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OREXIN-1 RECEPTOR-CANNABINOID CB1 RECEPTOR HETERO-DIMERIZATION RESULTS IN BOTH LIGAND-DEPENDENT AND -INDEPENDENT CO-ORDINATED ALTERATIONS OF RECEPTOR LOCALIZATION AND FUNCTION

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Following inducible expression in HEK293 cells the human orexin-1 receptor was targeted to the cell surface but became internalized following exposure to the peptide agonist orexin A. By contrast, constitutive expression of the human cannabinoid CB1 receptor resulted in a predominantly punctate, intracellular distribution pattern consistent with spontaneous, agonist-independent internalization. Expression of the orexin-1 receptor in the presence of the CB1 receptor resulted in both receptors displaying the spontaneous internalization phenotype. Single cell fluorescence resonance energy transfer imaging indicated the two receptors were present as hetero-dimers/oligomers in intracellular vesicles. Addition of the CB1 receptor antagonist SR-141716A to cells expressing only the CB1 receptor resulted in re-localization of the receptor to the cell surface. Although SR-141716A has no significant affinity for the orexin-1 receptor, in cells co-expressing the CB1 receptor, the orexin-1 receptor was also re-localized to the cell surface by treatment with SR-141716A. Treatment of cells co-expressing the orexin-1 and CB1 receptors with the orexin-1 receptor antagonist SB-674042 also resulted in re-localization of both receptors to the cell surface. Treatment with SR-141716A resulted in decreased potency of orexin-A to activate the MAP kinases ERK1/2 only in cells co-expressing the two receptors. Treatment with SB-674042 also reduced the potency of a CB1 receptor agonist to phosphorylate ERK1/2 only when the two receptors were co-expressed. These studies introduce an entirely novel pharmacological paradigm, whereby ligands modulate the function of receptors for which they have no significant inherent affinity by acting as regulators of receptor hetero-dimers.

Abbreviations: eYFP, enhanced yellow fluorescent protein; GPCR, G protein-coupled receptor; Image-iTTM, WGA-Alexa Fluor594 plasma membrane marker; ROI, region of interest; SB-408124, (1-(6,8-difluoro-2-methyl-quinolin-4-yl)-3-(4-dimethylamino-phenyl)-urea; SB-674042, (1-(5-(2-fluoro-phenyl)-2-methyl-thiazol-4-yl)-1-[(S)-2-(5-phenyl-[1,3,4]oxadiazol-2-ylmethyl)-pyrrolidin-1-yl]-methanone; SR-141716A, N-piperidinyl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide. WIN55, 221-2, (R)-

(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate.

Until recently it was widely believed that G protein-coupled receptors (GPCRs)¹ existed and functioned as monomers and therefore that ligands with high pharmacological selectivity would be expected to target a single receptor. However, in recent years data employing a wide range of approaches have indicated that most GPCRs exist as dimers or, potentially, as high-order oligomers (1-4) although there may be exceptions (5). Apart from providing an explanation for observations such as co-operativity in the binding of agonist ligands (5-9), homo-dimerization between two copies of the same GPCR has somewhat limited implications for either novel drug design or the function of previously well characterized GPCR ligands. However, GPCRs represent the largest family of transmembrane receptors in the human genome and some 400 genes encode GPCRs that are believed to respond to endogenously produced ligands (10). Greater than 90% of these GPCRs are expressed to some extent in the central nervous system (11) and it is believed that specific, key small nuclei and, indeed, even individual neurons may express a substantial number of distinct GPCRs (11). Although largely studied in detail in heterologous expression systems, there is little doubt that certain pairs of GPCRs can form and exist as hetero-dimers (1-4). There is also an expanding literature about the existence of GPCR hetero-dimers in physiological settings (12-16) and these may exhibit quite distinct pharmacology and function from the corresponding homo-dimers. As such, GPCR hetero-dimers may offer entirely novel sets of potential therapeutic targets (17). This concept has recently been given a substantial boost by the identification of a non-peptide, agonist ligand that appears to have significant selectivity to activate a κ -opioid peptide receptor- δ -opioid peptide receptor hetero-dimer, both in heterologous expression systems and *in vivo* (16).

GPCRs that respond to D⁹-tetrahydrocannabinol, the psychoactive principle of *Cannabis*, and a group of endogenously produced endo-cannabinoids, are termed CB1 and CB2 (18). The CB1 receptor is expressed predominantly in brain whilst the CB2 receptor is expressed largely in white cells of the immune system. The CB1 receptor is one of the most highly expressed GPCRs in both primate and rodent neurons and is widely expressed in neurons of the cortex, hippocampus, amygdala, basal ganglia and cerebellum (18-19). It has been appreciated for some time that the CB1 receptor can control conditioned drug-seeking (20-21) and antagonism of this receptor blocks the dopamine-releasing and motivational effects of nicotine (20-21). As such, a CB1 receptor antagonist/inverse agonist SR-141716A, also known as rimonabant, has been considered as a potential therapy in cessation of smoking programmes (22-24). Furthermore, as cannabinoid CB1 receptor agonists stimulate appetite, SR-141716A/rimonabant has also been assessed in weight reduction and anti-obesity programmes (25-26) and was recently approved for clinical use.

The orexigenic peptides orexin A and orexin B were named for their ability to stimulate feeding responses and function via the G protein-coupled orexin-1 and orexin-2 receptors (27). It has therefore been suggested that antagonists at one or either of these receptors might also be effective in weight loss programmes (28-29). The orexin-1 receptor is also widely expressed in brain (30-31), with distribution overlapping with the cannabinoid CB1 receptor in cerebral cortex, basal ganglia, thalamus and hippocampal formations as well as the lateral hypothalamus, a region of key importance in feeding and appetite control. The orexin system is also important in regulation of sleep, wakefulness and arousal (32) and low levels of orexin peptides are observed in some 90% of patients with sporadic narcolepsy-cataplexy (33). Although syndromes associated with narcolepsy are more usually associated

with effects at the orexin-2 receptor (34), alteration in levels of, or sensitivity to, the orexin peptides may generally provide links between wakefulness and feeding responses.

Co-expression of the CB1 receptor and the orexin-1 receptor in CHO cells was recently reported to enhance the potency of orexin A to stimulate ERK MAP kinase phosphorylation, and this effect was blocked by addition of SR-141716A/rimonabant (35). Although Hilaret et al., (35) suggested that these two receptors might interact to produce this effect, this was not explored directly. In the current study we show that the human CB1 and orexin-1 receptors can indeed hetero-dimerize/oligomerize. Although the cellular trafficking and recycling characteristics of the two receptors are very different when expressed individually, when co-expressed and in the absence of receptor ligands, the phenotype of the CB1 receptor is dominant in the CB1-orexin-1 receptor hetero-dimer in that both receptors recycle spontaneously. Because of this, in cells containing the CB1 receptor-orexin-1 receptor hetero-dimer, SR-141716A/rimonabant alters the cellular distribution not only of the CB1 receptor but also of the orexin-1 receptor although this ligand has no significant inherent affinity for the orexin-1 receptor. Moreover, in cells co-expressing the two receptors the orexin-1 antagonist SB-674042 (36) also alters the cellular distribution of the CB1 receptor, although this ligand has no inherent affinity for the CB1 receptor. Due to either their effects on receptor distribution or via direct allosteric modulation of the receptor hetero-dimer both SR-141716A and SB-674042 alter the effectiveness of agonists at the partner receptor. These studies introduce an entirely novel pharmacological paradigm, which may be significantly more widely applicable. We also speculate that this may contribute to the clinical effects of SR-141716A/rimonabant.

Experimental Procedures

Materials [³H]SR-141716A (50 Ci/mmol) was from Amersham Biosciences. [³H]SB-674042 (36) was the kind gift of Dr. C. Langmead, GlaxoSmithKline, Harlow, Essex, U.K. All tissue culture materials were from Invitrogen (Paisley, Lanarkshire, U.K.). Oligonucleotides were purchased from ThermoElectron, Ulm, Germany. Orexin A was produced by GlaxoSmithKline. WIN55, 212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) was from Tocris Bioscience (Bristol, U.K.). The VSV-G antiserum was generated by Sigma Genosys. IMAGEiT™ was supplied by (Invitrogen, Paisley, U.K.). Antibodies recognizing ERK1/2 MAP kinases and their phosphorylated forms were from Cell Signaling (Hitchin, Herts., UK). Flp-In T-REx HEK293 cells were from Invitrogen. All other materials were supplied by Sigma (Gillingham, Dorset, UK).

Molecular constructs- VSV-G-h-orexin-1-eYFP The VSV-G-human (h)-orexin-1-eYFP construct was created by splicing parts of two existing forms of the receptor (37). VSV-G-h-orexin-1 receptor in pcDNA3 was digested with *HindIII* and *AflIII* isolating the N-terminal portion of VSV-G-h-orexin-1 receptor. h-orexin-1 receptor-eYFP (also in pcDNA3) was digested with *HindIII* and *AflIII* removing the N-terminal section of h-orexin-1 receptor. The isolated VSV-G-h-orexin-1 receptor N-terminal fragment was then subcloned into the *HindIII* and *AflIII* sites of the digested h-orexin-1 receptor-eYFP construct. The resulting construct was digested with *HindIII* and *XhoI* to isolate VSV-G-h-orexin-1-eYFP that was then subcloned into pcDNA5/FRT/TO.

CB1-receptor-CFP - The h-CB1 receptor in pcDNA3 was used as PCR template with the following primers.

Sense primer:

5'CCCAAGCTTATGAAGTCGATCCTAGATGGCCTT3'

Anti-sense primer:

5'CGGGGTACCCAGAGCCTCGGCAGACGT3'

A *HindIII* site present in the sense primer and a *KpnI* site present in the anti-sense primer are underlined and the amplified fragment was digested and ligated into pcDNA3 upstream and in frame with CFP ligated between *KpnI* and *NotI*.

Generation of stable Flp-In T-REx HEK293 cells - Cells were maintained in Dulbeccos' modification of Eagles' medium without sodium pyruvate, 4500 mg/L glucose and L-glutamine, supplemented with 10% (v/v) foetal calf serum, 1% antibiotic mixture and 10 mg/ml blastacidin at 37°C in a humidified atmosphere of air/CO₂ (19:1). To generate Flp-In T-REx HEK293 cells (35-36) able to inducibly express VSV-G-h-orexin-1-eYFP, cells were transfected with a mixture containing the VSV-G-h-orexin-1-eYFP cDNA in pcDNA5/FRT/TO vector and the pOG44 vector (1:9) using lipofectAMINE (Invitrogen), according to the manufacturers' instructions. After 48 h, the medium was changed to medium supplemented with 200 mg/ml hygromycin B to initiate selection of stably transfected cells. To constitutively co-express a variant of the h-CB1 receptor in the inducible cell lines, the inducible VSV-G-h-orexin-1-eYFP expressing cell line was transfected with the appropriate cDNA construct as described above and resistant cells selected in the presence of 1 mg/ml G418. Resistant clones were screened for receptor expression by monitoring specific binding of [³H]SR-141716A in cell homogenates for the untagged h-CB1 receptor and by fluorescent microscopy for the h-CB1-CFP receptor. Cells were treated with 1 mg/ml doxycycline 24-96 h prior to assays to induce expression of the VSV-G-h-orexin-1-eYFP cloned into the Flp-In locus.

Cell surface expression of VSV-G-h-orexin-1-eYFP Monolayers of cells in 96-well plates were induced with 1 mg/ml doxycycline for varying times. Afterwards cell surface receptors were labelled with anti-VSV-G antibody (1:1000) in growth medium for 30 min at 37°C. The cells were washed once with 20 mM Hepes/Dulbeccos' modified Eagles' medium and then incubated for another 30 min at 37°C in growth medium supplemented with horseradish-peroxidase-conjugated anti-rabbit IgG as secondary antibody and 1 mM Hoechst nuclear stain (Sigma) to determine cell number. The cells were then washed twice with phosphate buffered saline and incubated with SureBlue (Insight Biotechnology) for 5 min in the dark at room temperature and the absorbance read at 620 nm in a Victor² plate reader (Packard Bioscience).

Immunoblot detection of VSV-G-h-orexin-1-eYFP Cells were grown in 12-well plates and receptor expression induced with 1 mg/ml doxycycline for varying times. Cells were then place on ice, washed twice with cold phosphate buffered saline and lysed in RIPA buffer. After 1 h at 4°C, the lysates were centrifuged for 10 min at 20,800 x g at 4°C to remove insoluble material. The samples were mixed with 5 x reducing loading buffer and heated for 3 min at 95°C. Cell lysates were resolved using SDS-PAGE gel electrophoresis. VSV-G-h-orexin-1-eYFP expression was detected by protein immunoblotting using anti-VSV-G antibody and horseradish-peroxidase-conjugated anti-rabbit IgG as secondary antibody for immunodetection.

ERK1/2 phosphorylation and immunoblots

Cells were grown in 12-well plates and serum starved overnight prior to treatment with ligands as indicated. Cell lysates were prepared as above. ERK1/2 phosphorylation was detected by protein immunoblotting using phospho-specific antibodies and horseradish-peroxidase-conjugated anti-rabbit IgG as secondary for immunodetection. After visualizing ERK1/2 phosphorylation, the PVDF membranes were stripped and re-probed using a total anti-ERK1/2 antibody.

Pharmacological redistribution assays using receptor inverse agonists/antagonists Cells were grown in 12-well plates and receptor expression induced with 1 mg/ml doxycycline for 72 h. Following this cells were incubated for 15 min at 4°C in Earle's buffer (140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgCl₂-6H₂O, 25 mM HEPES, pH 7.6) supplemented with 0.2 % bovine serum albumin and 0.01 % glucose. Cells were then incubated in supplemented Earle's buffer containing either 1 ¼M SR-141716A or 5 ¼M SB674042 for 30 min at 4°C, before being incubated at 37°C for 3 h prior to either imaging by epifluorescence microscopy or used in the ERK1/2 phosphorylation assays.

Cell membrane preparation Pellets of cells were resuspended in 10mM Tris, 0.1mM EDTA, pH 7.4 (TE buffer) and the cells homogenized using 40 strokes of a glass on Teflon homogenizer. Samples were centrifuged at 1000 x g for 10 min at 4°C to remove unbroken cells and nuclei. The supernatant fraction was removed and passed through a 25 gauge needle 10 times before being transferred to ultra-centrifuge tubes and subjected to centrifugation at 50,000 x g for 30 min. The supernatant was discarded and the pellet resuspended in TE buffer. Protein concentration was assessed, membranes diluted to 1mg/ml and stored at -80°C until required.

Radioligand binding - [³H]SR-141716A binding Reaction mixtures were established in a volume of 1000 ml containing 20-60 mg of membrane protein in binding buffer (50 mM Tris, 1 mM EDTA, 3 mM MgCl₂ and 0.3% BSA, pH 7.4) containing a range of concentrations (0.25 - 8 nM) of [³H]SR-141716A. Potential competing ligands (orexin A, 0.5 mM or SB-674042 5 mM) were diluted in binding buffer. Non-specific binding was determined using cold SR-141716A (2 mM). Samples were incubated for 90 min at 37°C prior to filtration through Whatman GF/C filters. Data was analysed using Graphpad Prism, and B_{MAX} and K_d values determined via saturation binding analysis.

[³H]SB-674042 binding Reaction mixtures were established in a volume of 500ml containing 5mg of membrane protein in binding buffer (25 mM HEPES, 0.5 mM EDTA, 2.5 mM MgCl₂, 0.5% BSA and 0.025% bacitracin, pH 7.4) supplemented with 5 nM [³H]SB-674042 (36). Non-specific binding was determined using SB-408124 (1-6,8-difluoro-2-methyl-quinolin-4-yl)-3-(4-dimethylamino-phenyl)-urea (3¼M). Samples were incubated for 60 min at 25°C prior to filtration through Whatman GF/C filters

Confocal laser scanning microscopy

Cells were imaged using a Zeiss LSM 5 Pascal laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY) equipped with a 63 x oil-immersion Plan Fluor Aplanachromat objective lens with a numerical aperture of 1.4. A pinhole of 20 and an electronic zoom of 1 or 2.5 was used. The excitation laser line for eYFP was the 514nm argon laser with detection via a 475-525nm band pass filter. The excitation laser line for CFP was the 458nm argon

laser with detection via a 505-530nm band pass filter. Cells grown on coverslips were washed 3 times with ice-cold PBS. For internalization studies cells were pre-treated with specific antagonist or control for 30 min at 37°C, the agonist was then added to these cells for further 30 min at 37°C. Cells were fixed for 10 min at room temperature using 4% paraformaldehyde in PBS/5% sucrose. The cells were washed a further 3 times in ice-cold PBS prior to being mounted onto microscope slides with 40% glycerol in PBS. The images were analyzed with Metamorph software, (version 6.3.3; Molecular Devices Corp., Downing, PA).

Epifluorescence microscopy - Dual imaging -h-CB1-CFP and VSV-G-h-orexin-1-eYFP receptor fusions

Paraformaldehyde fixed cells, expressing the appropriate receptor fusion protein were imaged in 2D or 3D using an inverted Nikon TE2000-E microscope (Nikon Instruments, Melville, NY) equipped with a x60, (NA=1.4), oil-immersion Plan Fluor Apochromat lens, a z-axis linear encoder and a cooled digital Cool Snap-HQ CCD camera (Roper Scientific/Photometrics, Tucson, AZ).

Epifluorescence excitation light was generated by an ultra high point intensity 75 W xenon arc Optosource lamp (Cairn Research, Faversham, Kent, UK) coupled to a computer controlled Optoscan monochromator (Cairn Research, Faversham, Kent, UK). Monochromator was set to 436/12 nm and 500/5 nm for the sequential excitation of CFP and eYFP respectively. CFP and eYFP excitation light was transmitted through the objective lens using the following single pass dichroics: 455DCLP for CFP and Q515LP for eYFP. CFP and eYFP fluorescence emission was controlled via a high speed filterwheel device (Prior Instruments) containing the following emitters: HQ480/40 nm for CFP; HQ535/30 nm for eYFP. Using these filter sets, the fluorophores were easily separated with no bleed through.

Images were collected using a Cool Snap-HQ digital camera operated in 12-bit mode. Computer control of all electronic hardware and camera acquisition was achieved using Metamorph software (version 6.3.3; Molecular Devices Corp., Downing, PA).

For 3D imaging, stacks of images, (2 x 2 binning, 200-300 msec exposure/image), with a 0.26- μ m Z step (~20-25 frames/stack) were sequentially acquired for each GFP variant.

VSV-G-h-orexin-1-eYFP receptor fusion and wheatgerm agglutinin-Alexa Fluor 594 fluorescent-labelled plasma membrane marker To fluorescently visualize the plasma membrane in live cells expressing the VSV-G-h-orexin-1-eYFP receptor construct cells were treated, (as specified by the manufacturer), with the reagents in the Image-iT plasma membrane and nuclear labeling kit (Invitrogen), in which the plasma membrane is specifically labeled with wheatgerm agglutinin (WGA)-Alexa Fluor 594 and nuclei are stained simultaneously with Hoechst 33342. eYFP was excited as previously described and Alexa Fluor 594 was excited at 575/12nm and imaged using the following filter set, (dichroic: Q595LP; emitter:HQ645/75 nm). Using these filters, no bleed through was observed. Sequential 12 bit images were collected as previously outlined above.

FRET imaging Cells were grown on poly-D-lysine treated coverslips and transiently transfected with appropriate CFP/eYFP constructs. Coverslips were placed into a microscope chamber containing physiological saline solution (130 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 10mM D-glucose, pH 7.4). Using an Optoscan monochromator, the CFP and eYFP fluorophores were excited sequentially at 500/5 and

430/12 nm respectively. Images were obtained individually for CFP, eYFP, and CFP-eYFP FRET filter channels using the appropriate excitation wavelength, a dual dichroic mirror, (86002v2bs; Chroma Inc., Rockingham, VT) and a high speed filterwheel device (Prior Instruments) containing the following band pass emitters: HQ470/30 nm for CFP; HQ535/30nm for eYFP. The illumination time was 250 ms and binning was set to 2x2. Metamorph imaging software, (version 6.3.3; Molecular Devices Corp., Downing, PA), was used to quantify the FRET images using the sensitized FRET method. Corrected FRET (FRET_c) was calculated using a pixel-by-pixel methodology using the equation $FRET_c = FRET - (\text{coefficient } B \times CFP) - (\text{coefficient } A \times eYFP)$, where CFP, eYFP and FRET values correspond to background corrected images obtained through the CFP, eYFP and FRET channels. B and A correspond to the values obtained for the CFP (donor) and eYFP (acceptor) bleed-through co-efficients respectively, calculated using cells singly transfected with either the CFP or eYFP protein alone. To correct the FRET levels for the varying amounts of donor (CFP) and acceptor (YFP), normalized FRET was calculated using the equation $FRET_n = FRET_c / CFP$, where FRET_c and CFP are equal to the fluorescence values obtained from single cells.

Image analysis - Co-localization - h-CB1-CFP and VSV-G-h-orexin-1-eYFP receptor constructs For the analysis of h-CB1-CFP and VSV-G-h-orexin-1-eYFP receptor constructs, a region of no fluorescence adjacent to the cell was used to determine the background level of fluorescence in the CFP and eYFP channels. The background amount was then subtracted from each pixel in each channel. Correlation coefficients were measured by comparing the amounts of fluorescence measured in each matched pixel of the two different channels using the Metamorph "correlation plot" application. The degree of co-localization was quantified by plotting the amount of background-subtracted yellow eYFP fluorescence from the pixels against the amount of back-ground cyan fluorescence in the corresponding pixels of the eYFP image. Correlation coefficients were quantified that described the degree by which CFP and eYFP fluorescence at each pixel within the region varied from a perfect correlation of 1.00.

VSV-G-h-orexin-1-eYFP receptor and plasma membrane Correlation coefficients were quantified by manually drawing a rectangular region of interest (ROI) on the WGA-Alexa Fluor 594 labelled plasma membrane. The ROI was then selected and transferred to the matched image acquired in the eYFP channel. Using the Metamorph "correlation plot" module, correlation coefficients were quantified that described the degree by which WGA-Alexa Fluor 594 and eYFP fluorescence at each pixel within the rectangular region varied from a perfect correlation of 1.00.

Quantification of surface versus intracellular receptors Fluorescent microscopy was used for measurement of surface expressed and intracellular receptors. To quantitatively measure the total h-CB1-CFP and VSV-G-h-orexin-1-eYFP receptor fluorescence intensity, images were deconvoluted using an iterative and constrained algorithm (Autodeblur software, version 9.3.6, Autoquant Imaging, Watervliet, NY) to produce high resolution images that were used to identify groups of adjacent pixels or "segments" that corresponded to receptors located on the cell surface and intracellular vesicles. For images with good contrast and low noise, intensity thresholding is the standard method of segmentation, however, it is extremely difficult to obtain well segmented objects especially 2D objects that fork and merge. The object counting module of Autoquant imaging software offers a powerful manual segmentation paint brush tool that allows users to segment complex objects and easily distinguish between signals in the plasma membrane and in the cytoplasm, by defining a region of the raw image to be segmented using this tool. The paintbrush has varying

diameters of 1, 3 or 5 pixels which the user can click to designate the region directly underneath the mouse pointer to be part of the threshold region or can click and drag to “paint” the region. As the mouse is dragged the region underneath it becomes part of the segmented region. This region is then displayed as a yellow mask over the grayscale data. Once manual thresholding is completed a new image is created in which all pixels of the raw image that are not part of the segmentation mask are set to zero, or effectively removed.

The manual segmentation method was used to quantify the mean total fluorescence intensity values corresponding to CFP or eYFP tagged receptors located at the membrane surface and cytoplasm of the cell was quantified using this method of manual segmentation. The total fluorescence pixel intensity measured from membrane and intracellular receptor segmented pixels was expressed as a percentage of the total fluorescent CFP or eYFP intensity. These values were exported into PRISM 4.03, (GraphPAD Software, San Diego, CA) and all data were expressed as the mean \pm S.E.M. The number of cells analysed from each experimental group was 6 and the statistical significance of any difference between mean values was determined using a Student's *t* test.

RESULTS

The human (h)-orexin-1 receptor (37) was modified at the N-terminus to introduce the VSV-G epitope tag and at the C-terminus by in-frame addition of enhanced yellow fluorescent protein (eYFP) to generate VSV-G-h-orexin-1-eYFP. A cDNA able to encode this modified construct was cloned into the Flp-In locus of HEK293 Flp-In T-REx cells to allow inducible expression under the control of the tetracycline/doxycycline-on promoter (38-39). Expression of VSV-G-h-orexin-1-eYFP in various clones of these cells was entirely dependent upon addition of doxycycline (**Figure 1** and data not shown). As monitored by confocal microscopy, following treatment with doxycycline (1 mg/ml, 72 h) eYFP fluorescence appeared to be located predominantly at the cell surface (**Figure 1a**). This was confirmed by co-staining of the cells with the WGA-Alexa Fluor594 plasma membrane marker, Image-iTTM. As shown in **Figure 1b** pixel by pixel analysis of a defined region of interest (ROI) resulted in a very high overlap correlation co-efficient ($r^2 = 0.92$). Quantitative pixel by pixel analysis of the distribution of cell surface versus intracellular eYFP fluorescence demonstrated 88.6 \pm 3.6 % of the receptor to be at the cell surface 72 h after induction of VSV-G-h-orexin-1-eYFP expression (**Table 1**), a proportion that was unaffected by maintenance of the presence of doxycycline for different times (data not shown). As the N-terminus of GPCRs is anticipated to be extracellular, intact cell anti-VSV-G ELISA assays (**Figure 1c**) confirmed delivery of the construct to the cell surface and appropriate integration of the construct into and across the plasma membrane. Time-courses of doxycycline treatment indicated that expression of VSV-G-h-orexin-1-eYFP could be detected immunologically within 12-18 h, increased up to 48 h and was maintained for at least 96 h (**Figure 1d**).

The expressed VSV-G-h-orexin-1-eYFP construct was functional but displayed no obvious constitutive activity because both induction of expression and addition of the peptide agonist orexin A were required to cause phosphorylation of the MAP kinases ERK1 and ERK2 (**Figures 2a, b**). As anticipated from previous work (37), the maintained presence of the peptide agonist orexin A (0.5 mM, 30 min) resulted in substantial internalization of VSV-G-h-orexin-1-eYFP (**Figure 2c**), a process also shown previously (37) to be b-arrestin and clathrin-dependent. This was blocked by prior addition of the orexin-1 receptor antagonist

SB-674042 (33) (**Figure 2d**) that had no detectable direct effect on the distribution of VSV-G-h-orexin-1-eYFP (**Figure 2d**).

The cells described above were further transfected with a cDNA encoding the human cannabinoid CB1 (h-CB1) receptor to cause stable, constitutive expression of this polypeptide and individual clones isolated. When VSV-G-h-orexin-1-eYFP expression was induced in these cells by addition of doxycycline the distribution of VSV-G-h-orexin-1-eYFP was now markedly different than in the absence of h-CB1 receptor expression (**Figure 3a**). Substantially less (61.3 +/- 2.9 %) of the eYFP was present at the cell surface 24 h after induction of VSV-G-h-orexin-1-eYFP and this was further reduced (to 47.2 +/- 2.6 %) 72 h after induction of expression (**Table 1**). Now the majority of the signal was present in punctate, intracellular vesicles with a pattern resembling that generated following addition of orexin A to cells expressing only the VSV-G-h-orexin-1-eYFP construct (compare **Figure 2c**). Ligand binding studies employing [³H]SR-141716A/rimonabant (**Figure 3b**) confirmed a lack of specific cannabinoid CB1 receptor binding sites in both parental HEK293 Flp-In T-REx cells and in those inducibly expressing only VSV-G-h-orexin-1-eYFP but demonstrated 2.8 +/- 0.11 pmol binding sites/mg membrane protein in clone 16 of the cells constitutively expressing h-CB1 receptor without induction of VSV-G-h-orexin-1-eYFP (**Table 2**). This level of expression was unaffected by induction of VSV-G-h-orexin-1-eYFP expression (3.0 +/- 0.06 pmol/mg membrane protein) as was the K_d for [³H]SR-141716A (**Table 2**).

Because the h-CB1 receptor in these cells was unmodified and therefore could not be observed directly we generated further cell lines in which the h-CB1 receptor with cyan fluorescent protein fused in frame to the receptor C-terminal tail (h-CB1-CFP) was expressed constitutively and where VSV-G-h-orexin-1-eYFP expression could be induced. In these cells, in the absence of VSV-G-h-orexin-1-eYFP expression, the pattern of h-CB1-CFP distribution was largely intracellular, 91.2 +/- 2.0%, (**Table 3**) and concentrated in punctate vesicles (**Figure 4a (ii)**). [³H]SR-141716A binding studies on cell membrane preparations indicated expression of 1.62 +/- 0.08 pmol/mg membrane protein (**Table 2**). Induction of expression of VSV-G-h-orexin-1-eYFP in these cells did not alter h-CB1-CFP expression levels, affinity to bind [³H]SR-141716A (**Table 2**), or the pattern of h-CB1-CFP distribution (**Figure 4a (v)**, **Table 3**). However, unlike in the absence of h-CB1 receptor, much (66.1 +/- 3.1 %) of the expressed VSV-G-h-orexin-1-eYFP now displayed a punctate, intracellular pattern of distribution (**Figure 4a (iv)**, **Table 3**) and merging of the signals corresponding to VSV-G-h-orexin-1-eYFP and h-CB1-CFP indicated marked overlap of distribution and potential co-localization (**Figure 4a (vi)**). Detailed pixel by pixel analysis of the patterns of distribution of the two receptors in individual cells demonstrated a very high intracellular overlap correlation coefficient ($r^2 = 0.87$) (**Figure 4b**). As the effect of h-CB1-CFP expression on the distribution of VSV-G-h-orexin-1-eYFP was entirely ligand-independent this was suggestive of direct protein-protein interaction and hence hetero-dimerization/oligomerization between the two GPCRs. To test this directly we performed single cell fluorescence resonance energy transfer (FRET) imaging studies (40-41) on cells expressing either h-CB1-CFP or VSV-G-h-orexin-1-eYFP alone or co-expressing these two constructs. Substantial FRET signals were obtained from intracellular sites of these cells only following co-expression of both receptor constructs (**Figure 4c**), consistent with direct h-CB1 receptor-h-orexin-1 receptor interactions in such vesicles.

The effect of the h-CB1 receptor on the cellular distribution of the orexin-1 receptor was not a general, non-specific effect. We generated further HEK293 Flp-In T-REx cell lines in which a h-m-opioid receptor C-terminally tagged with eYFP was expressed stably and

constitutively and in which h-CB1-CFP expression could be induced by addition of doxycycline. In these cells, h-m-opioid receptor-eYFP was located largely at the cell surface both in the absence and presence of h-CB1-CFP expression whilst, as anticipated from the foregoing, following induction of expression, h-CB1-CFP was present predominantly in punctate intracellular vesicles (**Figure 5**).

[³H]SR-141716A binding was specific for the h-CB1 receptor. Neither the peptide orexin A nor the selective non-peptide orexin-1 receptor antagonist SB-674042 displayed any significant ability to compete for h-CB1 receptor binding (**Figure 6a**). Equally, SR-141716A/rimonabant had no significant affinity for the orexin-1 receptor. Pre-treatment of cells expressing only VSV-G-h-orexin-1-eYFP with SR-141716A/rimonabant was unable to prevent orexin A-mediated internalization of VSV-G-h-orexin-1-eYFP (**Figure 6b**) and SR-141716A/rimonabant had no direct effect on the cellular distribution of VSV-G-h-orexin-1-eYFP (**Figure 6b**). Furthermore, SR-141716A/rimonabant was unable to compete with [³H]SB-674042 in ligand binding studies in membranes of cells induced to express VSV-G-h-orexin-1-eYFP (**Figure 6c**).

In cells expressing only h-CB1-CFP sustained treatment with SR-141716A/rimonabant (1 mM, 3 h) resulted in a substantial re-distribution of the h-CB1-CFP receptor to the cell surface (**Figure 7, Table 3**). In contrast, and as expected, treatment of cells expressing only h-CB1-CFP with SB-674042 (5 mM, 3 h) was without effect on the distribution of h-CB1-CFP (**Figure 7, Table 3**). In cells induced to express VSV-G-h-orexin-1-eYFP in the presence of constitutive expression of h-CB1-CFP, SR-141716A/rimonabant treatment re-distributed substantial fractions of both h-CB1-CFP and VSV-G-h-orexin-1-eYFP to the cell surface (**Figure 8a, Table 3**). As well as being able to block orexin-A mediated internalization of VSV-G-h-orexin-1-eYFP, sustained treatment of cells co-expressing VSV-G-h-orexin-1-eYFP and h-CB1-CFP with SB-674042 (5 mM, 3 h) resulted in re-distribution of VSV-G-h-orexin-1-eYFP from punctate intracellular vesicles back to the cell surface and also resulted in significant cell surface accumulation of h-CB1-CFP (**Figure 8a, Table 3**). Furthermore, FRET imaging studies on cells co-expressing h-CB1-CFP and VSV-G-h-orexin-1-eYFP and treated with SR-141716A/rimonabant (1mM, 3h) allowed detection of protein-protein interactions involving these two GPCRs at the surface of individual cells (**Figure 8b**) whereas no significant FRET signals were obtained in cells treated with SR-141716A/rimonabant when either receptor construct was expressed individually (**Figure 8b**).

Orexin A stimulated phosphorylation of the ERK1 and ERK2 MAP kinases in a concentration-dependent manner in all the cell lines in which VSV-G-h-orexin-1-eYFP expression was induced (**Figure 9**) but, as noted earlier (**Figure 2a**), this was not observed without induction of h-orexin-1 receptor expression. As anticipated, based on the lack of affinity of SR-141716A/rimonabant for the orexin-1 receptor (**Figure 6**), treatment of cells expressing only VSV-G-h-orexin-1-eYFP with SR141716A/rimonabant (1 mM, 3 h) did not alter the potency of orexin A to cause phosphorylation of the ERK MAP kinases (**Table 4**). However, in cells induced to co-express VSV-G-h-orexin-1-eYFP and h-CB1-CFP, SR-141716A/rimonabant pre-treatment substantially reduced the potency of orexin A (**Figure 9, Table 4**), consistent with binding of SR-141716A/rimonabant to the VSV-G-h-orexin-1-eYFP- h-CB1-CFP hetero-dimer altering signalling sensitivity via the partner polypeptide. Without SR-141716A/rimonabant pre-treatment, co-expression of VSV-G-h-orexin-1-eYFP and h-CB1-CFP resulted in a small, but statistically significant, increase in the potency of orexin A to stimulate ERK1 and ERK2 MAP kinase phosphorylation (**Table 4**). Pre-

treatment with SB-674042 (5 mM, 3 h), which has no significant affinity at the h-CB1 receptor (**Figure 6**), was without effect on the potency of the CB1 receptor agonist WIN55, 212-2 to stimulate phosphorylation of the ERK MAP kinases in cells expressing only h-CB1-CFP (**Table 5**). However, in cells induced to express VSV-G-h-orexin-1-eYFP in the presence of h-CB1-CFP pre-treatment with SB-674042 resulted in a 2 fold reduction in potency of WIN55, 212-2 to stimulate ERK1 and ERK2 MAP kinase phosphorylation (**Table 5**).

Levels of VSV-G-h-orexin-1-eYFP expression in HEK293 Flp-In T-REx cells constitutively expressing h-CB1-CFP could be modulated by altering the concentration of doxycycline used as inducer. This allowed us to explore the effect of differing levels of VSV-G-h-orexin-1-eYFP on ERK MAP kinase phosphorylation and effects of SR-141716A/rimonabant on this (**Table 6**). Induction of VSV-G-h-orexin-1-eYFP by treatment with low concentrations of doxycycline (0.025 or 0.1 ng/ml) reduced expression levels of VSV-G-h-orexin-1-eYFP to respectively 1 and 2.5% of that produced by treatment with 1 mg/ml doxycycline. Interestingly, following induction of lower levels of VSV-G-h-orexin-1-eYFP by treatment of the cells with 0.1 ng/ml doxycycline, the fraction of VSV-G-h-orexin-1-eYFP at the cell surface at steady-state was markedly lower (8.1 +/- 1.1 %) than when higher levels of the receptor were induced by treatment with 1 mg/ml doxycycline (33.9 +/- 3.1 %), whilst the distribution of h-CB1-CFP between cell surface and intracellular vesicles was unaltered. These data suggest that with low level expression, most of the VSV-G-h-orexin-1-eYFP was within a recycling hetero-dimeric/oligomeric complex with h-CB1-CFP. In cells lacking h-CB1-CFP expression, lower levels of VSV-G-h-orexin-1-eYFP reduced the potency of orexin A to stimulate ERK MAP kinase phosphorylation (**Table 6**) as expected from basic pharmacological principles. However, as observed with higher level VSV-G-h-orexin-1-eYFP expression, even with these lower levels of orexin-1 receptor expression, in the presence of h-CB1-CFP the potency of orexin A was always greater than in the absence of the CB1 receptor (**Table 6**) and, in all cases, this was reduced by pre-treatment with SR-141716A/rimonabant (**Table 6**).

DISCUSSION

Recent years have seen great advances in understanding the significance of GPCR quaternary structure. Although considered to exist and function as monomers for many years, it is now well established that most (1-4), but perhaps not all (5), GPCRs exist as dimers and/or higher-order oligomers and that such quaternary structure is required for plasma membrane delivery and function (42). As well as dimerization/oligomerization between monomers of the same GPCR, there has been growing evidence that at least certain pairs of co-expressed GPCRs have the potential to hetero-dimerize/oligomerize. Such interactions have been reported to modulate signal transduction cascades activated by the GPCRs (43), their pharmacology (44) and cellular trafficking (38, 45-46). Beyond the well established requirement for hetero-dimerization between the GABA_{BR1} and GABA_{BR2} gene products to generate the functional GABA_B receptor (47) and for hetero-dimerization between the T1R3 and either T1R1 or T1R2 receptors to generate respectively the umami and sweet taste receptors (48) there is growing interest in the concept that hetero-dimerization between co-expressed family A GPCRs is relevant to physiology and that such hetero-dimers might offer an entirely new portfolio of therapeutic targets to modulate disease (17). For this to be the case it is vital either that synthetic ligands can be identified that interact selectively with GPCR hetero-dimer pairs or that it is demonstrated that ligands with well established pharmacology can be shown to regulate the activity or function of a GPCR within a hetero-dimer for which it has

no inherent binding affinity. An example of a ligand with selective agonist action at a GPCR hetero-dimer appears to be 6'-guanidinonaltrindole as it is reported to display substantially higher potency and efficacy to generate signals in cells co-expressing the κ -opioid peptide and δ -opioid peptide receptors than when either of these GPCRs is expressed alone (16). We now show that antagonists at the cannabinoid CB1 and the orexin-1 receptors regulate the cellular distribution of both receptors and functional potency of agonists of the other receptor when the two GPCRs are co-expressed, and that this is despite the fact these antagonists have no inherent affinity to bind the partner receptor when each is expressed in isolation.

Particularly for the metabotropic glutamate-like, class C receptors, where chimeric receptors consisting of the extracellular ligand binding domain of one receptor and the seven transmembrane bundle and intracellular elements of a second can easily be produced, it is now clear that signal transduction subsequent to ligand binding can proceed via trans-activation via the second transmembrane element of the dimer as well as via cis-activation (49). Similar data is beginning to emerge for dimers of the rhodopsin-like, class A GPCRs and this is consistent with observations of co-operativity in effects of ligand binding (7, 9, 44). This is particularly relevant as interactions between pairs of GPCRs are expected to result in allosteric interactions between them, as recently observed following co-expression of the CCR2 and CCR5 chemokine receptors (50).

Previous studies indicated that co-expression of the cannabinoid CB1 receptor and the orexin-1 receptor in CHO cells increases the potency of the orexigenic peptide orexin A to cause stimulation of the ERK1/2 MAP kinases and that this effect is blocked by short term treatment with SR-141716A or by treatment of the cells with pertussis toxin to prevent CB1 receptor signalling (35). Both these observations are consistent with the concept that constitutive signalling from the CB1 receptor was responsible for this phenotype because SR-141716A has been described as an 'inverse agonist' at the CB1 receptor (35, 51). The current results are significantly different. We demonstrate that simple co-expression of these two receptors has a relatively small effect on the potency of orexin A to cause phosphorylation of the ERK1/2 MAP kinases. We do not, however, have an obvious, simple explanation of the differences in our data from those of Hilairet et al., (35). Certainly the experiments have been performed in different cell backgrounds and, perhaps more importantly, we observed that co-expression of the human forms of these two receptors markedly alters the cellular distribution of the orexin-1 receptor. This is because, as noted previously by others in both HEK293 cells (52) and isolated neurons (53), the human CB1 receptor recycles constitutively and, whilst when expressed on its own, the orexin-1 receptor is maintained predominantly at the cell surface until stimulated by the agonist orexin A, in the presence of the CB1 receptor the orexin-1 receptor adopts the phenotype of the CB1 receptor because the two receptors form a hetero-dimer/oligomer in which the recycling phenotype of the CB1 receptor is dominant. Hilairet et al., (35) reported only cell surface localization of the CB1 receptor and no alteration of the distribution of the orexin-1 receptor in the presence of the CB1 receptor. Importantly, however, co-expression with the h-CB1 receptor does not result in an altered distribution phenotype for all GPCRs that might have been suggestive of a non-specific effect. When we generated further cell lines to allow expression of the h-m-opioid receptor, this was at the cell surface in the absence of h-CB1-CFP expression and remained at the cell surface with induction of h-CB1-CFP expression.

Sustained treatment of cells expressing only the CB1 receptor with SR-141716A/rimonabant trapped a substantial proportion of the CB1 receptor at the cell surface and, when the orexin-1 receptor was also expressed, SR-141716A/rimonabant treatment also

moved this receptor to the cell surface. This is despite SR-141716A/rimonabant being unable to block orexin A-mediated internalization of the orexin-1 receptor and being unable to compete with [³H]SB-674042 to bind to the orexin-1 receptor. Likewise, treatment of cells expressing only the CB1 receptor with SB-674042 had no effect on cellular distribution of this receptor although this ligand caused re-distribution of the CB1 receptor to the cell surface when it was co-expressed with the orexin-1 receptor at which SB-674042 is an antagonist. In concert with single cell FRET imaging of interactions between CB1-CFP and orexin-1 receptor-eYFP, which we were able to observe in intracellular vesicles in the absence of SR-141716A/rimonabant and at the cell surface following treatment with this ligand, these pharmacological studies provide unambiguous identification of CB1 receptor-orexin-1 receptor hetero-dimerization/oligomerization.

These results may have important, more general implications than are currently appreciated for understanding the pharmacology and function of ligands. In ligand identification programmes in the pharmaceutical industry, interaction and/or functional screens are generally performed using cells or membranes of cells transfected to express a single molecular target of interest. Even in subsequent counter-screens to assess ligand selectivity for the target, potential interactions with related targets are usually assessed on a 'one target at a time' basis. Using such approaches SR-141716A/rimonabant is clearly defined simply as a CB1 receptor selective antagonist/inverse agonist and SB-674042 as a selective orexin-1 receptor blocker. Indeed, as shown herein SR-141716A/rimonabant has no inherent affinity to bind directly to the orexin-1 receptor or SB-674042 to bind the CB1 receptor. The distribution of these two GPCRs overlaps in the brain (18-19, 30-31) and although we show here that co-expressed orexin-1 and CB1 receptors form a hetero-dimeric/oligomeric complex there remains no direct evidence that these two receptors do interact *in vivo*. Efforts to assess this are beyond the scope of the current studies but clearly will be an important next step. Because binding of SR-141716A/rimonabant or SB-674042 to such a hetero-dimer complex alters the cellular distribution of both receptors and the potency of agonists at each receptor to activate signalling, these observations may have implications for the *in vivo* action of 'CB1 specific' antagonists including SR-141716A/rimonabant. Although SR-141716A/rimonabant is known to limit conditioned drug-seeking, and hence may well be useful as an anti-craving therapeutic, it is also known to produce weight loss via reduced food intake. Although both of these effects are consistent with reversing known effects of CB1 receptor stimulation, activation of the orexin-1 receptor is also a pro-feeding stimulus (54) and loss of weight and anorexia is associated with antagonists of this receptor (55). Because we demonstrate that treatment with SR-141716A/rimonabant, only of cells co-expressing the CB1 and the orexin-1 receptor, results in decreased potency of orexigenic signalling through the orexin-1 receptor it is clearly an interesting concept that at least some of the clinical capacity of SR-141716A/rimonabant to decrease feeding and hence cause weight loss may reflect its interaction with CB1/orexin-1 receptor hetero-dimers. Interestingly, orexin-containing neurones are also associated with reward seeking and activation of this system re-instates extinguished drug-seeking behaviours, effects that are also blocked by an orexin-1 receptor antagonist (56-57). Given the efficacy of SR-141716A/rimonabant as an anti-craving agent, an intriguing speculation is that part of this effect may also reflect binding to cannabinoid CB1/orexin-1 receptor hetero-dimers *in vivo*.

Both homo- and hetero-dimerization of cannabinoid receptor subtypes has previously been reported (58). In relation to the current studies, the observation of interactions between the CB1 and dopamine D2 receptor (59) may also have relevance to the actions of therapeutically-used medicines. Interestingly, co-expression of these two GPCRs was

reported to alter G protein-coupling selectivity (59) and similar effects have also been reported following co-expression of the dopamine D1 and D2 receptors (43). Alteration of G protein selectivity via GPCR hetero-dimerization may be an emerging theme in this area and hence provide mechanisms to further control signal strength and selectivity.

The concept that GPCR hetero-dimers may provide novel and selective therapeutic targets is still developing (16-17), however, the idea that a range of ligands may modulate GPCRs at which they have been considered to have no affinity due to hetero-dimerization offers a further novel scenario.

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FIGURE LEGENDS

Fig. 1 Doxycycline-dependent cell surface expression of an orexin-1 receptor construct

(a) Flp-In T-REx HEK293 cells with a VSV-G-h-orexin-1-eYFP construct at the Flp-In locus were imaged without (i) and following (ii) doxycycline (1 mg/ml, 72 h) treatment.

(b) Doxycycline-induced cells were labelled with 1 mM Hoechst 33342 nuclear stain (blue) (i-iii) and the plasma membrane marker WGA-Alexa Fluor 594 (red) (ii). Overlay of images i and ii (iii) showed co-localization of eYFP and WGA-Alexa Fluor 594. This was quantitated on a pixel by pixel basis (right hand side).

(c) Cell surface anti-VSV-G ELISA confirmed appearance of the VSV-G epitope tag on the extracellular surface following doxycycline treatment only of cells harbouring VSV-G-h-orexin-1-eYFP at the Flp-In locus and not of parental Flp-In T-REx HEK293 cells.

(d) The time course of expression of VSV-G-h-orexin-1-eYFP in response to treatment with doxycycline (1 mg/ml) was monitored following resolution of cell membrane preparations by SDS-PAGE and immunoblotting with anti-VSV-G.

Fig. 2 Orexin A causes phosphorylation of ERK MAP kinases via VSV-G-h-orexin-1-eYFP and internalization of this construct: SB-674042 is an orexin-1 receptor antagonist

(a, b) Cells harbouring VSV-G-h-orexin-1-eYFP at the Flp-In locus were treated with (+) or without (-) doxycycline as in Figure 1 for 72 h, and then treated with orexin A (0.5 mM) (+ OXA) or with vehicle (- OXA) for 5 min. Cell lysates were resolved by SDS-PAGE and immunoblotted to detect phospho- ERK1/ERK2 (a) or total levels of ERK1/ERK2 (b).

(c) Cells induced to express VSV-G-h-orexin-1-eYFP were treated with vehicle (CONTROL) or orexin A (0.5 mM, 30 min) (OXA).

(d) Cells as in (c) were pre-treated with SB-674042 (5 mM, 3 h), challenged with SB-674042 (5 mM) or orexin A (1 mM) + SB-674042 (5 mM) for 30 min and then imaged.

Fig. 3 Co-expression with h-CB1 alters the cellular distribution of VSV-G-h-orexin-1-eYFP

(a) Cells harbouring VSV-G-h-orexin-1-eYFP (**left hand panel**) or harbouring VSV-G-h-orexin-1-eYFP and constitutively expressing h-CB1 (**right hand panel**) were induced to express VSV-G-h-orexin-1-eYFP by treatment with doxycycline (1 mg/ml, 72 h). The distribution of VSV-G-h-orexin-1-eYFP was then imaged. (b) The specific binding of [³H]SR-141716A (5 nM) was measured in membrane preparations of parental Flp-In T-Rex HEK293 cells, and in Flp-In T-Rex cells induced to express VSV-G-h-orexin-1-eYFP in the absence and presence of constitutive expression of the h-CB1 receptor.

Fig. 4 In the presence of the h-CB1 receptor, VSV-G-h-orexin-1-eYFP adopts the cellular location of h-CB1: this reflects hetero-dimerization/oligomerization

Cells harbouring VSV-G-h-orexin-1-eYFP and constitutively expressing h-CB1-CFP were employed. (a) Images were taken corresponding to eYFP and CFP without (**i-iii**) and with (**iv-vi**) induction of VSV-G-h-orexin-1-eYFP expression for 72 h. (b) Single cell images for VSV-G-h-orexin-1-eYFP (**i**) and h-CB1-CFP (**ii**) were overlaid (**iii**) and colour overlap determined on a pixel by pixel basis (**iv**). (c) Direct images and corrected CFP to YFP FRET (cFRET) images were recorded after individual expression of VSV-G-h-orexin-1-eYFP (**upper panels**) and h-CB1-CFP (**middle panels**) or their co-expression (**lower panels**). Quantified cFRET values are detailed.

Fig. 5 m-opioid receptor distribution is unaffected by the presence of the h-CB1 receptor

Flp-In T-Rex HEK293 cells with h-CB1-CFP (blue) at the inducible Flp-In locus and constitutively expressing h-m-opioid receptor-YFP (yellow) were established and imaged both without (**a**) and with (**b**) doxycycline-induced (1 mg/ml, 24h) expression of h-CB1-CFP. In both situations the YFP signal was predominantly at the cell surface.

Fig. 6 SR-141716A and SB-674042 are highly selective pharmacological reagents

(a) The specific binding of [³H]SR-141716A (5 nM) in the absence or presence of orexin A (0.5 mM) or SB-674042 (5 mM) was assessed in membranes of cells constitutively expressing h-CB-1 without induction of VSV-G-h-orexin-1-eYFP.

(b) SR-141716A (1 mM, 3 h) was unable to internalize VSV-G-h-orexin-1-eYFP (i) or to antagonize orexin A (0.5 mM, 30 min) mediated internalization of this construct in cells induced to express VSV-G-h-orexin-1-eYFP in the absence of h-CB-1 (ii).

(c) The specific binding of [³H]SB-674042 (5 nM) in the absence or presence of SR-141716A (1mM) was assessed membranes of cells expressing only VSV-G-h-orexin-1-eYF as in (b).

Fig. 7 SR-141716A but not SB-674042 relocates h-CB1-CFP to the cell surface

Cells harbouring, but not induced to express VSV-G-h-orexin-1-eYFP and constitutively expressing h-CB1-CFP were untreated (**left hand panels**) or treated with either SR-141716A (1 mM, 3 h) (**middle panels**) or SB-674042 (5 mM, 3h) (**right hand panels**). Cells were subsequently imaged to detect YFP (top panels), CFP (centre panels) and the YFP and CFP images merged (bottom panels). Quantitative analysis is provided in Table 3.

Fig. 8 SR-141716A and SB-674042 relocate hetero-dimers of orexin-1-eYFP and CB1-CFP receptors to the cell surface when the two receptors are co-expressed

a. Cells harbouring VSV-G-h-orexin-1-eYFP and constitutively expressing h-CB1-CFP were treated with doxycycline (1 mg/ml, 72 h) to induce VSV-G-h-orexin-1-eYFP expression. The cells were untreated (**left hand panels**) or treated with either SR-141716A (1 mM, 3 h) (**middle panels**) or SB-674042 (5 mM, 3h) (**right hand panels**). Cells were subsequently imaged to detect eYFP (top panels), CFP (bottom panels). Quantitative analysis is provided in Table 3.

b. Direct images and corrected CFP to YFP FRET (cFRET) images were recorded after individual expression of h-CB1-CFP (**upper panels**), VSV-G-h-orexin-1-eYFP (**middle panels**) or their co-expression (**lower panels**) and treatment with SR-141716A (1 mM, 3 h). Quantified cFRET values are detailed. Data represent means +/- S.E.M. for 6 individual cells.

Fig. 9 SR-141716A decreases the potency of orexin A to stimulate ERK MAP kinase phosphorylation via the orexin-1 receptor only when the CB1 receptor is co-expressed

The ability of varying concentrations of orexin A to cause phosphorylation of the MAP kinases ERK1 and ERK2 (**top panels**) was assessed in Flp-In T-REx HEK293 cells induced to express VSV-G-h-orexin-1-eYFP without (**a, b**) and with (**c, d**) constitutive expression of h-CB1. Cells were pre-treated with (**b, d**) or without (**a, c**) SR-141716A (1 mM, 3 h) prior to performing the ERK MAP kinase assay. Total ERK1 and ERK2 amounts (**bottom panels**) provided loading controls.

a. Quantitation of data akin to Figure 8 a-d, means +/- SEM, n = 4.

TABLES

Table 1. Cellular distribution of VSV-G-h-orexin-1-eYFP after induction in the absence and presence of h-CB1 receptor

	VSV-G-OX1-YFP	VSV-G-OX1-YFP + h-CB1
Cell surface	88.6 +/- 3.6	47.2 +/- 2.6
Intracellular	11.4 +/- 3.6	52.8 +/- 2.6

The distribution of VSV-G-h-orexin-1-eYFP between the cell surface and intracellular compartments was assessed in Flp-In T-REx HEK293 cells following induction of expression by treatment with doxycycline (1 mg/ml, 72 h) in the absence or presence of constitutive expression of h-CB1. Data are means +/- S.E.M. from n = 6 individual cells. **Table 2.**

Expression levels of cannabinoid CB1 receptor constructs

	CB1 + OX1-YFP (- Dox)	CB1 + OX1-YFP (+ Dox)	CB1-CFP + OX1-YFP (- Dox)	CB1-CFP + OX1-YFP (+ Dox)
B_{max} (pmol/mg)	2.83 +/- 0.11	2.97 +/- 0.06	1.60 +/- 0.08	1.60 +/- 0.52
K_d (nM)	0.73 +/- 0.10	0.88 +/- 0.26	0.97 +/- 0.13	1.06 +/- 0.01

Saturation binding studies were performed on membrane preparations of Flp-In T-REx HEK293 that constitutively express either h-CB1 or h-CB1-CFP using varying concentrations of [³H]SR-141716A. Expression of VSV-G-h-orexin-1-eYFP was produced from the Flp-In locus by treatment with doxycycline (+ Dox) as in Table 1.

Table 3. Effects of SR-141716A and SB-674042 treatment on the cellular distribution of orexin-1 and cannabinoid CB1 receptors

CB1-CFP VSV-G-OX-1-YFP

+ CB1-CFP

	Control	SR-141716A (1 mM, 3 h)	SB-674042 (5 mM, 3 h)	Control	SR-141716A (1 mM, 3 h)	SB-674042 (5 mM, 3 h)
Cell surface YFP intensity (OX-1)	Not expressed	Not expressed	Not expressed	33.9 +/- 3.1	81.6 +/- 4.2 p < 0.001	81.7 +/- 3.3 p < 0.001
Intracellular	Not	Not	Not	66.1 +/- 3.1	18.4 +/- 4.2	18.3 +/-

YFP intensity (OX-1)	expressed	expressed	expressed		p < 0.001	3.3 p < 0.001
Cell surface CFP intensity (CB1)	8.8 +/- 2.0	54.4 +/- 11.1 p < 0.01	6.3 +/- 1.4	8.6 +/- 1.6	42.2 +/- 5.2 p < 0.001	34.3 +/- 3.9 p < 0.001
Intracellular CFP intensity (CB1)	91.2 +/- 2.0	45.6 +/- 11.1 p < 0.01	93.7 +/- 1.4	91.4 +/- 1.6	57.8 +/- 5.2 p < 0.001	65.7 +/- 3.9 p < 0.001

The distribution of VSV-G-orexin-1-h-eYFP and hCB1-CFP between the cell surface and intracellular compartments was assessed in Flp-In T-REx HEK293 cells constitutively expressing hCB1-CFP in the absence or presence of VSV-G-h-orexin-1-eYFP. Cells were untreated (**Control**) or pre-treated with either SR-141716A or SB-674042 for 3 h. Data are means +/- S.E.M. from n = 6 individual cells. **Table 4. The potency of orexin A to phosphorylate ERK1/ERK2 is decreased by SR-141716A /rimonabant only when the h-CB1 receptor is co-expressed with the orexin-1 receptor**

	OX1-YFP	OX1-YFP (+ SR-141716A)	CB1-CFP + OX1-YFP	CB1-CFP + OX1-YFP (+ SR-141716A)
EC₅₀ Orexin A (nM)	39.4 +/- 4.9	48.2 +/- 4.9 NS	23.0 +/- 1.6 p = 0.027	177.3 +/- 12.0 p = < 0.0001

The ability of varying concentrations of orexin A to stimulate phosphorylation of the MAP kinases ERK1 and ERK2 was quantitated in untreated cells and in those pre-treated with SR-141716A (1 mM, 3 h). Statistical differences in values are against untreated cells expressing VSV-G-h-orexin-1-eYFP. Data are mean +/- S.E.M. n = 4. NS = not significant.

These studies were performed by immunoblotting with a phospho-specific antibody to detect phosphorylated forms of ERK1 and ERK2. Phosphorylation of these kinases is considered synonymous with their state of activation. However, no attempt was made to ascertain the fraction of total cellular ERK1 and ERK2 that became phosphorylated in response to orexin-A. Similar caveats apply to the data of Tables 5 and 6.

Table 5 . The potency of WIN 55, 212-2 to phosphorylate ERK1/ERK2 is decreased by SB-674042 only when the h-CB1 receptor is co-expressed with the orexin-1 receptor

	CB1-CFP	CB1-CFP (+ SB-674042)	CB1-CFP + OX1- YFP	CB1-CFP + OX1- YFP (+ SB-674042)
EC₅₀ WIN 55, 212-2 (nM)	135.5 +/- 18.2	153.0 +/- 15.1 NS	144.3 +/- 7.5 NS	269.5 +/- 34.0 p = < 0.05

The ability of varying concentrations of the cannabinoid CB1 receptor agonist WIN 55, 212-2 to stimulate phosphorylation of the MAP kinases ERK1 and ERK2 in untreated cells and in those pre-treated with SB-674042 (5 mM, 3 h) was quantitated. Statistical differences in values are against untreated cells expressing CB1-CFP. Data are mean +/- S.E.M. n = 4. NS = not significant.

Table 6. Co-expression of the h-CB1 receptor and treatment with SR-141716A /rimonabant alters the potency of orexin A to phosphorylate ERK1/ERK2 over a wide range of orexin-1 receptor expression levels

	OX1-YFP	OX1-YFP + CB1	OX1-YFP + CB1 (+ SR-141716A)
EC₅₀ Orexin A (nM) (1.0 ¼g/ml Dox)	47.7 +/- 4.3	25.7 +/- 2.8 p = 0.0019	117.0 +/- 27.0 p = 0.0044
EC₅₀ Orexin A (nM) (0.0001 ¼g/ml Dox)	92.0 +/- 4.5 P = 0.0003	24.3 +/- 3.6 p = < 0.0001 NS	173.7 +/- 37.4 p = 0.0021 NS
EC₅₀ Orexin A (nM) (0.0000025 ¼g/ml Dox)	149.0 +/- 49.0 = 0.024	54.3 +/- 1.9 p = 0.029 P = < 0.0001	214.3 +/- 6.6 p = < 0.0001 P = 0.0038

The ability of varying concentrations of orexin A to stimulate phosphorylation of the MAP kinases ERK1 and ERK2 was quantitated in Flp-In T-REx HEK293 cells either lacking expression of h-CB1-CFP or constitutively expressing h-CB1-CFP. In each case the cells harbored VSV-G-h-orexin-1-eYFP at the Flp-In locus and were treated with different concentrations of doxycycline to induce expression of VSV-G-h-orexin-1-eYFP. [³H]SB-674042 binding studies indicated VSV-G-h-orexin-1-eYFP expression to varying 100 fold between cells treated with 0.0000025 mg/ml and 1 mg/ml doxycycline. Cells co-expressing VSV-G-h-orexin-1-eYFP and h-CB1-CFP were treated with or without SR-141716A (1 mM, 3 h).

Statistical differences in values are against untreated cells expressing VSV-G-orexin-1-eYFP (p), against untreated VSV-G-orexin-1-eYFP cannibinoid-1 co-expressing cells (p) and against cells expressing receptor following induction with 1.0 μ g/ml doxycycline for each cell line independently including cells pre-treated with SR-141716A (P). Data are mean \pm S.E.M. n = 3. NS = not significant.