	pIC ₅₀ value, M	Hill coefficient	pIC ₅₀ value, M	Hill coefficient
AM 251	4.9 ± 0.3	3.2 ± 0.2	4.8 ± 0.1	3.2 ± 1.1
Anandamide	4.4 ± 0.3	3.49 ± 0.3	4.3 ± 0.1	2.6±0.3
ACPA	5.3 ± 0.1	2.7 ± 0.5	4.8 ± 0.2	2.1 ± 0.5
2-AG	4.9 ± 0.1	3.5 ± 0.7	4.2 ± 0.1	4.1±0.4

Table 2

Saturation binding of [¹²⁵I] AB MECA to CHO membranes stably expressing the hA3 receptor in the presence or absence of increasing concentrations of 2-AG. Data was analysed using a one-way anova with a bonferonni post-hoc test. Significant differences in B_{max} as compared to the control condition are shown. No significant change in K_D was observed (p < 0.05 - *, P < 0.01 - **, P < 0.001 - ***).

	K_D, nM	B_{max}, pmol.mg⁻¹
Control	1.86 ± 0.69	1.16 ± 0.28
+ 1 μM 2-AG	1.16 ± 0.24	0.68 ± 0.08 *
+ 3 μM 2-AG	1.57 ± 0.27	0.56 ± 0.06 **
+ 10 μM 2-AG	0.93 ± 0.15	0.34 ± 0.04 ***

Table 3

The dissociation of [¹²⁵I] AB MECA from CHO membranes expressing the hA₃ receptor with or without the presence of either 2 AG or AM 251. The rate and half life observed for conditions in which cannabinoid ligands were present at a concentration of 10 μM were compared with the control (10 μM NECA only) condition using a one way ANOVA analysis with Bonferroni's post-hoc test, Graphpad prism 5. Data are means ± SEM of three experiments were performed in duplicate. (Significant differences as compared to the 10 μM NECA condition are noted as follows: *, P<0.05)

	k_{off}, min⁻¹	Half-Life, minutes
10 μM NECA	0.0069 ± 0.0005	100 ± 6
10 μM NECA + 2 AG	0.0109 ± 0.001 *	72 ± 3 *
10 μM NECA + Anandamide	0.0102 ± 0.003 *	80 ± 5 *
10 μM NECA + AM 251	0.0060 ± 0.0005	115 ± 9
10 μM NECA + WIN 55212	0.0067 ± 0.010	108 ± 10

Table 4

The dissociation of [¹²⁵I] AB MECA from CHO membranes expressing the hA₁ receptor with or without the presence of either 2-AG or AM 251

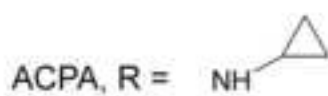
	10 μM NECA	10 μM NECA + 2-AG	10 μM NECA + AM251
k _{Fast}	0.26 ± 0.1	0.31 ± 0.06	0.27 ± 0.18
k _{Slow}	0.029 ± 0.016	0.028 ± 0.093	0.023 ± 0.004
Half life fast, min	2.98 ± 1.05	2.29 ± 0.42	3.21 ± 2.12
Half life slow, min	27.8 ± 11.5	23.6 ± 6.12	27.9 ± 1.22
% Fast	34 ± 2.61	22.3 ± 2.08	41 ± 17

Figure 1

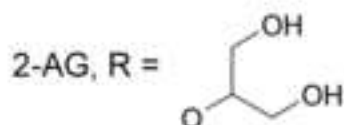
Eicosanoid cannabinoid receptor agonists



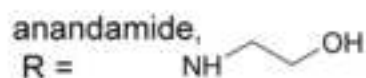
CB1 selective agonist



CB1 selective agonist

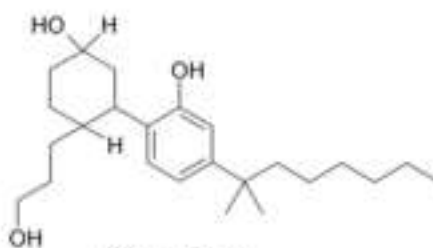


non-selective agonist

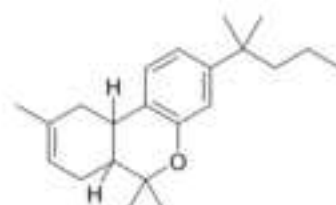


CB1 selective agonist

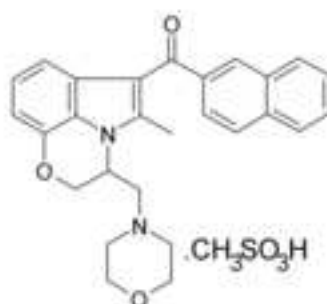
Non-eicosanoid cannabinoid receptor agonists



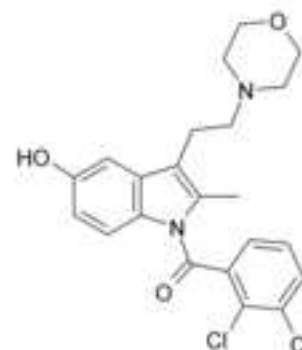
CP55940
non-selective agonist



JWH 133
CB2 selective agonist



WIN 55,212-2
non-selective agonist



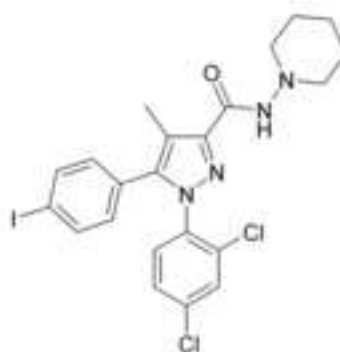
GW 405833
CB2 selective agonist

Non-eicosanoid cannabinoid receptor antagonists

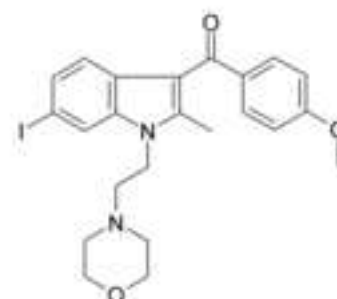
Non-eicosanoid lipid



Oleamide



AM 251
CB1 selective antagonist



AM 630
CB2 selective antagonist

Figure 2

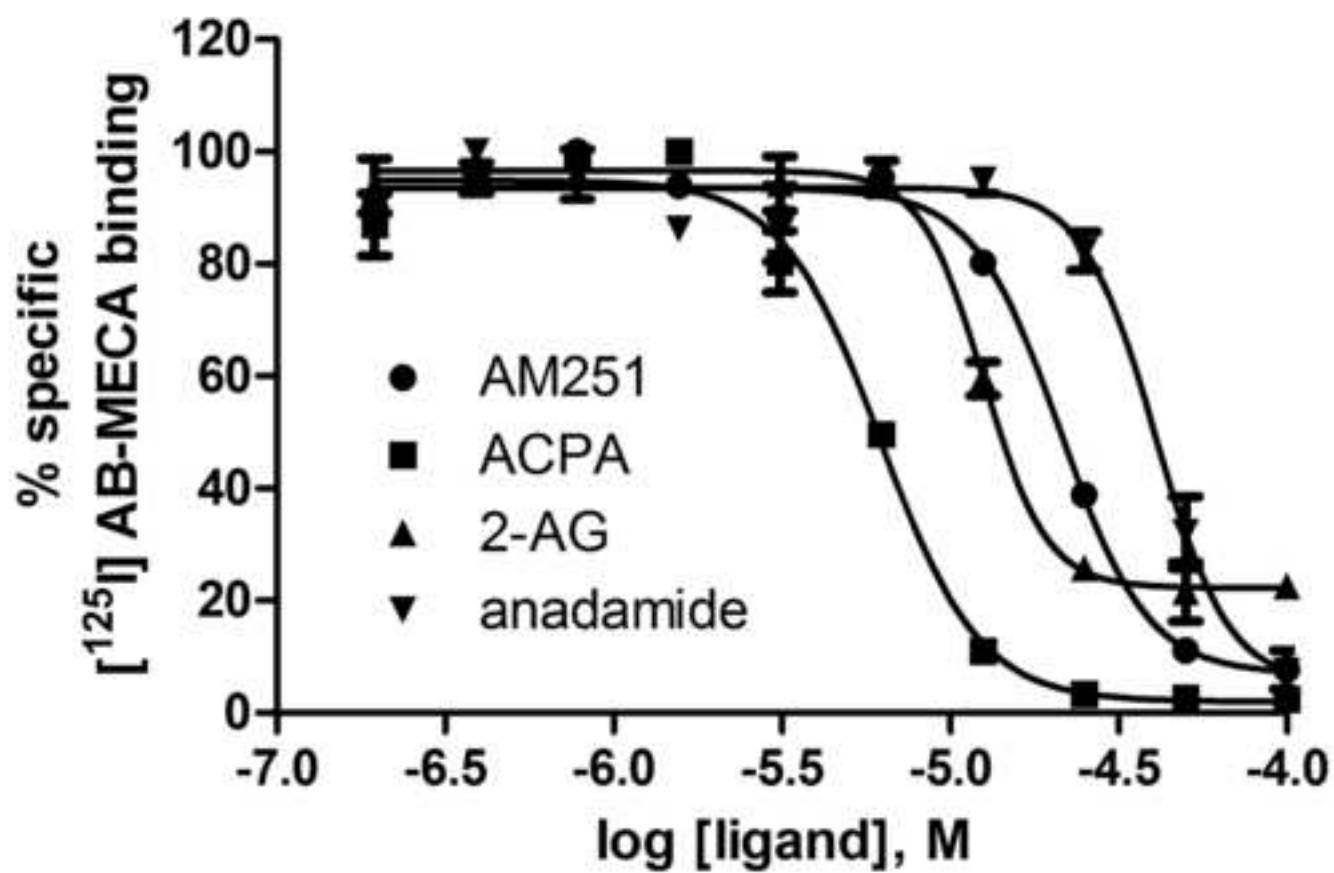


Figure 3

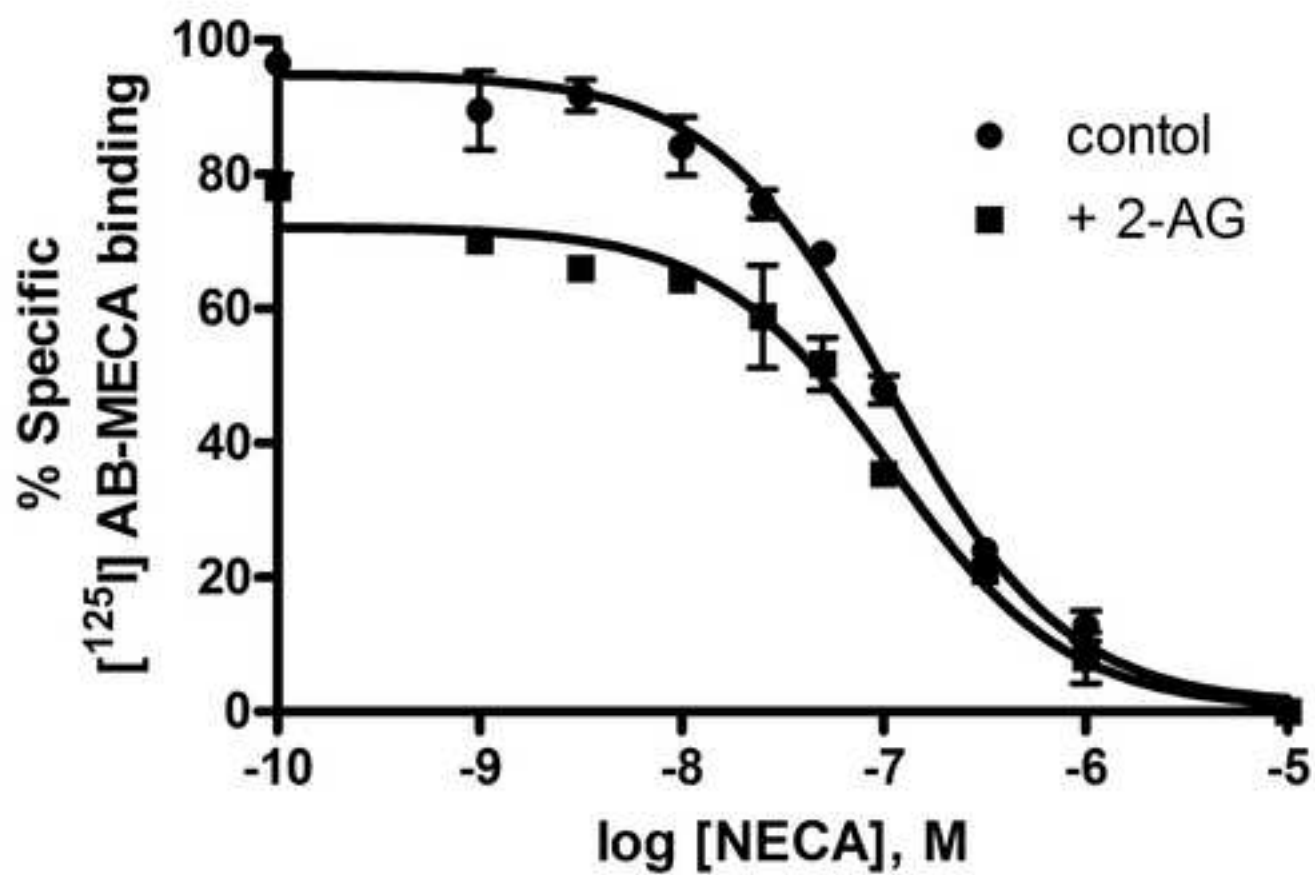


Figure 4

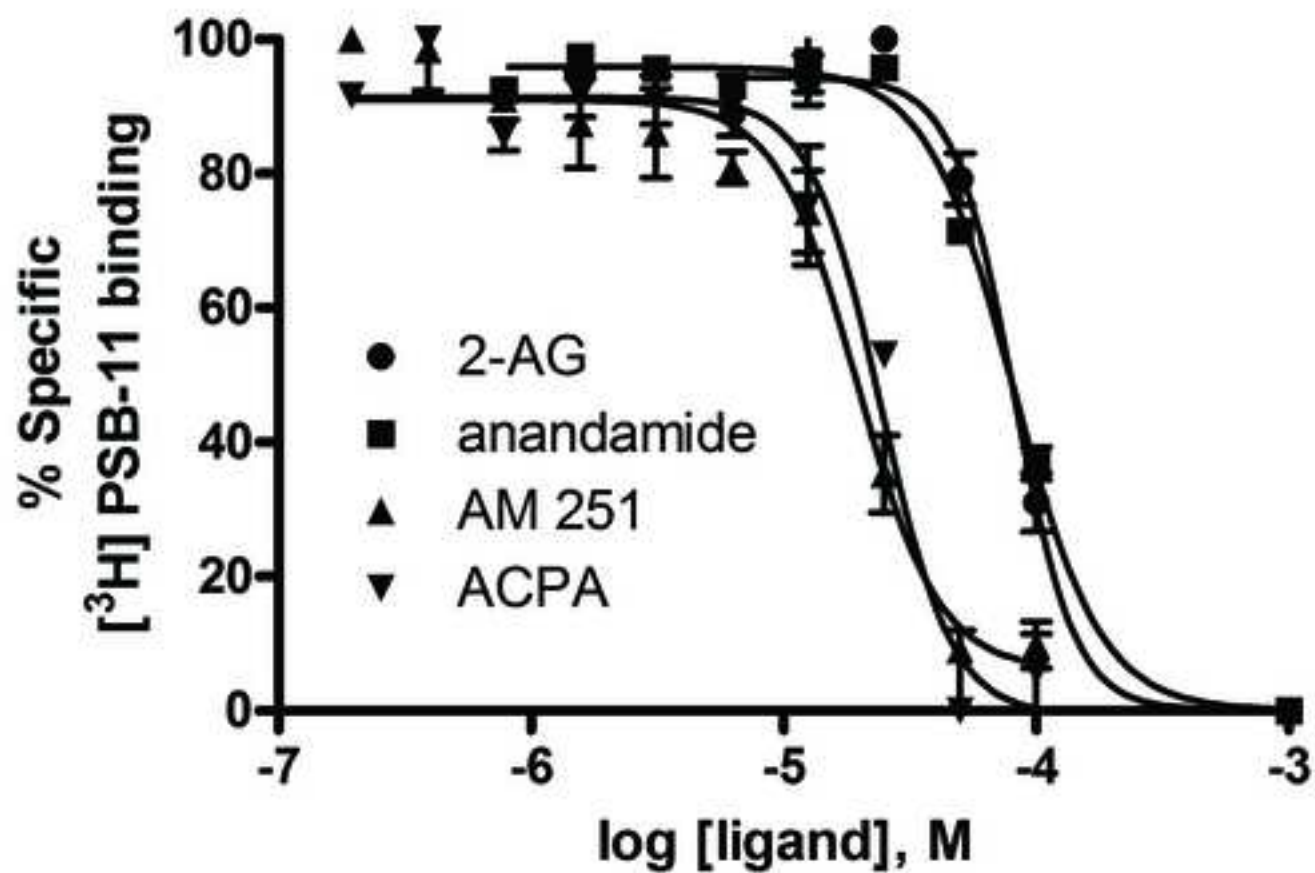


Figure 5

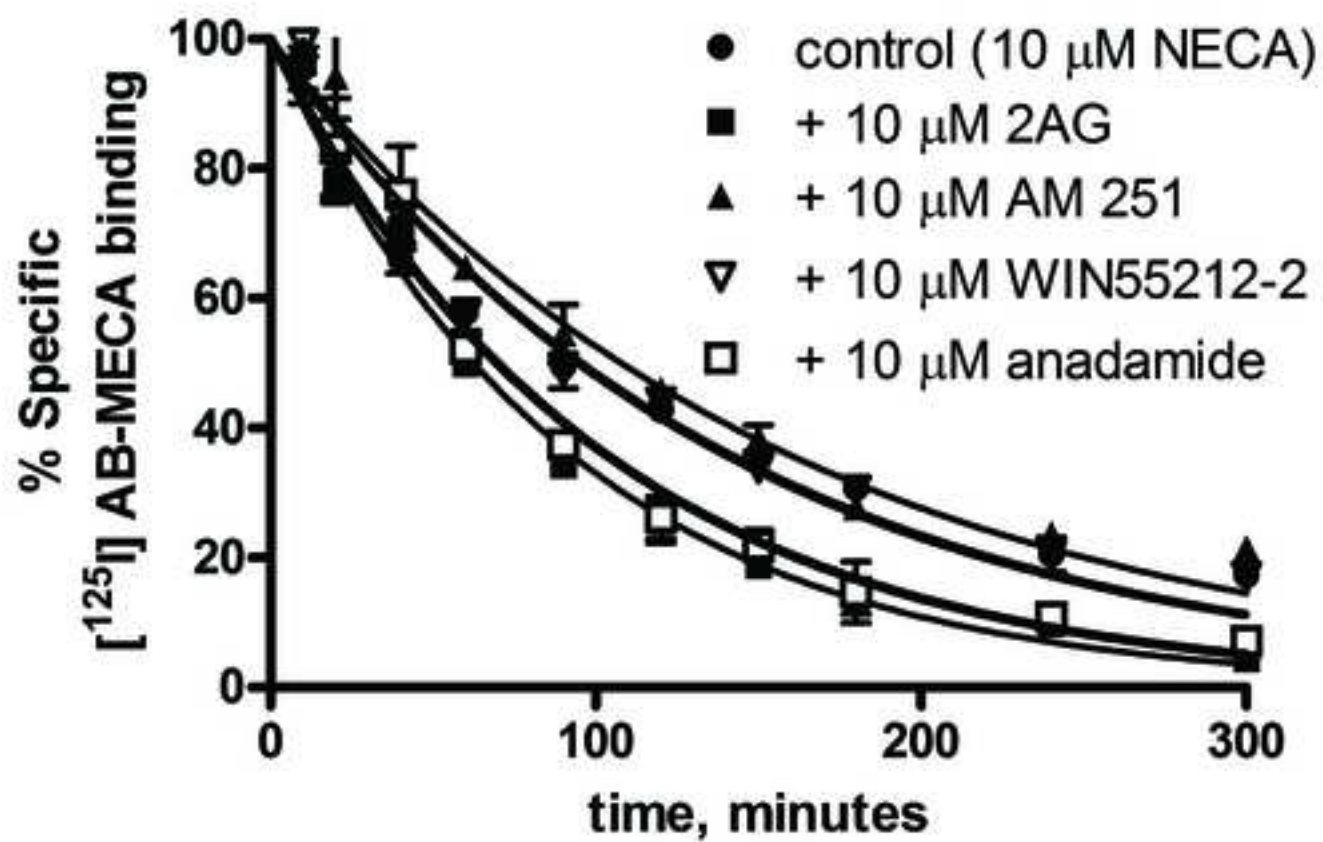
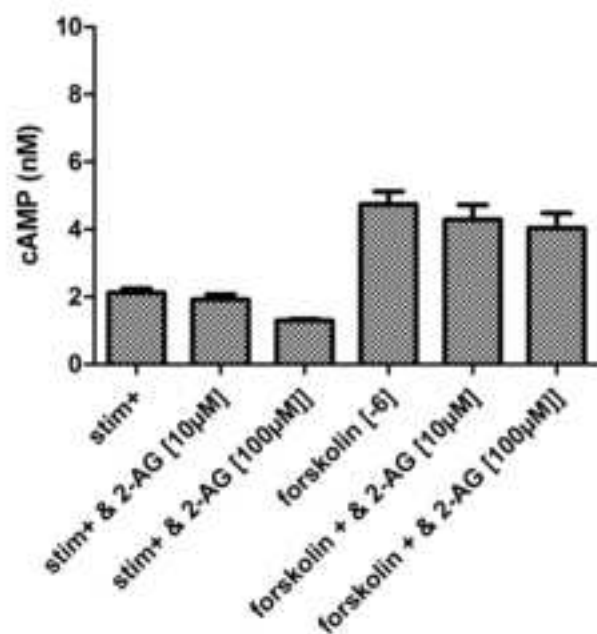


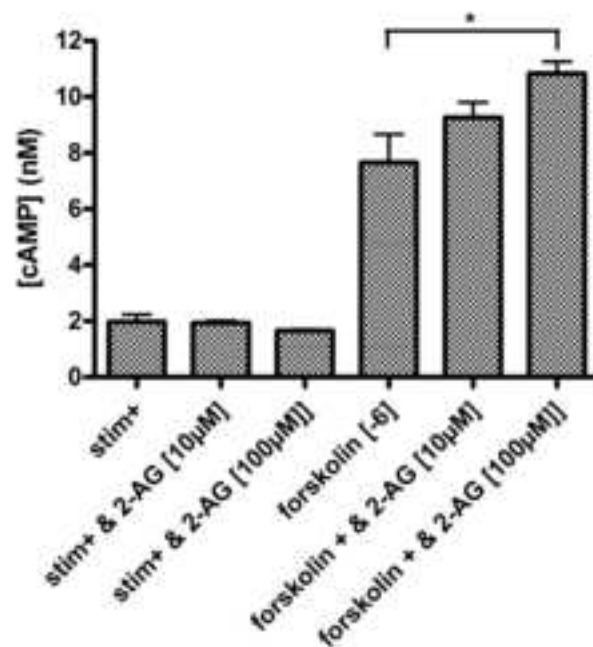
Figure 6

A)

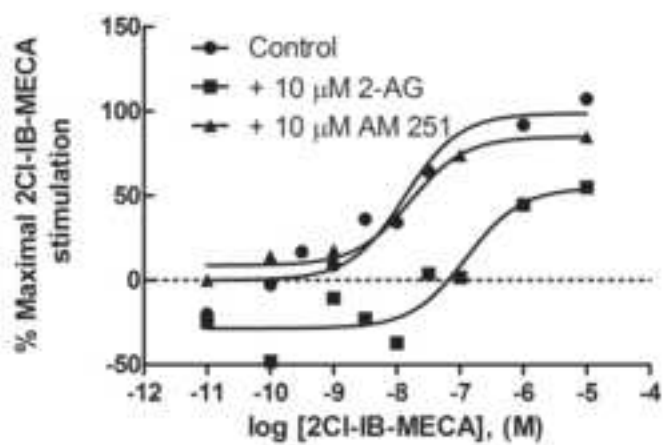
i)



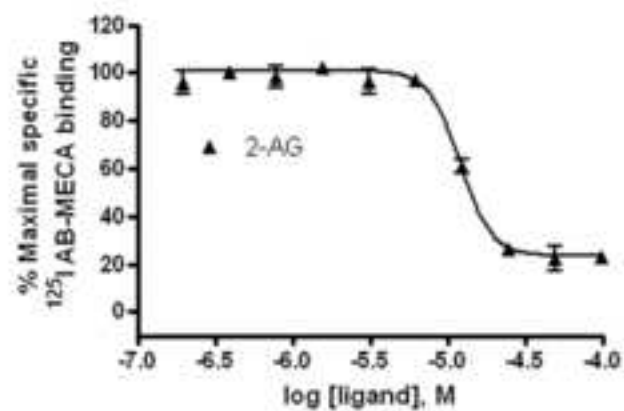
ii)



B)



1) 2-arachidonlyglycerol inhibits agonist binding at the hA₃ adenosine receptor



2) Kinetic binding studies and functional assays demonstrate that 2-AG is a negative allosteric modulator of the hA₃ receptor

