Autocrine endocannabinoid signaling through CB₁ receptors potentiates OX₁ orexin receptor signaling

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ABBRVIATIONS: 2-AG, 2-arachidonoyl glycerol; AM-251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide; CHO (cells), Chinese hamster ovary (cells); CNS, central nervous system; DAGL, diacylglycerol lipase; ERK, extracellular signal-regulated kinase; GFP, (enhanced) green fluorescent protein; GPCR, G-protein-coupled receptor; HBM, Hepes-buffered medium; HU-210, 3-(1,1'-dimethylheptyl)-6αR,7,10,10αR-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol; oxotremorine-M, N,N,N-trimethyl-4-(2-oxo-1-pyrrolidinyl)-2-butyn-1-ammonium iodide; PLA₂, PLC and PLD, phospholipase A₂, C and D, respectively; S-BSA, stripped bovine serum albumin; SB-334867, 1-(2-methylbenzoxazol-6-yl)-3-(1,5)naphthyridin-4-yl-urea HCl; SDS, sodium dodecyl sulfate; SR141716, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide; THL, tetrahydrolipstatin, N-formyl-L-
leucine-(1S)-1-[(2S,3S)-3-hexyl-4-oxo-2-oxetanyl]methyl)dodecyl ester; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto) butadiene; WB, Western blotting
ABSTRACT

It has been proposed that OX₁ orexin receptors and CB₁ cannabinoid receptors can form heteromeric complexes, which affect the trafficking of OX₁ receptors and potentiate OX₁ receptor signaling to ERK (extracellular signal-regulated kinase). We have recently shown that OX₁ receptor activity releases high levels of the endocannabinoid 2-arachidonoyl glycerol (2-AG) (Turunen et al., Mol Pharmacol 82(2): 156-167) suggesting an alternative route for OX₁-CB₁ receptor interaction in signaling, for instance in retrograde synaptic transmission. In the current study we set out to investigate this possibility utilizing recombinant Chinese hamster ovary K1 cells. 2-AG released from OX₁ receptor-expressing cells acted as a potent paracrine messenger stimulating ERK activity in neighboring CB₁ receptor-expressing cells. When OX₁ and CB₁ receptors were expressed in the same cells, OX₁ stimulation-induced ERK phosphorylation and activity were strongly potentiated. The potentiation but not the OX₁ response as such was fully abolished by specific inhibition of a) CB₁ receptors or b) the enzyme responsible for 2-AG generation, diacylglycerol lipase. Although the results do not exclude the previously proposed OX₁-CB₁ heteromerization, they nevertheless unequivocally identify diacylglycerol lipase-dependent 2-AG generation as the pivotal determinant of the OX₁-CB₁ synergism and thus suggest rather a functional than a molecular interaction of OX₁ and CB₁ receptors.
Neuropeptides orexin-A and -B are best known for their roles in the regulation of sleep/wakefulness and appetite, but many other prominent functions have also been described (reviewed in Aston-Jones et al., 2010; Scammell and Winrow, 2011; Perez-Leighton et al., 2012; Kukkonen, 2012a). Orexins mediate their actions via rhodopsin family G-protein-coupled receptors (GPCRs) named OX₁ and OX₂, which can be found in the central nervous system (CNS) but also in other parts of the body (reviewed in Scammell and Winrow, 2011; Kukkonen, 2012a). Orexin receptor activation leads to signaling via multiple intracellular pathways (reviewed in Kukkonen, 2012a). In neurons, activation of orexin receptors usually leads to depolarization through inhibition of K⁺ channels or activation of non-specific cation channels (reviewed in Kukkonen, 2012a). Intracellular Ca²⁺ concentration is elevated both through Ca²⁺ release and Ca²⁺ influxes, and other potently activated targets include phospholipases C, D and A₂ (PLC, PLD and PLA₂, respectively). Mitogen-activated protein kinase pathways ERK1/2 (extracellular signal-regulated kinase 1/2) and p38 may also be important signal relayers in orexin signaling (Hilairet et al., 2003; Ammoun et al., 2006a; Ammoun et al., 2006b; Shin et al., 2009; reviewed in Kukkonen, 2012a). The main G-protein pathway for orexin receptors is assumed to be G₉, but also other G-protein couplings have been seen (reviewed in Kukkonen, 2012a).

Endocannabinoids are locally released lipid metabolites that regulate a wide variety of processes in the central nervous system (CNS), including analgesia, mood, reward, memory, appetite, and energy metabolism (reviewed in Kano et al., 2009). The best known endocannabinoids are 2-arachidonoylglycerol (2-AG) and anandamide (reviewed in Di Marzo and Petrosino, 2007). The cannabinoid receptors CB₁ and CB₂ also belong to the GPCRs of the rhodopsin subfamily, and they predominantly couple to Gₛₒ proteins (reviewed
in Pertwee, 1997; Alexander and Kendall, 2007). CB1 receptors are mainly found in the CNS neurons. A central feature of the endocannabinoid system in the CNS is its involvement in retrograde synaptic transmission. In this process, PLC activation and/or Ca\(^{2+}\) elevation in the postsynaptic neuron induces local production and release of endocannabinoids that inhibit neurotransmitter release from the presynaptic neuron via action on presynaptic CB1 receptors (Ohno-Shosaku et al., 2001; reviewed in Kano et al., 2009). Like orexin receptors, CB1 receptors are known to utilize the ERK pathway in their signaling, in addition to the more often described inhibitory actions on adenylyl cyclase or voltage-gated Ca\(^{2+}\) channels, or activation of K+ channels (Bouaboula et al., 1995; Felder et al., 1995; reviewed in Alexander and Kendall, 2007; Kano et al., 2009; Turu and Hunyady, 2010).

The physiological functions regulated by endocannabinoids and orexins are partially overlapping as is their gross localization in the brain, for instance, in lateral hypothalamus (see, e.g. Herkenham et al., 1991; Nambu et al., 1999; Marcus et al., 2001; Wittmann et al., 2007; Palkovits et al., 2008; Suarez et al., 2011), although the endocannabinoid systems is much more widespread in the brain than the orexinergic system (Herkenham et al., 1991; Laitinen, 2004; reviewed in Kukkonen et al., 2002; Freund et al., 2003; Kano et al., 2009). Some studies implicate an interaction between orexinergic and cannabinoidergic systems (reviewed in Kukkonen, 2012a). For instance, in rat dorsal raphé nucleus, orexin-B-mediated inhibition of glutamate release can be ascribed to 2-AG release and action on CB1 receptors on presynaptic terminals (Haj-Dahmane and Shen, 2005). Orexin-A-induced analgetic response in rat periaqueductal gray relies on 2-AG-mediated retrograde inhibition of γ-aminobutyric acid release (Ho et al., 2011). Heterologous coexpression of OX1 and CB1 receptors in Chinese hamster ovary (CHO) cells very strongly potentiates OX1 receptor signaling to ERK (Hilairet et al., 2003). This potentiation has been interpreted to be due to heterodimerization of OX1 and CB1 receptors. Further studies have continued along this same
line and assessed, by different means, the heterodimerization/-oligomerization of these two receptors (Ellis et al., 2006; Ward et al., 2011).

We have recently shown that the endocannabinoid 2-AG is strongly produced and released in OX1 receptor signaling, and that this 2-AG is able to act as a potent paracrine messenger via CB1 receptors (Turunen et al., 2012). This, together with the studies in the CNS, suggests that 2-AG production is an important signal in orexin-mediated regulation of synaptic transmission. Therefore, the observed potentiation of OX1 receptor-mediated ERK phosphorylation upon OX1–CB1 receptor coexpression could be due to CB1 receptor activation by 2-AG instead of receptor di-/oligomerization. In the current study we set out to resolve this issue; the results show that 2-AG produced through OX1 receptor action is a central mediator of the potentiation.
Materials and Methods

Drugs.  1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (AM-251), 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (SR141716; a.k.a. SR-141716A or rimonabant), and 3-(1,1'-dimethylheptyl)-6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol (HU-210) were from Cayman Europe (Tallinn, Estonia). Human orexin-A and -B were from NeoMPS (Strasbourg, France), N,N,N,-trimethyl-4-(2-oxo-1-pyrrolidinyl)-2-butyn-1-ammonium iodide (oxotremorine-M) was RBI (Natick, MA) and N-formyl-L-leucine-(1S)-1-[(2S,3S)-3-hexyl-4-oxo-2-oxetanyl]methyl)dodecyl ester (tetrahydrolipstatin, THL, Orlistat), 1,4-diamino-2,3-dicyano-1,4-bis[o-aminophenylmercapto]butadiene (U0126) and 1-[2-methylbenzoxazol-6-yl]-3-[1,5]naphthyridin-4-yl-urea HCl (SB-334867) from Tocris Bioscience (Bristol, UK). Forskolin and 3-isobutyl-1-methylxanthine were from Sigma-Aldrich (St. Louis, MO) and [2,8-3H]adenine, myo-[2-3H]-inositol (PT6–271), [3H]SR141716 and [125I]orexin-A from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Cell Culture. CHO-hOX1, expressing human OX1 receptors, have been described previously as also their culture conditions (see, e.g. Turunen et al., 2010; Turunen et al., 2012). CHO-hCB1 cells, expressing human CB1a 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); the culture conditions for these have been described (Turunen et al., 2012). For Western blotting (WB) experiments, the cells were cultured on six-well plates (9.6 cm² well bottom area; Greiner Bio-One GmbH, Frickenhausen, Germany), for the luciferase assays, on either 24- or 96-well plates (Greiner),
and for the receptor binding experiments, on cell culture dishes (56 cm² bottom area) or 48-well plates (Greiner). Sf9 insect cells (for baculovirus propagation) were cultured in shaker culture as described in (Näreöja et al., 2011).

**Plasmids and transfection.** The plasmid pSG-GalElk-1 (fusion of the dimerization domain of the transcription factor Elk-1 and the DNA-binding domain of the yeast transcription factor Gal4; Kortenjann et al., 1994) was a kind gift from Dr. Peter E. Shaw (Queen’s Medical Centre, Nottingham, UK), pGL3 G5 E4 Δ38 (5×Gal4 binding site controlling firefly luciferase expression; Kamano et al., 1995) from Dr. Karl-Heinz Klempnauer (Westfälische-Wilhelms-Universität Münster, Germany) via Drs. Peter E. Shaw and Michael J. Courtney (University of Eastern Finland, Kuopio, Finland), pcDNA3.1-CB1-GFP (hCB1a receptor with C-terminal enhanced green fluorescent protein [(E)GFP]; D’Antona et al., 2006) from Dr. Debra Kendall (University of Connecticut, Storrs, CT), and pcDNA3.1-3HA-hCB1 (human CB1a receptor with 3×N-terminal hemagglutinin [HA] tag) from Missouri S&T cDNA Resource Center (Rolla, MO). pRL-TK (Renilla luciferase under herpes simplex virus thymidine kinase [TK] promoter) was from Promega (Madison, WI) and pcDNA3.1-hOX1 and pM1-GFP (human M1 muscarinic acetylcholine receptor [hM1]–EGFP-fusion) were constructed in-house (Holmqvist et al., 2002). Empty plasmids were used to keep DNA amount equal in all transfections.

Transient transfection was used for the Elk-1 activity assay in CHO-hOX1 and CHO-hCB1 cells, and for the PLC activity assay and binding experiments, which were to be performed in the same conditions. In addition to the reporter assay plasmids (pSG-GalElk-1, pGL3 G5 E4 Δ38, pRL-TK), either CB1 receptor (pcDNA3.1-3HA-hCB1 with CHO-hOX1 cells), OX1 receptor (pcDNA3.1-hOX1 with CHO-hCB1 cells) or M1 receptor (pM1-GFP with CHO-hCB1 cells) (or empty plasmid) was used. Cells on 96-well plates were grown to
40–50% confluence and transfected in Ham’s F12 with 0.312 µg/cm² DNA and 0.74 µl/cm² FugeneHD (Roche, Mannheim, Germany). The optimized transfections conditions used for DNA were 0.6% (w/v) pSG-GalElk-1, 50% pGL3 G5 E4 Δ38, 2.4% pRL-TK and 47% pcDNA3.1-3HA-hCB1/pcDNA3.1-hOX1/pM1-GFP/empty plasmid. Liposome-based delivery was used in this assay as several plasmids had to be cotransfected, which is more easily accomplished by this technique than using the baculovirus system (below).

**Elk-1 activity luciferase assay.** The cells were transfected as described above, and 5 h after transfection, washed and changed to serum-free medium to serum-starve the cells overnight. The following day the cells were stimulated. When inhibitors were used they (or the vehicle) were added to the cells 30 min prior to orexin-A, HU-210 or 2-AG. After 5 h of stimulation the cells were lysed and the assay continued essentially according to the instructions of the manufacturer of the assay kit (Dual-Luciferase Reporter Assay system, Promega, Madison, WI). Luminescence was measured with GloMax 20/20 luminometer (Promega). The Elk-1-specific signal (firefly luciferase) was normalized to Renilla luciferase in each sample. The Elk-1 reporter assay was specific for ERK since it was fully inhibited by U0126, an inhibitor of the upstream activator of ERK, MEK1 (MAPK/ERK kinase 1) (not shown).

Elk-1 activity assay was also utilized for measurement of paracrine communication from CHO-hOX₁ cells to CHO-hCB₁ cells via 2-AG release similar to a previously described cAMP assay (Turunen et al., 2012). Briefly, CHO-hCB₁ on cell culture dishes were transfected with the Elk-1 reporter plasmids (pSG-GalElk-1, pGL3 G5 E4-Δ38, pRL-TK). 5 h later, they were detached (PBS + 0.02% (w/v) EDTA), spun down, mixed with an equal amount of non-transfected CHO-hOX₁ and plated on a 24-well plate. The following evening, they were serum starved as above. The following morning (48 h after transfection), the "cell mixture" was stimulated and the luciferase activities measured as above.
Baculoviral vectors and transduction. Generation of baculovirus expression vectors for mammalian expression of GFP as well as human C-terminally GFP-tagged OX₁ receptor (OX₁-GFP) under cytomegalovirus (CMV) promoter has been previously described (Näsman et al., 2006). For a similar construct of CB₁ receptor (CB₁-GFP), the fragment of pcDNA3.1-CB₁-GFP containing the CMV promoter and the CB₁-GFP fusion was ligated in pFastBac with Rapid DNA ligation kit (Thermo Scientific, Rockford, IL). Recombinant baculovirus was obtained using the Bac-to-Bac expression system (Invitrogen, Carlsbad, CA). The virus stocks were propagated in Sf9 insect cells.

Baculovirus transduction was used for transient "second" receptor expression for WB analysis of ERK1/2 phosphorylation. Baculovirus was used for WB assays due to its ease, economy and 100% transduction efficiency. High-titer virus stocks obtained from Sf9 cells were cleared by centrifugation, resuspended in the cell culture medium and added to CHO cells on 6-well plates (Näsman et al., 2006). The cells were used 24 or 48 h later as described below.

ERK1/2 phosphorylation (activation) assay. CHO cells were transduced with recombinant baculovirus vectors containing OX₁-GFP or CB₁-GFP constructs; baculovirus expressing only GFP was used as a negative control. In the final experiments, only CHO-hCB₁ cells transduced with OX₁-GFP constructs were used. Transduction was done as follows. CHO-hCB₁ cells were plated on six-well plates (3×10⁶ cells/well) and cultured overnight (to 40–60% confluence). The following day, the cells were transduced with OX₁-GFP baculovirus. The virus-containing medium was removed 5 h later, and the cells were washed with PBS and left in serum-free medium to be used the following day.
The ERK phosphorylation experiments were performed largely as described in (Ammoun et al., 2006a) with some modifications. Inhibitors (THL, SR141716, vehicle control) were added to the cells in serum-free culture medium 30 min prior to stimulation. The cells were stimulated with orexin-A, 2-AG or HU-210 for 10 min, and rapidly lysed with 100 µl of lysis buffer (50 mM HEPES, 150 mM NaCl, pH 7.5, + 10% [v/v] glycerol, 1% [v/v] Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM Na⁺-pyrophosphate, 1 mM Na⁺-orthovanadate, 10 mM NaF, 250 µM p-nitrophenol phosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and PhosSTOP phosphatase inhibitor cocktail [Roche, Mannheim, Germany]). The samples were boiled in Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, + 1 mM dithiothreitol, 2% [w/v], sodium dodecyl sulfate [SDS], 10% [v/v] glycerol, and 0.1% [w/v] bromophenol blue), and separated on 10% polyacrylamide gel by electrophoresis. Protein was transferred to nitrocellulose membranes (Hybond-C Extra Nitrocellulose; Amersham Biosciences, Little Chalfont, Bucks, UK), which were then washed with PBS and blocked with a 50:50-mixture of Odyssey blocking buffer (Li-corr Biosciences, Lincoln, NE) and PBS + 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20) for 1 h. Membranes were then incubated with primary antibodies overnight at +4°C. Primary antibodies used were rabbit anti-active MAPK IgG (1:5000; #V803A; Promega, Madison, WI) and mouse p44/42 MAPK (ERK1/2) IgG (1:2500; #46965; Cell Signaling Technology, Danvers, MA) in a 50:50 mixture of Odyssey blocking buffer and PBS + 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20). The following day the membranes were washed with PBS (3 times, 10 min each) and incubated with infrared fluorescent secondary antibodies for 1 h at room temperature, protected from light. Secondary antibodies used were DyLight 800-conjugated goat-anti-rabbit IgG (1:10 000; #35571; Li-cor) and DyLight 680-conjugated goat-anti-mouse IgG (1:10 000; #35518; Li-cor). After incubation with secondary antibodies, the membranes were washed with PBS (3 times, 10
min each) before detecting the bands with Odyssey Infrared Imaging System (Li-cor). The images were analyzed using Nikon NIS-Elements AR.

Within an experiment, each gel included a standard (in duplicate) composed of 100 nM orexin-A in ctrl cells from the same stock material (see Supplemental Fig. 2). The intensity of each gel was additionally normalized according to these lanes to compensate for possibly uneven protein transfer.

**Adenylyl cyclase activity.** The measurements were performed essentially as described in (Turunen et al., 2012). CHO-hCB1 cells on 24-well plates were prelabeled with [3H]adenine for 2 h, after which they were washed once with 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) and then preincubated in HBM containing 500 µM 3-isobutyl-1-methylxanthine (a cyclic nucleotide phosphodiesterase inhibitor) and 0.5 mg/ml stripped bovine serum albumin (S-BSA; to help to retain 2-AG in solution; Savinainen et al., 2003) ± THL for 30 min at 37 ºC. The stimulants (forskolin, 2-AG) were added and the reactions allowed to proceed for 10 min, after which they were interrupted by rapid removal of the medium, addition of ice-cold perchloric acid and freezing. After thawing, cell debris was spun down and the [3H]ATP+[3H]ADP and [3H]cAMP fractions isolated from the cell extracts by sequential Dowex/alumina chromatography (see, e.g. Holmqvist et al., 2005). Radioactivity was determined using scintillation counting (Wallac 1414 liquid scintillation counter; PerkinElmer); the conversion of [3H]ATP to [3H]cAMP was calculated as a percentage of the total eluted [3H]ATP+[3H]ADP.

**Phospholipase C activity.** Total inositol phosphate release was measured essentially as described in (Jäntti et al., 2012). CHO-hCB1 cells were transiently transfected with either
OX₁ or M₁ cDNA and also the other vectors included in the Elk-1 luciferase assay in the same ratios as there to make the assays comparable. Five hours later, the cells were exposed to [³H]inositol to prelabel the membrane phosphoinositides. After 16 h of labeling, the cells were washed once with HBM and incubated in HBM containing 10 mM LiCl (to inhibit inositol monophosphatase) for 10 min at 37°C. The cells were then stimulated with orexin-A for 20 min. The reactions were stopped by rapid removal of the medium, addition of 0.2 ml of 0.4 M ice-cold perchloric acid and freezing. Thawed samples were neutralized with 0.1 ml of 0.36 M KOH + 0.3 M KHCO₃. The insoluble fragments were spun down and the total inositol phosphate fraction isolated by anion-exchange chromatography. The radioactivity of the inositol phosphate fraction was determined using scintillation counting as above.

**Receptor binding.** Receptor expression levels were determined by radioligand binding. [¹²⁵I]orexin-A was utilized for OX₁ receptors. The cells were plated on cell culture dishes and transfected (if relevant for the assay) the following day. The cDNA mixtures contained the different vectors in the same ratios as for the Elk-1 luciferase assay to make the assays comparable. After yet another 24 h the cells were detached (PBS + 0.02% EDTA), spun down and resuspended in HBM containing 0.1% (w/v) BSA. The binding assay was performed in this buffer in low protein-binding tubes (Eppendorf Nordic Aps, Horsholm, Denmark) with [¹²⁵I]orexin-A at room temperature. The non-specific binding was determined with 10 µM SB-334867 (preincubated for 10 min before orexin-A). The reaction was allowed to proceed for 10 min, after which the cells were rapidly spun down (+2°C, 14000 g, 30 s). The pellet was washed superficially (no resuspension) with ice-cold HBM and resuspended in HBM, and the radioactivity counted (Wallac Wizard 1480 Gamma Counter; PerkinElmer).

[¹²⁵I]orexin-A has several complicating properties (Kukkonen, 2012b; Kukkonen, 2012a), and the assay conditions were thus carefully optimized. Due to its high filter binding,
the separation of bound and free radioligand is not possible by filtration (Holmqvist et al., 2001; Kukkonen, 2012b; Putula and Kukkonen, unpublished), and centrifugation of cells in suspension was thus rather applied. BSA and low protein-binding tubes and pipette tips were used to reduce binding of orexin-A to plastic surfaces. Orexin-A (as well as $^{125}$Iorexin-A) is an agonist and stimulates receptor internalization. Cell surface-bound orexin-A was dissociated upon exposure to low-pH, urea-containing buffer (Chang et al., 1993) and the internalized radioligand seen after the NaOH incubation following this wash. At 10 min, very little internalization was seen. Thus, binding under these conditions rather reflects cell surface than total receptor expression.

CB$_1$ receptor expression was measured in cells attached on polyethylenimine-coated (25 µg/ml for 1 h at 37°C; Sigma-Aldrich) 48-well plates (Greiner). The cells were transfected as above and utilized 24 h later. The cells were incubated in HBM containing 0.1% (w/v) BSA + $[^3]$H$SR141716 (± 10 µM HU-210 to determine non-specific binding) for 60 min at room temperature. The plates were placed on ice, the medium rapidly removed, the wells washed rapidly twice with ice-cold HBM, the cells detached with NaOH and their radioactivity measured by scintillation counting (PerkinElmer). Both the radioligand and all available non-labeled ligands to determine non-specific binding are cell-permeable and can thus not separate cell surface and intracellular binding. $[^3]$H$SR141716$ binding reported is thus a measure of the total CB$_1$ receptor pool.

For both $^{125}$Iorexin-A and $[^3]$H$SR141716$ binding, the cells were preincubated with THL for 30 min before the addition of the radioligand, also to mimic the conditions in the Elk-1 assay.

**Data analysis.** All data are presented as mean ± S.E.M; $N$ refers to the number of batches of cells (= number of independent experiments). Each experiment was performed at least three
times and the averaged data presented are thus at least from three independent experiments. WB experiments were performed with two, Elk-1-luciferase measurements with three and cAMP measurements with four data points in parallel. Student’s paired or non-paired two-tailed $t$ test with Bonferroni correction was used in all pairwise comparisons.
The aim of the study was to investigate the molecular mechanism behind the previous finding of strong potentiation of OX₁ orexin receptor-induced ERK activity by CB₁ cannabinoid receptors coexpression. For the studies, we utilized two stable cell line expressing human OX₁ and CB₁ receptors, CHO-hOX₁ and CHO-hCB₁ cells, respectively. These cells where additionally transiently transfected or transduced to express CB₁ and OX₁ (or M₁) receptors, respectively (Supplemental Fig. 1). ERK activity was assessed by WB against the phosphorylated (active) ERK and downstream activity of the ERK target, transcription factor Elk-1.

**CHO-hCB₁ cells with transient expression of OX₁ receptors show CB₁ receptor signaling-dependent potentiation of OX₁ signaling to ERK.** In CHO-hCB₁ cells, CB₁ receptor stimulation with 2-AG or HU-210 produced an ERK1/2 phosphorylation response, as assessed by WB with antibodies against phosphorylated (active) ERK (Fig. 1A). There was no ERK phosphorylation in response to orexin stimulation in CHO-hCB₁ cells (Fig. 1A), but when OX₁ receptors were transiently expressed in these cells utilizing baculovirus, orexin-A produced a marked response (Fig. 1B). The same was seen in the Elk-1 activity assay (Fig. 1C).

Orexin-A produced a concentration-dependent stimulation of ERK phosphorylation in transiently OX₁-expressing CHO-hCB₁ cells (Fig. 2A and B, Supplemental Table 1, Supplemental Fig. 2). When CB₁ receptors were blocked with the CB₁ receptor antagonist/inverse agonist, SR141716, the maximum response to orexin-A was strongly reduced (Fig. 2A, B and C, Supplemental Fig. 2). An equal inhibition was seen with the diacylglycerol lipase (DAGL) inhibitor, THL (Fig. 2A, B and C, Supplemental Fig. 2). The
potency of orexin-A was also reduced 100–150-fold by SR141716 and THL (Fig. 2A, B and D, Supplemental Tables 1 and 2, Supplemental Fig. 2).

Elk-1 was activated by orexin-A with somewhat lower potency than ERK phosphorylation in CHO-hCB1 cells transiently expressing OX1 receptors (Fig. 3, Supplemental Table 1). The DAGL inhibitor THL and another CB1 receptor antagonist, AM-251, produced a 5–8-fold reduction in the potency of orexin-A (Fig. 3A and E, Supplemental Tables 1 and 2), but there was no difference in the basal (Fig. 3C) or the maximum response (Fig. 3D).

M1 muscarinic receptor response is more weakly potentiated than the OX1 receptor response. We expressed another strongly Gq-PLC-coupled receptor, human M1 muscarinic acetylcholine receptor, in CHO-hCB1 cells and assessed Elk-1 activity as for the OX1 receptor. Stimulation with the potent agonist oxotremorine-M produced a clear concentration-dependent Elk-1 activation (Fig. 3B). Inclusion of THL and AM-251 slightly reduced the maximum response as well as slightly shifted the concentration-response curve (Fig. 3BDE). The shift in the concentration-response curve by THL and AM-251 was significantly smaller for M1 than for the OX1 receptors (Fig. 3D). This indicates that the M1 response is more weakly potentiated by CB1 receptor signaling than the OX1 response, despite the fact that both receptors produced essentially equal PLC activation (Fig. 3E). AM-251 clearly reduced the basal Elk-1 activity in M1-expressing cells unlike the OX1-expressing cells (Fig. 3C).

CB1 expression on CHO-hOX1 background potentiates the ERK pathway. Orexin-A produced a concentration-dependent stimulation of Elk-1 activity in CHO-hOX1 cells (Fig. 4A, Supplemental Table 1). No response to the CB1 cannabinoid receptor stimulus (HU-210) was seen (not shown). Upon transient expression of CB1 receptors, HU-210 was able to
induce Elk-1 activity (not shown; see below for Fig. 6B). Transient expression of CB₁ receptors increased the potency of orexin-A 6-fold; this was fully blocked by THL or AM-251 (Fig. 4A and D, Supplemental Tables 1 and 2). CB₁ expression did not affect the maximum response (Fig. 4C). Interestingly, the basal Elk-1 activity was elevated by CB₁ receptor coexpression (Fig. 4B). This was clearly more efficiently inhibited by AM-251 than THL, and may thus relate to constitutive activity of CB₁ receptors (see, e.g. Turunen et al., 2012).

**DAGL inhibitor THL and CB₁ receptor antagonist/inverse agonist, AM-251, do not show non-specific effects.** As a control, we tested the drugs used for non-specific effects. THL did not have any direct blocking effect on CB₁ receptor signaling, and similarly, THL and AM-251 were devoid of an effect on OX₁ orexin receptor or M₁ muscarinic receptor signaling (Fig. 5), which is in agreement with previous findings (see Discussion).

**The impact of the receptor stoichiometry on the CB₁ receptor potentiation of OX₁ signaling.** Potentiation of the ERK signaling was obtained upon combination of OX₁ and CB₁ receptors on either cell background (CHO-hCB₁ and CHO-hOX₁, respectively), but the potentiation was somewhat more modest when CB₁ receptors were transiently expressed on the CHO-hOX₁ background (compare Figs. 3 and 4). Interestingly, the pEC₅₀-value of orexin-A for ERK phosphorylation or Elk-1 activation in both stable CHO cell clones was around 7.4–7.8 when CB₁ receptors were not present or when they were inhibited (THL or CB₁ antagonist; Supplemental Table 1).

When the responses to OX₁ and CB₁ receptor stimulation were compared between the cell types, it was clearly seen that the maximal CB₁ and OX₁ receptor activation equally strongly stimulated Elk-1 activity in CHO-hCB₁ + OX₁ cells (Fig. 6A and C), whereas OX₁
receptors much more strongly stimulated Elk-1 activity in CHO-hOX1 + CB1 cells (Fig. 6B and C). The efficacy of the synergistic signaling of OX1 and CB1 receptors could be imagined to relate to the receptor expression levels and stoichiometry. High OX1 expression might give a more robust 2-AG production, which is able to stimulate CB1 receptors even not in immediate vicinity of OX1 receptors. Efficient CB1 signaling, as indicated by the receptor's ability to connect to the ERK cascade when stimulated directly (HU-210), should suggest good ability to potentiate the orexin response. We thus measured the apparent expression levels of OX1 and CB1 receptors in the different cell systems utilizing the same expression of constructs as for the Elk-1 assay, to make the results as comparable as possible. The protocol applied (see Receptor binding) visualizes, for OX1 receptors, the cell surface expression. The binding was for CHO-hOX1 cells 460 ± 30 fmol/mg protein (N = 3) (Fig. 6D). Transient expression of CB1 receptor in these cells did not significantly alter the OX1 expression. CHO-hCB1 cells transiently transfected with OX1 receptor cDNA expressed very low levels of OX1 receptors (22 ± 3 fmol/mg protein; N = 5) (Fig. 6D). It should be pointed out that [125I]orexin-A binding only indicates the so-called agonist high-affinity sites and not the total receptor pool (Kukkonen, 2004). In contrast, the total CB1 receptor pool (both cell surface and internal, both agonist affinity states) is visualized by [3H]SR141716 binding (Fig. 6E). This binding amounted to 3.2 ± 0.3 pmol/mg protein in CHO-hCB1 cells. Transient expression of OX1 in these cells slightly elevated CB1 expression (Fig. 6E). Transient CB1 expression level in CHO-hOX1 cells was significantly lower than the stable expression in CHO-hCB1 cells (Fig. 6E). THL did not affect OX1 or CB1 expression levels (Fig. 6DF). Although the receptor expression levels (Fig. 6C) affect the responses obtained by stimulation of OX1 and CB1 receptors (Fig. 6F), the potentiation works equally well at very low OX1 receptor expression levels (CHO-hCB1 + OX1 cells) and thus also over a wide range of OX1–CB1 receptor stoichiometries (Fig. 6F).
**OX₁ receptor signaling relays endocannabinoid signals via CB₁ receptor to ERK also in paracrine fashion.** The results thus suggest that OX₁ receptor signaling releases 2-AG via DAGL (as we have recently shown in Turunen et al., 2012). 2-AG acts in an autocrine manner on the coexpressed CB₁ receptors, and these, synergistically with the OX₁ receptors, activate the ERK cascade. We have recently presented an artificial cell–cell communication assay between OX₁-expressing and CB₁-expressing cells via 2-AG release (i.e. retrograde synaptic transmission) using adenylyl cyclase regulation in CHO-hCB₁ cells as the output (Turunen et al., 2012). We wanted to test here, whether the 2-AG released would be able to activate the ERK cascade too. Indeed, the 2-AG released from CHO-hOX₁ cells upon exposure to orexin-A was an effective stimulant of the ERK cascade in nearby CHO-hCB₁ cells (Fig. 7). The response obtained was sensitive to THL and AM-251 (Fig. 7), as expected (Turunen et al., 2012).
We have recently shown that OX$_1$ receptor stimulation in CHO cells induces significant endocannabinoid 2-AG production by activating DAGL (Turunen et al., 2012). In the current study we show that this 2-AG is able to act both as auto- and paracrine messenger on CB$_1$ cannabinoid receptors. If the receptors are expressed in the same cells, OX$_1$ receptor signaling to the ERK cascade is strongly potentiated by autocrine CB$_1$ signaling. The potentiation is fully reversed by blocking CB$_1$ receptors with SR141716 or AM-251 or inhibition of DAGL with THL. This shows that the potentiation requires OX$_1$ receptor-induced release of 2-AG, which acts via CB$_1$ receptors.

Our findings are centrally based on the inhibitors THL, SR141716 and AM-251. THL was originally utilized as triglyceride lipase inhibitor, but was later found to be an even more potent inhibitor of DAGL$\alpha$ and $\beta$ (Bisogno et al., 2003; Szabo et al., 2006). Most importantly, we have recently shown that THL at 1 µM fully blocks 2-AG production in CHO cells (Turunen et al., 2012). THL displays weak affinity for CB$_1$ receptors too ($K_i \approx 4$ µM; Szabo et al., 2006), but this is too low to cause any significant inhibition of CB$_1$ receptors here (see also Fig. 5). We also tried overexpression of monoacylglycerol lipase, but did not obtain strong enough reduction in 2-AG levels (not shown). Whether this is due to low expression or lack of an activation signal remains elusive. SR141716 has mainly been used as CB$_1$ receptors antagonist/inverse agonist in the previous studies on OX$_1$–CB$_1$ interaction (Hilairet et al., 2003; Ellis et al., 2006; Ward et al., 2011), and we thus used it in the ERK phosphorylation assay. As an additional level of control, we utilized another well-known CB$_1$ receptors antagonist/inverse agonist, AM-251, in the Elk-1 activity assay. Both ligands behaved in a similar manner and showed no non-specific effects, despite the fact that AM-251 is actually an inverse agonist. DAGL block by THL and CB$_1$ receptor block with
AM-251 or SR141716 produce an essentially equal inhibition of the potentiation of ERK signaling, and thus the putative constitutive activity of CB$_1$ receptors seems largely redundant for the agonist-stimulated ERK activity.

The two assays utilized to measure ERK activation produced qualitatively equal potentiation – i.e. CB$_1$ receptor coexpression with OX$_1$ receptors always potentiated OX$_1$ signaling to ERK – but the level of potentiation was lower for the Elk-1 activity assay and the maximum response was not affected in CHO-hCB$_1$ cells. The assays are run for very different times, 10 min and 5 h, for pERK and Elk-1, respectively. One explanation for the result is that the temporal profile of ERK activation is different in the absence and presence of CB$_1$ receptor signaling, e.g. that CB$_1$ receptor contribution ceases faster than the OX$_1$ receptor component. Another explanation readily at hand is that Elk-1 response saturates at submaximal ERK activation levels. For ERK signaling there is the complicating factor of possible different cytosolic and nuclear ERK activity.

Potentiation of the ERK signaling of OX$_1$ receptors by CB$_1$ receptor coexpression has been previously observed in CHO cells (Hilairet et al., 2003). The potentiation was found to be blocked by the CB$_1$ antagonist SR141716 and pertussis toxin. The authors inferred, based on colocalization studies, that OX$_1$ and CB$_1$ receptors form heteromeric complexes with enhanced signaling capabilities (Hilairet et al., 2003). In the current study, we have assessed the ERK signaling cascade, and can show that 2-AG, produced by OX$_1$ receptor activity, signals to ERK via CB$_1$ receptors (Fig. 7), and also mediates the potentiation when the receptors are expressed in the same cells. Also the other findings of Hilairet et al. (2003) – i) CB$_1$ receptor stimulation-induced ERK phosphorylation not affected by OX$_1$ receptor coexpression and ii) OX$_1$-mediated PLC activation not potentiated by CB$_1$ coexpression – are likely explained by this signaling scheme: Since there is no PLC coupling of CB$_1$ receptors, PLC activity cannot be potentiated, and OX$_1$ receptor coexpression does not potentiate CB$_1$
receptor coupling to ERK, since CB₁ receptor activation does not produce any ligand for OX₁ receptors.

The concept of OX₁–CB₁ receptor heterodimerization was further refined by Milligan and coworkers in their work with HEK-293 cells (Ellis et al., 2006). In these cells, potentiation of ERK phosphorylation is very weak (Ellis et al., 2006). We have not investigated OX₁–CB₁ signaling in HEK-293 cells, but it is possible that the weak potentiation of the ERK response could be due to much lower or less potent 2-AG production upon OX₁ receptor activation in this cell type as compared to CHO cells, as is also indicated by our recent findings (Turunen et al., 2012); however, we have not had access to the same clone of HEK-293 cells. This group has also presented convincing FRET (Förster/fluorescence resonance energy transfer) and immunoprecipitation data on OX₁–CB₁ complex formation (Ward et al., 2011). Thus, there is little doubt that these receptors have the capacity to combine to heteromers, like many other GPCRs (reviewed in Birdsall, 2010; Rozenfeld and Devi, 2011). Previously, CB₁ receptors have been reported to interact with many other GPCRs either via dimerization or functional interaction (Glass and Felder, 1997; Rios et al., 2006; Harkany et al., 2007; Marcellino et al., 2008; Turu et al., 2009; Rozenfeld et al., 2011). We have no means of readily inhibiting receptor heteromerization process, and we therefore cannot assess the significance of this for the potentiation of the ERK signaling. However, we currently feel that the most important interaction between OX₁ and CB₁ receptors takes place via OX₁ receptor-stimulated 2-AG production, i.e. a functional interaction, which, theoretically, would not demand receptor complex formation. However, it is possible that functional interaction via endocannabinoids and receptor heteromerization collaborate. If receptor heteromers were placed in signaling complexes including PLC, DAGL and the putative 2-AG extrusion machinery, CB₁ receptors would be exposed to highest possible 2-AG levels. This would be an attractive hypothesis, but at least in CHO-
hOX₁ cells 2-AG production is high enough to even allow diffusion in the extracellular space (Fig. 7 and Turunen et al., 2012), as also should be the case for the retrograde synaptic transmission.

Potentiation of the ERK signaling by CB₁ receptors works well also at very low orexin receptor expression levels. The high OX₁ receptor level in CHO-hOX₁ cells may thus be to a large part superfluous with respect to 2-AG generation for autocrine CB₁ receptor stimulation. Orexin receptor coupling to the PLC cascade is very efficient, similar to what we have previously found for muscarinic receptors (Kukkonen et al., 1996). OX₁ receptors also potently coupled to the ERK-Elk-1 cascade even at low expression levels. CB₁ receptors were efficiently expressed both stably and transiently; some receptor reserve may exist also for CB₁ receptors, as indicated by the similar Elk-1 response in these different cell types. The cell surface receptor levels are likely significantly lower than measured here, as CB₁ receptors often show high degree of constitutive internalization (Ellis et al., 2006). We could not, unfortunately, directly assess this, as the binding assay does not allow separation of cell surface and intracellular receptors. However, local receptor levels in synaptic areas are also high.

If the potentiation of the ERK signaling by OX₁–CB₁ "collaboration" solely takes place via functional interaction of the signal pathways, what then are the molecular mechanisms? Orexin receptors are suggested to be able to interact with at least Gᵢₒ, Gᵣ and Gₛ family G-proteins (Randeva et al., 2001; Holmqvist et al., 2005; Karteris et al., 2005). However, both Gᵢₒ- and Gₛ-couplings appear rather weak, at least in CHO cells (Holmqvist et al., 2005). In contrast, CB₁ receptors strongly couple to Gᵢₒ proteins although also other couplings have been suggested (reviewed in Pertwee, 1997; Alexander and Kendall, 2007; see also Fig. 5). It is thus likely that the two receptors preferentially utilize different pathways to ERK, and these pathways could act synergistically to activate ERK. Interestingly, the
signaling of M1 muscarinic receptors was only weakly potentiated by CB1 receptor signaling, although both M1 and OX1 receptors equally strongly coupled to the PLC cascade in transient expression. Whether this is due to differences in complexing with CB1 receptors or efficacy of 2-AG production, remains unknown. However, it would be tempting to speculate that the coupling of OX1 receptors to receptor-activated Ca2+ influx may produce stronger DAGL stimulation than that obtained with muscarinic receptors (Magga et al., 2006; Johansson et al., 2007).

In conclusion, we have here shown that the critical determinant of the synergistic OX1–CB1 signaling to ERK is the communication cascade OX1 → DAGL → 2-AG → CB1 receptors, which allows both OX1 and CB1 receptors to simultaneously signal to ERK. Whether receptor heteromerization takes part in the process cannot be resolved, but the data indicate that this may not be needed. However, it is of interest, in future studies, to focus on the possible effects of 2-AG on receptor dimerization, trafficking and other properties described to be affected upon OX1–CB1 coexpression.
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Authorship Contributions

Participated in research design: Jäntti, Kukkonen

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Contributed new reagents or analytic tools: Jäntti, Putula, Lindqvist, Näsman

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Distribution of type 1 cannabinoid receptor (CB1)-immunoreactive axons in the
Footnote

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Legends for Figures

**Fig. 1.** pERK and Elk-1 activity responses in CHO-hCB1 cells expressing OX1 receptors upon transduction or transfection. A, ERK phosphorylation in response to CB1 receptor stimulation with HU-210 or 2-AG or orexin receptor stimulation with orexin-A in native CHO-hCB1 cells and, B, in cells transduced with OX1-GFP baculovirus. Please observe that the results come from infrared fluorescence and thus the figures appear inverted as compared to film images. Also, the dynamic range is much wider than on the film or can be shown here; a compromise what concerns the intensity had to be made in order to present the data here. C, responses in CHO-hCB1 cells transfected with OX1 cDNA with respect to the Elk-1 activity assay (N = 5). Comparison is to basal; **, p < 0.01.

**Fig. 2.** Quantitated pERK1/2 WB data from CHO-hCB1 cells transduced with OX1-GFP baculovirus. A and B, representative concentration-response curves from the experiment presented in Supplementary Fig. 2. The basal ERK phosphorylation was very low (see also Fig. 1A and B and Supplementary Fig. 2) and thus the basal is normalized to 0 for the curves. C and D, average responses (N = 3). The data were normalized to the non-inhibited CB1–OX1 control in each batch of cells before averaging. Comparisons are to the corresponding controls; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Fig. 3.** Elk-1 (A–D) and PLC (E) activity in CHO-hCB1 cells expressing OX1 or M1 receptors upon transient transfection. A and B, representative concentration-response curves for OX1- and M1-expressing cells. C, D and E, average responses (N = 3–6). The data were normalized to the non-inhibited CHO-hCB1 + OX1 or CHO-hCB1 + M1 control in each batch of cells before averaging. Comparisons are to the corresponding controls. F, maximum PLC
activation by OX₁ and M₁ receptors in CHO-hCB₁ cells (N = 4). oxo-M = oxotremorine-M.
The comparison is to the basal in CHO-hCB₁ + OX₁ cells, to which the values also are normalized similar as above. ns (not significant), p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Fig. 4.** Elk-1 activity in CHO-hOX₁ cells expressing CB₁ receptors upon transient transfection. A, representative concentration-response curves. B–D, average responses (N = 3). The data were normalized to the non-inhibited CB₁–OX₁ control in each batch of cells before averaging. The first comparisons are to the corresponding controls; ns (not significant), p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001. In D, the second comparison is to the ctrl CHO-hOX₁ cells (no CB₁ expression); NS, not significant.

**Fig. 5.** THL and AM-251 do not show non-specific effects on CB₁, OX₁ or M₁ receptors. A, the effect of THL on CB₁ receptor action in CHO-hCB₁ cells measured on inhibition of forskolin-stimulated adenylyl cyclase activity by 2-AG. B, same data as in A normalized to basal and the forskolin response. C, the effect of THL and AM-251 on OX₁ receptor stimulation of Elk-1 activity in CHO-hOX₁ cells. D, the effect of THL and AM-251 on M₁ muscarinic receptor stimulation of Elk-1 activity in CHO-hCB₁ + M₁ cells. Representative experiments are presented. There are no significant differences between control and inhibitor-treated cells.

**Fig. 6.** The impact of the receptor stoichiometry. A, CHO-hCB₁ cells transfected with OX₁ cDNA. B, CHO-hOX₁ cells transfected with CB₁ cDNA. C, efficacy ratios of OX₁ and CB₁ receptor stimulation. The ratios were calculated in each batch of cells before averaging. The data in A and B are normalized to the basal. Orexin-A and 2-AG were applied at 1 µM and
HU-210 at 10 nM concentration. D, relative cell surface OX₁ receptor expression levels (high-affinity agonist binding sites) in different cell systems as determined by [¹²⁵I]orexin-A binding. E, relative total CB₁ receptor expression levels in different cell systems as determined by [³H]SR141716 binding. N = 3–5. Comparisons are to the normalization controls; ns (not significant), p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001. F, correlation of the apparent [¹²⁵I]orexin-A and [³H]SR141716 binding levels in the cell systems. Please note, that these ratios are not direct measures of the relative cell surface receptor levels, as the two radioligands work in a different way.

Fig. 7. Elk-1 data from CHO-hOX₁ cell–CHO-hCB₁ cell communication assay (see Elk-1 activity luciferase assay.). Elk-1-driven luciferase activity was measured in CHO-hCB₁ cells interspersed among CHO-hOX₁ cells stimulated with orexin-A. Inhibition of the luciferase activity with THL and AM-251 indicates that the Elk-1 activity is induced by the cascade orexin-A → OX₁ receptors (on CHO-hOX₁ cells) → 2-AG (extracellular) → CB₁ receptors (on CHO-hCB₁ cells). Please also see (Turunen et al., 2012). N = 4. Comparisons are to the basal; ns (not significant), p > 0.05; ***, p < 0.001.
**Figure 2**

**(A)** Graph showing pERK1 (times max ctrl) against log[orexin-A] (M) for different concentrations of THL and SR141716.

**(B)** Graph showing pERK2 (times max ctrl) against log[orexin-A] (M) for different concentrations of THL and SR14176.

**(C)** Bar graph comparing max (times ctrl) for pERK1 and pERK2 across ctrl, THL, and SR141716.

**(D)** Bar graph showing EC50 (times ctrl) for ctrl, THL, and SR141716.