OX₁ Orexin/Hypocretin Receptor Signaling via Arachidonic Acid and Endocannabinoid Release

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Running title: Orexin-induced arachidonic acid and endocannabinoid release

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# of text pages: 41
# of tables: 0
# of figures: 9
# of references: 60
# words in the Abstract: 245
# words in the Introduction: 605
# words in the Discussion: 1486

ABBREVIATIONS: 2-AG, 2-arachidonoyl glycerol; AA, arachidonic acid; AM-251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide; BEL, bromoenol lactone; BSA, bovine serum albumin; CAY10499, [4-(5-methoxy-2-oxo-1,3,4-oxadiazol-3(2H)-yl)-2-methylphenyl]-carbamic acid phenylmethyl ester; CAY10593, N-[2-[4-(5-chloro-2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]-1-methylethyl]-2-naphthalenecarboxamide); CCPA, N-cyclohexanecarbonylpentadecylamine; cPLA₂, cytosolic phospholipase A₂; DAG, diacylglycerol; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; FKGK11, 1,1,1,2,2-pentafluoro-7-phenyl-heptan-3-one); HBM, Hepses-buffered medium; HU-210, 3-(1,1'-dimethylheptyl)-6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-
6H-dibenzo[b,d]pyran-9-methanol; IBMX, 3-isobutyl-1-methylxanthine; iPLA₂, Ca²⁺-independent phospholipase A₂; JZL184, 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl) piperidine-1-carboxylate; MAFP, methyl arachidonyl fluorophosphonate; MAGL, monoacylglycerol lipase; NAAA/PEAase, N-acylethanolamine-hydrolyzing acid amidase / acidic palmitoyl ethanolamidase; pyrrophenone, \( N\-[(2S,4R)-1-[(2,4-

difluorobenzoyl)benzoyl]-4-[(triphenylmethyl)thio]-2-pyrrolidinyl]methyl]-4-[(Z)-(2,4-
dioxo-5-thiazolidinylidene)methyl]-benzamide; PA, phosphatidic acid; PC; phosphatidylcholine; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D; probenecid, \( p\)-
(dipropylsulfamoyl) benzoic acid; PUFA, polyunsaturated fatty acid; RHC-80267, 1,6-
bis[cyclohexyloximinocarbonylamino]hexane; SB-334867, 1-(2-methylbenzoxazol-6-yl)-3-
(1,5)naphthyridin-4-yl-urea HCl; S-BSA, stripped BSA; THL, tetrahydrolipstatin, (1S)-1-
[(2S,3S)-3-hexyl-4-oxo-oxetan-2-yl]methyl] dodecyl \( (2S)-2\)-formamido-4-methyl-pentanoate;
TLC, thin layer chromatography; U-73122, 1-[6-\( [(17\)-3-methoxyestra-1,3,5 (10)-trien-17-
yl]amino)hexyl]-1H-pyrrole-2,5-dione; URB597, \( [3-(3\)-carbamoylphenyl]phenyl \) N-
cyclohexylcarbamate
ABSTRACT

We have previously shown that OX₁ orexin receptor stimulation produces a strong ³H-overflow response from [³H]arachidonic acid (AA) -labeled cells. We have here attacked this issue with a more novel set of tools and methods to distinguish enzyme pathways responsible for this. Chinese hamster ovary-K1 (CHO-K1) cells, heterologously expressing human OX₁ receptors were used as a model system. Using selective pharmacological inhibitors, we can here show that in orexin-A-stimulated cells, the AA-derived radioactivity is released as two distinct components, free AA and the endocannabinoid 2-arachidonoyl glycerol (2-AG). Two orexin-activated enzymatic cascades are responsible for this, namely the Ca²⁺-sensitive phospholipase A₂ (cPLA₂) and diacylglycerol lipase (DAGL); the former cascade is responsible for a part of the AA release while the latter is fully responsible for 2-AG release and a part of the AA release. Interestingly, essentially only diacylglycerol released by phospholipase C but not by phospholipase D was implicated as a substrate for 2-AG production, although both phospholipases are strongly activated. The 2-AG released acted as a potent paracrine messenger via cannabinoid CB₁ receptors in an artificial cell–cell-communication assay developed. The cPLA₂ cascade, in contrast, was involved in the activation of the orexin receptor-operated Ca²⁺ influx. 2-AG was also released upon OX₁ receptor stimulation in recombinant HEK-293 and neuro-2a cells. The results, for the first time, directly show that orexin receptors are able to, in addition to the arachidonic acid signaling, also generate potent endocannabinoid signals, which may explain the proposed orexin–cannabinoid interaction, e.g. in neurons.
Introduction

Peptide transmitters orexins/hypocretins, acting via G-protein-coupled OX₁ and OX₂ receptors, are engaged in the regulation of homeostatic brain functions, especially wakefulness and sleep pattern, and appetite (reviewed in Kukkonen et al., 2002; Scammell and Winrow, 2011). Orexin receptor expression and signaling are also found in the periphery of the body, but the physiological significance of this is not known. Molecular mechanisms of orexin receptor signaling are very diverse (reviewed in Kukkonen and Åkerman, 2005). Especially prominent seems to be the coupling to the production of messengers via phospholipase action (Johansson et al., 2008; Turunen et al., 2010a; Jäntti et al., 2012).

Endocannabinoids are phospholipid-derived messengers containing arachidonic acid (AA). The best established of endocannabinoids are 2-AG (2-arachidonoylglycerol) and anandamide (N-arachidonoyl-ethanolamine) (reviewed in Kano et al., 2009). Endocannabinoids act via G-protein-coupled CB₁ and CB₂ receptors, of which CB₁ is expressed in central neurons. In addition to the receptors, the endocannabinoid system includes the enzymes producing and metabolizing endocannabinoids. Endocannabinoids are generally thought to be produced on demand and not stored in membrane vesicles like traditional neurotransmitters. Endocannabinoid signaling has been a subject of much interest both from the basic physiological perspective as well as medical one. In the central nervous system, endocannabinoids are involved in the regulation of, e.g. appetite, nociception, memory, reward, and mood (reviewed in Kano et al., 2009). On the synaptic level, endocannabinoids engage in retrograde transmission, where postsynaptically produced endocannabinoids act on the inhibitory, presynaptic CB₁ receptors. This function may also act in homosynaptic feedback but the assumed major function is
heterosynaptic. Cannabinoid receptors couple to G\(_i\)-family G-proteins, and thus the presynaptic inhibition is likely to occur via G\(\beta\gamma\)-mediated inhibition of voltage-gated Ca\(^{2+}\) channels and inward rectifier K\(^+\) channels (reviewed in Howlett, 2005; Kano et al., 2009).

There is much circumstantial evidence for interaction between orexinergic and cannabinoidergic systems. These show significant overlap on the gross neuroanatomical level, especially in particular nuclei of the hypothalamus. However, the possibility of the interaction at cellular level is not verified for most nuclei. More direct evidence is so far scarce. In the lateral hypothalamus, exogenous CB\(_1\) stimulation inhibits orexinergic neurons by reducing excitatory drive on these (Huang et al., 2007). On the other hand, CB\(_1\) receptor block attenuates orexin-A-induced feeding (Crespo et al., 2008). It has also been proposed that CB\(_1\) and OX\(_1\) receptors make heteromeric complexes, which may enhance orexin signaling (Hilairet et al., 2003; Ellis et al., 2006; Ward et al., 2011).

We have previously shown that OX\(_1\) orexin receptor activation strongly stimulates \(^3\)H-overflow from [\(^3\)H]AA-labeled cells (Turunen et al., 2010a). The full sensitivity of the response to the reputed PLA\(_2\) (phospholipase A\(_2\)) inhibitor, MAFP (methyl arachidonyl fluorophosphonate), in addition to partial sensitivity to inhibitors of other enzymes and intracellular signal pathways (Turunen et al., 2010a), would suggest that this likely is mediated by PLA\(_2\) enzymes. However, although MAFP is very commonly used as a PLA\(_2\)-inhibitor, it is an analogue of AA, and is able to inhibit a number of other serine hydrolases hydrolyzing ester- or amide-bound AA (De Petrocellis et al., 1997; Deutsch et al., 1997; Dinh et al., 2002; Savinainen et al., 2010). Thus, does the \(^3\)H-overflow observed solely take place through PLA\(_2\), and in that case, which isoform? Another question left open was whether the \(^3\)H-overflow would only be composed of AA or could other water-soluble and secreted metabolites be involved. In
the current study, we set out to resolve these questions. The results indeed demonstrate that both free AA as well as AA incorporated in the endocannabinoid 2-AG are released upon orexin receptor stimulation. Both AA and 2-AG are also shown to have a signaling role of their own.

Materials and Methods

Drugs. Human orexin-A and -B were from NeoMPS (Strasbourg, France). AM-251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide), CAY10499, CAY10593 ([4-(5-methoxy-2-oxo-1,3,4-oxadiazol-3(2H)-yl)-2-methylphenyl]-carbamic acid phenylmethyl ester; compound 69 of Scott et al., 2009), CCPA (N-cyclohexanecarbonylpentadecylamine; compound 17 of Tsuboi et al., 2004), FKGK11 (1,1,1,2,2-pentafluoro-7-phenyl-heptan-3-one; compound 10a of Baskakis et al., 2008), HU-210 (3-(1,1'-dimethylheptyl)-6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b, d]pyran-9-methanol), JZL184 (4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate), lipid standards (2-AG and AA), MAFP (methyl arachidonyl fluorophosphonate), pyrophenone (N-[(2S,4R)-1-[2-(2,4-difluorobenzoyl)benzoyl]-4-[(tri phenylmethyl)thio]-2-pyrrolidinyl)methyl]-4-[(Z)-(2,4-dioxo-5-thiazolidinylidene)methyl]-benzamide; compound 6 of Seno et al., 2001), and URB597 ([3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate) were from Cayman Europe (Tallinn, Estonia). RHC-80267 (1,6-bis[cyclohexyloximinocarbonylamino]hexane), SB-334867 (1-[2-methylbenzoxazol-6-yl]-3-[1,5]naphthyridin-4-yl-urea HCl), THL (tetrahydrolipstatin, (1S)-1-[(2S,3S)-3-hexyl-4-oxo-oxetan-2-yl]methyl] dodecyl] (2S)-2-formamido-4-methyl-pentanoate) and U-73122 (1-[6-([(17b)-3-methoxyestra-1,3,5 (10)-tri en-17-yl]amino)hexyl]-1H-pyrrole-2,5-dione) were from
Tocris Cookson Ltd (Avonmouth, UK), fura-2 acetoxymethyl ester from Molecular Probes (Eugene, OR) and \[^{3}H\]AA ([5,6,8,9,11,12,14,15-\(^{3}\)H]arachidonic acid), \[^{14}\]C\]AA ([1-\(^{14}\)C]-arachidonic acid) and \[^{3}\]H\]oleic acid ([9,10-\(^{3}\)H]oleic acid) from New England Nuclear Corp. GesmbH (Vienna, Austria). Forskolin, probenecid (\(p-\)dipropylsulfamoyl) benzoic acid) and IBMX (3-isobutyl-1-methylxanthine) were from Sigma Chemical Co. (St. Louis, MO).

**Cell Culture.** CHO-hOX\(_{1}\) cells, expressing ~500 fmol/mg protein of high-affinity human OX\(_{1}\) receptors (as determined from the high-affinity \(^{125}\)I-orexin-A binding; unpublished) have been described previously as also their culture conditions (Lund et al., 2000; Turunen et al., 2010a). CHO-hCB\(_{1}\) cells, expressing human CB\(_{1}\)a receptor, (Grimsey et al., 2010) were a kind gift from Dr. Michelle Glass (University of Auckland, Auckland, New Zealand) via Drs. Jarmo Laitinen and Juha Savinainen (University of Eastern Finland, Kuopio, Finland); these were propagated under same conditions as CHO-hOX\(_{1}\) except that 0.25 mg/ml zeocin (Gibco, Paisley, UK) was included. Neuro-2a-hOX\(_{1}\), PC12-hOX\(_{1}\) and wild-type HEK-293 cells were propagated in as described in (Holmqvist et al., 2002; Putula and Kukkonen, 2012) except that PC12 cells received an additional supplement of 5% horse serum (Gibco). For the \(^{3}\)H-overflow experiments with \[^{3}H\]AA- and \[^{3}H\]oleic acid-labeled cells as well as for the 2-AG reporter assay, the CHO-hOX\(_{1}\) cells were cultivated on 24-well plates (1.77 cm\(^{2}\) well bottom area; Greiner Bio-One GmbH, Frickenhausen, Germany) coated with polyethyleneimine (25 \(\mu g/ml, 1\) h, 37°C; Sigma) and for \(Ca^{2+}\) imaging, on polyethyleneimine-coated circular glass coverslips (diameter 13 mm; Menzel-Gläser, Braunschweig, Germany). For the TLC (thin layer chromatography) assay, the cells were cultured on 6-well plates (bottom area 9.6 cm\(^{2}\); Greiner). For cAMP measurements (direct CB\(_{1}\) receptor assay or the 2-AG reporter assay), CHO-hCB\(_{1}\) cells were culture on plastic
HEK-293 cells were transiently transfected with hOX₁ receptor cDNA utilizing Fugene HD (Roche Diagnostics GmbH, Mannheim, Germany) as described in (Putula and Kukkonen, 2012).

**3H-overflow from [3H]arachidonic or [3H]oleic acid-labeled cells.** The experiments were largely performed as described in (Turunen et al., 2010a). CHO-hOX₁ cells were plated on 24-well plates (20 000 cells / well) and left to grow for 24 h. The wells were precoated with polyethyleneimine, which very much reduced cell detachment during the washes. 0.1 µCi [3H]AA (or 0.2 µCi [3H]oleic acid – to compensate for the lower release and lower 3H-content), was added in each well and the cells cultured for another 20 h. The incubation medium was removed and the cells were washed twice with the Hepes-buffered medium (HBM; composition in mM: NaCl 137, KCl 5, CaCl₂ 1, MgCl₂ 1.2, KH₂PO₄ 0.44, NaHCO₃ 4.2, glucose 10 and Hepes 20, adjusted to pH 7.4 with NaOH) supplemented with 2.4 mg/ml stripped bovine serum albumin (S-BSA; see Turunen et al., 2010b), and finally left in HBM with S-BSA at 37°C. The cells were then immediately stimulated with orexin-A or thapsigargin for 7 min, after which 200 µl of the total volume of 250 µl in each well was transferred to Eppendorf tubes on ice. These samples were spun down for 1.5 min at 4°C, 100 µl of the medium transferred to a scintillation tube, scintillation cocktail (HiSafe 3, Wallac-PerkinElmer, Turku, Finland) added and the radioactivity measured with Wallac 1414 liquid scintillation counter. It should be pointed out that total radioactivity released in the supernatant from the 3H-preloaded cells is measured in this assay without any separation to molecular species.

In some cases, orexin stimulation was preceded by some inhibitor preincubation. In such cases, the inhibitor used was added to the cells after one wash with HBM + S-BSA and the cells
preincubated for 30 min in HBM in the absence of S-BSA. HBM + 10× S-BSA was added to
final concentration of S-BSA of 2.4 mg/ml for the last 5 min to chelate the radioactivity leaked
during this period. The incubation solution was removed, fresh HBM + S-BSA with the inhibitor
added and the cells stimulated immediately with orexin-A for 7 min. The control cells were
treated in the same way. The procedure effectively removed the radioactivity leaked during the
preincubation period and the S-BSA did not interfere with the inhibitor entry into the cells.

**Lipid extraction and TLC.** CHO-hOX1, neuro-2A-hOX1, PC12-hOX1 or transiently hOX1-
transfected HEK-293 cells grown on 6-well plates were labeled with $[^{14}\text{C}]$AA (0.2 µCi/ml) in
cell culture medium (Ham's F12) 16 h prior to the experiments. $[^{14}\text{C}]$AA loading medium was
removed, the cells were washed twice with HBM + S-BSA (2.4 mg/ml) and stimulated with
orexin-A for 7 min. Supernatants from the cells were rapidly removed and spun down (13500 g,
+4°C, 2 min) to remove detached cells. The lipids were extracted from the supernatant with a
slightly modified Bligh and Dyer method (Bligh and Dyer, 1959). Briefly, 800 µl of the total
supernatant (1000 µl) was transferred to a Kimax tube (Kimble Glass, Inc., Vineland, NJ), 2 ml
of methanol was added followed by 1 ml of chloroform and the tubes were shaken thoroughly.
After adding 1 ml of water and 1 ml of chloroform, the tubes were shaken again and centrifuged
at 500 g (5 min, room temperature). The lower phase was collected and dried under a stream of
N2. The dried lipids were dissolved in chloroform and the samples applied onto TLC plates
(Silicagel 60, Merck, Darmstadt, Germany), which had been dried in 110°C for 1 h. Plates were
developed in ethylacetate:methanol (90:10) (Glass et al., 2005) in a chromatography tank lined
with filter paper. Non-labeled lipid standards were run on each TLC plate either on separate
lanes or together with the samples; the movement of the lipids of interest (2-AG and AA) in the
samples was not affected by the presence of the standards. The average $R_f$-values (retardation factors) for AA and 2-AG were $0.58 \pm 0.01$ and $0.67 \pm 0.01$, respectively.

TLC plates were quantitated both by imaging plate analysis and scraping and scintillation counting, both of which gave comparable results (Jäntti et al., 2012). For this reason, only the results from the imaging plate analysis are presented. Briefly, after the development, the plates were vacuum-dried and an imaging plate (BAS-MS, Fujifilm, Tokyo, Japan) exposed overnight. The imaging plate was scanned with Fujifilm FLA 5100 scanner and the band areas and intensities measured with ImageJ (http://rsbweb.nih.gov/ij/). The plate background was subtracted from the band intensities.

**2-AG reporter assay and cAMP measurements.** 2-AG release from CHO-hOX$_1$ cells upon orexin receptor stimulation was detected and quantitated utilizing CHO-hCB$_1$ cells as detector cells; the assay is conceptually reminiscent of, for instance, a NO production assay (Hu and el-Fakahany, 1993). Briefly, CHO-hCB$_1$ cells on cell culture dishes were prelabeled with 5 µCi/ml $[^3]$H]adenine for 2 h in the culture medium, after which the cells were washed with PBS and detached with PBS + 0.2 % (w/v) EDTA. The cells were spun down, resuspended in HBM containing 500 µM IBMX (a cyclic nucleotide phosphodiesterase inhibitor) and dispensed on top of CHO-hOX$_1$ cells growing on 24-well plates previously washed with HBM. Different densities of CHO-hCB$_1$ cells were tested, but we finally settled for 150 000 cells / well. The plates were then gently spun (100 g, 3 min, room temperature) to sediment CHO-hCB$_1$ cells on top of CHO-hOX$_1$ cells in HBM. The cells were allowed to rest at 37°C for 10 min, after which they were stimulated for 7 min with orexin-A or HU-210. The final volume in each well was 250 µl. In case some inhibitors were included, these cells (and the control cells) were preincubated with the
inhibitor/vehicle for 10 min (SB-334867, AM-251) or 30 min (THL) before orexin stimulation. The reactions were interrupted by rapid removal of the medium, addition of 300 µl of 0.33 M, ice-cold perchloric acid and freezing. The insoluble fragments of the thawed samples were spun down (10 min, 1100 g, room temperature) and the $[^3\text{H}]\text{ATP} + [^3\text{H}]\text{ADP}$ and $[^3\text{H}]\text{cAMP}$ fractions of the cell extracts isolated by sequential Dowex/alumina chromatography (see, e.g. Holmqvist et al., 2005). Radioactivity was determined using scintillation counting; the conversion of $[^3\text{H}]\text{ATP}$ to $[^3\text{H}]\text{cAMP}$ was calculated as a percentage of the total eluted $[^3\text{H}]\text{ATP} + [^3\text{H}]\text{ADP}$. Since only CHO-hCB$_1$ cells were prelabeled with $[^3\text{H}]$adenine, the radioactivity isolated here is derived from these cells alone.

CHO-hCB$_1$ cells were also subjected to cAMP measurements in the absence of CHO-hOX$_1$ cells. The assay was similar as above, except that the detached cells were first preincubated in HBM + IBMX for 10 min (+ inhibitors, if these were used), and then dispensed on 96-well plates (100 000 cells / well) where the stimulants (orexin-A, HU-210, 2-AG) were already present. The final volume was 150 µl / well. On the plates, also 0.5 mg/ml S-BSA was included, as this helps to retain 2-AG in solution (Savinainen et al., 2003). After 10 min stimulation, the reactions were interrupted by rapidly spinning down the cells (1100 g, 3 min, +4°C), removal of the medium and addition of 150 µl of 0.33 M, ice-cold perchloric acid and freezing. The samples were thereafter treated as above.

**Ca$_{2+}$ measurements.** Cells plated on polyethyleneimine-coated glass coverslips were loaded with 4 µM fura-2 acetoxyethyl ester (Invitrogen, Carlsbad, CA) for 20 min at 37°C in HBM containing 1 mM probenecid, rinsed once, and used immediately. Ca$_{2+}$ measurements were performed at 35°C using Nikon TE2000 fluorescence microscope (20×/0.75 air objective) and
Andor iXon 885 EM-CCD camera under the control of Nikon NIS Elements AR software with 6D extension. For Ca\(^{2+}\) imaging, the cells were excited with alternating 340 and 380 nm light (Sutter DG4 Plus) and the emitted light collected through a 400 nm dichroic mirror and a 450 nm long-pass filter. Additions were made by constant perfusion (HBM + probenecid). When the inhibitors THL and pyrrophenone were tested, the cells were pretreated with these for 20 min, and these were also included in the perfusion medium throughout the experiment. Regions of interest were defined in NIS software and the data extracted to Microsoft Excel for visualization and quantitation. More than 30 cells were measured in each experiment, and each experiment was repeated altogether 4 times or more.

**Data analysis and statistical procedures.** All the data are presented as mean ± S.E.M.; N refers to the number of batches of cells. Each experiment was performed at least three times. The AA-release experiments were performed with 6, imaging with 30 or more, cAMP measurements with 3–4 and TLC with 2 data points in parallel. Student’s two-tailed \(t\) test with Bonferroni correction was used in all pairwise comparisons, except for Fig. 8, where, because of the non-parametric nature of cell counting, also \(\chi^2\) test was used (Ekholm et al., 2007). Significances are as follows: ns (not significant), \(p > 0.05\); *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\). For the second comparison, NS and the symbol "†" are used in the same way. Microsoft Excel was used for the nonlinear curve-fitting for the determination of the EC\(_{50}\)-values. The effect of "inhibitors" on the orexin-stimulated AA and 2-AG release was calculated from the values using a formula that compensates for the possible effect of the inhibitors also on the basal release: release (in % of the ctrl [non-inhibited]) = (orexin\(_{\text{inhibitor}}\) − basal\(_{\text{inhibitor}}\))/(orexin\(_{\text{ctrl}}\) − basal\(_{\text{ctrl}}\)) × 100% (Figs. 2 and 4).
In this manner, the non-treated controls (basal, 1 nM orexin-A, 100 nM orexin-A) are set to 100% and the full inhibition to 0%.
Orexin receptor stimulation induces $^3$H-AA-derived $^3$H-overflow via distinct cPLA$_2$ (Ca$^{2+}$-sensitive phospholipase A$_2$) and DAGL (diacylglycerol lipase) cascades. Our previous studies have shown strong release of radioactivity from $[^3]$HAA-labeled CHO-hOX$_1$ cells upon OX$_1$ receptor stimulation (see also Fig. 1), but the enzymes responsible for this could not be resolved due to low selectivity of the inhibitors available (Turunen et al., 2010a). We have previously shown that OX$_1$ receptors also potently activate both phospholipase C and D (PLC and PLD, respectively) cascades (Johansson et al., 2008; Jäntti et al., 2012), producing diacylglycerol (DAG) and phosphatidic acid (PA), respectively. Both of these could act as substrates for AA release by DAGL $\rightarrow$ MAGL (monoacylglycerol lipase) and PA-PLA$_2$ cascades, respectively, and PA can also be hydrolyzed to DAG by PA-phosphohydrolase (reviewed in Kukkonen, 2011). Only PLD1 but not PLD2 is activated by orexin receptor stimulation in CHO cells, and this isoform can be fully inhibited by CAY10593 (Jäntti et al., 2012). CAY10593 produced a very weak (10–20%) inhibition of the $^3$H-overflow (Fig. 2A). In contrast, the PLC inhibitor U-73122 produced a full inhibition (Fig. 2A).

THL (tetrahydrolipstatin) is used as a pancreatic triglyceride lipase inhibitor, but it is an even more potent inhibitor of some other related enzymes, including DAGL (Lee et al., 1995; Bisogno et al., 2003), and can be used at 1 µM as a relatively selective DAGL inhibitor (Bisogno et al., 2006; also V. Di Marzo, personal communication). THL produced a significant inhibition of the orexin response already at 1 µM (Fig. 2B). RHC-80267, a less potent DAGL inhibitor, showed a similar trend of inhibition (Fig. 2B).
Selective inhibitors for particular PLA2 isoforms have been produced during the last 10 years. The best characterized (for, e.g. selectivity) of these are different pyrrolidine inhibitors for cPLA2α (Seno et al., 2000; Seno et al., 2001). A commercially available inhibitor of this type, pyrrophenone (Seno et al., 2001), at 1 µM, produced a significant inhibition of the orexin response (Fig. 2C). Interestingly, the inhibition "profile" was the opposite of that of THL; while THL apparently produced a weaker inhibition at 3 nM orexin-A and stronger at 100 nM, pyrrophenone was stronger at 3 nM orexin-A. We therefore hypothesized that DAGL and cPLA2 could represent two components of the orexin-stimulated 3H-overflow from [3H]AA-labeled cells. Indeed, combination of THL and pyrrophenone produced full inhibition of the orexin-A response (Fig. 2D). In contrast, the response to thapsigargin showed no sensitivity to THL but was almost fully inhibited by pyrrophenone alone (Fig. 2E), in agreement with Ca2+-activation of cPLA2 (reviewed in Ghosh et al., 2006). While Ca2+ elevation should also stimulate DAGL (Bisogno et al., 2003), Ca2+ elevation (at least when triggered by thapsigargin or ionomycin) is a poor stimulant of PLD and even poorer stimulant of PLC in CHO cells (Lund et al., 2000; Johansson et al., 2007; Jäntti et al., 2012); thus, in the absence of DAG production, there is also no DAGL activity.

Thus, orexin response appears to rely on two different components. The relative contribution of the components may become clearer when normalized raw data are shown instead of inhibition (Fig. 3A). The same is also seen in more complete concentration response curves (Fig. 3B; representative data). The inhibition with THL and pyrrophenone was essentially not overlapping at all (Fig. 3A, black bars; Fig. 3B, dotted line). In the analyses of the concentration-response, the control pEC50 was 8.7 ± 0.1 and the pEC50-values after pyrrophenone (putative DAGL component) and THL (putative cPLA2 component) treatment 8.4
± 0.1 and 9.3 ± 0.1, respectively (N = 3–5). Thus, the putative cPLA₂ component would be activated with significantly higher potency (see Discussion).

We have previously shown that ³H-overflow from [³H]oleic acid-labeled cells is also stimulated by OX₁ receptor activation in CHO-hOX₁ cells, though with significantly lower potency and efficacy than from [³H]AA-labeled cells (Turunen et al., 2010a). The potency of this ³H-overflow seems to overlap better with the DAGL component of AA release than AA release in its entity. Indeed, orexin-induced ³H-overflow from [³H]oleic acid-labeled cells was fully inhibited by THL but not at all by pyrrophenone (Fig. 4). [³H]oleic acid is likely to mainly end up in the sn1-position not hydrolyzed by cPLA₂. Even if some oleic acid would be found in the sn2-position, cPLA₂α, in contrast to other PLA₂ isoforms (including cPLA₂ζ), is rather specific for AA in the sn2-position (Ghosh et al., 2007; reviewed in Ghosh et al., 2006).

Endocannabinoid-hydrolyzing enzymes, hormone-sensitive lipase and iPLA₂ in orexin-induced AA-derived ³H-overflow. DAG lipases are thought to show sn1-selectivity (3–8-fold in vitro; Bisogno et al., 2003); therefore, DAGL might be thought to be unlikely to release AA from its usual sn2-position. DAGL, however, works as a "gatekeeper" for MAGL, as no MAG should be produced in the absence of DAGL activity. We therefore tested a selective MAGL inhibitor, JZL184 (Long et al., 2009). JZL184 (10 µM) produced a weak but significant inhibition (20 ± 12% and 11 ± 10 % for 3 nM and 100 nM orexin-A, respectively; $p < 0.01$ and $p < 0.05$, respectively; see also Supplemental Fig. 1). We similarly tested inhibitors of fatty acid amide-type endocannabinoid-hydrolyzing enzymes, URB597 (100 nM) (fatty acid amide hydrolase [FAAH] inhibitor) and CCPA (100 µM) (N-acylethanolamine-hydrolyzing acid amidase / acidic palmitoyl ethanolamidase [NAAA/PEAase] inhibitor) (Kathuria et al., 2003; Tsuboi et al., 2004;
Neither URB597 nor CCPA significantly inhibited orexin-induced \(^{3}\text{H}\)-overflow from \(^{3}\text{H}\)AA-labeled cells (Supplemental Fig. 1). Similarly, the hormone-sensitive lipase/FAAH/MAGL inhibitor, CAY10499 (30 µM), and the \(\text{iPLA}_2\) (intracellular \(\text{Ca}^{2+}\)-independent \(\text{PLA}_2\)) inhibitor, FKGK11 (10 µM) (Baskakis et al., 2008; Muccioli et al., 2008; Minkkilä et al., 2009), were incapable of inhibiting the orexin response (Supplemental Fig. 1).

\(^{3}\text{H}\)-overflow from \(^{3}\text{H}\)AA-labeled cells consists in part of free AA and in part of the endocannabinoid 2-AG. The \(^{3}\text{H}\)-overflow method utilized here is well-established and much used of AA-release studies. However, it does not as such specifically indicate \(^{3}\text{H}\)AA release, only that \(^{3}\text{H}\)AA-derived radioactivity is released from the cells. We therefore conducted TLC separation of the molecular species released; to allow use of imaging plate-identification of multiple molecular species, the cells were labeled with \(^{14}\text{C}\)AA instead. The results show that the radioactivity released upon orexin receptor stimulation is found both in free AA and in the endocannabinoid 2-AG (Fig. 5A); AA was released with significantly higher potency by \(\text{cPLA}_2\) (AA release in the presence of THL) than 2-AG by DAGL (\(\text{pEC}_{50} = 9.8 \pm 0.1\) and 8.3 \(\pm\) 0.1, respectively [\(\text{N} = 3–9\)]; Fig. 6). THL fully blocked orexin-stimulated 2-AG release but only partly reduced AA release (Fig. 5BEF, Fig. 6). In contrast, pyrrophenone only inhibited AA but essentially not 2-AG release (Fig. 5CEF). Inclusion of both THL and pyrrophenone fully inhibited orexin-stimulated release of both free AA and 2-AG (Fig. 5DEF).

Other \(\text{OX}_1\) receptor-expressing cells showed distinct responses to orexin-A stimulation. Neuro-2a and HEK-293 cells showed strong 2-AG release at 100 nM orexin-A (Supplemental Fig. 2). In these cells also AA release was seen; orexin-stimulation of 2-AG and AA release was eliminated by THL (not shown), suggesting that AA was released through 2-AG breakdown and
not by PLA2 action on phospholipids. PC12 cells showed high basal AA release with no apparent stimulation with orexin-A and no 2-AG release (Supplemental Fig. 2).

**Orexin receptor activation is able to induce strong paracrine signaling to cannabinoid receptors via 2-AG.** 2-AG is a potent endogenous ligand for cannabinoid receptors (reviewed in Di Marzo and Petrosino, 2007; Kano et al., 2009). We thus went on to test whether 2-AG released upon orexin receptor signaling is able to activate cannabinoid receptors. For this, we designed a cell–cell communication assay between CHO-hOX1 and CHO-hCB1 cells as described in **2-AG reporter assay**. Briefly, CHO-hCB1 cells were plated on top of the CHO-hOX1 cell culture, CHO-hOX1 cells were stimulated with orexin-A, and 2-AG production was detected as a selective inhibition of adenylyl cyclase in CHO-hCB1 cells. Indeed, orexin-A in this assay caused a strong and potent inhibition of adenylyl cyclase activity in CHO-hCB1 cells, though the efficacy was somewhat lower than that of direct stimulation with the CB1 agonist HU-210 (Fig. 7AB). The response to orexin-A was fully inhibited by the OX1 receptor antagonist SB-334867, by THL and by the CB1 receptor antagonist/inverse agonist AM-251 (Fig. 7A), indicating that inhibition of forskolin-stimulated adenylyl cyclase activity in CHO-hCB1 cells by orexin-A was mediated by the cascade orexin-A → OX1 (on CHO-hOX1) → 2-AG (by DAGL in CHO-hOX1) → CB1 (on CHO-hCB1). AM-251 but neither THL nor SB-334867 enhanced the forskolin-stimulated cAMP production (Fig. 7A), suggesting that CB1 receptors show some constitutive activity. SB-334867 alone weakly inhibited the forskolin response (Fig. 7A), which may suggest that SB-334867 shows some weak partial agonist activity on OX1 receptors, which is in line with some previous observations (Bengtsson et al., 2007; also own unpublished
observations in recombinant systems). 10 µM AM-251 strongly but incompletely inhibited the response to 1 µM HU-210 (Fig. 7A; see also below).

In CHO-hCB₁ cells alone, exogenous 2-AG was nearly equally efficacious as HU-210 as CB₁ receptor activator (Fig. 7CD). AM-251 elevated adenylyl cyclase activity as compared to forskolin alone (Fig. 7C), as also seen in the reporter assay (Fig. 7A) and fully blocked the response to 2-AG (Fig. 7C). In contrast, the response to 1 µM HU-210 was not fully blocked (Fig. 7C), similar to the reporter assay (Fig. 7A), which likely reflects the fact that the affinity/potency of HU-210 is too high (~100–1000-fold higher than that of 2-AG) to be fully blocked at this concentration by 10 µM AM-251.

To get an estimate of the 2-AG levels reached upon orexin receptor stimulation, we measured the potency of exogenous 2-AG to activate CB₁ receptors in CHO-hCB₁ cells (Fig. 7D). Using these data (inhibitionₘₐₓ = 86 ± 3%, pEC₅₀ = 7.40 ± 0.03, nₑₕₐₙ = 1.1 ± 0.2; N = 5) as a "standard curve" to calculate 2-AG level from adenylyl cyclase inhibition by orexin-A in the reporter assay (inhibitionₘₐₓ = 60 ± 3%, pEC₅₀(orexin-A) = 9.1 ± 0.1, nₑₕₐₙ = 1.5 ± 0.1; N = 4), the average orexin-A-induced 2-AG elevation sensed by the CB₁ cells may be around 87 nM and the pEC₅₀ of orexin-A for 2-AG production approximately 8.8.

cPLA₂ but not DAGL activity is pivotal for OX₁ receptor activation of receptor-operated Ca²⁺ influx. We have previously shown that MAFP can strongly inhibit OX₁ receptor-operated Ca²⁺ influx (Turunen et al., 2010a), which is central for orexin receptor-mediated Ca²⁺ elevation as it also "drives" the PLC activity at low orexin concentrations (Lund et al., 2000; Johansson et al., 2007). Since MAFP is a potent inhibitor of both the (c)PLA₂ and DAGL pathways, we tested the selective inhibitors of these pathways, pyrrophenone and THL in Ca²⁺ imaging.
Pyrrophenone (Fig. 8AB) produced an inhibition equally strong as MAFP (not shown; see also Turunen et al., 2010a) whereas THL was incapable of inhibiting the Ca\textsuperscript{2+} influx response (Fig. 8AC), clearly showing that the cPLA\textsubscript{2} pathway is involved in the orexin receptor-operated Ca\textsuperscript{2+} influx. Inclusion of both pyrrophenone and THL did not produce stronger inhibition than pyrrophenone alone (Fig. 8ABD).
Discussion

We targeted the $[^3\text{H}]\text{AA}$-derived $^3\text{H}$-overflow using a number of established and novel inhibitors of lipases and amidases capable of releasing AA. Most importantly, THL, an inhibitor of DAGL, and pyrrophenone, an inhibitor of cPLA$_2^\alpha$ and $-\zeta$ (Seno et al., 2001; Ghomashchi et al., 2010) produced complementary inhibition, together reaching full inhibition. THL was selected as the DAGL inhibitor for detailed investigations due to its higher potency, efficacy and stability and also likely higher selectivity than RHC-80267. The pharmacological selectivity of the inhibitors in CHO cells was further confirmed with respect to i) thapsigargin-induced $^3\text{H}$-overflow from $[^3\text{H}]\text{AA}$-labeled cells (fully inhibited by pyrrophenone), and ii) orexin-A-induced $^3\text{H}$-overflow from $[^3\text{H}]\text{oleic}$ acid-labeled cells (fully inhibited by THL). The conclusive evidence was obtained from TLC separation of $[^1\text{C}]\text{AA}$-labeled lipid species, showing that the apparent AA-overflow was indeed composed of free AA as well as 2-AG. 2-AG release was fully dependent on DAGL (inhibited by THL), whereas the free AA-component was dependent on both the cPLA$_2$ and DAGL pathways (additively inhibited by pyrrophenone and THL, respectively). The results are summarized in Fig. 9. The DAGL pathway is likely to follow from PLC activity, as suggested both by the inhibition by the PLC inhibitor U-73122 and only a minor inhibition by the PLD inhibitor CAY10593. 2-AG production closely mirrors PLC activity, as judged from total inositol phosphate release and DAG generation with PLD inhibited (Johansson et al., 2008). It is, however, unclear, whether DAGL, in general, passively follows PLC activity or whether it is actively regulated by, e.g. Ca$^{2+}$ elevation (Bisogno et al., 2003) or phosphorylation. MAGL or another component with similar activity (Long et al., 2009), degrading 2-AG to glycerol and AA, is likely to be responsible for the inhibition of free AA-release by THL. We also utilized a
number of other inhibitors of enzymes with possible AA-releasing capacity, including the MAGL-inhibitor JZL184, FAAH-inhibitor URB597, MAGL/FAAH/hormone-sensitive lipase inhibitor CAY10499, iPLA2 inhibitor FKGK11 and NAAA/PEAase inhibitor CCPA. While JZL184, URB597 and CAY10499 are rather well characterized, FKGK11 and CCPA are not – they have been used in only a handful of studies – so it was difficult to estimate the effective concentration. However, none of the inhibitors, except JZL184, produced any inhibition, which also is logical in the light of the studies clearly pointing at the involvement of cPLA2 and DAGL only. It is unclear, why JZL184 produced a small but significant inhibition of 3H-overflow. At this concentration, CAY10499 is an effective inhibitor of MAGL too (Muccioli et al., 2008; Minkkilä et al., 2009), but it does not show any inhibition here; it is thus possible that the inhibition seen with JZL184 is due to an effect on some other target than MAGL.

cPLA2 is activated with a high potency by orexin receptors, and may thus also be a potent physiological signal. In the current study, we can verify that it indeed is cPLA2 and not DAGL signaling that is required for the activity of the receptor-operated Ca\textsuperscript{2+} influx pathway. Our results do, however, not reveal whether it is AA or some other polyunsaturated fatty acid, the other product of the cPLA2 activity, lysophosphatidylcholine, or some metabolite of these that mediates the response. All these metabolites could be active on TRP (transient receptor potential) or ARC channels (reviewed in Kukkonen, 2011).

cPLA2 enzymes are known to be sensitive to Ca\textsuperscript{2+} elevation. The PLC inhibitor U-73122 fully inhibited 3H-overflow from [3H]AA-labeled cells, suggesting that PLC activity was required for both cPLA2 and DAGL pathways. U-73122 is known to be toxic to CHO cells (see, e.g. Taylor and Broad, 1998; Lund et al., 2000), and it can fully block orexin receptor-mediated Ca\textsuperscript{2+} influx (Smart et al., 1999) although IP\textsubscript{3} (inositol-1,4,5-trisphosphate) -dependent Ca\textsuperscript{2+} influx
is not involved in this response (Ekholm et al., 2007). In addition, cPLA_2 activity occurs with a higher potency than PLC or DAGL activity (see below). We therefore feel doubtful as to the conclusion of involvement of PLC in cPLA_2 activation. Unfortunately, there are no other effective (and non-toxic) PLC inhibitors available (for at least CHO cells), and the activation mechanism of cPLA_2 thus remains unclear here. However, we know that Ca^{2+} elevation (likely influx), induced by thapsigargin is a potent stimulator of cPLA_2 in these cells (the present study and Turunen et al., 2010a), and Ca^{2+} influx is required for effective stimulation of ^3H-overflow from [^3H]AA-labeled cells by orexin-A (Turunen et al., 2010a). It is thus possible that there is a feed-forward cycle in orexin receptor signaling involving cPLA_2 and receptor-operated Ca^{2+} influx. However, further experiments are required to resolve this and alternative options.

Potency of orexin-A for cPLA_2 and DAGL activation was determined in several ways. 2-AG would be released by DAGL with pEC_{50} of 8.4 (^3H-overflow in the presence of pyrrophenone), 8.3 (TLC) and 8.8 (2-AG release reporter assay). AA, instead, would be released by cPLA_2 with pEC_{50} of 9.3 (^3H-overflow in the presence of THL) or 9.8 (TLC in the presence of THL). Some of the variation may dependent on the resolution of the assays; for instance, TLC separation shows much less variation and thus greater sensitivity than the ^3H-overflow assay. Nevertheless, cPLA_2 is clearly activated with a significantly higher potency than DAGL in CHO cells. In the other examined cell types, the situation was different. It is interesting that in all these cell types, orexin receptors couple to PLC (Holmqvist et al., 2002; Putula and Kukkonen, 2012), so the differences in 2-AG release could be related to another component, for instance lack of DAGL or the putative 2-AG transporter, or effective alternative metabolism of DAG or degradation of 2-AG.
Our previous results, using the reputed iPLA₂ inhibitor, BEL (bromoenol lactone), suggested partial involvement of iPLA₂ in the orexin-stimulated \(^3\)H-overflow (Turunen et al., 2010a). BEL is known to act on different serine hydrolases (reviewed in Balsinde and Balboa, 2005), forming a soluble, reactive molecular species, which can also form cysteine adducts with bystander proteins (Song et al., 2006). We are thus convinced that the previous result with BEL does not indicate involvement of iPLA₂ but rather is a result of non-specific interaction of this inhibitor. This is supported by the fact that a novel iPLA₂ inhibitor, FKGK11, did not inhibit \(^3\)H-overflow.

Orexin–endocannabinoid interaction has been investigated in a few studies. CB₁ receptor mRNA and preproorexin mRNA are expressed in close apposition in the lateral hypothalamus, in part in the same cells (Cota et al., 2003). The physiological function of orexins and endocannabinoids in appetite stimulation is similar, and leptin, a satiety messenger and negative regulator of orexinergic neurons (Håkansson et al., 1999; Lopez et al., 2000), is also a negative regulator of endocannabinoid levels (Di Marzo et al., 2001). Thus, these systems may take part in the same pathways. A recent study indeed indicates endocannabinoid action in orexin-mediated stimulation of appetite (Crespo et al., 2008), and similar is indicated for the analgetic action of orexin-A (Ho et al., 2011). On the other hand, endocannabinoids inhibit orexin signaling in the lateral hypothalamus by reducing the excitatory glutamatergic drive to orexinergic neurons (Huang et al., 2007). Endocannabinoids may have different function in lateral hypothalamus and in upstream regulatory centra of orexinergic neurons: while endocannabinoids in lateral hypothalamus may suppress orexinergic neurons (Huang et al., 2007), endocannabinoids in nucleus accumbens shell may stimulate activation of orexinergic neurons (Kirkham et al., 2002; Zheng et al., 2003; Soria-Gomez et al., 2007), and orexinergic
projections to nucleus accumbens shell (Mori et al., 2011) may cause a positive feedback cycle. Thus, the few studies performed suggest interactions between these systems, but the conclusions are not straight-forward. Some of the problems arise from methodological obstacles, for instance unreliable orexin receptor antibodies (own finding). Secondly, the studies conducted are either performed with intact animals or with ex vivo slice preparations, where the neuronal circuitry involved may cause problems in identifying the actual site of action of orexins and endocannabinoids.

In conclusion, the present study identifies two enzyme cascades, cPLA$_2$ and DAGL, as potent orexin receptor targets, and as the enzymes entirely responsible for the previously reported orexin-induced $^3$H-overflow from $[^3]$HAA-labeled cells (Turunen et al., 2010a). The activation mechanisms for either enzyme species remain unclear. Each enzyme activity produces a signal of its own. While cPLA$_2$ appears essential for the receptor-operated Ca$^{2+}$ influx, DAGL produces 2-AG, which is able to activate CB$_1$ cannabinoid receptors in paracrine manner. This indicates that the suggested interaction between orexinergic and cannabinoidergic systems in the brain could take place via paracrine endocannabinoid signaling, and that the regulation of partially same physiological responses in, e.g. appetite may take place via an arrangement like this. Orexin receptors have previously been suggested to heterodimerize with CB$_1$ receptors in heterologous co-expression systems (Hilairet et al., 2003; Ellis et al., 2006; Ward et al., 2011). Our view is that the interaction (rather or additionally) takes place via 2-AG production, representing a much more flexible signal system which does not require any molecular interaction between orexin and endocannabinoid receptors.
Acknowledgements

We gratefully acknowledge Dr. Michelle Glass (University of Auckland, Auckland, New Zealand) and Dr. Jarmo Laitinen and Dr. Juha Savinainen (University of Eastern Finland, Kuopio, Finland) for the CHO-hCB₁ cells, Dr. Vincenzo Di Marzo (Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, Pozzuoli, Italy) and Dr. Jarmo Laitinen for advice with the experiments and Pirjo Puroranta for technical assistance.
Authorship Contributions

*Participated in research design:* Turunen, Jäntti, and Kukkonen

*Conducted experiments:* Turunen and Jäntti

*Contributed new reagents or analytic tools:* Kukkonen

*Performed data analysis:* Turunen, Jäntti, and Kukkonen

*Wrote or contributed to the writing of the manuscript:* Turunen, Jäntti, and Kukkonen
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Footnote

This study was supported by the Academy of Finland, the Magnus Ehrnrooth Foundation, the University of Helsinki Research Funds, the Biomedicum Helsinki Foundation and the Research Foundation of the University of Helsinki.
Figure Legends

**Fig. 1.** Orexin-A-stimulated $^3$H-overflow from $[^3]$HAA-labeled CHO-hOX$_1$ cells.

**Fig. 2.** Molecular mechanism of orexin-A-and thapsigargin-stimulated $^3$H-overflow from $[^3]$HAA-labeled CHO-hOX$_1$ cells. A, orexin response is inhibited by the PLC inhibitor U-73122 but not by the PLD1 inhibitor CAY10593. B–C, orexin response is partially inhibited by the DAGL inhibitors THL and RHC-80267 (B) and the cPLA$_{2\alpha/\zeta}$ inhibitor pyrrophenone (C). D, combination of THL and pyrrophenone produces a full inhibition of the orexin response. E, response to 1 µM thapsigargin is nearly fully blocked by pyrrophenone alone. The data are normalized so that each control response (basal, 3 nM orexin-A, 100 nM orexin-A) amounts to 100% (see Data analysis and statistical procedures). Comparisons are to the corresponding controls. N = 3–7. Some difference in the inhibitory efficacy can be seen between B, C and D; this is because in D only experiments where all the inhibitors had been tested were included in the analysis to allow direct comparison of the efficacy of THL and pyrrophenone.

**Fig. 3.** Normalized raw data of the effect of the DAGL inhibitor THL and the cPLA$_{2}$ inhibitor pyrrophenone on the $^3$H-overflow from $[^3]$HAA-labeled CHO-hOX$_1$ cells. A, summarized data (same as in Fig. 2D; N = 5). The significances are the same as in Fig. 2D. B, a representative experiment. The data are normalized so that each basal is 0 and the maximum response to 100 nM orexin-A in the absence of inhibitors is 100% (response = (orexin$_{inhibitor}$ − basal$_{inhibitor}$)/(orexin$_{100nM-ctrl}$ − basal$_{ctrl}$) × 100%). "Non-inhibited components together" refers to a procedure where the orexin responses remaining after THL and pyrrophenone, respectively, were
added together. Essentially, light grey bar (A) or triangle (B) + medium grey bar/triangle gives black bar/dotted line.

**Fig. 4.** Orexin-A-induced $^3$H-overflow from [3H]oleic acid-labeled CHO-hOX$_1$ cells is in its entity mediated by DAGL. Data are normalized as explained in **Data analysis and statistical procedures** and Fig. 2. Comparisons are to the corresponding controls. N = 5.

**Fig. 5.** TLC separation of the [14C]AA-labeled lipid species released from CHO-hOX$_1$ cells. A, release of both free AA and 2-AG upon orexin stimulation; N = 8. B–C, the inhibitory actions of THL and pyrrophenone, respectively, and, D, the combined effect of THL and pyrrophenone on AA and 2-AG release upon OX$_1$ receptor stimulation. E–F, the summarized data for inhibition of AA and 2-AG release, respectively; N = 3–5. The data in bar diagrams are normalized to the AA-release response to 100 nM orexin-A (100%). Comparisons are to the corresponding controls.

**Fig. 6.** Concentration-response curves for orexin-A with respect to [14C]AA-labeled lipid species released from CHO-hOX$_1$ cells (as in Fig. 5.). 2-AG release was fully blocked by THL (1 µM) and is thus not shown.

**Fig. 7.** OX$_1$–CB$_1$ receptor communication via OX$_1$ receptor-induced 2-AG production. A–B, OX$_1$–CB$_1$ communication assay; C–D, CHO-hCB$_1$ cells alone. A, communication between OX$_1$ and CB$_1$ receptors as summarized data (N = 5–6). The data are normalized to basal (0) and the ctrl forskolin response (100%). On the left, adenylyl cyclase inhibition with 100 nM orexin-A
and direct CB1 receptor stimulation with 1 µM HU-210. The first comparison is to forskolin alone and the second to forskolin+orexin-A. On the right, sensitivity of the orexin response to the OX1 receptor antagonist SB-334867, the DAGL inhibitor THL and the CB1 receptor antagonist/inverse agonist AM-251. The first comparison is to forskolin alone. The second comparison is to forskolin in the presence of the inhibitor (forskolin+SB-334867 for forskolin+SB-334867+orexin-A, forskolin+THL for forskolin+THL+orexin-A, forskolin+AM-251 for forskolin+AM-251+orexin-A). B, concentration-response curve for orexin-A in OX1–CB1 communication assay as representative data from a single experiment. C, CB1 receptor responses in CHO-hCB1 cells alone as summarized data (N = 4–6). The data are normalized as in A. On the left, direct stimulation of CB1 receptors with 1 µM 2-AG or 1 µM HU-210. The first comparison is to forskolin alone and the second to forskolin+2-AG. On the right, sensitivity of the 2-AG response to the CB1 receptor antagonist/inverse agonist AM-251. The first comparison is to forskolin alone and the second to forskolin in the presence of AM-251. D, concentration-response curve for 2-AG as representative data from a single experiment.

**Fig. 8.** Dependence of the Ca2+ signaling of orexin receptors on cPLA2. A, ctrl CHO-hOX1 cells; B–C, cells pretreated with and run in the presence of 1 µM pyrrophenone and 1 µM THL, respectively; D, cells pretreated with and run in the presence of both 1 µM pyrrophenone and 1 µM THL. Each subfigure is a representative average trace from one cover slip with 31–53 cells. Standard errors are only shown for every 8th point for the sake of clarity. The black bars under the traces indicate successive additions of 0.3 nM, 3 nM and 30 nM orexin-A. Significances, in comparison to the control, are indicated for the maximum response (t test; first comparison) and for the number of cells responding (χ² test; second comparison).
Fig. 9. A signaling scheme based on the results and suggestions of the present study. Orexin receptor stimulation leads to activation of both PLC (PLCβ?) → DAGL and cPLA₂ cascades. The former produces the endocannabinoid 2-AG, which is able to act either from the membrane face or from the extracellular side on CB₁ receptors expressed in the same cells. The CB₁ receptor-dependent and other OX₁-triggered signal cascades may interact within the cell. 2-AG may also exit the cells and stimulate CB₁ receptors on nearby cells. The other product of DAGL activity, mainly saturated free fatty acids (FFA), may not have an important signaling role. cPLA₂ likely acts on phosphatidylcholine (PC) leading to production of lysophosphatidylcholine and polyunsaturated fatty acids (PUFAs), like AA. Either lysophosphatidylcholine or PUFAs or their metabolites centrally contribute to the activation of the orexin receptor-operated Ca²⁺ influx channel.
Fig. 1

The graph shows the 
$^3$H-overflow (times basal) in response to different concentrations of orexin-A.

- Basal: 
- 3 nM orexin-A: 
- 100 nM orexin-A:
Fig. 2

A

B

C

D

E


Fig. 4

The figure shows a bar graph comparing the 

\[ ^{3}H \text{-overflow (\% of ctrl)} \]

across different conditions:

- **Basal**
- **3 nM orexin-A**
- **100 nM orexin-A**

Each condition is represented by three bars:

- White bar: control (ctrl)
- Light gray bar: 1 μM THL
- Dark gray bar: 1 μM pyrrophenone

Significance levels are indicated as follows:

- **ns**: Not significant
- *p < 0.05
- **p < 0.01
- ***p < 0.001
Fig. 6

The graph shows the percentage of maximum AA release stimulated by orexin-A at different concentrations. The x-axis represents the logarithm of orexin-A concentration (M), and the y-axis represents the percentage of maximum AA release. Different conditions are indicated by different symbols:

- Open circles (○) represent AA.
- Filled circles (●) represent AA + THL.
- Open triangles (△) represent 2-AG.
- Filled triangles (◆) represent 2-AG + THL.
- Dashed line (--) represents AA + 2-AG.

The graph demonstrates the efficacy of each condition in stimulating AA release.

Molecular Pharmacology Fast Forward. Published on May 1, 2012 as DOI: 10.1124/mol.121.078063
Fig. 7

A. OX₁-CB₁

- ctrl
- 10 μM SB-334867
- 1 μM THL
- 10 μM AM-251

B. OX₁-CB₁

Adenyl cyclase activity (% of forskolin stimulation)

C. CB₁

- ctrl
- 10 μM AM-251

D. CB₁

Adenyl cyclase activity (% of conversion)
Fig. 8

A. ctrl

B. 1 μM pyrrophenone

C. 1 μM THL

D. 1 μM pyrrophenone + 1 μM THL