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Effect of repeated systemic administration of selective inhibitors of endocannabinoid inactivation on rat brain endocannabinoid levels

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Abstract

Several selective inhibitors of endocannabinoid inactivation via either the fatty acid amide hydrolase (FAAH) or the putative endocannabinoid transporter have been developed so far. Here, we have studied the effect in rats of a subchronic intraperitoneal treatment with three recently developed selective inhibitors of endocannabinoid uptake (VDM-11, UCM-707 and OMDM-2) or with a selective FAAH inhibitor (*N*-arachidonoyl-serotonin, AA-5-HT), on the brain levels of anandamide and 2-arachidonoylglycerol (2-AG) measured by means of isotope dilution LC–MS 1, 5 and 12 h after the last treatment. OMDM-2 was the most efficacious compound at enhancing the levels of anandamide at all time points, with a maximal effect (1.9-fold enhancement) after 5 h. This compound also enhanced 2-AG levels by ~1.3-fold, but only 5 and 12 h from administration. VDM-11 slightly, albeit significantly, enhanced anandamide levels (1.3-fold) only at 1 h from administration and 2-AG levels (1.3-fold) only after 5 h. Finally, UCM-707 only affected 2-AG levels (by two-fold) at only 1 h from administration. FAAH inhibition by AA-5-HT significantly enhanced the levels of both anandamide (between 1.3- and 1.5-fold, maximal effect after 1 h) and 2-AG (between 1.3- and 1.6-fold, maximal effect after 12 h) at all time points. Brains from rats treated with AA-5-HT did never exhibit enhanced levels of serotonin, thus pointing to the metabolic stability of this FAAH inhibitor. These data indicate that: (1) the pharmacological effects reported so far for the four compounds under study in animal models of diseases may be due to enhancement of both anandamide and 2-AG levels; (2) 2-AG seems to need a longer time after the last administration in order to be augmented; (3) OMDM-2 and AA-5-HT should be regarded as enhancers of endocannabinoid levels suitable for use in vivo.

1. Introduction

The discovery of the metabolic pathways for endocannabinoid inactivation does not simply represent an important step towards the understanding of the endocannabinoid system. In fact, the knowledge of the mechanisms through which the endogenous levels of the endocannabinoids, and hence the state of activation of cannabinoid receptors, are regulated might have an enormous impact also on the development of new therapeutic drugs. This concept is based on the original assumption, now being supported by an ever increasing number of experimental reports, that the symptoms of several central and peripheral disorders are due to, or are causative of, changes in endocannabinoid biosynthetic and degradative pathways, and, subsequently, of pathologically altered activation of either CB_1 or CB_2 cannabinoid receptors [1]. If it is found that the symptoms of a certain disorder are caused, for example, by defective endocannabinoid levels or that endocannabinoids are produced in order to counteract the symptoms or the progress of the disorder, then substances selectively inhibiting endocannabinoid inactivation might produce beneficial effects by prolonging endocannabinoid life-span and enhancing endocannabinoid levels. There is, indeed, an increasing number of reports in the literature of diseases where this happens, at least in animal models, including: multiple sclerosis [2,3]; Huntington's disease [4]; glutamate excitotoxicity and epilepsy [5,6]; glutamatergic hyperactivity in Parkinson's disorder [7]; anxiety [8]; inflammatory pain [9]; cholera toxin-induced

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Table 1
Summary of the previously reported pharmacological properties in vitro and in vivo of the fours compounds tested in this study

	Uptake (IC_{50}, μM)	Rat brain FAAH (IC_{50}, μM)	Rat CB_1 (K_i, μM)	Rat CB_2 (K_i, μM)	Behavioural activity of acute administration in healthy animals	Activity in animal models of CNS disorders
OMDM-2	3.0[a] [28]	>50 [28]	5.1 [28]	>10 [28]	No inhibition of locomotion (open field, 1–10 mg/kg, i.p.) in rats. Weak analgesia (hot plate, 5 mg/kg, i.p.) in rats [25]	Inhibition of spasticity in CREAE (acute, 5 mg/kg, i.v.) [25]. Amelioration of clinical score in Theiler's virus-induced EAE (chronic, 2–7 mg/kg, i.p.) in mice [15]
VDM-11	10.2[a] [26]	>50 [26]	>5 [26]	>10 [26]	No inhibition of locomotion (open field, 1–10 mg/kg, i.p.) in rats. Weak analgesia (hot plate, 5 mg/kg, i.p.) in rats [23]	Inhibition of spasticity in CREAE (acute, 5 mg/kg, i.v.) [25]
UCM-707	0.8[b] [27], 25[a] [43]	30 [27]	4.7 [27]	0.067 [27]	Inhibition of locomotion (open field, 10 mg/kg, i.p.) but no effect on analgesia (hot plate, 1–10 mg/kg, i.p.) in rats [23]	Inhibition of seizures in kainate-treated mice (acute, 3 mg/kg, i.p.) [5]
AA-5-HT	>25[a] [29]	5.6 [29]	>25 [29]	Not tested	Induction of immobility (ring test, 10 mg/kg, i.p.) but no effect on analgesia (hot plate), locomotion (open field), or rectal temperature (10 mg/kg, i.p.) in mice [29]	Enhancement of non-opioid stress-induced analgesia (acute, 10 mg/kg, i.p.) in mice [44]

EAE, experimental allergic encephalomyelitis; CREAE, chronic relapsing EAE.
[a] Tested in rat basophilic leukaemia (RBL-2H3) cells.
[b] Tested in human lymphoma U937 cells.

diarrhea and intestinal inflammation [10,11]; some types of cancer [12], to quote just a few. In these cases, there are already numerous examples of inhibitors of endocannabinoid inactivation being capable of ameliorating the symptoms and even the course of the disorders [8–10,13–15]. Such inhibitors are theoretically capable of influencing endocannabinoid levels by acting at either of the two major steps whereby endocannabinoids are usually inactivated: (1) cellular re-uptake via a putative membrane transporter and/or alternative/additional cellular processes specific for endocannabinoids [16,17]; (2) intracellular hydrolysis via the enzymes fatty acid amide hydrolase (FAAH), which is capable of recognizing as substrate both anandamide and 2-AG, and monoacylglycerol lipase, specific for 2-AG [18,19].

Despite the wide use of inhibitors of endocannabinoid inactivation against the symptoms and the progress of disorders in animal models, only in a few cases the actual effect of these compounds on endocannabinoid levels has been investigated. Indeed, these investigations have been limited so far to one inhibitor of endocannabinoid reuptake, AM404 [20], which is not selective over vanilloid (TRPV1) receptors and FAAH [21,22], and to two FAAH inhibitors, URB-597 and OL-135 [8,9], whose selectivity towards phospholipase A_2 and endocannabinoid reuptake, respectively, has not been assessed. However, to date, no inhibitor of anandamide reuptake or FAAH has ever been shown to be effective at raising 2-AG levels after systemic administration. Furthermore, no data exist on the effect on endocannabinoid levels of compounds that have proven to be selective towards TRPV1 receptors and FAAH (in the case of uptake inhibitor) or towards phospholipase A_2 (in the case of FAAH inhibitors), and are still efficacious in vivo under both physiological and pathological conditions [5,10,13–15,23–25]. These compounds are VDM-11, UCM-707 and OMDM-2, three second generation uptake inhibitors [26–28] and *N*-arachidonoyl-serotonin (AA-5-HT), a FAAH inhibitor developed in the late 1990's [29] (Table 1). In the present study, we have aimed at: (1) understanding whether these four selective uptake or FAAH inhibitors do enhance brain anandamide levels and (2) testing new conditions of administration, i.e. systemic treatment over 2 days, with sampling at different times from the last administration, in the attempt to obtain the enhancement of the brain levels also of 2-AG, which is the most abundant, efficacious and selective endocannabinoid identified so far.

2. Materials and methods

2.1. Drugs

VDM-11, UCM-707, OMDM-2 and AA-5-HT were synthesized in our laboratories as previously described [26–29].

2.2. Animals, treatment and sampling

Male Wistar rats were housed in a room with controlled photoperiod (08:00–20:00 light) and temperature (23 + 1 °C). They had free access to standard food and water

and were used at adult age (3-month-old; 250–300 g weight) in experiments conducted according to European rules (directive 86/609/EEC). Rats were administered intraperitoneally with the compounds at a dose of 5 mg/kg. The treatment included five injections done every 12 h over a total period of 48 h. Rats injected with vehicle (Tween 80-saline solution) were used as controls. The animals were sacrificed after 1, 5 and 12 h from the last administration and their brains were immediately removed and frozen to avoid the post mortem rise in the levels of long-chain *N*-acylethanolamines [30].

2.3. Endocannabinoid level measurements

Brains were homogenized in 5 vol. of chloroform/methanol/Tris–HCl 50 mM (2:1:1) containing 500 pmol of d_8-anandamide and d_8-2-arachidonoylglycerol. Deuterated standards were synthesized from d_8-arachidonic acid and ethanolamine or glycerol as described previously [31,32]. Homogenates were centrifuged at 13,000 × *g* for 16 min (4 °C), the aqueous phase plus debris were collected and extracted again twice with 1 vol. of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized samples were then stored frozen at −80 °C under nitrogen atmosphere until analyzed. Lyophilized extracts were resuspended in chloroform/methanol 99:1 (v/v). The solutions were then purified by open bed chromatography on silica as described in Fontana et al. [33]. Fractions eluted with chloroform/methanol 9:1 (v/v) (containing anandamide and 2-AG) were collected and the excess solvent evaporated with a rotating evaporator and aliquots analyzed by liquid chromatography–atmospheric pressure chemical ionisation–mass spectrometry (LC–MS) using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface. MS analyses were carried out in the selected ion monitoring (SIM) mode as described previously [34] and allowing the separations of 2-AG and anandamide. MS detection was carried out in the selected ion monitoring mode using *m*/*z* values of 356 and 348 (molecular ions + 1 for deuterated and undeuterated anandamide), 384 and 379 (molecular ions + 1 for deuterated and undeuterated 2-AG). The area ratios between signals of deuterated and undeuterated anandamide varied linearly with varying amounts of undeuterated anandamide (30 fmol–100 pmol). The same applied to the area ratios between signals of deuterated and undeuterated 2-AG in the 100 pmols–20 nmol interval. Anandamide and 2-AG levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. Two LC–MS peaks for both deuterated and undeuterated mono-AG were found, corresponding to 2-AG and 1(3)-AG, in agreement with the previous observation that 2-AG undergoes isomerization during the purification procedure [35]. Therefore, the amounts of 2-AG were calculated by adding the amounts of the two isomers. The amounts of endocannabinoids are expressed as pmols or nmols per gram of wet tissue extracted.

2.4. Brain serotonin measurements

Brains were homogenized in 20–40 vol. of cold 150 mM potassium phosphate buffer, pH 6.8, and used to determine the amounts of serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) using HPLC coupled to electrochemical detection. An aliquot of each homogenate was used to analyze protein concentration. The remaining homogenates were diluted (1:2) with 0.4N perchloric acid containing 0.4 mM sodium disulfite, 0.90 mM EDTA and *N*-methyl-serotonin (as an internal standard). Afterwards, samples were centrifuged for 3 min (15,000 × *g*) and the supernatants directly injected into the HPLC system. This consisted of the following elements. The pump was an isocratic Spectra-Physics 8810. The column was a RP-18 (Spherisorb ODS-2; 150 mm, 4.6 mm, 5 μm particle size; Waters, MA, USA). The mobile phase, previously filtered and degassed, consisted of 100 mM citric acid, 100 mM sodium acetate, 1 mM EDTA and 7% methanol (pH 4.2). The flow rate was 0.8 ml/min. The effluent was monitored with a Metrohm bioanalytical system amperometric detector using a glassy carbon electrode. The potential was 0.80 V relative to an Ag/AgCl reference electrode with a sensitivity of 50 nA (approximately 2 ng per sample). The signal was recorded on a Spectra-Physics 4290 integrator. The results were obtained from the peaks and calculated by comparison with the area under the corresponding internal standard peak. Values were expressed as ng/mg of protein.

2.5. Statistical analyses

Means of the amounts of either anandamide or 2-AG from different groups, as well as the data on 5-HT and 5-HIAA contents, were compared by means of one-way analysis of variance followed by the Bonferroni's post hoc test (Statmost®). The threshold for statistical significance was set at $P < 0.05$.

3. Results

The effects of a repeated intraperitoneal administration of a 5 mg/kg dose of the four compounds on rat brain endocannabinod levels at different times after the last administration are shown in Figs. 1–4. This dose was selected based on the several previous in vivo studies carried out with these compounds [5,10,13–15,23–25]. Of the three uptake inhibitors tested, OMDM-2 was the most efficacious since it significantly enhanced the levels of anandamide at all time points, with a maximal effect (∼1.9-fold enhancement)

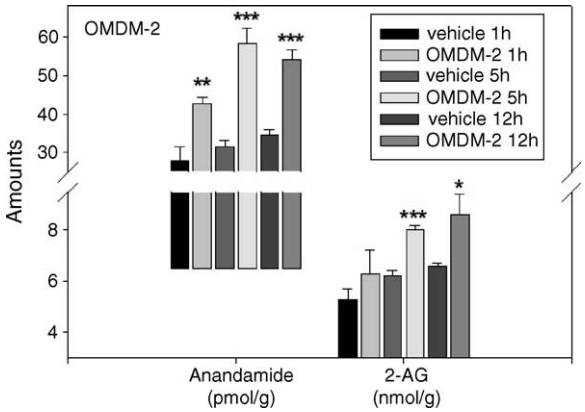

Fig. 1. Effect of the endocannabinoid uptake inhibitor OMDM-2 on rat brain endocannabinoid levels. The inhibitor (5 mg/kg) was administered twice a day and five times over 48 h. Brains were dissected 1, 5 and 12 h after the last administration. Data are means + S.E.M. of $N = 4$ determinations. Means were compared by ANOVA followed by Bonferroni's post hoc analysis. $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, $^{***}P \leq 0.001$.

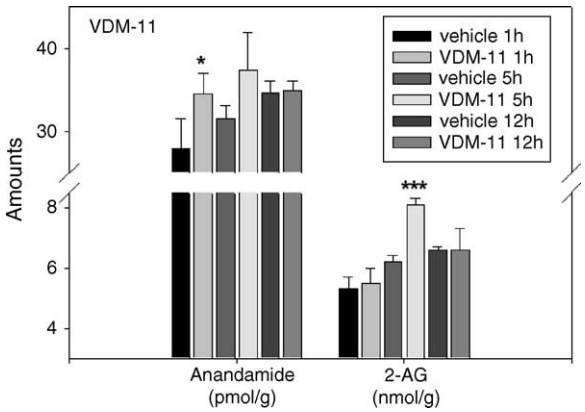

Fig. 2. Effect of the endocannabinoid uptake inhibitor VDM-11 on rat brain endocannabinoid levels. The inhibitor (5 mg/kg) was administered twice a day and five times over 48 h. Brains were dissected 1, 5 and 12 h after the last administration. Data are means + S.E.M. of $N = 4$ determinations. Means were compared by ANOVA followed by Bonferroni's post hoc analysis. $^{*}P \leq 0.05$, $^{***}P \leq 0.001$.

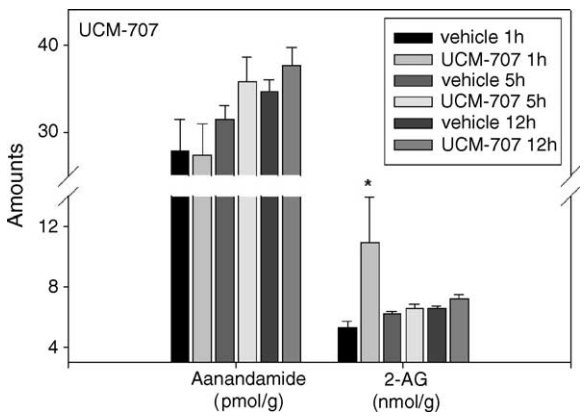

Fig. 3. Effect of the endocannabinoid uptake inhibitor UCM-707 on rat brain endocannabinoid levels. The inhibitor (5 mg/kg) was administered twice a day and five times over 48 h. Brains were dissected 1, 5 and 12 h after the last administration. Data are means + S.E.M. of $N = 4$ determinations. Means were compared by ANOVA followed by Bonferroni's post hoc analysis. $^{*}P \leq 0.05$.

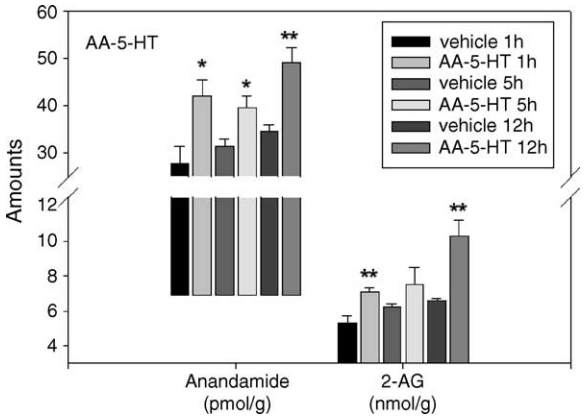

Fig. 4. Effect of the fatty acid amide hydrolase inhibitor AA-5-HT on rat brain endocannabinoid levels. The inhibitor (5 mg/kg) was administered twice a day and five times over 48 h. Brains were dissected 1, 5 and 12 h after the last administration. Data are means + S.E.M. of $N = 4$ determinations. Means were compared by ANOVA followed by Bonferroni's post hoc analysis. $^{*}P \leq 0.05$, $^{**}P \leq 0.005$.

after 5 h (Fig. 1). OMDM-2 also enhanced 2-AG levels, although to a lesser extent (∼1.3-fold) and only 5 and 12 h from administration. VDM-11 was less efficacious than OMDM-2, but equally effective on anandamide and 2-AG levels although at different times from administration. In fact, it significantly enhanced anandamide levels (∼1.3-fold) at 1 h and 2-AG levels (∼1.3-fold) at 5 h from administration (Fig. 2). UCM-707 only affected 2-AG levels (by ∼two-fold) at 1 h from administration (Fig. 3). Finally, FAAH inhibition by AA-5-HT significantly enhanced the levels of both anandamide (between ∼1.3- and ∼1.5-fold, maximal effect after 1 h) and 2-AG (between ∼1.3- and ∼1.6-fold, maximal effect after 12 h) at almost all time points (Fig. 4).

Measurement of serotonin levels in one of the hemispheres of the brains treated with AA-5-HT revealed no enhancement of the levels of this neurotransmitter, and its metabolite 5-HIAA, at all time points (Table 2), thus indicating that this inhibitor is not hydrolyzed following repeated systemic administration.

Table 2
Effect of the fatty acid amide hydrolase inhibitor AA-5-HT on rat brain serotonin (5-HT) and 5-HIAA contents

Time after the last administration (h)	5-HT (ng/mg protein)	5-HIAA (ng/mg protein)
Vehicle[a]	27.3 ± 2.8	13.3 ± 1.7
1	20.9 ± 3.0	12.2 ± 2.1
5	27.5 ± 3.1	11.5 ± 1.5
12	18.7 ± 2.4	12.6 ± 2.3

The inhibitor (5 mg/kg) was administered twice a day and five times over 48 h. Brains were dissected 1, 5 and 12 h after the last administration. Data are means + S.E.M. of $N = 5$ and 6 determinations. Means were compared by ANOVA followed by Bonferroni's post hoc analysis.

[a] This group corresponds to the combination of the different vehicle-treated rats for each time-point, which did not exhibit any statistically significant differences.

4. Discussion

We have reported here that four inhibitors of endocannabinoid inactivation via either the putative membrane transporter (OMDM-2, VDM-11 and UCM-707) or FAAH (AA-5-HT) significantly enhance basal brain endocannabinoid levels following subchronic systemic administration. The previously reported pharmacological properties of these compounds are summarized in Table 1. Although it was already known that inhibitors of endocannabinoid uptake and hydrolysis, after a single systemic administration, could elevate anandamide levels, the present study represents the first report that these two classes of compounds: (1) are effective also when administered repeatedly during 48 h; (2) produce elevations of basal endocannabinoid levels that may last for up to 12 h from the last administration; (3) can cause statistically significant elevations of the basal levels also of the other major endocannabinoid, 2-AG.

Of the three uptake inhibitors tested, OMDM-2 was undoubtedly the most efficacious at elevating the brain levels of both anandamide and 2-AG levels, particularly when the animals were sampled 5 h after the last administration. At this time point as well as after 12 h, the percent elevation effected on 2-AG (about 30%) concentrations may seem not so high, particularly if one looks at the almost 100% elevation exerted on anandamide levels. However, it must be remembered that the total brain levels of 2-AG are almost two orders of magnitude higher than those of anandamide [35,36]. Most of the basal levels of 2-AG are probably intracellular and are employed as metabolic intermediate in housekeeping reactions concerning phosphoglycerides, triacylglycerols and diacylglycerols. Only a part of the micromolar amounts of brain 2-AG levels probably act as extracellular activators of cannabinoid receptors, whose activity would otherwise be permanently elevated by this compound. Since we postulate that OMDM-2 acts by preventing the uptake and intracellular degradation of endocannabinods, a 30% enhancement produced by this inhibitor will probably concern uniquely the extracellular population of 2-AG, which acts entirely as an endocannabinoid. VDM-11 was significantly less efficacious than OMDM-2 at elevating the brain levels of both endocannabinoids. This compound produced a significant, albeit relatively small (30%), elevation of anandamide levels, which did not appear to be long-lasting, and a similar effect on 2-AG levels only 5 h from the last administration, which, however, for the reasons outlined above, may still have a strong impact on cannabinergic signalling in the CNS. While the different efficacy between OMDM-2 and VDM-11 can be easily explained by the fact that the former compound is significantly more metabolically stable than the latter one, both in vitro and in vivo [25,28], we have no explanation for the fact that the third uptake inhibitor tested here, UCM-707, never exerted any significant effect on anandamide levels, and it caused a strong elevation of 2-AG levels only 1 h after administration. Since with the other two uptake inhibitors we observed that the effect on 2-AG levels tended to have a slower onset than that on anandamide levels, we can only hypothesize that the pharmacokinetic properties of UCM-707 are such that this compound has a much earlier onset and shorter duration of action than either OMDM-2 or VDM-11. No data exist on the metabolic stability of UCM-707, which, however, is chemically similar to VDM-11 in as much as both compounds are derived from arachidonic acid and, hence, are more susceptible to oxidation than OMDM-2, which instead is derived from oleic acid.

Also with AA-5-HT, the only FAAH inhibitor tested here using our new conditions of administration, we observed a high efficacy with both endocannabinoids, and a slower onset of action for what concerns the enhancement of the brain levels of 2-AG. Indeed, this is the first report of a FAAH inhibitor being capable of elevating brain 2-AG levels following systemic administration. Previous studies carried out with FAAH inhibitors significantly more potent than AA-5-HT in vitro, i.e. URB-597 and OL-135 [8,9] showed that a single systemic administration of these compounds only elevates the brain levels of anandamide and its fatty acid ethanolamide congeners. On the other hand, AA-5-HT had been previously found to enhance both anandamide and 2-AG levels in: (1) tumours, after local (i.e. intra-tumour) administration repeated over 5 weeks [14], and in isolated cancer cells [12]. It is possible that a protocol with repeated administration of the FAAH inhibitor, such as that used in the present study, and the sampling of tissue up to several hours after the last administration, are both needed to observe an effect on 2-AG. Furthermore, AA-5-HT is very stable to enzymatic hydrolysis in vitro [29], and also in vivo, as shown here by the fact that no increase in serotonin and its metabolite was produced from this compound even after repeated administration. Enzymatic data suggest that this inhibitor forms a stable, although not covalent, complex with the enzyme [29]. These unique features of AA-5-HT may help explaining why this compound is also effective at inhibiting 2-AG inactivation and at raising brain 2-AG levels.

We observed that three out of the four compounds tested here affect the levels of anandamide earlier after treatment than those of 2-AG. In fact, with OMDM-2, VDM-11 and AA-5-HT, at least 5 h are necessary to observe a maximal elevation of the concentrations of this latter endocannabinoid, whereas the increase of anandamide levels is observed already after 1 h from the last administration, in agreement with previous studies with OL-135 and URB-597 [9,37]. The delayed onset of the effect on 2-AG levels may indicate that inhibitors of reuptake mechanisms and FAAH start acting on 2-AG only when they have finished inhibiting anandamide inactivation, which in turn implies that the putative anandamide transporter and FAAH recognize 2-AG as a substrate only after they have acted on anandamide, and that other mechanisms are

mostly responsible for 2-AG inactivation. This possibility is supported by the finding of another hydrolase, the monoacylglycerol lipase (MGL), which plays a major role in 2-AG inactivation [18] and by the fact that the putative transporter has higher affinity for anandamide than 2-AG [38]. However, this possibility is not supported by the fact that we used a 2-day, repeated administration protocol, which should have minimized any differences with which anandamide and 2-AG levels are affected by the inhibitors. It is also possible that the observed elevation of 2-AG levels is a consequence of the elevation of anandamide levels, because anandamide may inhibit both the reuptake of 2-AG (as observed previously [32,38–41]) and its inactivation via other mechanisms [41]. This explanation, however, is less likely in view of the several data pointing to blockade of 2-AG reuptake in vitro by inhibitors of anandamide reuptake [38–41], and of the fact that anandamide cannot inhibit 2-AG hydrolysis when this is catalysed by the MAGL [18], nor 2-AG esterification into phospholipids [41]. Finally, the delayed onset of the effect of the inhibitors raises the possibility that 2-AG levels were increased not because of inhibition of the putative transporter and FAAH, but because of the progressive conversion of the inhibitors into metabolites that could act as 2-AG precursors. In fact, theoretically, VDM-11, UCM-707 and AA-5-HT could be converted into arachidonic acid, which might be re-esterified into membrane phospholipids and subsequently increase the levels of 2-AG biosynthetic precursors. However, this possibility, which would have been difficult to test here because many phospholipid classes act as 2-AG precursors in the brain [42], does not explain why OMDM-2, which contains in its structure a fatty acid that cannot act as 2-AG precursor, is also capable of significantly enhancing 2-AG levels, nor why the effects of UCM-707 and AA-5-HT on 2-AG levels were observed also after 1 h from the last administration. Furthermore, this possibility is not supported by the observation that the levels of serotonin, the other possible hydrolysis product of AA-5-HT, and of its metabolite 5-IHAA, were not enhanced following repeated treatment with this compound, thus pointing to the metabolic stability in vivo of at least this inhibitor. In summary, further studies are needed in order to understand the pharmacokinetics of the elevation of brain 2-AG concentrations by uptake and FAAH inhibitors.

In conclusion, we have provided in this study conclusive evidence for the fact that certain widely used endocannabinoid uptake and hydrolysis inhibitors elevate both anandamide and 2-AG basal brain levels, at least when administered systemically and repeatedly. The overall "indirect" effect of these compounds on cannabinoid receptor activity under the administration protocol used here appears to be long-lasting, although, with elevations of endocannabinoid levels ranging between 1.3- and 2-fold, it does not cause necessarily dramatic changes in basal cannabinergic signalling. As summarized in Table 1, the four compounds, when acutely administered per se, exert little, if any, central cannabimimetic actions in healthy rodents. However, no behavioural studies have been carried out under physiological conditions and using the protocol of sub-chronic administration used here. Such studies are now required in view of the fact that this protocol does indeed result in significant increases of endocannabinoid levels. It is possible that the increases we have observed here were minimized by the fact that we analyzed the whole brain and not small brain areas, thus possibly "diluting" an effect that might have been localized only to those brain regions with high basal endocannabinoid turnover. It is also possible that a stronger enhancement of anandamide or 2-AG tissue concentrations levels by the inhibitors used here occurs under pathological conditions in which these compounds are over-produced, for example to exert protective actions [2,5,11,13]. In these cases, enhanced endocannabinoid signalling was shown to occur only in those tissues affected by the disorder and to be further enhanced by the compounds used here [5,34] (Table 1). Therefore, these compounds, like other inhibitors of endocannabinoid inactivation, are likely to produce selective effects in tissues where there is an ongoing turnover of endocannabinoids and may be employed as a therapeutic alternative to the use of "direct" agonists, which, when administered systemically, activate cannabinoid receptors in all tissues, thereby producing stronger undesired psychotropic and immune-depressant side-effects.

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