Phenotypic screening of cannabinoid receptor 2 ligands shows different sensitivity to genotype

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ABSTRACT

The Cannabinoid Receptor 2 (CB$_2$R) is a G protein-coupled receptor (GPCR) investigated intensively as therapeutic target, however no drug has reached the market yet. We investigated personal differences in CB$_2$R drug responses using a label-free whole-cell assay (xCELLigence) combined with cell lines (Lymphoblastoid Cell Lines) from individuals with varying CB$_2$R genotypes. Responses to agonists, partial agonists and antagonists of various chemical classes were characterized. Endogenous cannabinoids such as 2-AG induced cellular effects vastly different from all synthetic cannabinoids, especially in their time-profile.

Secondly, the Q63R polymorphism affected CB$_2$R responses in general. Agonists and especially partial agonists showed higher efficacy in a Q63R minor homozygote versus other genotypes. Non-classical cannabinoid CP55940 showed the most pronounced personal effects with highly reduced potency and efficacy in this genotype. Contrarily, aminoalkylindole compounds showed less individual differences.

In conclusion, a label-free whole-cell assay combined with personal cell lines is a promising vehicle to investigate personal differences in drug response originating from genetic variation in GPCRs. Such phenotypic screening allows early identification of compounds prone to personal differences ('precision medicine') or more suited as drugs for the general population.

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1. Introduction

The Cannabinoid Receptor 2 (CB$_2$R) is a class A G Protein-Coupled Receptor (GPCR) which has been investigated intensively,

for instance as therapeutic target for novel immunomodulators [1]. The Cannabinoid receptor family consists of CB$_1$R, CB$_2$R and as of late, the former orphan receptors GPR55 and GPR18. Together with their endogenous ligands, they form part of the endocannabinoid system which is involved in many physiological processes. CB$_2$R is a (predominantly) Gα$_i$-coupled receptor which is expressed mainly in cells of the immune system, such as T- and B-lymphocytes, as well as the central and peripheral nervous system and the gastrointestinal tract [1–3]. As such, the CB$_2$R is involved in a wide range of pathological conditions ranging from atherosclerosis [4], neuropathic pain [5], neurodegenerative diseases [6], osteoporosis [7] and autoimmune diseases [8] to cancer [9–11]. Hence, the CB$_2$R has been in the focus of drug development efforts for over a decade. However, no selective drug targeting the CB$_2$R has made it to the market as of yet. There can be several reasons as to why drugs fail in clinical trials, one of which is

differences in individuals' responses to the drug. In fact, even the most widely prescribed and sold drugs, the so-called big 'blockbuster' drugs, only work in 35–75% of all patients [12], as individual drug response varies due to differences in genetics, lifestyle and environment. Therefore, personalized or precision medicine aims to personalize drug prescriptions based on a patient's individual characteristics, e.g. genetic information, and thereby decreases risks of ineffective dosing or side-effects [13,14]. An abundant source of genetic variation in humans is Single Nucleotide Polymorphism (SNP), which can lead to an alteration in the amino acid sequence of a protein [15]. Many polymorphisms have been documented in the CB$_2$R, including three that change the amino acid sequence and occur highly frequently in the population, namely Q63R, Q66R and H316Y [16]. Of these, both Q63R and H316Y have been linked to various pathological conditions. Q63R is special, as it can be caused by a SNP (rs2501432) as well as a dinucleotide polymorphism (rs35761398). Q63R has been shown to be involved in schizophrenia and depression [17–19], alcoholism [20], eating disorders [21], early menarche in obesity [22] and various immune system related disorders [23–25], while H316Y has been associated with lowered bone mineral density [26].

We investigated personal differences in CB$_2$R drug responses using a sensitive *in vitro* assay, i.e. a label-free cellular assay using the xCELLigence system, in combination with personal cell lines. With the xCELLigence, whole-cell responses are measured non-invasively allowing for the investigation of drug responses in an unbiased way, i.e. without selecting one signaling pathway or effect. The personal cell lines used in this study were Lymphoblastoid Cell Lines (LCLs) obtained from participants of the Netherlands Twin Register (NTR), which are derived from B-lymphocytes and thus endogenously express the CB$_2$R [27,28]. Using LCLs from individuals with different CB$_2$R genotypes, we tested a number of ligands ranging from agonists and partial agonists to antagonists (Fig. 1), which have potential use in different pathological indications. Firstly, endogenous cannabinoids are fatty acid derivatives

such as the eicosanoids 2-AG (2-Arachidonoylglycerol), the main endogenous ligand for CB$_2$R, and AEA (anandamide) [29,30]. Synthetic cannabinoids can be divided into classical and non-classical, such as JWH133 and CP55940, respectively. Another large class of synthetic cannabinoid receptor ligands are the aminoalkylindoles, of which WIN55212-2 is the most studied agonist and AM630 is one of the most utilized CB$_2$R antagonists [1,31]. Several classes also contain partial agonists, such as aminoalkylindole GW405833 or BAY59-3074, which belongs to a separate chemical class.

In this study, we show that the xCELLigence in combination with these personal cell lines can be successfully applied to investigate personal differences in drug response originating from, for instance, genetic variation in GPCRs. We furthermore demonstrate that while certain classes of CB$_2$R ligands show individual differences, others deliver consistent effects independent of genotype. Thus while taking personal medical effects into account, it is still possible to identify potential 'blockbuster' drugs by using such phenotypic screening methods with personal cell lines.

2. Material and methods

2.1. Chemicals and reagents

Fibronectin from bovine plasma, Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (25 mM HEPES and NaHCO$_3$) and Pertussis Toxin (PTX) were purchased from Sigma Aldrich (Zwijndrecht, NL). CB$_2$R ligands AM630, GW405833 and CP55940 were purchased from Sigma Aldrich, BAY59-3074, WIN55212-2 mesylate, JWH133 and AEA from Tocris Bioscience (Bristol, UK) and 2-AG from Cayman Chemicals (Ann Arbor, MI, USA). All other chemicals and reagents were of analytical grade and obtained from commercial sources, unless stated otherwise.
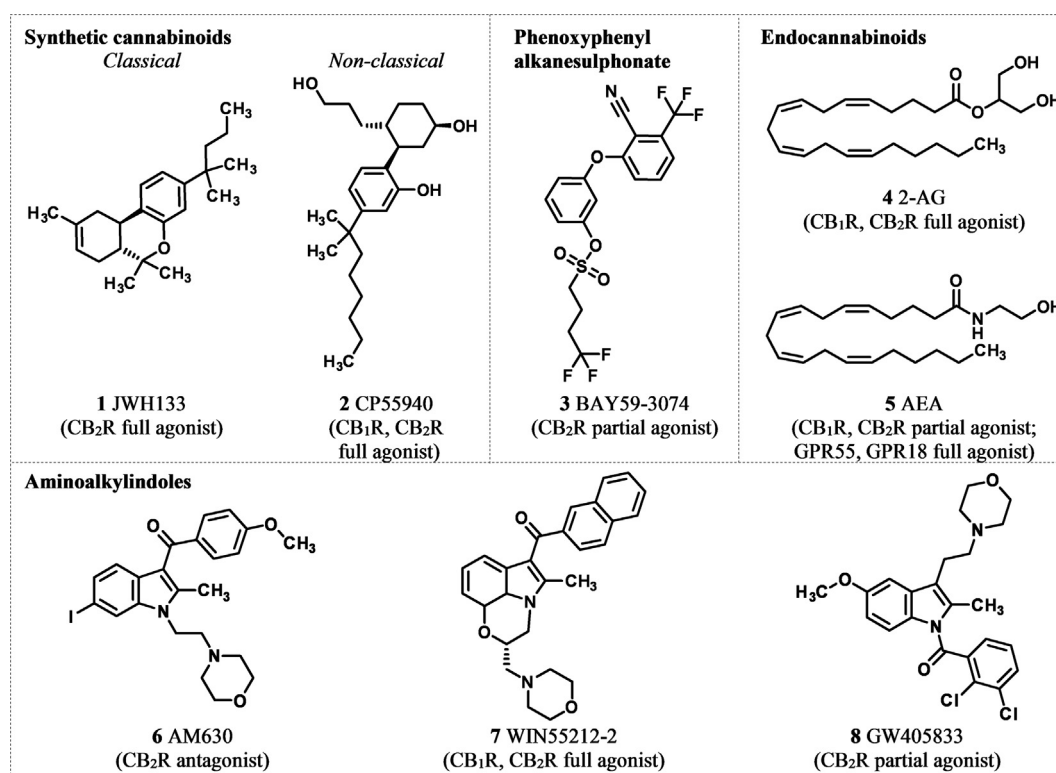

Fig. 1. Chemical structures of CB$_2$R ligands characterized in this manuscript.

2.2. Lymphoblastoid cell line generation

For all participants of the Netherlands Twin Register (NTR, VU, Amsterdam, NL) [27] included in this study, lymphoblastoid cell lines (LCLs) were generated in accordance with a previous publication [32,33] by the Rutgers University Cell and DNA Repository (Department of Genetics, Piscataway, NJ, USA). According to a standard transformation protocol [27], peripheral B-lymphocytes were transformed with Epstein-Barr Virus (EBV) by treatment with filtered medium from a Marmoset cell line in the presence of phytohemagglutinin during the first week of culture [34–36]. EBV transformed lymphocytes were expanded by culture for 8–12 weeks and subsequently cryopreserved.

2.3. Cell culture

LCLs from a family of four individuals, two parents (i.e. genetically unrelated; individual 2 and 3) and their monozygotic twin children (i.e. genetically equal; individual 4 and 5), as well as one other unrelated individual (individual 1) were used for the experiments presented in this manuscript. Individual 2 and 3 have been part of a previous article [32], where we published effects of JWH133, AM630 as well as PTX inhibition of JWH133. These data were incorporated in the current manuscript to allow direct comparison to effects of other compounds, individuals and genotypes. The LCLs were cultured as described previously [32]. In short, LCLs were cultured as suspension cells in RPMI 1640 (25 mM HEPES and NaHCO$_3$) supplemented with 15% Fetal calf serum (FCS), 50 mg/ml streptomycin, 50 IU/ml penicillin, at 37 °C and 5% CO$_2$. Cells were subcultured twice a week at a ratio of 1:5 on 10 cm ø plates and disposed after maximally 120 days.

2.4. qPCR

For qPCR analysis of receptor expression, RNA of three independent samples of each cell line was isolated by RNeasy Plus Mini (QIAGEN, Venlo, the Netherlands) and cDNA was randomly primed from 500 ng of total RNA using ReverstAid H Minus First Strand cDNA synthesis Kit (ThermoFisher, Breda, The Netherlands). The primer list is included in Table 1. Real-time qPCR was performed in triplicate for each sample using SYBR Green PCR (Applied Biosystems, part of ThermoFisher) on a 7500 Real-Time PCR System (Applied Biosystems). qPCR data were collected and analyzed using SDS2.3 software (Applied Biosystems). Household gene β-actin was used as internal control to normalize receptor expression and compare between individuals. Relative mRNA amounts after correction for β-actin control mRNA were expressed using the $2^{-\Delta\Delta Ct}$ method.

2.5. Label-free whole-cell analysis (xCELLigence RTCA system)

2.5.1. Instrumentation principle

Cellular assays using the xCELLigence RTCA system [37] were performed in accordance with previously published protocols [32,38]. The real-time cell analyzer (RTCA) uses a detection system based on electrical impedance to measure the whole-cell responses. Cell attachment to gold electrodes embedded on the bottom of the microelectronic E-plates changes the local ionic environment at the electrode-solution interface, which generates impedance. Relative changes in impedance (Z) are recorded in real-time and summarized in the Cell Index (CI). This CI, which is a dimensionless parameter, is defined at any given time point as $(Z_i - Z_0) \Omega/15 \Omega$. Z_i is the impedance at each individual time point, whereas Z_0 is defined as 0, as it represents the baseline impedance in the absence of cells measured prior to the start of the experiment. Impedance and the corresponding CI increase proportionally as cell adhere to the electrodes. The impedance profile directly reflects any changes in degree of adhesion, cell number, viability and morphology [37,39]. As such cellular parameters are also affected upon activation of GPCR signaling, this allows real-time monitoring of cellular signaling events [37].

2.5.2. General protocol

xCELLigence assays on LCLs were performed as described previously [32] with some minor modifications. Briefly, cells were seeded onto fibronectin-coated E-plates (10 µg/ml) at 50,000 cells/well, unless stated otherwise. Cell counts were performed with Trypan blue staining on a BioRad TC10 automated cell counter. E-plates were clicked in the xCELLigence recording station in an incubator (37 °C, 5% CO$_2$). Impedance was measured overnight for 18 h, after which the cells were stimulated with a cannabinoid receptor agonist or vehicle control in 5 µl, unless specified otherwise. As compound solubility required addition of dimethylsulfoxide (DMSO) or acetonitrile (ACN), the final concentration upon ligand or vehicle addition was kept at 0.25% DMSO or respectively 1% ACN for all wells and assays.

For agonist screening purposes, cells were stimulated with agonist concentrations corresponding to approximately $100 \times$ published pK$_I$ values for hCB$_2$R [40,41]. Agonist or partial agonist concentration-effect curves were generated by stimulating cells with increasing concentrations of the respective compound. For antagonist assays, cells were pre-incubated for 30 min with 5 µl of vehicle control or the respective antagonist at increasing concentrations. Subsequently, cells were challenged with a submaximal agonist concentration of reference full agonist JWH133 equal to the agonist's EC$_{80}$ concentration (100 nM) or vehicle control. Of note, for partial agonist curves, fibronectin coating was increased (50 µg/ml) and cells were seeded at a higher density of 100,000 cells/well in order to achieve a sufficient window. To allow comparison, full agonist JWH133 was always tested alongside all partial agonists under equal conditions. For endocannabinoids, addition of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) to prevent possible degradation was tested, but as this did not change the responses or time-profile it was further omitted (*data not shown*).

For studies on Gα$_i$ coupling, cells were seeded in assay medium containing 100 ng/ml Pertussis Toxin (PTX) or vehicle control, and stimulated after 18 h with agonist at corresponding EC$_{80}$ concentration or vehicle control.

2.6. Data analysis

Data were analyzed as published previously [32]. Experimental data were captured and processed with RTCA Software 1.2 (ACEA,

Table 1
Primers for qPCR.

Gene	Forward	Reverse
β-actin	ATTGCCGACAGGATGCAGAA	GCTGATCCACATCTGCTGGAA
CNR1	GAGAAGATGACTGCGGGAGA	GTTGTAAAATTCTGTAATGTTCACCTG
CNR2	CATGCTGTGCCTCATCAACT	GATCTCGGGGCTTCTTCTTT
GPR55	GGAAAGTGGAAAAATACATGTGC	CAGCGGGAAGAAGACCTTG
GPR18	AACGGGGGAGAACAGTTACA	AACTTTTTCTGCGCATGCTT

San Diego, CA, USA), in which ligand responses were normalized to the last time point prior to compound addition resulting in the Δ Cell Index (Delta Cell Index or Δ CI). Data were exported to GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) for further analysis. Correction for any ligand-independent effects was achieved by subtracting vehicle control as baseline. Peak responses were defined as highest Δ CI (Max Δ CI) observed within 30 min after compound addition. For negative impedance responses of 2-AG, Max-Min Δ CI within 1 h was used, which is the amplitude between the highest and lowest Δ CI. Peak values and experimental Δ CI traces were used for construction of bar graphs or concentration–effect curves by nonlinear regression and calculation of IC_{50} (half maximal inhibitory concentration), EC_{50} (half maximal effective concentration) and EC_{80} (80% maximal effective concentration) values. E_{max} (maximum effect) values of compounds were derived from maximal responses within the analyzed timeframe. Agonist and partial agonist curves of all individuals as well as the derived E_{max} values were normalized to E_{max} of CB_2R-selective agonist JWH133 response on individual 1, first as this individual also showed the highest response for all agonists with the exception of CP55940, and secondly as this was also the only case of a single individual per genotype (only minor homozygote for Q63R, *R63*).

All values obtained are means of at least three independent experiments performed in duplicate, unless stated otherwise. When comparing multiple means or multiple instances of two means, statistical significance was calculated using a two-way analysis of variance (ANOVA) with Fisher's LSD test, for example comparison of multiple EC_{50} values or antagonist inhibition of multiple compounds. Comparison of multiple means to one value was performed with a two-way ANOVA with Dunnett's post hoc test, for instance comparison of JWH133 Peak Δ CI response after preincubation with various antagonists.

2.7. Processing of SNPs and genetic data

As stipulated in a previous publication [33], SNP data for the NTR individuals included in this study were obtained from the Genomes of the Netherlands consortium (GoNL; http://www.nlgenome.nl/) of which the NTR is part of [42] and analyzed in-house using PLINK, an open-source whole genome association analysis toolset (PLINK v1.07, http://pngu.mgh.harvard.edu/purcell/plink/) [43]. All SNPs within the boundaries of the *CNR2* gene (Ensembl gene: ENSG00000188822) as defined by human genome overview GRCh37 were analyzed further. Based on GRCh37 and dbSNP information (http://www.ncbi.nlm.nih.gov/SNP/), SNPs were annotated according to position (e.g. coding sequence, exon) and SNP type (e.g. missense).

2.8. Data access

The LCLs used in this study were kindly provided within the framework of this collaboration [27] and are part of the Netherlands Twin Register (NTR; http://www.tweelingenregister.org/en/), and part of the Center for Collaborative Genomic Studies on Mental Disorders (NIMH U24 MH068457-06). Data and biomaterials (such as cell lines) are available to qualified investigators, and may be accessed by following a set of instructions stipulated on the National Institute of Mental Health (NIMH) website (https://www.nimhgenetics.org/access_data_biomaterial.php).

3. Results

3.1. LCLs predominantly express CB_2R

To confirm the suitability of LCLs for studies of CB_2R function alone, RNA expression levels of the four receptors belonging to the cannabinoid family were assessed by qPCR. These results showed that mRNA of all four cannabinoid receptors is present in LCLs to a similar degree, both compared between receptors and between individuals. There were however some differences. For instance, GPR18 was expressed higher in many individuals, though not statistically significant in all. The corresponding expression data are summarized in Fig. 2. We used the xCELLigence to further confirm the presence or absence of the different cannabinoid receptor subtypes, specifically CB_2R, by testing selective and non-selective cannabinoid agonists and antagonists using the LCL of one exemplary individual (individual 4). To ensure full receptor occupancy, we tested the compounds at concentrations corresponding to approximately $100\times$ their K_i value at the respective receptor [40,41]. The agonists tested included selective CB_2R agonist JWH133 as well as non-selective agonists CP55940 and WIN55212-2, which are both known to activate CB_1R as well as CB_2R. Neither of these three compounds are GPR18 agonists [44]. These agonists were also chosen as they represent three distinct chemical classes (Fig. 1). Ligand-induced changes in impedance were recorded in real-time, of which an example of resulting xCELLigence traces is shown in Fig. 3. A full real-time trace of a complete experiment is shown in Fig. 3A, and the corresponding vehicle-corrected compound responses are summarized in Fig. 3B. LCL seeding resulted in an initial quick increase in impedance related to cell adhesion, after which cells were allowed to proliferate and adjust for 18 h (Fig. 3A). Subsequent addition of the agonists induced an immediate increase of impedance to a peak which gradually decreased towards a plateau within 30 min (Fig. 3B). The responses of all three agonists were highly similar
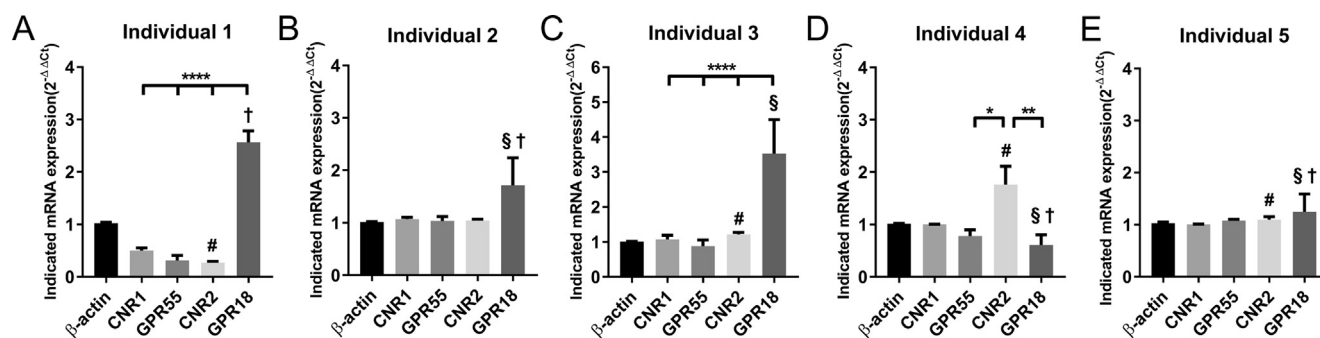

Fig. 2. Cannabinoid receptor subtype mRNA expression in LCLs. Results of real-time qPCR (three independent samples measured in triplicate, mean ± SEM) show mRNA expression of four cannabinoid receptor genes per individual (A–E for individual 1–5, respectively). Significant differences in expression were determined with a two-way ANOVA Fisher's LSD test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001. Expression differences within each individual are indicated in the figure. Expression differences between individuals were for CNR2: # = individual 1 with *to 3,5 and ***to 4. For GPR18 these were: † = individual 1 with *to 2; **to 5; ****to 4 and § = individual 3 with ****to 2,4,5.
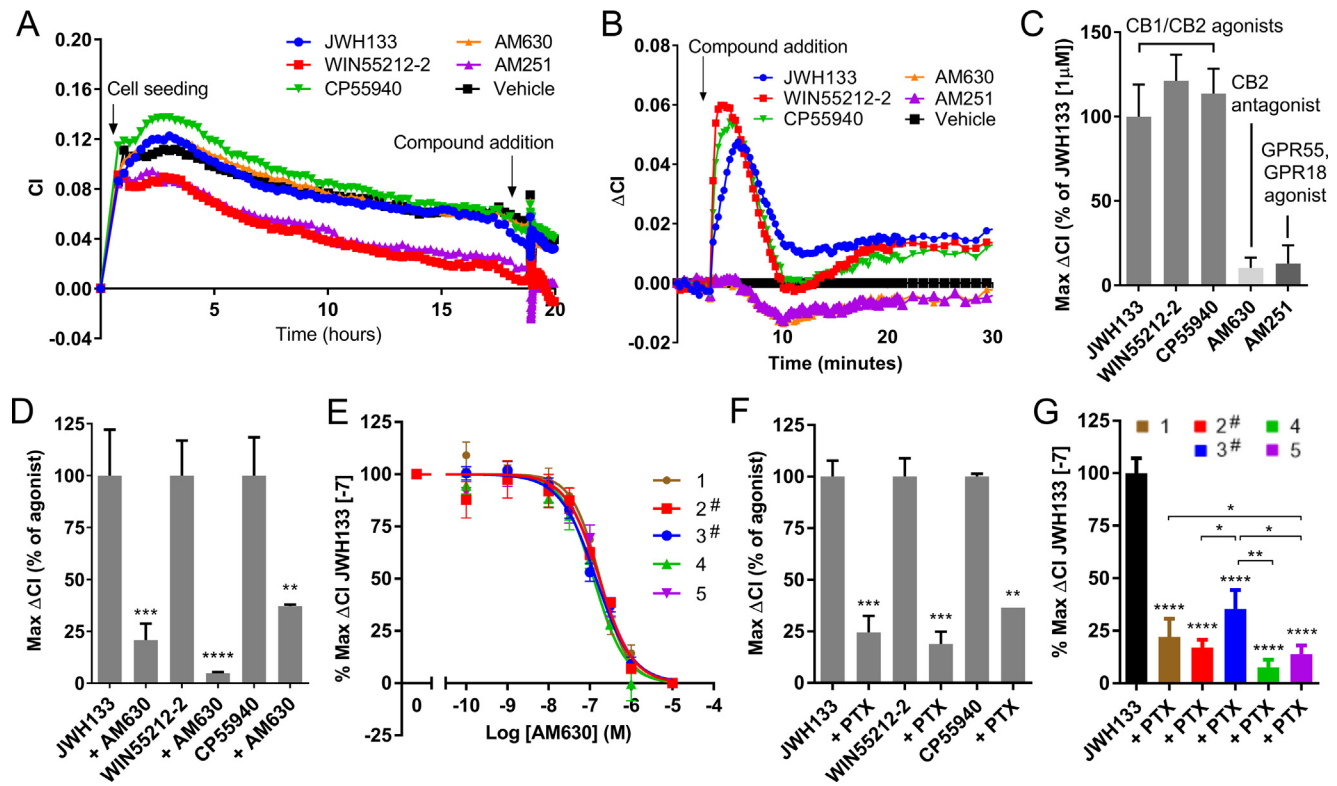
Fig. 3. Cannabinoid receptor ligand screen to determine receptor subtype expression. Representative real-time traces of (A) a full experiment and (B) baseline-corrected responses in a screen of cannabinoid agonists and antagonist. (C) Corresponding maximal responses of the screen (Max Δ Cell Index or Max Δ CI), normalized to response to CB$_2$R-selective agonist JWH133. Concentrations used were JWH133 [1 μM], WIN55212-2 [10 μM], CP55940 [1 μM], AM630 [10 μM] and GPR55 agonist AM251 [10 μM]. All data shown were obtained with the LCLs of individual 4. (D) Inhibition of agonist effects by CB$_2$R specific antagonist AM630 in LCLs of individual 4, normalized to peak Δ CI of untreated agonist response. LCLs were pre-incubated with AM630 [10 μM] 30 min before stimulation with agonist at EC$_{80}$ (JWH133 [100 nM], WIN55212-2 [10 nM], CP55940 [10 nM]). Degree of inhibition did not differ significantly between agonists, as determined by two-way ANOVA with Fisher's LSD post-hoc test. (E) Antagonist AM630 concentration-effect curve obtained from peak Δ CI of the baseline-corrected JWH133 response antagonized by increasing concentrations of AM630. Antagonist potency values were 6.76 ± 0.04, 6.77 ± 0.06, 6.85 ± 0.04, 6.90 ± 0.05 and 6.77 ± 0.04 for individuals 1–5, respectively. No statistically significant differences between individuals were observed as determined by two-way ANOVA with Fisher's LSD post-hoc test. (F) Inhibition of agonist-induced Gα_i downstream signaling by pretreatment with PTX in LCLs of individual 4, normalized to peak Δ CI of untreated agonist response. LCLs were seeded in presence or absence of PTX [100 ng/ml] and treated with agonist at EC$_{80}$ after 18 h growth. Degree of inhibition did not differ significantly between agonists, as determined by two-way ANOVA with Fisher's LSD post-hoc test. (G) Individual effect of Gα_i inhibition by PTX on CB$_2$R response to agonist JWH133. Response in the presence of PTX versus JWH133 alone was highly significantly reduced within each individual (****). Statistical differences between individuals were determined by two-way ANOVA with Fisher's LSD post-hoc test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Data represent mean ± SEM obtained from three or four (B, C, E, G) independent experiments of performed in duplicate. For (D, F) data represent mean ± SD from two independent experiments performed in duplicate from individual 4, used as representative example here, while results on other individuals (2, 3, 5) were comparable (*data not shown*). #AM630 curves and PTX inhibition for individuals 2 and 3 had been previously established [32] but were incorporated to allow direct comparison.

both in shape and height (Fig. 3B, C), indicating that the effects were mediated through the same receptor. AM251, which is known to be a GPR55 full agonist, partial GPR18 agonist and CB$_1$R antagonist [44], gave little to no response. This indicates that the actual protein expression of these receptors is absent or too low to contribute to any of the compound responses measured here.

Furthermore, a CB$_2$R-selective antagonist, aminoalkylindole AM630 was tested as well to confirm that agonist responses were indeed CB$_2$R-mediated. While AM630 gave little to no response on its own, it was able to significantly block responses of all agonists at a concentration of $100 \times$ K$_i$. The level of blockade did not differ significantly between agonists, irrespective of their receptor selectivity (Fig. 3D). Furthermore, comparable AM630 effects were observed on LCLs from other individuals. For instance, AM630 showed strong inhibition with a clear concentration-effect relationship that did not differ in potency between the five individuals tested and ranged from 6.76 ± 0.04 to 6.90 ± 0.05 (Fig. 3E).

Finally, the effect of pertussis toxin (PTX) pre-treatment was investigated to confirm downstream signaling through Gα_i. PTX caused a significant decrease in cellular responses of all three agonists for individual 4, which was to a similar degree as AM630

(Fig. 3F). In addition, inhibition of the agonist JWH133 by PTX was strong in all five individuals, with some differences in the level of remaining effects ranging from 7.6 ± 3.6% up to 35.5 ± 8.9% (Fig. 3G). Taken together, the agonist, antagonist and PTX effects confirm that CB$_2$R signaling can be measured sensitively and specifically in these LCLs.

3.2. Individual differences in CB$_2$R synthetic agonist responses in LCLs

Following the confirmation that cellular effects were specifically CB$_2$R-related, agonist concentration-effect curves were studied on LCLs from five individuals. Individuals 2 and 3 are the parents of individuals 4 and 5, their monozygotic twin children, while individual 1 is unrelated. Examining their genotypes from DNA sequence data revealed that individual 1 is a homozygote for the minor allele (genotype *GG* thus *R63*) for Q63R polymorphism (rs35761398), while individuals 2 and 3 are heterozygotes and individuals 4 and 5 are homozygotes for the major allele (genotype *AA* thus *Q63*) (see also Table 2, 3), representing the most common genotype among the human population (http://www.ncbi.nlm.nih.gov/SNP/).

Table 2
Individual potencies of all CB_2R (partial) agonists. Statistically significant differences between individuals were determined by two-way ANOVA with Fisher's LSD post-hoc test. * = p < 0.05; ** = p < 0.01; **** = p < 0.0001.

Individual	JWH133 $pEC_{50} \pm SEM$	WIN55212-2 $pEC_{50} \pm SEM$	CP55940 $pEC_{50} \pm SEM$	2-AG $pEC_{50} \pm SEM$	AEA $pEC_{50} \pm SEM$	GW405833 $pEC_{50} \pm SEM$	BAY59-3074 $pEC_{50} \pm SEM$
1 (Unrelated; R63)	7.73 ± 0.06	8.73 ± 0.09	8.12 ± 0.06 ****to all	5.98 ± 0.02 *to 2,4	6.76 ± 0.08	7.99 ± 0.09 *to 2 **to 3	7.67 ± 0.09 *to 4 ****to 2,5
2 (Parent 1; Q/R63)	7.82 ± 0.07[a]	8.65 ± 0.12	8.93 ± 0.05 ****to 1	5.65 ± 0.06 *to 1,5 ****to 3	6.91 ± 0.09 *to 3	7.69 ± 0.09 *to 1,5	8.27 ± 0.10 *to 4,5 **to 3 ****to 1
3 (Parent 2; Q/R63)	7.71 ± 0.04[a]	8.75 ± 0.11	8.97 ± 0.05 ****to 1	6.19 ± 0.09 ****to 2,4	6.64 ± 0.05 *to 2	7.56 ± 0.15 **to 1,5	7.92 ± 0.08 **to 2, ****to 5
4 (Twin 1; Q63)	7.90 ± 0.08 *to 5	8.62 ± 0.18	8.90 ± 0.04 ****to 1	5.69 ± 0.02 *to 1,5 ****to 3	6.75 ± 0.07	7.76 ± 0.08	8.00 ± 0.04 *to 1,2 ****to 5
5 (Twin 2; Q63)	7.67 ± 0.05 *to 4	8.72 ± 0.23	9.11 ± 0.03 ****to 1	5.95 ± 0.05 *to 2,4	6.76 ± 0.16	7.95 ± 0.13 *to 2 **to 3	8.54 ± 0.12 *to 2 ****to 1,3,4

[a] JWH133 curves for individuals 2 and 3 have been previously established [32] and data were incorporated to allow direct comparison.

Table 3
Individual efficacies of all CB_2R (partial) agonists. All efficacies are in reference to the standard CB_2R-selective agonist JWH133 tested on the individual that was the only minor homozygote for Q63R (R63), individual 1, with exception of 2-AG which was normalized to maximal 2-AG effect on individual 1 as explained in text. Statistically significant differences between individuals were determined by two-way ANOVA with Fisher's LSD post-hoc test. * = p < 0.05; ** = p < 0.01; *** = p < 0.001, **** = p < 0.0001.

Individual	JWH133 $E_{max} \pm SEM$	WIN55212-2 $E_{max} \pm SEM$	CP55940 $E_{max} \pm SEM$	2-AG $E_{max} \pm SEM$	AEA[a] $E_{max} \pm SEM$	GW405833[a] $E_{max} \pm SEM$	BAY59-3074[a] $E_{max} \pm SEM$
1 (Unrelated; R63)	**100 ± 4.2** *to 4 **to 3 ***to 2	110.1 ± 5.4 *to 4,5 ****to 2,3	50.1 ± 3.2 *to 4	100 ± 13.2 ****to 2,3	26.8 ± 2.3 **to 3	53.8 ± 1.5 *to 2,4,5 **to 3	71.9 ± 5.9 *to 2,4 **to 3,5
2 (Parent 1; Q/R63)	62.4 ± 1.8[b] ***to 1,5	38.5 ± 5.3 ***to 4 ****to 1,5	39.3 ± 6.3 ***to 4	47.5 ± 7.1 **to 5 ***to 4 ****to 1	18.2 ± 2.1 ***to 3	28.4 ± 6.0 *to 1	50.5 ± 11.9 *to 1
3 (Parent 2; Q/R63)	72.5 ± 7.7[b] **to 1,5	42.4 ± 4.3 ***to 4 ****to 1,5	47.0 ± 5.8 **to 4	27.8 ± 3.1 ****to 1,4,5	58.7 ± 9.4 ***to 1 ***to 2 ****to 4,5	24.9 ± 3.1 **to 1	39.2 ± 1.3 **to 1
4 (Twin 1; Q63)	77.9 ± 4.1 *to 1,5	77.3 ± 9.9 *to 1 ***to 2,3	74.1 ± 6.8 *to 1,5 **to 3 ***to 2	82.9 ± 4.4 ***to 2 ****to 3	9.5 ± 2.3 ****to 3	27.6 ± 3.9 *to 1	46.4 ± 3.8 *to 1
5 (Twin 2; Q63)	98.8 ± 10.9 *to 4 **to 3 ***to 2	83.2 ± 13.3 *to 1 ****to 2,3	52.4 ± 10.9 *to 4	81.0 ± 18.4 **to 2 ****to 3	9.8 ± 1.7 ****to 3	26.9 ± 1.7 *to 1	38.9 ± 3.5 **to 1

[a] As opposed to the full agonists, experiments with partial agonists AEA, GW405833 and BAY59-3074 were performed with more coating (Fibronectin 50 μg/ml) and higher cell density (100,000 cells/well) in order to obtain a sufficient window.
[b] JWH133 curves for individuals 2 and 3 have been previously established [32], data were incorporated to allow direct comparison.

First, full concentration-response curves were made for three compounds, typically referred to as full agonists, from different chemical classes, JWH133, WIN55212-2 and CP55940. Example xCELLigence traces of the JWH133 concentration-effect relationship are given in Fig. 4A. The resulting concentration-effect curves are summarized in Fig. 4B–D. Corresponding pEC_{50} values are summarized in Table 2 while E_{max} values are given in Table 3.

As can be observed from the curves and pEC_{50} values (Table 2), potencies for the three agonists were similar for all individuals, with a notable exception for CP55940 on individual 1 (Fig. 4D). For this individual, who is the only minor homozygote for Q63R, CP55940 showed a significantly increased EC_{50} value of approximately 10-fold. In contrast, the efficacy of all three agonists was much more divergent on the different cell lines. Interestingly, WIN55212-2 which showed no significant differences in potency, showed a significant spread in efficacy corresponding to genotype (Fig. 4C, Table 3). WIN55212-2 had the lowest efficacy on the two heterozygous individuals 2 and 3, which in fact made it a partial agonist on these cell lines in comparison to JWH133 (Table 3). For the other three individuals, WIN55212-2 had a similar efficacy to JWH133, and both compounds had the highest efficacy on the LCLs of individual 1. The two synthetic cannabinoids JWH133 and CP55940 showed differences in efficacy that did not correlate with genotype. However, compared to JWH133, CP55940 had a lower efficacy in all individuals making it a partial agonist, with exception of individual 4. Even on individual 1 CP55940 was a partial agonist, where for all other tested agonists the highest efficacy

was found. Taken together, CP55940 was the only synthetic agonist with clear individual differences related to genotype (i.e. a decreased potency and efficacy in presence of R63), while aminoalkylindole WIN55212-2 was the least prone to individual variation.

3.3. Endogenous agonist induces different cellular response than synthetic agonists

To test whether signaling caused by endogenous agonists also showed individual differences, the response induced by the two main endogenous CB_2R ligands, eicosanoid 2-AG and AEA, known as full and partial CB_2R agonists respectively, were examined. In order to allow a sufficient response window to characterize partial agonist AEA, conditions were optimized by seeding more cells (100,000 cells/well) and coating with more fibronectin (50 μg/ml). Both full agonist JWH133 and 2-AG were also tested under these adjusted conditions, and the responses of JWH133 were used as reference compound to determine the level of partial agonism. Interestingly, the resulting real-time trace differed significantly from all synthetic agonists, as shown in Fig. 5A, B. While all synthetic agonists induced an immediate positive impedance change, which was characterized by a fast peak and subsequent decline to baseline in around 30 min, the endogenous 2-AG induced a negative change in impedance with a much slower onset after about 20 min, and a much more prolonged response that still persisted after 180 min (Fig. 5A). Interestingly, AEA showed a similar time-
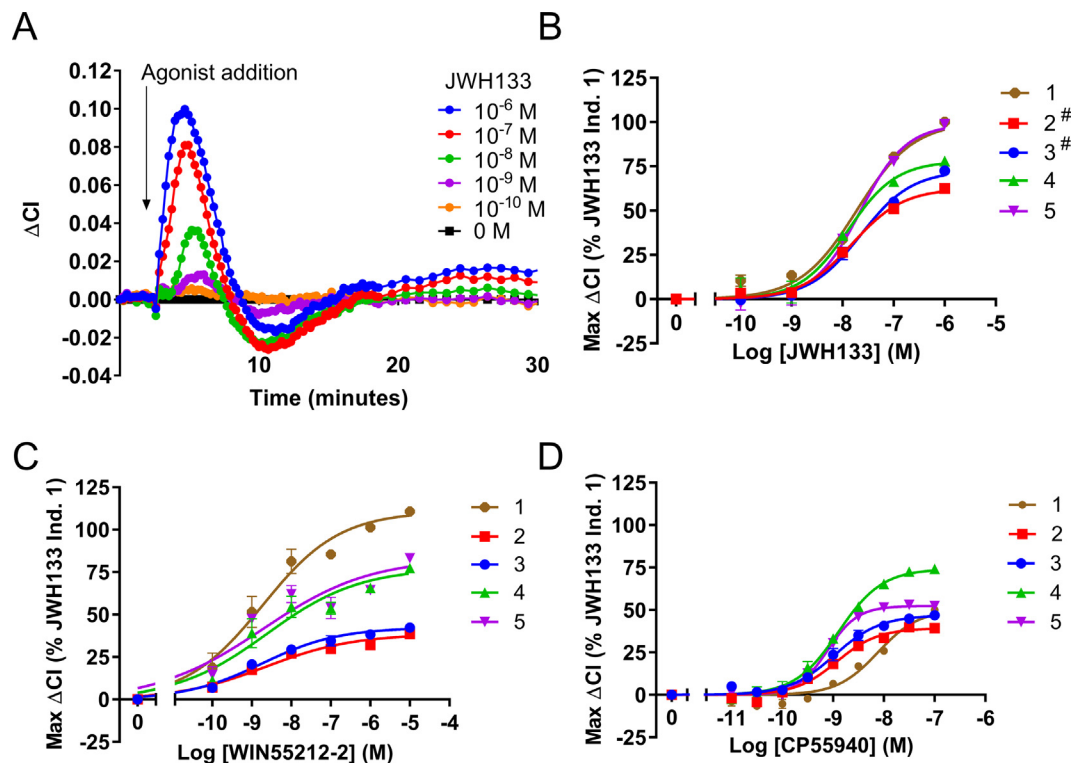
Fig. 4. Individual CB_2R responses to full agonists of three distinct chemical classes. Cell lines were stimulated with different concentrations of full agonist JWH133, WIN55212-2 or CP55940 18 h after seeding (50,000 cells/well). (A) Representative graph of the baseline-corrected JWH133 response [1 μM – 100 pM] from individual 1. Concentration-effect curves of all individuals (1–5) of (B) classical cannabinoid JWH133 (C) aminoalkylindole WIN55212-2 and (D) non-classical cannabinoid CP59940 obtained from Max Δ CI, normalized to E_{max} of CB_2R-selective agonist JWH133 response on individual 1. Data represent mean ± SEM obtained from three or four independent experiments performed in duplicate. #JWH133 curves for individuals 2 and 3 have been previously established [32] but were incorporated to allow direct comparison.

profile as 2-AG with slower onset and prolonged response, but induced a positive impedance change like the synthetic cannabinoids, albeit with a different shape (Fig. 5B). Thus, endogenous agonist signaling through CB_2R lead to vastly different cellular changes than any of the synthetic agonists. To confirm whether these effects were also CB_2R-mediated, we showed that the 2-AG response is blocked by CB_2R-selective antagonist AM630, similar to the synthetic agonists (Fig. 5E). Moreover, downstream signaling via $G\alpha_i$ was inhibited by PTX pre-treatment as well (Fig. 5F). Of note, AM630 blockade and PTX inhibition did not differ significantly between individuals, even with opposing Q63R genotype, as demonstrated in the LCLs of individuals 1 and 4 (Fig. 5E, F).

Furthermore, the concentration-effect relationship of 2-AG showed significant differences between the five individuals, which were within half a log-unit and therefore smaller than those observed for CP55940. However, these differences in potencies were not consistent with the presence of Q63R (Fig. 5C, Table 2). Interestingly, the differences in efficacy of 2-AG were consistent with genotype (Table 3), as the efficacy in heterozygous individuals 2 and 3 was significantly lower than for all other individuals. Any differences observed for AEA were not CB_2R genotype-related (Fig. 5D, Table 2, 3). In summary, especially signaling by the main CB_2R endogenous ligand 2-AG lead to different cellular changes as opposed to synthetic agonists, and showed a genotype effect on efficacy as it appeared to be highest in the *R63* homozygote, but lowest in Q63R heterozygotes.

3.4. Partial agonist responses differ between individuals

Subsequently, two partial CB_2R agonists were tested on all five individuals to investigate the presence of any differences in individual effects possibly linked to the Q63R genotype. Once again,

conditions were adjusted to more cells (100,000 cells/well) and fibronectin (50 μg/ml) to allow a sufficient response window for these partial agonists. JWH133 was also tested under these adjusted conditions as reference compound to determine the level of partial agonism. The two partial agonists tested were aminoalkylindole GW405833 and BAY59-3074, which belongs to a separate chemical class (Fig. 1). In all individuals, both agonists induced positive impedance responses like the synthetic full agonists, and demonstrated clear partial agonistic behavior in comparison to JWH133, irrespective of genotype (Fig. 6A and B). The concentration-effect curves are represented in Fig. 6C and D, while the resulting pEC_{50} and E_{max} values are summarized in Tables 2 and 3, respectively. GW405833 showed significant differences in potency which were within half a log-unit and were not entirely consistent with genotype. However, the individual potencies for BAY59-3074 showed a larger spread close to a full log-unit. The lowest potency was observed on individual 1, though this statistical difference was not genotype consistent. In terms of efficacy, BAY59-3074 had a higher efficacy than GW405833 for all individuals. Interestingly, the E_{max} value of GW405833 on the LCLs of individual 1 (i.e. presence of *R63*) was significantly higher than that on all other individuals (Table 3), which was also observed for BAY59-3074. Taken together, the partial agonists showed personal differences in response, which (in part) appeared to be compound specific and less pronounced for the aminoalkylindole GW405833.

4. Discussion

CB_2R is considered a potential therapeutic target for immune system related disorders such as multiple sclerosis and allergy [45], neuropathic pain [46], cancer and osteoporosis [1,45]. As genetic differences between individuals can induce large variations
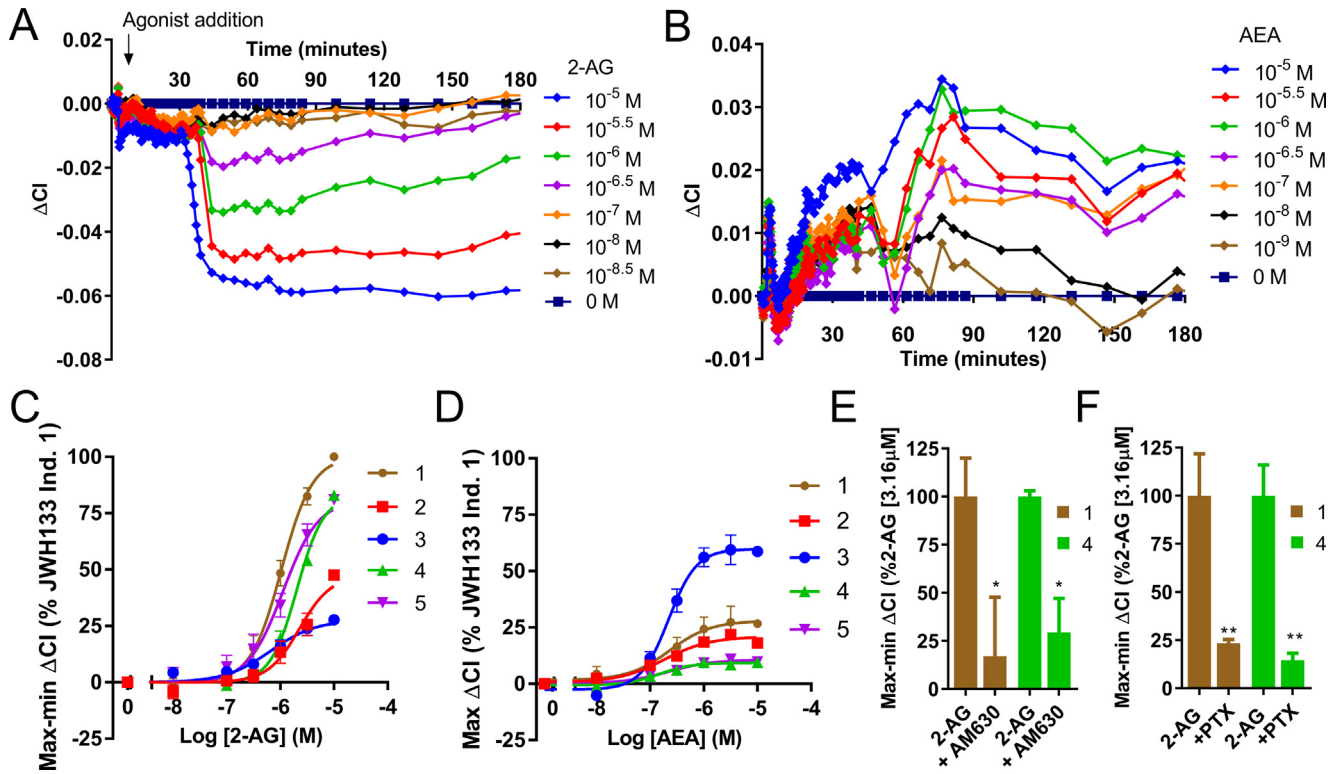
Fig. 5. Individual CB$_2$R responses to endocannabinoids. Cell lines were stimulated with 2-AG (50,000 cells/well, fibronectin 10 µg/ml) or AEA (100,000 cells/well, fibronectin 50 µg/ml) 18 h after seeding. Representative graphs of the baseline-corrected (A) 2-AG [10 µM – 3.16 nM] and (B) AEA [10 µM – 1 nM] response from individual 1. (C) Concentration-effect curves of 2-AG were obtained from Max-Min Δ CI within 1 h of stimulation were normalized to E$_{max}$ on individual 1. (D) Concentration-effect curves of AEA were obtained from Max Δ CI normalized to E$_{max}$ of CB$_2$R-selective agonist JWH133 response on individual 1. Next, bar graphs show the inhibition of the 2-AG effect by (E) CB$_2$R-selective antagonist AM630 [10 µM] and (F) Gα_i-inhibitor PTX normalized to 2-AG's effect at EC$_{80}$ (3.16 µM). Data represent the means ± SEM from three or four (C, D) or means ± SD of two (E, F) independent experiments performed in duplicate. Significance of inhibitor effect versus 2-AG response only was determined with a two-way ANOVA Fisher's LSD test * = p < 0.05, ** = p < 0.01. AM630 and PTX inhibition did not differ significantly between individuals 1 (*R63*) and 4 (*Q63*) as determined using a two-way ANOVA with a Sidak post-hoc test.
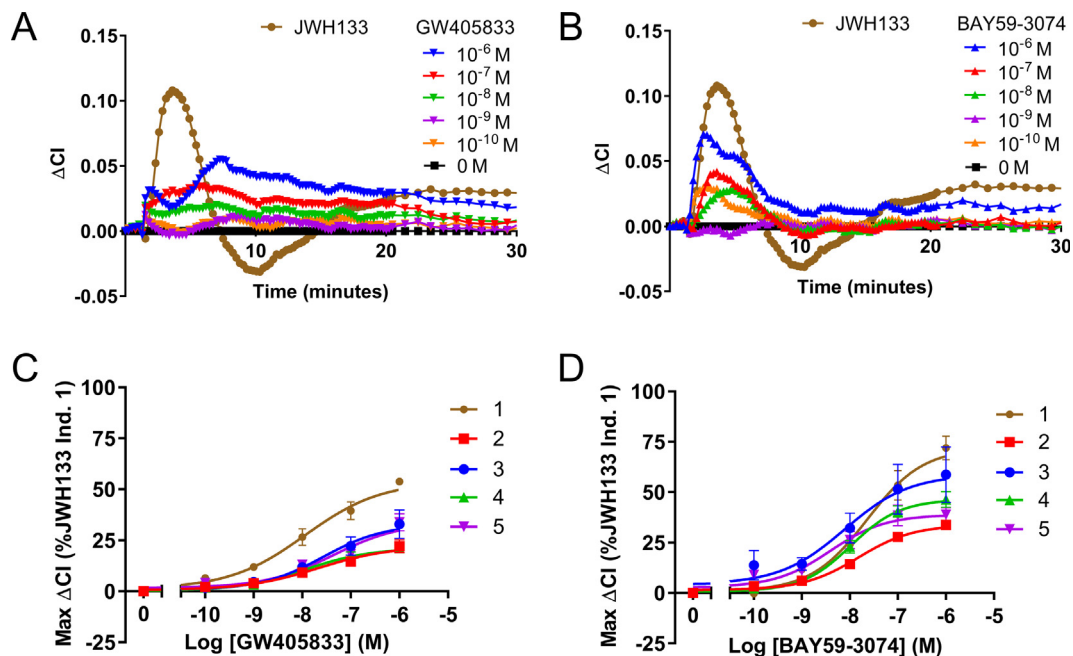

Fig. 6. Individual CB$_2$R responses from two partial agonists. Cell lines were stimulated with agonist 18 h after seeding (100,000 cells/well, fibronectin 50 µg/ml). Representative graph of the baseline-corrected response to (A) GW405833 and (B) BAY59-3074 [1 µM – 100 pM] from individual 1. Resulting concentration-effect curves of (C) GW405833 and (D) BAY59-3074 obtained from peak Δ CI normalized to JWH133 [1 µM] effect on individual 1. Data represent mean ± SEM obtained from three independent experiments performed in duplicate.

in drug response, we studied such personal effects on a variety of CB$_2$R ligands with a panel of personal cell lines, the LCLs, from individuals with varying CB$_2$R genotypes. These included genetically unrelated individuals as well as monozygotic twins, who are deemed genetically identical. Hence, confirming the comparability of their responses is a standard way to control for genotype-unrelated effects [27,47]. The individuals in this study represent all possible genotypes for the polymorphism Q63R. Even though this polymorphism is present in roughly half of the population and thus is extremely common, it has also been associated with various pathological disorders [17–19,22–25]. This makes characterizing the impact of this polymorphism on drug responses an important issue for CB$_2$R drug discovery.

We characterized the genotype-effect on responses of several individuals by applying label-free cellular assay technology, namely the impedance-based xCELLigence apparatus. Such technologies allow sensitive non-invasive assays that enable the investigation of GPCRs in endogenous cell systems, including LCLs for which we recently published an optimized protocol [32]. The combination of such a non-invasive assay with a personal cell line offers many advantages over traditional GPCR methodologies. In general, potencies of all CB$_2$R compounds tested in our research on the LCLs were within one log-unit range of previously published values (Table 2) [40,41]. Notable exceptions were 2-AG and GW405833, which differed from published pEC$_{50}$ values by up to 17-fold (pEC$_{50}$ of 6.91 by Gonsiorek et al. [48]) and 43-fold (pEC$_{50}$ of 9.19 ± 0.09 by Valenzano et al. [49]), respectively. This discrepancy is most likely due to differences in cell lines and assay type. Valenzano et al. [49] used a typical endpoint cAMP accumulation assay in combination with a CHO-K1 system overexpressing recombinant CB$_2$R, while LCLs represent a more physiological cell system with endogenous receptor expression. Furthermore, rather than just being a human cell line with endogenous expression, LCLs are even one step closer to the physiological situation as they are directly derived from individual persons. The use of a label-free whole-cell assay is preferable over typical endpoint assays to minimize bias [50], especially when investigating a GPCR with functional selectivity such as the CB$_2$R, in which multiple pathways can be activated to a different extent [51,52].

Before starting CB$_2$R functional investigations in LCLs, we studied expression levels and screened functional responses to confirm receptor subtype presence. All cannabinoid receptors are expressed in LCLs at mRNA level (Fig. 2) with some differences between individuals. However, these did not correspond to the general differences we observed in compound potency or efficacy (Tables 2, 3). For example for CB$_2$R, mRNA expression differed for individual 1, especially as opposed to individual 4. However, both individuals were among the highest responders on average for CB$_2$R compounds (Table 3). Furthermore, most individuals showed high GPR18 mRNA levels, but AM251 which targets GPR18 and GPR55 but not CB$_2$R, showed no response (Fig. 3) [48]. This indicates that functional GPR18 levels were in fact not high, if at all present in these LCLs, which shows that mRNA expression levels do not necessarily correlate with functional protein expression on the cellular membrane, a feature well appreciated in literature [53,54]. Taken together, the data shown in Fig. 3 prove that CB$_2$R is in fact the major receptor responsible for compound responses, which is in accordance with previous literature that states CB$_2$R is the highest expressed receptor in LCLs [28]. Of note, any of the full agonists tested in this manuscript such as WIN5512-2, JWH133, CP55940 and 2-AG are not known as agonists of GPR18 [44].

After confirming that CB$_2$R is well expressed in LCLs and that CB$_2$R signaling can be measured sensitively and specifically in LCLs (Figs. 2, 3), we characterized responses of five individuals to various CB$_2$R ligand types and classes (Fig. 1) which revealed that certain chemical classes of compounds were more sensitive to genotype than others (Figs. 4–6, Tables 2, 3). All tested aminoalkylindole compounds as well as the classical cannabinoid JWH133 showed the least differences between individuals, in comparison to compounds of other chemical classes. The notion that aminoalkylindole compounds showed the least genotype-related effects was strengthened by testing three pharmacological types of ligands of this chemical class. Similar to the aminoalkylindole agonist, no individual differences were observed for the CB$_2$R-selective antagonist AM630 (Fig. 3D). Even a partial agonist of this class (GW405833) was less prone to individual differences than a partial agonist of another class. It has been suggested that partial agonists are more sensitive to system-related differences in receptor function, for instance receptor expression or downstream coupling, than full agonists or antagonists [55]. Consequently, they may be more prone to genotype-related effects. In fact, we have demonstrated in a previous publication that a partial agonist on the adenosine A$_{2A}$ receptor showed a clear genotype-related difference in LCLs, while full agonists did not [33]. The two synthetic partial agonists for the CB$_2$R that we tested here exhibited similar sensitivity (Fig. 6, Tables 2, 3). In efficacy, they showed the clearest genotype-related effect as it was only significantly elevated for the R63 individual, as opposed to the full agonists where more individuals differed.

Overall, CP55940 showed the most pronounced personal effects with highly reduced potency and efficacy in presence of R63, while all other agonists and partial agonists showed the highest efficacy in presence of this genotype. Interestingly, Q63R has been reported to cause diminished WIN55212-2 efficacy in HEK293hCB$_2$R cells while CP55940 was not affected [56]. Our results contradict these findings, which may be due to the difference in model systems used. HEK293 cells are recombinant and receptor-overexpressing, whereas LCLs are personal cell lines with endogenous levels of receptor expression, and therefore may represent a more physiologically relevant system.

When investigating genotype effects on endogenous cannabinoid response, we noted that 2-AG showed vastly different cellular effects than any other ligand tested here, despite being clearly CB$_2$R-mediated (Fig. 5). Another endocannabinoid, AEA, showed a similarly changed time-profile as 2-AG, even though the direction of impedance change was more similar to synthetic cannabinoids. These differences in cellular effects between endogenous and synthetic cannabinoids may originate from downstream signaling differences resulting in a different cellular response as measured by xCELLigence. For instance, Shoemaker et al. [51] found that 2-AG was a more potent activator of MAPK whereas synthetic ligands more potently inhibited adenylyl cyclase activity. Moreover, our experiments with 2-AG do not suggest that Q63R influences its responses, which contrasts with previous reports of Carrasquer et al. [56] and Ishiguro et al. [17], where recombinant overexpressing cell systems, HEK293 and CHO cells, were used. However, our findings are confirmed by Sipe et al. [8] who used a more physiological setting of T-lymphocytes, as is the case in this study. Taken all of the above together, this once more highlights the importance of using primary or derived (i.e. endogenous immortalized) cell systems that offer more physiological relevance versus recombinant systems.

There are several mechanisms by which a polymorphism may influence receptor signaling. Q63R in the CB$_2$R results from a dinucleotide conversion of AA to GG that exchanges a glutamine for an arginine at position 63 in the intracellular loop 1, and as such it is not in proximity of the putative CB$_2$R ligand binding site [56,57]. Therefore, its position suggests that Q63R does not directly influence ligand binding. Rather, its effects on drug responses may originate from differences in downstream signaling [17,56]. CB$_2$R has been shown to signal through multiple pathways such as

cAMP, β-arrestin, pERK and GIRK, to which various agonists may be differently biased [30,58,59]. Moreover, it has been well established that agonists can activate the various G protein-dependent and –independent pathways modulated by CB_2R to a different extent [51,52]. In our LCLs, all CB_2R agonists signaled strongly through $G\alpha_i$ coupling as was demonstrated by potent inhibition through PTX (Figs. 3, 5), which on some instances showed differences in the levels of remaining response (Fig. 3D). While $G\alpha_i$ signaling therefore clearly represents the predominant signaling pathway for CB_2R in all individuals, the varying remaining responses could indicate individual differences in coupling to other signaling pathways. Hence, Q63R related differences observed between CP55940 and other agonists may be related to their specific bias. Q63R could potentially affect coupling to one signaling pathway more than others, an effect which is then only noted for agonists that preferably and potently activate that pathway, in this case CP55940. Alternatively, Q63R could affect the bias of a particular ligand as CP55940 towards different signaling pathways.

Another interesting genotype-related effect was that in overall efficacy (Table 3), *R63* homozygous individual 1 generally ranked highest. Q63R heterozygotes (ind. 2 and 3) appeared to have the lowest efficacy for CB_2R agonists, even compared to *Q63* homozygotes (ind. 4 and 5), rather than an intermediate or mixed cellular effect. This was most pronounced for WIN55212-2 and 2-AG (Table 3). The effect could arise from, for instance, a difference in signaling pathway bias between the two receptor forms. In a heterozygote, where both receptor forms are present that each have different efficiencies in pathway-coupling, the overall signaling and cellular effect may be lower as opposed to either receptor form as homozygote, that works synergistically.

In conclusion, our results demonstrate that aminoalkylindole compounds exhibited the least sensitivity to genotypes while non-classical cannabinoid CP55940 showed the most. *R63* genotype influenced CB_2R ligand effects leading to higher efficacy of agonists and especially partial agonists, but decreased potency and efficacy of the non-classical cannabinoid CP55940, which was also the most pronounced 'personal' effect measured here. The LCLs, as personal cell lines, in combination with the sensitive label-free impedance-based technology have the potential to represent a more physiologically relevant model system to investigate individual differences in drug response. Their combination provided novel insights into the impact of CB_2R polymorphism on drug response, which demonstrates on the one hand the ability of this phenotypic screening method to identify 'blockbuster' drug candidates that are less prone to individual differences. On the other hand, this approach may advance precision medicine and stratify patient groups. Altogether, this will help in reducing attrition rates of drugs in clinical trials.

Disclosure declaration

The authors declare that no competing interests exist.

Acknowledgements

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