A complete endocannabinoid signaling system modulates synaptic transmission between human induced pluripotent stem cell-derived neurons

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**Abbreviations**: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; 2-AG, 2-arachidonoyl glycerol; CB₁/₂Rs, cannabinoid type 1 and 2 receptors; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; DAG, diacylglycerol; DHPG, dihydroxyphenylglycine; DO34, (3-(Phenylmethyl)-4-[[4-[(4-(trifluoromethoxy)phenyl)-1H-1,2,3-triazol-1-yl]carbonyl]-1-piperazinecarboxylic acid, 1,1-dimethylethyl ester, (2-Benzyl-4-[[2-(2-methyl-2-propyl)oxy]carbonyl]piperazinyl)[4-[(4-trifluoromethoxy)phenyl]-1H-1,2,3-triazol-1-yl]methanone); eCB, endocannabinoid; ECS, eCB system; [Mg²⁺]₀, extracellular Mg²⁺ concentration; HBSS, HEPES buffered Hanks' salt solution; hiPSC, human induced pluripotent stem cell; [Ca²⁺]ᵢ, intracellular Ca²⁺ concentration; MK801, [(+)-5-methyl-10,11-dihydroxy-5H-dibenzo(a,d)cyclohepten-5,10-imine]; NESS 0327, N-piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo [6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide]; Δ⁹-THC, Δ⁹-tetrahydrocannabinol; Win-2, [R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo [1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate] (WIN 55,212-2)

**Key words**: human induced pluripotent stem cells, CB₁ receptor, 2-arachidonoyl glycerol, endocannabinoid, diacylglycerol lipase, monoacylglycerol lipase, GRABₑCB₂,₀ sensor
Abstract

The endocannabinoid system (ECS) modulates synaptic function to regulate many aspects of neurophysiology. It adapts to environmental changes and is affected by disease. Thus, the ECS presents an important target for therapeutic development. Despite recent interest in cannabinoid-based treatments, few preclinical studies are conducted in human systems. Human induced pluripotent stem cells (hiPSCs) provide one possible solution to this issue. However, it is not known if these cells have a fully functional ECS. Here we show that hiPSC-derived neuron/astrocyte cultures exhibit a complete ECS. Using Ca\(^{2+}\) imaging and a genetically-encoded endocannabinoid sensor we demonstrate that they not only respond to exogenously applied cannabinoids but also produce and metabolize endocannabinoids. Synaptically driven [Ca\(^{2+}\)]i spiking activity was inhibited (EC\(_{50}=48\pm13\) nM) by the efficacious agonist Win55,212-2 ([R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol [1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate]) and by the endogenous ligand 2-arachidonoyl glycerol (2-AG; EC\(_{50}=2.0\pm0.6\) µM). The effects of Win55212-2 were blocked by a CB\(_1\) receptor-selective antagonist. Δ\(^9\)-tetrahydrocannabinol acted as a partial agonist, maximally inhibiting synaptic activity by 47±14% (EC\(_{50}=1.4\pm1.9\) µM). Carbachol stimulated 2-AG production in a manner that was independent of Ca\(^{2+}\) and blocked by selective inhibition of diacylglycerol lipase. 2-AG returned to basal levels via a process mediated by monoacylglycerol lipase as indicated by slowed recovery in cultures treated with JZL-184 (4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester). Win 55,212-2 markedly desensitized CB\(_1\) receptor function following a 1 d exposure, while desensitization was incomplete following 7 d treatment with JZL-184. This human cell culture model is well suited for functional analysis of the ECS and as a platform for drug development.
Significance Statement

Despite known differences between the human response to cannabinoids and that of other species, an in vitro human model demonstrating a fully functional endocannabinoid system has not been described. hiPSCs can be obtained from skin samples and then reprogrammed into neurons for use in basic research and drug screening. Here we show that hiPSC-derived neuronal cultures exhibit a complete endocannabinoid system suitable for mechanistic studies and drug discovery.
Introduction

The endocannabinoid (eCB) system (ECS) regulates processes ranging from appetite and emesis to mood and memory (Lowe et al., 2021; Lu and Mackie, 2021). As our understanding of the ECS in health and disease continues to develop, there is increasing interest in this system as a therapeutic target (Di Marzo, 2018; Wilkerson et al., 2021). The ECS presents several sites for drug action. It is composed of cannabinoid type 1 and 2 receptors (CB$_{1/2}$R), eCB ligands, and lipases that produce eCBs on demand from membrane lipids or hydrolyze them to terminate signaling.

CB$_1$Rs are primarily expressed in neurons and are widely distributed throughout the CNS (Glass et al., 1997). The principal active ingredient in cannabis, Δ$^9$-tetrahydrocannabinol (Δ$^9$-THC ), exerts its psychoactive effects via CB$_1$R (Matsuda et al., 1990). CB$_1$R agonists are approved for use as antiemetics and appetite stimulants (Garcia and Shamliyan, 2018) and show potential for treating seizures (Rosenberg et al., 2017), pain (Fowler, 2021), and anxiety (deRoon-Cassini et al., 2020). On-target adverse effects have generated considerable interest in developing agonists biased towards desired therapeutic signaling pathways (Leo and Abood, 2021). Much of the preclinical work on CB$_1$R ligands is performed on rodent neurons because human brain tissue is relatively unavailable. However, rodent models have limitations. For example, ECS neurodevelopment differs between rodents and humans influencing the effects of cannabinoids on synaptic plasticity (Bara et al., 2021), and human neurons are more sensitive than rodent neurons to seizure-causing drugs (Tukker et al., 2020). It is of particular importance to pharmacological studies that human and rodent CB$_1$Rs differ in their distribution and their affinities to a range of ligands (McPartland et al., 2007).

In vitro models with a complete ECS are useful for understanding how ECS components are affected by CB$_{1/2}$R ligands as well as how the ECS adapts to disease conditions. For example, the ECS is affected by and modulates stress in the human brain (deRoon-Cassini et al., 2020). Furthermore, when the ECS as a whole is considered as a therapeutic target,
modulating eCB metabolism emerges as an important strategy which preserves the spatial and temporal aspects of endogenous signaling. Indeed, clinical trials for inhibitors of metabolic enzymes, which are thought to be less likely than receptor agonists to produce tolerance or psychoactive side effects, are ongoing (Egmond et al., 2020). While exciting progress has been made, obstacles to clinical drug development remain. In vitro models suitable for long term study are lacking and human models with a fully functional ECS have not been described.

Human induced pluripotent stem cells (hiPSCs) are one potential solution to this problem. hiPSCs are derived from somatic cells, such as skin fibroblasts, which can be obtained much more easily and less invasively than brain tissue. Exogenous expression of factors necessary for pluripotency reprograms fibroblasts into pluripotent stem cells, capable of differentiating into all three embryonic germ layers (Takahashi et al., 2007). These iPSCs are then differentiated into cultures resembling target cell type(s). iPSC-derived neurons offer a minimally invasive in vitro human model for basic research and drug development, with the potential for long-term and high-throughput studies.

hiPSC-derived neuronal cultures therefore show promise as a tool for studying the ECS. Previous studies have shown that CB1R is expressed in hiPSCs and hiPSC-derived neurons (Bobrov et al., 2017) and that ∆9-THC can affect development (Stanslowsky et al., 2017) and gene expression (Guennewig et al., 2018) in these cells. In cortical spheroids derived from hiPSCs, the components of the ECS are expressed and CB1Rs appear functional because rimonabant, an inverse agonist of CB1Rs, altered the development of excitatory synapses (Papariello et al., 2021). However, to our knowledge there have been no functional studies of cannabinoid agonists on neural activity in hiPSC-derived neurons, and whether the components of an ECS beyond CB1Rs are functional is not known.

In this study, we demonstrate the presence of a fully functional ECS in a commercially available line of hiPSC-derived neuron/astrocyte cultures. In this model system CBs modulate synaptic activity, and 2-AG is synthesized by diacylglycerol (DAG) lipase upon stimulation and
metabolized via monoacylglycerol (MAG) lipase. We characterize the Ca^{2+} sensitivity of 2-AG synthesis, demonstrating the utility of these cultures for mechanistic studies. We also compare a receptor agonist to a MAG lipase inhibitor for their ability to desensitize CB_{1}R-mediated inhibition of synaptic activity, showing the feasibility of long term (7d) treatment protocols with these cultures. This human cell culture model is well suited for functional analysis of the ECS and for screening drugs for actions on its components.
Materials and Methods

Reagents

Penicillin/streptomycin (catalog number: 15140) was from Thermo Fisher Scientific (Carlsbad, CA, USA); BrainPhys Neuronal Medium with SM1 (catalog number: 05792), human recombinant brain-derived neurotrophic factor (hBDNF; catalog number: 78005), and human recombinant glial cell-derived neurotrophic factor (hGDNF; catalog number: 78058) were from Stem Cell Technologies (Vancouver, BC, Canada); NeuralX medium with cortical supplement (catalog number: 500005-250) was from StemoniX (Maple Grove, MN, USA); HEPES (catalog number: H4034), calcium chloride (CaCl₂, catalog number: C3881), and WIN 55,212-2 (catalog number: 131543-23-2) were from Millipore Sigma (St. Louis, MO, USA); 2-arachidonoyl glycerol (2-AG, catalog number: 62610), NESS 0327 (catalog number: 10004184), JZL 184 (catalog number: 13158) and Win 55,212-2 (Win-2, catalog number: 10009023) were from Cayman Chemical (Ann Arbor, MI, USA); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, catalog number: 1045), dihydroxyphenylglycine (DHPG, catalog number: 0805), carbachol (catalog number: 2810), and MK 801 (catalog number: 0924) were from Tocris Biosciences (Minneapolis, MN, USA); FLIPR Calcium 6 dye (catalog number: R8190) was from Molecular Devices (San Jose, CA, USA); Δ9-tetrahydrocannabinol (Δ9-THC ) was from the National Institute on Drug Abuse Drug Supply Program (Research Triangle Institute, Research Triangle Park, NC, USA). DMEM/F12 medium (catalog number: 11330-032), Neurobasal Medium (catalog number: 21103-049), B27 Supplement (catalog number: 17504-044), N2 Supplement (catalog number: 17502-048) GlutaMax (catalog number: 35050-061) were from Life Technologies (Carlsbad, CA, USA). Matrigel (catalog number: 356273) was from Corning (Glendale, AZ, USA). For experiments using cells obtained from BrainXell, brain-derived neurotrophic factor (BDNF, catalog number: 450-02), glial cell derived neurotrophic factor (GDNF, catalog number: 450-10), and transforming growth factor beta 1 (TGF-β1, catalog number: 100-21C) were from PeproTech (East Windsor, NJ, USA).
Human iPSC-derived neuronal cultures

Human iPSC-derived neuron/astrocyte cultures in 96-well plates (catalog number: BCARX-AA-0096) were obtained from StemoniX, Inc. (Maple Grove, MN, USA) and received 8-10 weeks after plating. Upon receipt, transfer medium was replaced with BrainPhys medium supplemented with 1% SM1 and hBDNF and hGDNF (both at 20 ng/mL) via one 75% media change followed by one 50% media change. Culture medium was exchanged 50% with fresh BrainPhys medium every 2-3 days. Cultures were maintained in a humidified atmosphere of 5% CO₂/95% air (pH 7.4) at 37°C. To allow for recovery from shipping, cells were maintained for a minimum of 7 days before starting experiments. These mixed neuronal-astrocyte cultures contain 90% glutamatergic and 10% GABAergic neurons (StemoniX, Inc. Technical Summary: Cellular Identity in microbrain assay ready cultures).

In a subset of experiments hiPSC-derived neurons were obtained from BrainXell (Madison, WI, USA) and Applied StemCell, Inc. (Milpitas, CA, USA) (BX-0300 and ASE-9741-C, respectively). Cultures were produced according to manufacturers’ instructions. Briefly, BrainXell hiPSCs were suspended in seeding media comprised of a 1:1 mixture of DMEM/F-12:Neurobasal supplemented with B27, N2, seeding supplement, supplement K, 0.5 mM GlutaMax, 15 μg/mL Matrigel, 10 ng/mL BDNF, 10 ng/mL GDNF, and 10 ng/mL TGF-β1 and plated (45,000 Cells/well) in a 96-well plate (Greiner; catalog number 655892) precoated with poly-D-lysine. Four days after plating, cells were fed with media without Matrigel and in which the seeding supplement was replaced with day 4 supplement. After the day 4 feeding, cells were fed every 3 days with media comprised of a 1:1 mixture of DMEM/F-12:Neurobasal supplemented with B27, N2, supplement K, 0.5 mM GlutaMax, 10 ng/mL BDNF, and 10 ng/mL GDNF. Experiments were performed 14-17 d after plating. Applied StemCell, Inc. hiPSCs (30,000 Cells/well) were suspended in proprietary cortical neuron culture media (ASE-9741MM), spun at 250 x g, re-suspended in cortical neuron culture media, and plated on a Matrigel-coated 96-well plate. Cultures were fed every 2 days and experiments performed 14-15 d after plating.
Viral vectors

PHP.eB Adeno-associated virus (AAV) vectors were produced by the Viral Vector and Cloning Core facility at the University of Minnesota following standard packaging procedures (Chen et al., 2019). The pRC-PHP.eB packaging plasmid was a gift from Dr. Viviana Gradinaru (California Institute of Technology; Chan et al., 2017).

The GRAB\textsubscript{eCB2.0} plasmid was described previously (Dong et al., 2022); AAV\textsubscript{PHP.eB-hSYN-GRAB\textsubscript{eCB2.0}} virus was used at a titer of 3.68 x 10\textsuperscript{11} genome copies/mL. The hSyn-eGFP plasmid was a gift from Dr. Bryan Roth (Addgene plasmid #50465); AAV\textsubscript{PHP.eB-hSYN-GFP} virus was used at a titer of 1.30 x 10\textsuperscript{11} genome copies/mL. The GFAP-mCherry plasmid was produced by the University of Minnesota Viral Vector Core from pAAV-GFAP-hM4D(Gi)-mCherry, which was a gift from Bryan Roth (Addgene plasmid # 50479). AAV\textsubscript{PHP.eB-GFAP-mCherry} virus was used at a titer of 1.41 x 10\textsuperscript{11} genome copies/mL. Viruses were added to cultures at the indicated titers immediately after a routine media change 6-12 days before imaging.

Image acquisition

All images were acquired on a Nikon A1 laser scanning confocal system with a Nikon ECLIPSE Ti inverted microscope (Nikon, Melville, NY, USA) using Nikon Elements software (version 5.02.01). An infrared z-positioning device (Nikon Perfect Focus System) was used to prevent drift in the z-dimension during acquisition of time courses. Cultures were maintained at 37°C and 5% CO\textsubscript{2} in a stage-top incubator (Chamlide) modified to hold 96-well plates. Breathe-Easy gas-permeable plate sealing membranes (Research Products International, Mt. Prospect, IL, USA) were used to prevent contamination of wells which were not actively being imaged.

hSYN-eGFP and GFAP-mCherry expression

Cultures expressing hSYN-eGFP and GFAP-mCherry were imaged 6 days after infection. Culture medium was replaced with HHSS (0.9 mM [Mg\textsuperscript{2+}]) before imaging to reduce background fluorescence. 12-bit 1024x1024 pixel images were acquired using a Nikon Plan
Apochromat λ 60x oil objective (numerical aperture = 1.4, refractive index = 1.515). eGFP was excited at 488 nm and emission detected at 550 nm (50 nm band pass); mCherry was excited at 561 nm and emission detected at 600 nm (50 nm band pass). The pinhole was set to 1 Airy unit with one-directional imaging. Channels were captured sequentially using a GaAsP detector and no averaging was performed. Each image was captured as a 20 µm z-stack (voxel size = 0.2072x0.2072x1 µm³). The example image in Figure 1 is represented as a maximum intensity z-projection.

**Calcium imaging**

To visualize calcium events in the hiPSC-derived cultures, 25% of the media in the well was replaced with pre-warmed FLIPR Calcium 6 dye (Molecular Devices) dissolved in HBSS. Cells were incubated in dye at 37°C and 5% CO₂ for 1.5-2 hours before imaging. 2 min before imaging, the culture medium was fully exchanged twice with HHSS containing either 0.9 mM (normal) or 0.1 mM (low) Mg²⁺.

12-bit 512x512 pixel images (pixel size = 1.1525x1.1525 µm²) were acquired with a Nikon Plan Apochromat VC 20x DIC N2 20x air objective (numerical aperture = 0.75). The Calcium 6 dye was excited at 488 nm and emission detected at 550 nm (50 nm band pass) and the pinhole set to 1 Airy unit with one-directional imaging. Images were recorded using a GaAsP detector and no averaging was performed.

Calcium imaging data were analyzed with a custom ImageJ macro. Briefly, background was subtracted using the rolling ball method (radius 250 µm) and frames were registered using ImageJ's “Correct 3D Drift” function. A maximum intensity projection of the registered image was thresholded and segmented into ROIs using ImageJ's “Analyze particles” function. Average intensity for each ROI in each frame of the image was calculated and converted to ΔF/F₀ values, using the minimum value for each ROI as F₀. Traces were manually quality checked and ROIs were eliminated if they 1) were not fully in the field of view for the entire recording; 2) did not
show activity in 0.1 mM Mg\(^{2+}\) (raw intensity amplitude of at least 50 to distinguish from noise) during experiments in which changes in this activity were of interest rather than the presence or absence of activity, *i.e.* those other than Figures 1 and 8; or 3) had an unstable baseline, defined as a change of more than 20% within the baseline epoch before addition of drug. Wells with fewer than 10 ROIs after quality checking were excluded from further analysis. The area under the curve (AUC) was calculated by summing the ΔF/F\(_0\) values in each imaging epoch. The response to drugs and treatments is expressed as a percent change in AUC before and after adding drug.

**GRAB eCB imaging**

Cultures expressing GRAB\(_{eCB2.0}\) were imaged 6-12 days after infection. The GRAB\(_{eCB2.0}\) sensor was excited at 488 nm with emission detected at 550 nm (50 nm band pass). The pinhole was set to 1 Airy unit with one-directional imaging. Images were recorded using a GaAsP detector and no averaging was performed. Before imaging, the media was replaced with HHSS (0.9 mM [Mg\(^{2+}\)\(_0\)] ) to reduce background fluorescence. In order to better capture multiple fine processes in focus, each frame was acquired as a 3-slice z-stack with slices 1 µm apart (voxel size 0.2072x0.2072x1 µm\(^3\)), and ROIs were chosen and analyzed using maximum intensity z-projections.

For initial proof of concept and time course of GRAB\(_{eCB2.0}\) activation (figure 5), 12-bit 1024x1024 pixel images were acquired using a Nikon Plan Apochromat λ 60x oil objective (numerical aperture = 1.4, refractive index = 1.515). Each frame consisted of one 3-slice z-stack (voxel size = 0.2072x0.2072x1 µm\(^3\)) and frames were automatically acquired every 20 s during each epoch as follows: baseline, starting 2 min after a media change to HHSS and continuing for 1 min (4 frames); carbachol, starting 10 s after the addition of 1 µM carbachol and continuing for 3 min (10 frames); Win-2, starting 10 s after the addition of 300 nM Win-2 and continuing for 3 min (10 frames). The total time elapsed between each epoch was 30 s.
In order to quickly image a larger field of view containing processes from multiple cells (figures 6-8), lower-magnification and lower-resolution 12-bit 512x512 pixel images (voxel size = 1.1525 x 1.1525 x 1 µm³) were acquired with a Nikon Plan Apochromat VC 20x DIC N2 20x air objective (numerical aperture = 0.75). In preliminary studies, carbachol-induced GRAB fluorescence consistently peaked by 50 seconds after addition of carbachol and Win-2-induced GRAB fluorescence reached a steady state after 2-3 minutes. Therefore, five frames were acquired ten seconds apart for each epoch, with baseline starting 2 min after a complete media change to HHSS buffer to reduce background fluorescence, carbachol starting 10 s after addition of carbachol, and Win-2 starting 2.5 min after addition of Win-2. Cells were incubated with drug or vehicle in media/Calcium 6 dye mixture for 1 h (DO34 and JZL 184) or 30 min (thapsigargin) before imaging and the drug concentration was maintained in HHSS throughout image acquisition.

Images were analyzed using ImageJ. Briefly, frames were registered using ImageJ's "Correct 3D drift" plugin and regions of interest were drawn manually with the polygon tool around processes which expressed the GRAB sensor (i.e., showed robust fluorescence in the presence of Win-2). Only the Win-2 epoch was considered when selecting ROIs; the investigator was blinded to treatment condition and to the appearance of the processes during the carbachol epoch. Average intensity in each frame was calculated for four ROIs plus one ellipsoid background region per image, with the four ROIs selected from different quadrants of the image wherever possible. Background-corrected average intensities were used to calculate ΔF/F₀ for each ROI in each frame, with the baseline epoch average used as F₀. Because brightness varied considerably between individual processes, data are reported as a percentage of the Win-2 epoch average. The average peak ΔF/F₀ (normalized to Win-2) of all ROIs in a well was considered as n=1.
Desensitization

To test for receptor desensitization, cells were pretreated with 0.1% DMSO, 1 µM Win-2, or 1 µM JZL 184 (added with a half media change containing 2x drug during regular feeding) for 15 minutes (acute treatment) or 1, 3, or 7 days (chronic treatments). For 7-day treatments, drugs were included in fresh media at 1x during scheduled feedings; 1- and 3-day treatments were not long enough to require feeding. Immediately before imaging, the pretreated drugs were washed out using four 5-minute washes in 0.9 mM Mg^{2+} HHSS containing 0.5% fatty acid free bovine serum albumin (75% media change with each wash). After washing, cells were placed in 0.1 mM Mg^{2+} HHSS and imaged before and after the addition of Win-2 as described in "Calcium Imaging" above.

Experimental design and statistical analysis

Controls and experimental groups were run in parallel to minimize effects of time or individual experiments. For all experiments, an individual sample (n = 1) is defined as the average of all ROIs in a single well, and each experiment was replicated on 2-3 independent platings of cells. For desensitization experiments, sample size was predetermined using GPower software (version 3.1) for an effect size of 0.5 and a power of 0.8; other sample sizes were not predetermined but conform to similar studies. For ROI selection in GRABeCB2.0 imaging experiments the investigator was blinded to treatment condition and to the culture’s response during the experimental carbachol epoch; calcium imaging experiments relied on automated algorithms and objective exclusion criteria (described in “Calcium Imaging” above) without blinding. Cultures used in figures 1-9 were derived from a single adult male donor.

Statistical analyses and hypothesis testing were performed using GraphPad Prism (version 9.2.0), aside from concentration-response curve fitting which was performed using OriginPro 2019 (version 9.6.0). Data are presented as mean ± SD. The Shapiro-Wilk test was used to test for normality and Levene's test for homogeneity of variance. Non-parametric data
were analyzed with the Mann-Whitney U test. For normally distributed data with homogeneous variance, Student's t-test (unpaired, two-tailed) was used for two-group comparisons, one- or two-way ANOVA with Tukey's post hoc test for comparisons of multiple groups, and two-way repeated measures ANOVA with Tukey's post hoc test for time courses. The Brown-Forsythe ANOVA with Dunnet's T3 multiple comparisons test was used for comparisons of multiple groups with non-homogeneous variance. Statistical significance was defined as p<0.05. No tests for outliers were performed.
Results

hiPSC-derived neurons form a glutamatergic signaling network in vitro

hiPSC-derived neuronal cultures consisted of a mix of neuron-like cells and astrocyte-like cells, as evidenced by morphology and the fact that they expressed fluorescent markers from the neuron-selective human synapsin (hSyn-GFP) and astrocyte-selective glial fibrillary acidic protein (GFAP-mCherry) promoters, respectively (Fig. 1A). The neuronal cells extended fine processes that in a previous study were shown to express synaptic markers (Green et al., 2019). In this study we examined these cultures for the elements of a functional eCB system.

To study synaptic transmission between hiPSC-derived neurons we evoked synaptically driven $[\text{Ca}^{2+}]_i$ spiking activity. Previous studies of synaptic networks in primary cultures of rat neurons have shown that reducing the extracellular Mg$^{2+}$ concentration ($[\text{Mg}^{2+}]_o$) will evoke paroxysmal epileptiform bursts of action potentials that produce $[\text{Ca}^{2+}]_i$ spikes that are driven by glutamatergic synaptic activity (McLeod et al., 1998). Bathing the culture in buffer containing 0.1 mM Mg$^{2+}$ evoked repetitive $[\text{Ca}^{2+}]_i$ spikes detected by confocal imaging of cultures loaded with FLIPR Calcium 6 dye (Molecular Devices). Ca$^{2+}$ levels in the cell body of neuronal cells were measured from somatic regions of interest (ROIs) defined by applying a threshold-based segmentation to the fluorescence image as described in Methods. The individual ROI traces shown in Figure 1C showed that the $[\text{Ca}^{2+}]_i$ oscillated in a seizure-like synchronized pattern. In 0.1 mM $[\text{Mg}^{2+}]_o$ the $[\text{Ca}^{2+}]_i$ spiking frequency was $3 \pm 0.4$ events/min (Fig. 1D). This activity was blocked completely by 10 µM tetrodotoxin, which blocks action potentials, and by 18-h pre-incubation with 2.5 µM tetanus toxin, which prevents neurotransmitter release. Thus, the 0.1 mM $[\text{Mg}^{2+}]_o$-evoked repetitive $[\text{Ca}^{2+}]_i$ spiking, while recorded from the soma, represents synaptic activity between networked hiPSC-derived neurons.

To study the pharmacology of this network activity the following treatment protocol was employed (Fig. 2A). Cell culture media was replaced with HEPES buffered Hank’s salt solution (HBSS) containing 0.1 mM $[\text{Mg}^{2+}]_o$ and the cultures were allowed to stabilize for 2 min. A 5 min
baseline epoch was recorded which served as control for the experiment. Then 50% of the well volume was exchanged with 0.1 mM [Mg\(^{2+}\)]\(_o\) containing drug or vehicle at twice the final concentration and was incubated for 15 min. Finally a 5 min treated epoch was recorded. [Ca\(^{2+}\)]\(_i\) spiking activity was quantified by measuring the area under the curve (AUC) for the average change in fluorescence (ΔF/F\(_o\)) of the FLIPR Calcium 6 dye for all active ROIs in the imaging field. Drug effects were quantified by determining the change in AUC during the treated epoch relative to the initial baseline epoch (% change in AUC).

Synchronous [Ca\(^{2+}\)]\(_i\) spiking activity was stable in vehicle treated wells (Fig. 2B-C). The NMDA receptor antagonist MK 801 (10 µM) inhibited [Ca\(^{2+}\)]\(_i\) spiking by 71 ± 11%, while the AMPA receptor antagonist CNQX (100 µM) inhibited the synaptic activity by 57 ± 18%. Thus, glutamatergic synaptic transmission drives a large portion of the [Ca\(^{2+}\)]\(_i\) spiking activity. CNQX significantly inhibited both frequency (U=4; p<0.05) and amplitude (t\(_{60}\)= 3.7; p<0.01) of the [Ca\(^{2+}\)]\(_i\) spiking. MK801 significantly inhibited [Ca\(^{2+}\)]\(_i\) spiking frequency (t\(_{60}\)=4.3; p<0.01) but did not significantly affect [Ca\(^{2+}\)]\(_i\) spike amplitude (t\(_{60}\)=1.9; p=0.1). Thus, we used AUC as a measure of [Ca\(^{2+}\)]\(_i\) spiking activity in order to capture both effects. The contributions of NMDA and AMPA receptors are consistent with research showing that the low [Mg\(^{2+}\)]\(_o\) model of epileptiform activity depends on NMDA receptor activation with a smaller contribution from AMPA receptors (Gulyás-Kovács et al., 2002). These results show that hiPSC-derived neuronal cultures contain cells which resemble neurons morphologically and functionally, and which form a glutamatergic signaling network \textit{in vitro} that could potentially be manipulated by cannabinoids.

**hiPSC-derived neurons are sensitive to exogenous cannabinoids**

We next tested whether the low [Mg\(^{2+}\)]\(_o\)-induced synaptic activity between hiPSC-derived neurons was sensitive to cannabinoid receptor agonists. The potent synthetic agonist WIN 55,212-2 (Win-2) produced a concentration-dependent decrease in both the amplitude and frequency of [Ca\(^{2+}\)]\(_i\) spikes (Fig. 3 A and D). The IC\(_{50}\) for Win-2-mediated inhibition of [Ca\(^{2+}\)]\(_i\)
spiking was 48 ± 13 nM, an 18-fold lower potency relative to that described for inhibition of synaptic activity in rodent models (Shen et al., 1996). The inhibition of \([\text{Ca}^{2+}]_i\) spiking by Win-2 was completely blocked by the selective CB1R antagonist NESS 0327 (Ruiu et al., 2003) (Fig. 2C & E), indicating that Win-2 acted on functional CB1Rs in these neurons. Application of 100 nM NESS 0327 did not consistently increase \([\text{Ca}^{2+}]_i\) spiking activity (t(14)=1.9 p=0.07), suggesting that eCB tone was not sufficiently elevated under these conditions to suppress synaptic transmission.

An endogenous ligand for CB1Rs, 2-arachidonoyl glycerol (2-AG), also inhibited 0.1 mM \([\text{Mg}^{2+}]_o\) evoked \([\text{Ca}^{2+}]_i\) spiking (Fig 2 B & D). The IC\(_{50}\) for 2-AG-mediated inhibition of \([\text{Ca}^{2+}]_i\) spiking was 2.0 ± 0.6 µM. The lower potency relative to Win-2 is consistent with that previously described in rodent models (Straiker and Mackie, 2005). The potency of 2-AG of glutamatergic synaptic activity in hiPSC cultures is 5-fold lower than that described for inhibition of synaptic activity in rat hippocampal cultures (Wu and Thayer, 2020). Overall, these pharmacological studies indicate that hiPSC-derived neuronal cultures respond to both a synthetic cannabinoid and an eCB.

**Δ⁹-THC acts as a partial agonist on CB₁R in hiPSC-derived neurons**

Δ⁹-THC, the principal psychoactive ingredient in marijuana, is a cannabinoid commonly used both medicinally and recreationally, which makes it important to test in this model system. Furthermore, Δ⁹-THC acts as a partial agonist on both rodent and human CB₁/₂Rs (Govaerts et al., 2004; Roloff and Thayer, 2009). Δ⁹-THC inhibited 0.1 mM \([\text{Mg}^{2+}]_o\) evoked \([\text{Ca}^{2+}]_i\) spiking in a manner consistent with action as a partial agonist (Fig. 4). The IC\(_{50}\) of Δ⁹-THC-mediated inhibition of \([\text{Ca}^{2+}]_i\) spiking was 1.4 ± 1.9 µM, a potency lower than that observed for Win-2 and comparable to that observed for 2-AG. A maximally effective concentration of Δ⁹-THC elicited only 47 ± 14 % inhibition (Fig. 4B), consistent with its partial agonist properties, and in contrast to the full agonists Win-2 and 2-AG (Fig. 3). The inhibition of \([\text{Ca}^{2+}]_i\) spiking evoked by 1 µM Δ⁹-
THC was significantly reduced in the presence of the CB₁R antagonist NESS 0327 (Fig. 4 A and D).

Because partial agonists occupy all the receptors at a maximally effective concentration, they can inhibit the activity of more efficacious agonists by competing for receptor binding sites. Indeed, 1 µM Δ⁹-THC antagonized the activity of 1 µM Win-2 when added to the cultures 15 min before Win-2 (Fig. 4 C and E), confirming that Δ⁹-THC acts as a partial CB₁R agonist in hiPSC-derived neuronal cultures.

**hiPSC-derived neuronal cultures produce 2-AG via diacylglycerol lipase**

We next investigated whether hiPSC-derived neuronal cultures produce eCBs, in addition to responding to exogenously applied CBs. To detect eCB production, we used a GPCR-activation based eCB (GRABₑCB₂.₀) sensor (Dong et al., 2022). The sensor consists of a modified CB₁R fused to circularly permutated GFP such that it fluoresces upon ligand binding. We infected the hiPSC-derived neuronal cultures with an adeno-associated virus (AAV) vector that expressed GRABₑCB₂.₀ under control of the neuron-specific human synapsin promoter (AAV_PHP.eB-hSyn-GRABₑCB₂.₀). Six days after infection, the cultures expressed functional GRABₑCB₂.₀ sensor, as evidenced by a large increase in fluorescence upon the addition of Win-2 (Fig. 5). The increase in GRABₑCB₂.₀ fluorescence evoked by 1 µM Win-2 was completely reversed by 100 nM NESS 0327 (n=4).

Metabotropic suppression of excitation (MSE) is a form of synaptic modulation mediated by eCBs produced following activation of G₉-coupled receptors that increase phospholipase C activity, leading to increased production of diacylglycerol (DAG) from membrane phospholipids. Hydrolysis of DAG by DAG lipase increases levels of the eCB 2-AG which subsequently activates presynaptic CB₁ receptors (Maejima et al., 2001a; Straiker and Mackie, 2007). MSE can be triggered by multiple G₉-coupled receptors including muscarinic M1 receptors, which can be activated by the agonist carbachol (Martin et al., 2015). When carbachol (1 µM) was added
to hiPSC-derived neuronal cultures expressing the GRAB\textsubscript{eCB2.0} sensor, fluorescence increased over 30-60 s to peak at 95 ± 43 % of the maximum response to Win-2 (Fig. 5C and Fig. 6A). This indicates that the cultures are producing eCBs, likely 2-AG. To determine if carbachol was stimulating the production of 2-AG we blocked DAG lipase activity with the potent inhibitor DO34. Pre-incubation with 10 or 30 nM DO34 for 60 min abolished the GRAB sensor response to carbachol (Fig. 6 A-B). This both confirms that the GRAB\textsubscript{eCB2.0} sensor is measuring eCB levels and indicates that 2-AG is the main eCB produced by carbachol stimulation of hiPSC-derived neuronal cultures.

The eCB system

hiPSC-derived cultures metabolize 2-AG via monoacylglycerol lipase

After signaling, 2-AG is degraded by metabolic enzymes such as monoacylglycerol (MAG) lipase, which make up the final piece of a basic eCB system. Treating GRAB\textsubscript{eCB2.0} expressing hiPSC-derived neuronal cultures with 1 µM carbachol for 1 min, followed by drug wash out, produced a transient increase in fluorescence that returned to basal levels over approximately 5 min (Fig. 7A). This decay was not due to photobleaching of the GRAB\textsubscript{eCB2.0} sensor, because the signal induced by 1 µM Win-2 remained constant throughout the same imaging protocol (data not shown). To determine the role of MAG lipase in this recovery process cultures were pretreated with vehicle (0.1% DMSO) or the irreversible MAG lipase inhibitor JZL184 for 1 h before recording (Fig. 7A). When cultures were pretreated with JZL 184, the decay of the carbachol-induced GRAB\textsubscript{eCB2.0} signal was significantly slower (significant effect of time x treatment, 2-way repeated measures ANOVA, $F_{(29, 290)}=3.064$, $p<0.0001$). To compare recovery kinetics across multiple recordings the recovery phase of each recording was normalized to peak and then averaged. The decay process was well fit by an exponential equation as shown by the linear regression fit to the data on a semi-log plot (Fig. 7B). As shown in Fig. 7C the time constant for recovery increased significantly from $\tau=112 \pm 28$ s in vehicle-treated cultures to $\tau=227 \pm 55$ s in cultures treated with JZL184 (excluding one JZL 184
recording in which the decay was too slow to calculate a time constant; Student’s t-test, $t_{(9)}=4.5$, $p<0.01$). This indicates that MAG lipase is degrading 2-AG produced by stimulating hiPSC-derived neuronal cultures with carbachol. Taken together, these results show that hiPSC-derived neuronal cultures contain a complete and functional eCB system.

**Activation of muscarinic receptors in hiPSC-derived cultures can produce 2-AG independent of $[\text{Ca}^{2+}]_i$.**

The enzymes responsible for 2-AG production, phospholipase C and DAG lipase (Fig. 5B), are stimulated by increases in $[\text{Ca}^{2+}]_i$ (Hashimotodani et al., 2005; Hashimotodani et al., 2008). Here, we examined the $\text{Ca}^{2+}$ sensitivity of 2-AG production in hiPSC-derived neuronal cultures following activation of muscarinic receptors. Carbachol stimulates phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate ($\text{PIP}_2$) to inositol trisphosphate ($\text{IP}_3$) and DAG. $\text{IP}_3$ releases $\text{Ca}^{2+}$ from endoplasmic reticulum (ER) stores. Thus, it was not surprising that neither the carbachol evoked increase in $[\text{Ca}^{2+}]_i$ nor the evoked increase in 2-AG required extracellular $\text{Ca}^{2+}$ (Fig. 8).

However, in cells pretreated for 30 min in nominally $\text{Ca}^{2+}$-free buffer with 1 µM thapsigargin, an agent that depletes ER $\text{Ca}^{2+}$ stores by inhibiting the sarcoplasmic/endoplasmic reticulum $\text{Ca}^{2+}$ ATPase, the carbachol-induced increase in $[\text{Ca}^{2+}]_i$ was completely blocked while the evoked production of 2-AG was not affected (Fig. 8). Thus, $\text{Ca}^{2+}$ mobilized from the ER does not appear to be required for 2-AG synthesis by this route, suggesting that a maximally effective concentration of carbachol triggers eCB production in a $\text{Ca}^{2+}$-independent manner in hiPSC-derived neuronal cultures.
CB₁R desensitization is less pronounced with a metabolic inhibitor than a receptor agonist in hiPSC-derived neuronal cultures

Although ∆⁹-THC and other CB₁R agonists show promise for treating a number of conditions, they carry the risk of tolerance and/or psychoactive side effects (Gorelick et al., 2013; Issa et al., 2014). One potential solution to avoid these adverse effects is to slow eCB metabolism instead, thus enhancing the endogenous spatial and temporal patterns of 2-AG signaling rather than activating all CB₁Rs for a prolonged period. We therefore tested whether the cannabinoid receptor agonist Win-2 and the MAG lipase inhibitor JZL 184 desensitized CB₁R-mediated presynaptic inhibition in hiPSC-derived neuronal cultures.

We used the 0.1 mM [Mg²⁺]₀ evoked [Ca²⁺]ᵢ spiking assay to test Win-2 mediated inhibition after treating the culture with vehicle, 1 µM Win-2, or 1 µM JZL184 for varying times (Fig. 9A), followed by a wash protocol described in Methods. A short (15 min) treatment was used to validate the wash protocol. This brief treatment with vehicle, Win-2, or JZL 184 did not significantly affect the subsequent Win-2 test response. Thus, the wash protocol was sufficient to prevent any acute effects on CB₁R signaling resulting from carryover of the drug pretreatment.

The Win-2 test response was significantly attenuated in cultures pretreated with Win-2 for 1, 3, or 7 days before testing, as opposed to those pretreated with vehicle (Fig. 9B). This indicates that Win-2 desensitizes the CB₁R in hiPSC-derived neurons. Exposing cultures to JZL 184, on the other hand, caused less pronounced desensitization of the Win-2 response. JZL 184 is an irreversible inhibitor of MAGL, so its duration of action is a function of the stability of JZL 184 as well as the turnover of MAGL protein. In control experiments we found the effects of JZL 184 were not significantly different when added once every 3 days with regular media changes or refreshed daily (t₁₀=0.4 p=0.70). Thus, for these experiments fresh drug was only added at regular media changes. A 1 d exposure to JZL184 significantly attenuated the test response, but did not completely block the CB₁R-mediated response, and even after 7 d the
desensitization was incomplete. This suggests MAG lipase inhibition may be an effective strategy for avoiding tolerance when targeting the eCB system therapeutically.

**hiPSC-derived neuronal lines from multiple sources are sensitive to CB₁R agonists**

The results presented thus far were generated from multiple platings of cells differentiated from a single iPSC line obtained from Stemonix, Inc. In Figure 10 we show recordings from cultures obtained from additional commercial sources. In cells obtained from BrainXell we observed asynchronous spontaneous [Ca²⁺]i spiking activity that was not increased or synchronized by bathing the cells in 0.1 mM Mg²⁺ (Fig. 10 A). However, in those cells that exhibited spontaneous [Ca²⁺]i spiking, 1 µM Win-2 inhibited the synaptic activity in a manner partially reversed by the CB₁R antagonist Ness 0327 (100 nM)(Fig. 10C). hiPSC-derived neuronal cultures produced from cells obtained from Applied StemCell exhibited synchronized [Ca²⁺]i spiking activity in 0.1 mM Mg²⁺ that was markedly inhibited by 1 µM Win-2 (Fig. 10B and C). The effects of Win-2 were significantly reversed by Ness 0327 (100 nM). Thus, each of the three hiPSC derived neuronal cultures exhibited synaptic activity that was inhibited by activation of CB₁Rs, suggesting that the results presented can be generalized, at least qualitatively, to multiple hiPSC-derived neuronal lines.
Discussion

Despite recent interest in the role of the ECS in neurological disorders, there are few options for studying pathophysiological changes in the ECS or evaluating drugs that act on the ECS in human neurons. In this study, we show that hiPSC-derived neurons contain a functional ECS, providing a readily available human in vitro model for studying ECS neurophysiology and for drug discovery. hiPSC-derived neurons were amenable to useful functional assays including Ca\(^{2+}\) imaging and a genetically encoded fluorescent cannabinoid sensor. Using these assays in combination with pharmacological tools we demonstrate that the ECS is fully functional in hiPSC-derived neuronal cultures.

While many studies to date involving cannabinoids and hiPSCs have examined the role of the CB\(_{1/2}\)Rs in stem cell maintenance, differentiation, and synaptogenesis (Miranda et al., 2020; Shum et al., 2020; Stanslowsky et al., 2017), only a few have looked at the ECS in fully differentiated neurons. For example, Guennewig et al. (Guennewig et al., 2018) examined \(\Delta^9\text{-THC}\) -induced gene expression changes in hiPSC-derived neurons, finding that genes affected by \(\Delta^9\text{-THC}\) exposure overlap partially with those that are altered in neuropsychiatric conditions including schizophrenia. Because these cells responded to \(\Delta^9\text{-THC}\), it suggests that they express functional receptors although the signal transduction pathways were not characterized in this study. Our study showed for the first time that CB\(_1\)R agonists inhibit synaptic transmission between hiPSC-derived neurons. Taken together, these studies suggest that it may be possible to relate cannabinoid induced changes in synaptic function to altered gene expression in human neurons. We also determined the concentration dependence of CB1R agonists. They were less potent in hiPSC-derived neuronal cultures than in primary rat hippocampal cultures previously studied by our laboratory (Figs. 3-4; (Shen et al., 1996; Wu and Thayer, 2020). This may result from species differences in receptor sensitivity, in agreement with previous studies showing Win-2 acted on human CB\(_1\)Rs with lower affinity than on rat receptors (McPartland et al., 2007).
There could also be effects of culture type (iPSC-derived versus primary), brain region (cortex-like versus hippocampus), or sex (one male human donor versus a mix of male and female rats).

We demonstrate for the first time that hiPSC-derived neuronal cultures express a fully functional ECS that modulates synaptic transmission via CB1Rs and synthesizes and metabolizes 2-AG. Our study, which focuses on pharmacological characterization and evaluation of ECS function, compliments another recent study in a hiPSC-derived cortical spheroid model which demonstrated expression of the ECS components we assessed functionally. Using immunofluorescence and quantitative reverse transcription PCR, Papariello et al. (2021) found CB1R, DAG lipase, and MAG lipase were expressed in cortical spheroids. Together with this study, our [Ca2+]i imaging and eCB sensor data provide solid evidence that the ECS is both present and functional in hiPSC-derived neurons. Importantly, the cortical spheroids used by Papariello et al. (2021) used dual-SMAD inhibition (Chambers et al., 2009) for neural induction, while our 2D cultures used an embryonic body-based induction protocol (Marchetto et al., 2010). This, along with our observation that hiPSC-derived neurons from multiple commercial sources are sensitive to CB1R agonists, suggests that ECS expression and function are present in a variety of different hiPSC-derived neurons, and not peculiar to one cell line, method, or source.

In addition to responding to exogenously applied cannabinoids, hiPSC-derived neuronal cultures also produced eCBs in response to carbachol. 2-AG is likely the main carbachol-evoked eCB in this system because its synthesis and degradation were blocked by inhibitors of DAG lipase and MAG lipase, respectively. This does not, however, rule out the possibility that the hiPSC-derived cultures can produce other eCBs, perhaps with different stimuli. Furthermore, the carbachol-evoked GRABeCB2.0 response continued to recover in the presence of JZL 184 despite being significantly slowed, suggesting that other degradative enzymes such as Alpha/Beta-Hydrolase Domain-Containing 6 (Zhang et al., 2021) might be present in hiPSC-derived neuronal cultures.
There are multiple ways to evoke eCB synthesis, with different levels of Ca\(^{2+}\) dependence. In general, depolarization-induced suppression of excitation, in which voltage-gated Ca\(^{2+}\) channels are activated, requires an influx of extracellular Ca\(^{2+}\) (Ohno-Shosaku et al., 2001), while metabotropic suppression of excitation (MSE), in which Gq-coupled receptors activate PLC, is either Ca\(^{2+}\)-independent (Maejima et al., 2001b) or partially dependent on Ca\(^{2+}\) release from intracellular ER stores (Robbe et al., 2002). Our data support the hypothesis that MSE is calcium-independent in hiPSC derived neurons, based on the fact that nominally calcium-free buffer combined with thapsigargin abolished the carbachol-evoked [Ca\(^{2+}\)] response but not carbachol-evoked eCB production. Because we did not add a Ca\(^{2+}\) chelator to our Ca\(^{2+}\)-free media, we cannot entirely rule out the possibility that trace amounts of Ca\(^{2+}\) are required for eCB synthesis; however, the carbachol-evoked increase in [Ca\(^{2+}\)] was undetectable by the Calcium 6 dye. Submaximal stimulation of the metabotropic receptor and voltage-gated Ca\(^{2+}\) influx pathways display synergistic activation of 2-AG synthesis (Hashimotodani et al., 2005). However, our experiments were not designed to detect enhancement of submaximal DAG lipase activation. Our study does illustrate the utility of hiPSC-derived neuronal cultures for mechanistic study of the ECS.

It is important to study the complete eCB system to understand how drugs that act selectively on a single element of the ECS affect the other components indirectly. The desensitization experiments performed in this study illustrate this point. CB\(_1\)Rs are downregulated or desensitized with prolonged exposure to ligand (Kouznetsova et al., 2002), making tolerance an obstacle to drug development. Indeed, heavy cannabis use can lead to \(\Delta^9\)-THC tolerance and dependence in humans (Colizzi and Bhattacharyya, 2018) and rodents (González et al., 2005), and the CB\(_1\)R is downregulated in postmortem human brain tissue from frequent cannabis users relative to non-users (Villares, 2007). Previous studies have investigated JZL 184-induced CB\(_1\)R desensitization in rodents, with mixed results. Schlosburg et al. (2010) found that a 6-day treatment with JZL 184 led to tolerance in mice as measured by
antinociceptive effects, and CB1R binding sites were reduced in several brain regions including cortex. Conversely, Feliszek et al. (2016) saw only mild receptor desensitization in the hippocampus after 14 d exposure to the same dose; they also noted that the amount of desensitization was dose- and age-dependent. Our results in adult human iPSC-derived neuronal cultures support the hypothesis that JZL 184 causes slower and/or less pronounced desensitization than a CB1R agonist. Future studies using multiple concentrations and longer pretreatment times could further clarify the best way to administer inhibitors of 2-AG metabolism for the most benefit with the least tolerance. The desensitization experiment also highlights the stability of the hiPSC cultures. While our treatment protocol was 7 d in duration, we have used cultures as old as 15 weeks, suggesting that extended drug exposures are feasible in hiPSC-derived neuronal cultures that are not possible in rodent primary cultures.

hiPSC-derived neuronal cultures show promise for studying human neurological disorders because they can be derived from patient tissue samples (Okano and Morimoto, 2022). Understanding how the ECS is affected in inherited human disease may identify novel insights into pathophysiological changes. In a study of fetal brain development, comparing cells derived from children with autism to those derived from typically developing controls revealed that expression of both DAGL and MAGL were increased in autism, highlighting the usefulness of hiPSC-derived neurons that express the ECS for studying neurodevelopmental disorders (Papariello et al., 2021).

Here, we focused on the effects of cannabinoids on a glutamatergic network; however, the CB1R is expressed at very high levels in GABAergic interneurons (Marsicano and Lutz, 2006) and at lower levels in astrocytes (Navarrete and Araque, 2008). These other cell types contribute to more complex activity patterns which are outside the scope of this study. In addition, MAGL inhibitors do not only increase eCB signaling—they also reduce inflammation by blocking the production of arachidonic acid from 2-AG (Long et al., 2009). Microglia play a large role in neuroinflammation but are not present in the cultures used in this study. Although
microglia arise from a yolk sac lineage and cannot easily be produced from the same
differentiation protocol as neurons and astrocytes, they can be derived separately and co-
cultured with other cell types of interest (reviewed in Hasselmann and Blurton-Jones, 2020).
Development of co-culture models may help to elucidate the full effects of MAGL inhibitors in
the future.

In conclusion, human iPSC-derived neuronal cultures recapitulate a diverse suite of eCB
functions, providing a minimally-invasive and potentially high-throughput human platform for
eCB research and drug discovery.
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Authorship contributions

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References


Shum C, Dutan L, Annuario E, Warre-Cornish K, Taylor SE, Taylor RD, Andreae LC, Buckley NJ, Price J, Bhattacharyya S and Srivastava DP (2020) Δ(9)-tetrahydrocannabinol and
2-AG decreases neurite outgrowth and differentially affects ERK1/2 and Akt signaling in hiPSC-derived cortical neurons. *Mol Cell Neurosci* **103**: 103463.


Footnotes

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Figure legends

Figure 1. hiPSC-derived neuronal cultures exhibit synaptically driven \([\text{Ca}^{2+}]\) spiking. (A) hiPSC-derived neuronal cultures expressing mCherry from the GFAP promoter (magenta) and GFP from the synapsin promoter (green). Scale bar = 50 µm. (B) Representative image of hiPSC-derived cultures in Calcium 6 dye (left) and ROIs derived from this image using threshold-based segmentation in ImageJ (right). Scale bar = 50 µm. (C) Individual \([\text{Ca}^{2+}]\) traces representing ΔF/F₀ for each ROI in (B). Scale bar = 30 s. (D) Representative traces show mean \([\text{Ca}^{2+}]\) (solid line) from a single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg²⁺ buffer containing H₂O vehicle (top), 10 µM tetrodotoxin (TTX, middle), or pretreated (18 h) with 2.5 µM tetanus toxin (TeNT, bottom). Scale bars: horizontal = 30 s, vertical = 1.0 ΔF/F₀. Bar graph summarizes \([\text{Ca}^{2+}]\) spiking activity (events per minute) in cultures treated with H₂O vehicle (CTL), TTX, or TeNT as depicted in traces. *, p<0.05 relative to CTL. Kruskal-Wallis test with Dunn’s multiple comparisons correction, p = 0.024 for TTX and 0.013 for TeNT relative to vehicle, n=3-4 wells per treatment.

Figure 2. hiPSC-derived neuronal cultures form a glutamatergic synaptic network. (A) Schematic shows drug treatments sequence for this and following figures. (B) Representative traces show mean \([\text{Ca}^{2+}]\) (solid line) for a single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg²⁺ buffer before (baseline epoch) and after (treated epoch) the addition of vehicle (H₂O, top), 100 µM CNQX (middle), or 10 µM MK801 (bottom). Scale bars: horizontal = 30 s, vertical = 1.0 ΔF/F₀. (C) Bar graph summarizes the change in \([\text{Ca}^{2+}]\) spiking activity (% change in AUC) before and after adding vehicle (H₂O for CNQX and DMSO for MK 801), 100 µM CNQX, or 10 µM MK 801. **, p<0.01 relative to vehicle control; ***, p<0.001 relative to vehicle. Data are presented as mean ± SD. Student’s t-test: t = 4.9 and p = 0.002 for CNQX relative to H₂O; t = 7.0 and p = 0.0004 for MK 801 relative to DMSO; n = 4-5 wells per condition.
Figure 3. Cannabinoid agonists inhibit synaptic activity between hiPSC-derived neurons via CB1R. (A-C) Representative traces show mean (solid) [Ca2+]i (ΔF/F0) of single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg2+ buffer before and after the addition of 300 nM Win-2 (A), 3 µM 2-AG (B), or 1 µM Win-2 in the presence of 100 nM NESS 0327 (C). Scale bars: horizontal = 30 s, vertical = 1.0 ΔF/F0. (D) Concentration-response curves for Win-2 (circles) and 2-AG (triangles) were fit with a logistic equation of the form: % change in AUC = A1 + [(A2-A1)/(1+10(logxₒ-x)p)], where xₒ = EC50, x = log[drug], A1 = % change in the absence of drug, A2 = % change at a maximally effective drug concentration, and p = slope factor. The following values were calculated using a non-linear, least-squares curve fitting program: A1 = -25% for Win-2 and -21% for 2-AG; A2 = -84% for Win-2 and -86% for 2-AG; EC50 = 48 ± 13 nM for Win-2 and 2.0 ± 0.6 µM for 2-AG; p = -3.0 ± 1.4 for Win-2 and -2.0 ± 1.0 for 2-AG. n = 4-6 wells per concentration. (E) Bar graph summarizes change in [Ca2+]i spiking induced by 1 µM Win-2 in wells treated with vehicle (0.1% ethanol, left) or 100 nM NESS 0327 (right). Data are presented as mean ± SD. **, p<0.01. Welch’s t-test, t(6.4)=5.4, p=0.0014, 6 wells per condition.

Figure 4. ∆9-THC acts as a partial agonist to inhibit synaptic transmission in hiPSC-derived neuronal cultures. (A) Representative traces show mean (solid line) [Ca2+]i (ΔF/F0) of a single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg2+ buffer before and after the addition of 1 µM ∆9-THC in the absence (upper) or presence of 100 nM NESS 0327 (lower). Scale bars: horizontal = 30 s, vertical = 1.0 ΔF/F0. (B) Concentration-response curve for ∆9-THC was fit with a logistic equation of the form % change in AUC = A1 + [(A2-A1)/(1+10(logxₒ-x)p)], where xₒ = EC50, x = log[∆9-THC ], A1 = % change in the absence of drug, A2 = % change at a maximally effective drug concentration, and p = slope factor. The following values were calculated using a non-linear, least-squares curve fitting program: A1 = -17 ± 11%; A2 = -47 ± 14%; EC50 = 1.4 ± 1.9 µM; p = -0.9 ± 1.2; n = 4-7 wells per concentration. (C)
Representative traces show mean (solid line) [Ca$^{2+}$], (ΔF/F$_0$) of single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg$^{2+}$ buffer before and after the addition of 1 µM Win-2 in the presence of vehicle (0.01% ethanol, top) or 1 µM ∆$^9$-THC (bottom). Scale bars: horizontal = 30 s, vertical = 1.0 ΔF/F$_0$. (D) Bar graph shows change in [Ca$^{2+}$] spiking relative to baseline (AUC) after addition of 1 µM ∆$^9$-THC in wells treated with vehicle or 100 nM NESS 0327. Data are presented as mean ± SD. Unpaired Student’s t-test, t$_{(10)}$=2.3, *p<0.05, n = 6 wells per condition. (E) Bar graph shows change in [Ca$^{2+}$] spiking relative to baseline (AUC) after addition of 1 µM Win-2 in wells treated with vehicle or ∆$^9$-THC. Data are presented as mean ± SD. Unpaired Student's t-test, t$_{(5.5)}$=18.8, **p<0.0001, n = 6 wells per condition.

Figure 5. eCB GRAB sensor imaging in hiPSC-derived neuronal cultures. (A) Left: GRAB$_{eCB2.0}$ fluorescence (ΔF/F$_0$) in cells treated with 300 nM Win-2. Scale bar = 10 µm. Right: enlarged images of the boxed region at baseline (top) and after the addition of 1 µM carbachol (middle) and 300 nM Win-2 (bottom). Scale bar = 10 µm. (B) Schematic showing the mechanism of carbachol-triggered 2-AG synthesis. Cch = carbachol, mAChR = muscarinic acetylcholine receptor, PIP$_2$ = phosphatidylinositol bisphosphate, PLC-β = phospholipase C-β, DAG = diacylglycerol, DAGL = diacylglycerol lipase, 2-AG = 2-arachidonoylglycerol. (C) Time course of GRAB$_{eCB2.0}$ fluorescence (ΔF/F$_0$) for the ROI highlighted in (A) expressed as a percentage of the response in saturating Win-2 (mean of final 40 s of recording). Arrowheads mark the addition of 1 µM carbachol (Cch) and 300 nM Win-2.

Figure 6. hiPSC-derived neuronal cultures synthesize eCBs via DAG lipase. (A) Time courses showing mean GRAB$_{eCB2.0}$ fluorescence (ΔF/F$_0$, expressed as a percentage of the Win-2 response) from representative wells pretreated for 1 h with vehicle (0.1% DMSO) or 10 or 30 nM DO34. Arrowheads mark the addition of 1 µM carbachol (Cch) and 300 nM Win-2. n = 4 ROIs per well. (B) Bar graph summarizes peak carbachol-evoked GRAB$_{eCB2.0}$ fluorescence (ΔF/F$_0$, expressed as a percentage of the Win-2 response) in wells pretreated for 1 h with
vehicle (0.1% DMSO), 10 nM DO34, or 30 nM DO34. Data are presented as mean ± SD.

* p<0.05 relative to vehicle. Brown-Forsythe ANOVA test with Dunnet’s T3 multiple comparisons test, F(2.0, 3.1)= 17.45, p = 0.045 for 10 nM DO34 and 0.040 for 30 nM DO34 vs vehicle. n = 4-5 wells; each well is the average of 4 ROIs.

**Figure 7. hiPSC-derived neuronal cultures metabolize 2-AG via MAG lipase. (A) Traces showing average GRAB<sub>eCB2.0</sub> fluorescence from representative wells pretreated with vehicle or 1 µM JZL 184. Arrowheads show addition of 1 µM carbachol (Cch), washout of carbachol (wash) and addition of 300 nM Win-2. Each trace is the average of 4 ROIs from a single well. Scale bars: horizontal = 30 s, vertical = 20% of saturating Win-2 response (ΔF/F<sub>0</sub>). (B) Time course showing the decay of carbachol-evoked GRAB<sub>eCB2.0</sub> fluorescence after washout of carbachol (ΔF/F<sub>0</sub>, expressed as a percentage of the peak fluorescence on a log scale) in cells pretreated for 1 h with vehicle (0.1% DMSO, filled circles) or 1 µM JZL 184 (open squares). One JZL 184 well decayed too slowly to calculate a time constant and is not included. n = 6 wells for vehicle and 5 wells for JZL 184. Data are presented as mean ± SD. Curves were fit with a linear regression. (C) Bar graph shows time constants for GRAB<sub>eCB2.0</sub> fluorescence decay calculated from the individual recordings averaged in (B). Individual curves were fit with an exponential decay function constrained to a y-intercept of 100 and an asymptote approaching 0. Data are presented as mean ± SD. **, p<0.01. Unpaired Student’s t-test, t(9)=4.5, n = 6 wells for vehicle and 5 wells for JZL 184.

**Figure 8. Carbachol-evoked 2-AG production is independent of [Ca<sup>2+</sup>]i. (A) Top: representative carbachol-evoked [Ca<sup>2+</sup>]i traces from wells pretreated for 30 min with vehicle (0.1% DMSO) or 1 µM thapsigargin in the absence or presence of Ca<sup>2+</sup> as indicated. Horizontal bar=30 s. Bottom: representative traces showing GRAB<sub>eCB2.0</sub> fluorescence in wells pretreated as indicated. Horizontal bar=30 s. (B) Bar graph summarizes peak increases in [Ca<sup>2+</sup>]i evoked by 1 µM carbachol in the absence or presence of 1 µM thapsigargin. Recordings were performed in...
the absence (nominally Ca\textsuperscript{2+}-free) or presence of 1.3 mM extracellular Ca\textsuperscript{2+} as indicated. n = 6 wells per condition. Data are presented as mean ± SD. Brown-Forsythe ANOVA \((F_{(2, 8.4)} = 12.9, p = 0.0028)\) with Dunnet’s T3 multiple comparisons test **\(p<0.01\), relative to vehicle + Ca\textsuperscript{2+} + carbachol. (C) Peak carbachol-evoked GRAB\textsubscript{eCB2.0} fluorescence in cells treated as indicated. n = 4 wells per condition; each well value is the average of 4 ROIs. Data are presented as mean ± SD. Brown-Forsythe ANOVA \(F_{(2, 8.9)} = 0.3, p = 0.75\).

**Figure 9. Comparison of Win-2 to JZL184-induced desensitization of CB\textsubscript{1}R-mediated inhibition of synaptic activity.** (A) Timeline of pretreatment and imaging for desensitization experiments. (B) Representative traces show mean (solid line) [Ca\textsuperscript{2+}], \((\Delta F/F_0)\) of a single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg\textsuperscript{2+} buffer before and after the addition of 1 µM Win-2 (arrowheads) in cells pretreated for 7 d with either vehicle (0.1% DMSO, top), 1 µM Win-2 (middle), or 1 µM JZL 184 (bottom). Scale bars: horizontal = 30 s, vertical = 1.0 \(\Delta F/F_0\). (C) Plot shows inhibition of [Ca\textsuperscript{2+}], spiking activity produced by 1 µM Win-2 in cells pretreated for the indicated times with vehicle (0.1% DMSO, black circles), 1 µM Win-2 (red squares), or 1 µM JZL 184 (green triangles). Data are presented as mean ± SD. n = 6 wells per condition. * \(p<0.05\) and **** \(p<0.0001\) relative to vehicle; † \(p<0.05\), †† \(p<0.001\), and †††† \(p<0.0001\) relative to Win-2. Two-way repeated measures ANOVA with Tukey’s multiple comparisons test. Pretreatment time \(F_{(3, 59)} = 16.1, p<0.0001\); pretreatment drug \(F_{(2, 59)} = 67.2, p<0.0001\), interaction \(F_{(6, 59)} = 4.3, p=0.0012\).

**Figure 10. CB\textsubscript{1}R activation inhibits [Ca\textsuperscript{2+}], spiking in hiPSC-derived neuronal cultures from multiple sources.** (A) Individual [Ca\textsuperscript{2+}], traces representing \(\Delta F/F_0\) for spontaneously active ROIs in a cell culture produced from cells obtained from BrainXell. Recording was performed in 0.1 mM Mg\textsuperscript{2+} buffer and cells were treated with 1 µM Win-2 at the time indicated by the arrow. Scale bar: horizontal = 30 s, vertical = 1.0 \(\Delta F/F_0\). (B) Representative traces show mean (solid line) [Ca\textsuperscript{2+}] \((\Delta F/F_0)\) and SD (denoted by blue shading) from a single field of hiPSC-derived
neuronal cells (obtained from Applied StemCell) in 0.1 mM Mg\(^{2+}\) buffer treated with 1 µM Win-2 at the time indicated by the arrow. Scale bars: horizontal = 30 s, vertical = 1.0 $\Delta F/F_0$. (C) Bar graph summarizes change in [Ca\(^{2+}\)], spiking induced by 1 µM Win-2 in wells treated with vehicle (0.1% ethanol, open bars) or 100 nM NESS 0327 (filled bars) from cells obtained from BrainXell or Applied StemCell as indicated. Data are presented as mean ± SD. *, p<0.05; **, p<0.01 Unpaired Student’s t-test. For BrainXell hiPSCs $t_{(10)}=3.8$, p=0.003, 6 wells per condition. For Applied StemCell hiPSCs $t_{(6)}=2.3$, p = 0.049, 4 wells per condition.
Figure 3

A) Win-2 (300 nM)

B) 2-AG (3 μM)

C) NESS (100 nM)  Win-2 (1 μM)

D) 

\[
\text{[Ca}^{2+}]_{\text{i}} \text{ Spiking (\% change in AUC)}
\]

\(10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}\)

E) 

\[
\text{[Ca}^{2+}]_{\text{i}} \text{ Spiking (\% change in AUC)}
\]

Vehicle+Win-2  NESS+Win-2

**
Figure 4

A

NESS
THC

Veh

B

[Ca^{2+}]_{\text{spiking}} (% change in AUC)

[THC] (µM)

10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}

C

Win-2

THC

D

THC + vehicle
THC + NESS

Win-2 + vehicle
Win-2 + THC

E

% change in AUC
Figure 5

A. [Image of a fluorescence microscopy image showing a neural network with labeled receptors and channels.]

B. Diagram showing the intracellular signaling pathway:

- Cch → mACHR
- Gq → PLC-β → PIP₂ → DAG → DAGL → 2-AG

C. Graph showing the time course of the response:

- Baseline
- Carbachol
- Win-2

Graph shows GRABcB2.0 ΔF/F₀ (%) of Win-2 vs. Time (s) with data points for Cch and Win-2.
Figure 6

A

GRAB_{eCB2.0, ΔF/F₀} (% of Win-2)

Time after adding Cch (s)

Vehicle
10 nM DO34
30 nM DO34

Cch
Win-2

B

Peak Cch-evoked GRAB_{eCB2.0, ΔF/F₀} (% of Win-2)

Vehicle
DO34 (10 nM)
DO34 (30 nM)

* *
Figure 7

A  Vehicle
   Wash  Win-2
   Cch

JZL 184
   Wash  Win-2
   Cch

B

GRABeCB2.0 ΔF/F₀ (% of peak)

Time (s)

C

T (s)

Vehicle  JZL 184

**
**Figure 9**

**A**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Wash</th>
<th>Baseline epoch</th>
<th>Win-2</th>
<th>Treated epoch</th>
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<td>15 min - 7 d</td>
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<td>5 min</td>
<td>15 min</td>
<td>5 min</td>
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</tbody>
</table>

**B**

- 7-day Vehicle
- 7-day Win-2
- 7-day JZL 184

**C**

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
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<tr>
<td>Win-2</td>
</tr>
<tr>
<td>JZL 184</td>
</tr>
</tbody>
</table>

Win-2 evoked change in [Ca^{2+}]_{i}, Spiking (% change in AUC)

- Pretreatment time (d): 0, 1, 3, 7
- Statistical significance:
  - **:** p < 0.0001
  - ++: p < 0.01
  - ++++: p < 0.001
  - +++: p < 0.005
  - *: p < 0.05
  - **: p < 0.01