CB₁ antagonism increases excitatory synaptogenesis in a cortical spheroid model of fetal brain development

Alexis Papariello¹, David Taylor¹, Ken Soderstrom¹* & Karen Litwa²*

The endocannabinoid system (ECS) plays a complex role in the development of neural circuitry during fetal brain development. The cannabinoid receptor type 1 (CB₁) controls synaptic strength at both excitatory and inhibitory synapses and thus contributes to the balance of excitatory and inhibitory signaling. Imbalances in the ratio of excitatory to inhibitory synapses have been implicated in various neuropsychiatric disorders associated with dysregulated central nervous system development including autism spectrum disorder, epilepsy, and schizophrenia. The role of CB₁ in human brain development has been difficult to study but advances in induced pluripotent stem cell technology have allowed us to model the fetal brain environment. Cortical spheroids resemble the cortex of the dorsal telencephalon during mid-fetal gestation and possess functional synapses, spontaneous activity, an astrocyte population, and pseudo-laminar organization. We first characterized the ECS using STORM microscopy and observed synaptic localization of components similar to that which is observed in the fetal brain. Next, using the CB₁-selective antagonist SR141716A, we observed an increase in excitatory, and to a lesser extent, inhibitory synaptogenesis as measured by confocal image analysis. Further, CB₁ antagonism increased the variability of spontaneous activity within developing neural networks, as measured by microelectrode array. Overall, we have established that cortical spheroids express ECS components and are thus a useful model for exploring endocannabinoid mediation of childhood neuropsychiatric disease.

The endocannabinoid system (ECS) classically regulates synaptic plasticity via inhibitory presynaptic feedback in the adult brain. Constituents of the ECS are also expressed in the human brain during fetal gestation, where they direct numerous neurodevelopmental processes, including neural progenitor proliferation, differentiation, neuronal migration, and axonal growth cone directionality. While the role of the ECS in the adult brain is well defined, the role of the ECS in the initial establishment of synapses during fetal brain development is not. In the following research, we investigate whether ECS disruption via CB₁ antagonism impacts synaptogenesis in a cortical spheroid model of fetal brain development.

Synaptogenesis begins when nascent pre- and post-synaptic surfaces contact and adhere to one another. This process requires the coordinated activity of multiple subcellular systems including cell adhesion molecules, scaffold proteins and receptors, and cytoskeletal regulators. Disruption of these systems which govern synapse selection and maintenance can cause altered excitatory/inhibitory (E/I) synapse balance. Effective information transfer in the brain relies on homeostatic balance between excitatory and inhibitory synapses, thus, changes to the E/I balance during critical periods of development may negatively impact behavior and cognition. Altered E/I balance is a phenotype which has been implicated in neuropsychiatric ailments that lack clear etiologies including epilepsy, schizophrenia, and autism spectrum disorder (ASD). Thus, while neural circuits are pliant during early development, they are also particularly vulnerable to genetic and environmental disruption.

Many features of neurodevelopmental disorders are difficult to adequately characterize in animal models. This stems from the heterogeneous genetic nature of many neurodevelopmental disorders, the timing of critical periods, and diagnostic criteria that is not easily translated into animal research (such as verbal and nonverbal communication). Recent advances in the use of induced pluripotent stem cells (iPSC) and the creation of...
organoid and spheroid model systems promise progress21,22. These models, which replicate brain tissue structure better than two-dimensional cell culture21,22, provide an avenue for drug testing in a genetically relevant paradigm23,24 and allow for the study of human disease processes without complicated in vivo work20,22. Using iPSCs from neurotypical control patients, we are able to grow cortical spheroids which have functional synapses, spontaneous activity, an astrocyte population, and pseudo-laminar organization which resembles the dorsal telencephalon of the human fetus at 19–24 weeks post conception35-37.

To address how the ECS impacts synapses formation, we focused on the role of endocannabinoid receptor CB1. CB1 is not only the predominant endocannabinoid receptor in the brain38, it is also the most abundant G-protein coupled receptor in the vertebrate central nervous system39. Activation of presynaptic CB1 by the endogenous, endocannabinoid agonist 2-arachidonoylglycerol (2-AG) elicits activity-dependent, G1-linked effects40,41 that decrease presynaptic neurotransmitter release and weakens synaptic strength42,43. The biosynthetic enzyme for 2-AG, diacylglycerol lipase (DAGLα, the principal CNS isoform), along with the metabolic enzyme monoacylglycerol lipase (MAGL), control the local distribution of 2-AG, which is the principal endocannabinoid during gestation44,45. In addition to the classic, paracrine signaling of 2-AG during CB1-mediated presynaptic feedback inhibition, 2-AG also exhibits distinct, autocrine signaling during development46,47. DAGLα colocalization with CB1 within the growth cone promotes neuronal polarization and subsequent radial migration by preventing premature synaptogenesis through autocrine, CB1-mediated inhibition of presynaptic vesicle exocytosis48,49. Once synaptogenesis commences, DAGLα expression in the growth cone decreases while MAGL expression in the nascent presynapse increases50. Ultimately, DAGLα localization is redistributed to postsynaptic sites around the somatodendritic axis of mature neurons51. This change in enzyme localization is necessary for the switch from autocrine to paracrine 2-AG signaling and facilitates the ability of CB1 to regulate synaptic strength. Other important effects mediated by the activation of CB1 by 2-AG include neurite retraction52,53 and repulsive axonal head movement54. Global CB1 knockouts in mice, as well as specific interneuron and pyramidal cell CB1 knockouts, are viable but show axonal guidance errors and impaired postsynaptic target selection55-57. Interestingly, pharmacological treatment with both CB agonists and antagonists creates axon fasciculation errors during development in mice58-60. It is clear from this research that the ECS is necessary for correct axonal targeting and the subsequent establishment of synapses in the developing brain.

Due to the prominent role of the ECS in synapse establishment and maintenance, endogenous or exogenous disruptions to this system during fetal neurodevelopment can impact synaptogenesis and early circuit building. We have developed a model of altered ECS function in cortical spheroids to monitor the effects on synapse formation and the development of synaptic activity in neural circuits. Within our cortical spheroids, we found abundant expression of CB1 and ECS-associated enzymes FAAH, MAGL, and DAGLα. Through acute pharmacological treatment with both CB1 agonists and antagonists creates axon fasciculation errors during development in mice58-60. It is clear from this research that the ECS is necessary for correct axonal targeting and the subsequent establishment of synapses in the developing brain.

Methods

IPSC culture information and techniques. Control WTC-11-ActBmeGFP iPSCs were obtained under MTA from the Coriell Institute. The parental WTC-11 iPSC line was developed by Bruce Conklin of the Gladstone Institute, and was further gene-edited by the Allen Institute for Cell Science using CRISPR/Cas9 to tag endogenous β-actin with monomeric Green Fluorescent Protein (GFP)59. Control 7545 19B iPSCs were generated by Dr. Mike McConnell (Lieber Institute for Brain Development) from fibroblasts obtained under MTA from the Coriell Institute. ASD patient iPSCs (UMB#: 5278, 5403, and 797) were obtained under MTA from the NICHD Brain and Tissue Bank for Developmental Disorders. iPSCs were cultured in Matrigel-coated plates and maintained at 37 °C and 5% O2 in mTeSR or Essential 8 media. ROCK inhibitor Y-27632 (10 µM) was added to the media during the first 24 h of plating after thawing or splitting cells.

Cortical spheroid generation and feeding schedule. This protocol is adapted from the Pasca protocol55. On day 0, iPSCs were enzymatically lifted off the plate and pelleted for 5 min at 300 rpm. The pellet was disrupted and transferred into 3 wells of a low attachment plate with ESDMEM media (DMEM/F12, 1.5% HEPES, 1% GlutaMAX, 1% NEAA, 10% Knock-Out Serum, and 1% Pen/Strep). ROCK inhibitor Y-27632 (20 µM) and dual SMAD inhibitors SB431542 (10 µM) and Dorsomorphin (10 µM) were added to the media. On day 2, old ESDMEM was replaced with fresh ESDMEM and both SMAD inhibitors were replaced. This protocol continued for the next 3 days. On day 6, dual SMAD inhibitors were substituted with EGF (20 ng/mL) and FGF2 (20 ng/mL) in Neuronal Medium (Neurobasal-A, 2% Gibco B-27 serum substitute without vitamin A, 1% GlutaMax, 1% Pen/strep). Media was replaced every day with fresh FGF2 and EGF for the first 10 days and then every other day for the following 9 days. On day 25, BDNF (20 ng/mL) and NT3 (20 ng/mL) supplementation began and media was changed every other day until day 42. On day 43 all supplements were removed and the Neuronal Media was changed out every 4 days. All cultures underwent regular mycoplasma testing.

Quantitative real-time polymerase chain reaction. Three independent sets of cortical spheroids from control- and autism derived-iPSCs were used to isolate RNA and synthesize cDNA. For total RNA extraction, spheroids were homogenized in guanidinium-acid-phenol reagent. RNA quality was confirmed by gel electrophoresis. Total RNA (200 ng) was used to synthesize cDNA using an iScript synthesis kit (Bio-Rad). Completed reactions were diluted to a total volume of 200 µL using nuclease-free water and 5 µL was used for...
Dosing with SR141716A (SR) for confocal imaging. For confocal imaging, 90-day old cortical spheroids were treated with vehicle, 30 nM, or 300 nM of SR141716A. This CB₁-selective antagonist/inverse agonist was chosen due to experiments with it in prior work\(^4\). Concentrations employed were determined following dose–response experiments measuring neurite length of IPSC-derived neuronal monolayers (Supplemental Fig. S4). The SR141716A was dissolved in DMSO which also served as the vehicle control. Our vehicle control group was bathed in a final concentration of 0.00001% DMSO, equal to the amount of DMSO added to our 300 nM SR141716A dose groups. Each treatment group consisted of 3–5 midsized (diameter > 1 mm) cortical spheroids. Treatment occurred for 24 h and spheroids were kept in low-attachment plates at 37 °C. This experiment was repeated 3 times with independently grown sets of cortical spheroids. A treatment period of 24 h was chosen to selectively perturb synaptogenesis (rather than migration or differentiation) and prior research from our lab has shown that this is an effective period of treatment for altering synaptogenesis\(^2\). 

Fixation and cryosection. After 24 h of treatment, spheroids were fixed in 4% paraformaldehyde and cryo-protected in 30% sucrose solution. Spheroids were then placed in OCT embedding medium overnight at 4 °C in a 24 well plate. Spheroids were transferred to a disposable base mold and frozen with dry ice and 2-methyl butane slush. Once frozen, spheroids were sliced 10 µm thick on a cryostat with the objective temperature set at −7 °C. Mount sections were thawed and transferred on to slides treated with 2% 3-Aminopropyl Triethoxysilane.

Immunofluorescent staining. A Sequenza rack system was used to stain sectioned cortical spheroids. Slides were blocked in 5% normal goat serum for 30 min. Primary antibodies were diluted in 2% normal goat serum and applied to the slides overnight at 4 °C. Secondary antibodies were diluted in 2% normal goat serum and applied to the slides for 1 h at room temperature in the dark. Slides were removed from the Sequenza rack system and 1.5 mm coverslips were affixed to the slide using Fluorogel with or without DAPI. Slides were placed on a plate warmer for 15 min to ensure coverslip attachment. Cortical spheroids were kept in the dark during the immunofluorescent staining protocol. All antibodies used can be found in Supplemental Table S2. Our CB₁ antibody was raised to target a 16-amino acid region within the intracellular tail portion of the receptor. The predicted epitope is FRSMFP, corresponding to amino acids 409–414 of human CB₁. This well-conserved sequence is identical across human, rat, mouse and zebra finch orthologs. Anti-CB₁ specificity was demonstrated previously by western blot labeling of appropriately sized proteins, expected histological CNS staining patterns and absence of both types of labeling following preincubation with 20 µM of the immunizing peptide\(^2\). 

Confocal imaging settings and equipment. Imaging took place using ZEN Black Software on a Zeiss Laser Scanning Microscope 700 with a 40 × objective (Plan-Apochromat/1.4 Oil DIC M27). Only cortical spheroids larger than 1 mm were imaged and analyzed. Nine total images per treatment group were taken. Each image consisted of a 4 × 4 tile scan (592.16 × 592.16 µm, 3789 × 3789 pixels) as well as a z-stack. The z-stack was compiled of 5 slices with 1 µm between each slice, making a 4 µm thick stack. Confocal images in Figs. 1 and 2 were pseudocolored from their original, 8-bit, greyscale format. Brightness and contrast have been enhanced equally across controls and experimental groups. Figures were assembled in Adobe Photoshop version 21.2.

STORM staining and imaging. The preparation of slides for the Nikon STORM microscope follows the same protocol as described above for the confocal with the addition of a 10-min, formaldehyde (4%) and glutaraldehyde (0.1%) post-fix after application of the secondary antibody. Slides were mounted with Vectashield and put onto a plate warmer for 20 min then sealed with nail polish to ensure coverslips were anchored. Stained slides were used within 2 weeks of staining to ensure a robust signal. STORM images were acquired using Nikon NIS-Elements AR software and an Apo TIRF 100 × objective (1.49 NA) on a Nikon Ti-E inverted microscope equipped with N-STORM. Period count was set to 20,000 and laser intensity was set to 100% for all channels.

Dissociation of cortical spheroids for microelectrode array (MEA) recording. Cytoview 24-well MEA plates with 16 electrodes per well (Axion Biosystems) were prepared with polyethyleneimine which was incubated at 37 °C for 1 h. Wells were then washed 4 times with sterile water and allowed to dry out overnight in a sterile hood. Wells were treated with laminin (5 µM) overnight at room temperature after which laminin was replaced with HBBS prior to plating. Cortical spheroids were dissociated onto 24-well MEA plates after 90 days of growth. Between 4 and 6 cortical spheroids were placed into a 1.5 mL tube and washed with ice cold HBBS. Neuronal Isolation Enzyme with papain (Pierce Primary Neuron Isolation Kit, ThermoFisher) was added to the spheroids and incubated at 37 °C for 30 min. Spheroids were dissociated into a single-cell suspension via vigorous pipetting with a 1000 µL micropipette. A cell count was performed with trypan blue. Cells were plated at a density of 250,000 cells per well. The MEA plate was placed in a 37 °C incubator for an hour before MEA media (Neurobasal-A, 2% B27 Plus, 1% GlutaMAX, 1% Pen/Strep) was added to each well. Half of the media was replaced every 3 to 4 days with fresh media.
a) DAGLα activation leads to CB1 activation, which results in retraction and repulsion of growth cone.

b) DAGLα activation inhibits vesicle release, leading to decreased synaptic strength.

VGLUT1 = green
PSD95 = red
CB1 = blue

VGAT = cyan
Gephyrin = red
CB1 = green
ECS constituents are expressed in 90-day old cortical spheroids. CB₁, MAGL, DAGLα, and FAAH have increased transcription in cortical spheroids derived from patients with the neurodevelopmental disorder ASD. (a) Primary developmental endocannabinoid 2-AG is produced by DAGLα within the growth cone and activates CB₁ in an autocrine manner during fetal neurodevelopment. (b) Paracrine signaling mediated by 2-AG at mature synapses regulates both excitatory and inhibitory synaptic plasticity. (c) Expression levels of DAGLα, FAAH, and MAGL, are elevated in cortical spheroids derived from patients with ASD compared to controls (DAGLα: p = 0.007, FAAH: p = 0.014, MAGL: p = 0.012). Expression levels were assessed by qRT-PCR and are expressed as fold differences from TATA-BP. No significant gene expression differences were observed between control- and ASD-derived samples for CB₁. Error bars = SEM, compared using student’s t-test. (d) Confocal images of 10 µm thick cryosections of 90-day old control cortical spheroids expressing ECS constituents CB₁, DAGLα, MAGL, and FAAH. All cortical spheroids express endogenous GFP-linked β-actin (actin-GFP). (e) Example of an excitatory synapse captured using STORM microscopy. Dashed line represents the synaptic cleft, with CB₁ (blue) and VGLUT1 (green) on the presynaptic side and PSD95 (red) on the postsynaptic side. (f) Example of inhibitory synapse captured via STORM microscopy with CB₁ (green) located at the presynapse with VGAT (cyan), opposite of gephyrin (red). (g) The median distance between CB₁ and presynaptic marker VGLUT-1 (0.060 µm) is significantly less (p < 0.0001, n = 126 synapses) than the distance between CB₁ and postsynaptic marker PSD-95 (0.141 µm), indicating presynaptic localization. (h) The median distance between CB₁ and PSD-95 (0.046 µm) was significantly shorter (p = 0.014, n = 47 synapses) than the distance between gephyrin and CB₁ (0.062 µm), indicating presynaptic localization of CB₁ at inhibitory synapses. (i) DAGLα was presynaptic, with a median distance between DAGLα and VGLUT1 (0.052 µm) that was significantly shorter (p = 0.006, n = 77 synapses) than the distance between DAGLα and PSD95 (0.086 µm). (j) MAGL was postsynaptic, with a significantly shorter (p < 0.001, n = 105 synapses) median distance between MAGL and PSD95 (0.045 µm) compared to the distance between MAGL and VGLUT1 (0.072 µm). Distances were compared using the Mann Whitney test, significance was defined as p < 0.05. Illustrations in panel a and b were created by Alexis Papariello.

MEA recording and SR141716A dosing. MEA recording was performed on day 40 after plating and 48 h after the last feeding. Extracellular recordings were performed in AxIS Navigator software using an Axion Maestro Edge set at 37 °C and 5% CO₂. The recording stream was configured for spontaneous neural bursting activity with network burst detection. Data underwent DC offset filtering and Butterworth band-pass filtering with 0.1 Hz and 5 kHz cutoffs prior to spike detection. A “spike” was defined as a short, extracellular, electrical event with a peak voltage 6 times or greater than the standard deviation of the estimated “noise” signal. A “burst” was defined as 5 or more spikes with no more than 100 ms separating each spike. The MEA plate was placed into the Maestro Edge and activity was allowed to normalize for 5 min prior to a 10-min basal recording. The plate was then removed from the instrument and dosed with vehicle (0.00001% DMSO), 3 nM, 30 nM, or 300 nM of SR141716A in a sterile hood (n = 6 wells per treatment group per independent replicate). The plate was then returned to the instrument and one 10-min recording was taken every hour for 24 h. Recording took place 48 h after the last feeding. Experiment repeated in triplicate with independently grown and plated spheroids.

Data analysis software and settings. Confocal imaging analysis. All .czi images were exported from the ZEN Black software as a greyscale .tif files. Using ImageJ, individual channel z-stacks were consolidated into a maximum intensity z projection. Each channel had a threshold applied to it. Synapse masks were defined by the colocalization plugin using the thresholded presynaptic and postsynaptic images. For Fig. 2, synapse characteristics of area, size, and number were determined using the particle analysis function. For Fig. 3, we used ratiometric image analysis by using the image calculator function in Image J to divide the active RhoA area by the total RhoA area. This area was then overlayed with VGLUT1 area to determine the ratio of activated RhoA at excitatory presynapses. Synapse measurements were taken along the outer edge of the cortical spheroid using 10 × 100 µm diameter circles. Only the outer 100 µm was measured because this is the area of active synaptogenesis in our model at 90 DIV. Within each circle the pre-synaptic, post-synaptic, co-localized pre- and post-synaptic, and CB₁ staining area was measured. All area values were normalized to the internal GFP-tagged actin. Images per dose group were analyzed and this process was independently repeated in triplicate. A total of 6–7 cortical spheroids were analyzed per treatment group.

MEA analysis. MEA video recording streams (.raw files) were batch processed in AxIS Navigator to detect spontaneous neural spiking activity (.spk). These files were further processed into .csv files by Axion Neural Metrics software. Active electrodes were defined by having 5 or more spikes per minute. The weighted mean firing rate is a measure of spikes per minute within a well and is weighted by the number of active electrodes within that well.

STORM analysis. In the NIS-Elements AR program, raw .nd2 files had a constant threshold applied corresponding to the lowest “blink” value in each channel. These files were batch processed into .czi files by Axion Neural Metrics software. Active electrodes were defined by having 5 or more spikes per minute. The weighted mean firing rate is a measure of spikes per minute within a well and is weighted by the number of active electrodes within that well.

Figure 1. ECS constituents are expressed in 90-day old cortical spheroids. CB₁, MAGL, DAGLα, and FAAH have increased transcription in cortical spheroids derived from patients with the neurodevelopmental disorder ASD. (a) Primary developmental endocannabinoid 2-AG is produced by DAGLα within the growth cone and activates CB₁ in an autocrine manner during fetal neurodevelopment. (b) Paracrine signaling mediated by 2-AG at mature synapses regulates both excitatory and inhibitory synaptic plasticity. (c) Expression levels of DAGLα, FAAH, and MAGL are elevated in cortical spheroids derived from patients with ASD compared to controls (DAGLα: p = 0.007, FAAH: p = 0.014, MAGL: p = 0.012). Expression levels were assessed by qRT-PCR and are expressed as fold differences from TATA-BP. No significant gene expression differences were observed between control- and ASD-derived samples for CB₁. Error bars = SEM, compared using student’s t-test. (d) Confocal images of 10 µm thick cryosections of 90-day old control cortical spheroids expressing ECS constituents CB₁, DAGLα, MAGL, and FAAH. All cortical spheroids express endogenous GFP-linked β-actin (actin-GFP). (e) Example of an excitatory synapse captured using STORM microscopy. Dashed line represents the synaptic cleft, with CB₁ (blue) and VGLUT1 (green) on the presynaptic side and PSD95 (red) on the postsynaptic side. (f) Example of inhibitory synapse captured via STORM microscopy with CB₁ (green) located at the presynapse with VGAT (cyan), opposite of gephyrin (red). (g) The median distance between CB₁ and presynaptic marker VGLUT-1 (0.060 µm) is significantly less (p < 0.0001, n = 126 synapses) than the distance between CB₁ and postsynaptic marker PSD-95 (0.141 µm), indicating presynaptic localization. (h) The median distance between CB₁ and PSD-95 (0.046 µm) was significantly shorter (p = 0.014, n = 47 synapses) than the distance between gephyrin and CB₁ (0.062 µm), indicating presynaptic localization of CB₁ at inhibitory synapses. (i) DAGLα was presynaptic, with a median distance between DAGLα and VGLUT1 (0.052 µm) that was significantly shorter (p = 0.006, n = 77 synapses) than the distance between DAGLα and PSD95 (0.086 µm). (j) MAGL was postsynaptic, with a significantly shorter (p < 0.001, n = 105 synapses) median distance between MAGL and PSD95 (0.045 µm) compared to the distance between MAGL and VGLUT1 (0.072 µm). Distances were compared using the Mann Whitney test, significance was defined as p < 0.05. Illustrations in panel a and b were created by Alexis Papariello.
**Results**

**Cortical spheroids derived from human iPSCs express CB$_1$, DAGLα, MAGL, and FAAH.** The ECS plays a dual role in the developing brain by modulating both growth cone directionality$^{7,37,39}$ (Fig. 1a) and presynaptic feedback inhibition at mature synapses$^{28,46}$ (Fig. 1b). Disruptions to this system during critical periods of development may have lasting impacts on neural circuitry building. We first examined ECS gene expression levels in cortical spheroids derived from control vs autism samples were assessed using unpaired two-tailed t-tests. Mann Whitney tests were utilized for STORM distance data. For confocal image analysis, a one-way ANOVA with multiple comparisons was performed across the vehicle control group and both SR dose groups. Stains were normalized to the internal standard of GFP-tagged β-actin. A Kolmogorov-Smirnoff test was performed for the cumulative distribution in Fig. 2. One way ANOVA with multiple comparisons was used for RhoA data. One-way ANOVA with multiple comparisons was utilized for MEA data across time. Dunnett’s T3 multiple comparisons test was used for comparison of MEA interquartile ranges. A p-value of less than 0.05 was considered statistically significant. All values are reported as mean ± SEM.

Statistical analysis and reporting. Statistical analysis was performed in GraphPad Prism 8. Differences in endocannabinoid gene expression levels in spheroids derived from control vs autism samples were assessed using unpaired two-tailed t-tests. Mann Whitney tests were utilized for STORM distance data. For confocal image analysis, a one-way ANOVA with multiple comparisons was performed across the vehicle control group and both SR dose groups. Stains were normalized to the internal standard of GFP-tagged β-actin. A Kolmogorov-Smirnoff test was performed for the cumulative distribution in Fig. 2. One way ANOVA with multiple comparisons was used for RhoA data. One-way ANOVA with multiple comparisons was utilized for MEA data across time. Dunnett’s T3 multiple comparisons test was used for comparison of MEA interquartile ranges. A p-value of less than 0.05 was considered statistically significant. All values are reported as mean ± SEM.

Using immunofluorescent staining and imaging, we observed abundant cytosolic, synaptic, and neurite expression of CB$_1$ in our 90-day old, control patient-derived cortical spheroids (Fig. 1d). We also observed the 2-AG enzymatic regulators, MAGL and DAGLa, in our 90-day old cortical spheroids, as well as FAAH. Interestingly, in concordance with our qRT-PCR results, we found that the area of DAGLa and MAGL, the principal synthetic and metabolic enzymes for 2-AG respectively, were expressed at significantly higher levels in cortical spheroids derived from autistic patient relatives to controls (DAGLa: p = 0.007, MAGL: p = 0.012) (Fig. 1c). Across 3 independent cortical spheroid experiments, DAGLa expression increased from 0.102 ± 0.02 to 0.258 ± 0.05 -fold mRNA expression relative to TATA-BP. Even more dramatically, MAGL expression increased from 0.056 ± 0.01 to 0.351 ± 0.11 -fold mRNA expression relative to TATA-BP. Additionally, the enzyme responsible for anandamide metabolism, FAAH, had significantly higher expression (p = 0.014) in ASD spheroids relative to controls and increased from 0.050 ± 0.01 to 0.159 ± 0.04-fold mRNA expression relative to TATA-BP. Significant expression differences were not observed for CB$_1$, DAGLβ or NAPE-PLD. We attempted to amplify CB$_2$ receptor cDNA but no amplification was observed, leading us to conclude that this receptor is not expressed at detectable levels in our model. The lack of CB$_2$ serves as an internal control for our model system because the use of dual-SMAD inhibition blocks mesodermal and endodermal differentiation and therefore the cortical spheroids do not generate microglia, the cell type which primarily expresses CB$_2$ in the brain$^{47,48}$.

Figure 2. CB$_1$ antagonist SR141716A (SR) increases excitatory synaptogenesis in a cortical spheroid model of human brain development. (a) Work flow of synapse analysis. (b) Representative confocal images of cortical spheroids stained with excitatory presynaptic marker VGLUT1 and excitatory postsynaptic marker PSD95. (c) Representative confocal images of cortical spheroids stained with inhibitory presynaptic marker VGAT and inhibitory postsynaptic marker gephyrin. All cortical spheroids endogenously express GFP-β-actin (GFP-β-actin). This was used as the internal control for synaptic area measurements. (d) Excitatory presynaptic (VGLUT1) and postsynaptic (PSD95) area after SR treatment. Both high and low doses of SR increased VGLUT1 area compared to vehicle control (0 vs 30 and 0 vs 300: p = 0.0001). SR increased PSD95 area in a dose dependent manner (30 vs 300: p = 0.0001). (e) Box plot of inhibitory presynaptic (VGAT) and postsynaptic (gephyrin) area after SR treatment. The low dose but not the high dose of SR increased VGAT area (0 vs 30: p = 0.0035). (f) SR increases excitatory synapse area in a dose dependent manner (30 vs 300: p = < 0.0001). SR also increased inhibitory synapse area when compared to control (0 vs 30: p = 0.0121, 0 vs 300: p = 0.0301) (g) SR increases the number of inhibitory synapses in a dose dependent manner (30 vs 300: p = 0.0030) and increases the number of inhibitory synapses at the low dose when compared to vehicle control (0 vs 30: p = 0.0011). (h) SR increases the area of CB$_1$-positive excitatory synapses at the low dose when compared to vehicle control (0 vs 30: p = 0.0001) but has no effect on the ratio of CB$_1$-positive inhibitory synapses. (i) Cumulative distribution plot of individual excitatory synapse size after treatment with SR. Analyzed by Kolmogorov–Smirnov tests. Data from graphs d-h was analyzed by ordinary one-way ANOVA with multiple comparisons. Data represented as mean ± SEM. n = 90 areas analyzed per dose group. Significance (*) defined as p < 0.05.

We wanted to further analyze whether ECS components exhibit synaptic localization characteristic of fetal autocrine signaling. However, confocal microscopy is limited by resolution and prevents us from determining whether ECS components such as CB$_1$ correctly localizes to the presynaptic compartment in our system. To overcome this limitation, we used stochastic optical reconstruction microscopy (STORM) which has a resolution...
of up to ~20 nm in x,y, allowing us to resolve individual synapses which have a synaptic cleft distance of ~20 nm\textsuperscript{26,51}. Examples of excitatory and inhibitory synapses can be found in Fig. 1e, f, respectively. Using STORM, we analyzed 126 excitatory synapses and confirmed presynaptic localization of CB\textsubscript{1} in our cortical spheroid model, consistent with previous findings in other models\textsuperscript{7,52,53}. The median distance between CB\textsubscript{1} and presynaptic marker VGLUT-1 was 0.060 µm, which is significantly smaller (p < 0.0001) than the median distance between CB\textsubscript{1} and postsynaptic marker PSD-95 (0.141 µm) (Fig. 1g). This indicates that CB\textsubscript{1} is closer to the presynaptic marker than the postsynaptic marker and preferentially localizes to the presynapse. The median distance between VGLUT-1 and PSD-95 (0.131 µm) was similar to distance between CB\textsubscript{1} and PSD-95 and is consistent with our previous STORM measurements of synaptic cleft size\textsuperscript{26,27}. Additionally, we investigated CB\textsubscript{1} localization at inhibitory synapses (n = 47) and observed a significantly shorter (p = 0.014) median distance between CB\textsubscript{1}/presynaptic marker VGAT (0.046 µm) compared to CB\textsubscript{1}/postsynaptic marker gephyrin (0.062 µm) (Fig. 1h). Further, we observed presynaptic localization of DAGL\textalpha (Fig. 1i) and postsynaptic localization of MAGL (Fig. 1j) at excitatory synapses. The median distance between DAGL\textalpha and VGLUT1 (0.052 µm) was significantly shorter (p = 0.006, n = 77 synapses) than the distance between DAGL\textalpha and PSD95 (0.086 µm). MAGL was postsynaptic, with a significantly shorter (p > 0.001, n = 105 synapses) median distance between CB\textsubscript{1}/presynaptic marker VGAT (0.046 µm) compared to CB\textsubscript{1}/postsynaptic marker gephyrin (0.062 µm) (Fig. 1h).

Since the number of CB\textsubscript{1} molecules at inhibitory versus excitatory synapses could impact the effect of pharmacological treatment, we used STORM microscopy to analyze the distribution of CB\textsubscript{1} receptor count at excitatory and inhibitory synapses. We found that CB\textsubscript{1} receptors are more abundant at excitatory synapses (530 ± 25 CB\textsubscript{1} molecules/synapse) than at inhibitory synapses (262 ± 14 CB\textsubscript{1} molecules/synapse) in the outer, 100 µm of the cortical spheroid (p > 0.001, unpaired t-test) (Supplemental Fig. S2). Association of the presynaptic terminal with a postsynaptic process is indicative of synapse formation, and increased postsynaptic area is indicative of synaptic strengthening\textsuperscript{54}. Our model recapitulates synaptic scaling at both excitatory and inhibitory synapses as demonstrated by the positive relationship between the molecular count of pre- and postsynaptic markers at a given synapse (Supplemental Fig. S2). We therefore sought to determine whether the number of CB\textsubscript{1} molecules scaled with increased postsynaptic association. We observed a positive relationship between CB\textsubscript{1} receptor count and postsynaptic marker count at both excitatory (PSD95) and inhibitory (gephyrin) synapses (Sup Fig. S2), suggesting that CB\textsubscript{1} receptors exhibit synaptic scaling.

Thus, we have determined that human cortical spheroids express ECS machinery, and that CB\textsubscript{1}, the predominant ECS receptor type in the brain, localizes to presynaptic compartments at both excitatory and inhibitory synapses. We also observed DAGL\textalpha localization to the presynapse and MAGL localization to the postsynapse.

Figure 3. CB\textsubscript{1} selective antagonism by SR141716A in 90-day old cortical spheroids decreases activity of actin regulator GTPase RhoA (a) Representative confocal images of cryosectioned cortical spheroids stained with Active RhoA, Total RhoA, and excitatory presynaptic marker VGLUT1. Activated RhoA was distinguished from total RhoA by an antibody bound to the GTP form of RhoA (activated). An antibody bound to the GDP form of RhoA distinguished the “total” RhoA. All cortical spheroids express endogenous GFP-linked β-actin (actin-GFP). (b) Shown are the normalized ratios of activated RhoA to total RhoA as measured by ratiometric image analysis. Treatment of cortical spheroids with 30 nM and 300 nM SR141716A decreased the relative intensity of gray values at sites colocalized with excitatory presynaptic marker VGLUT1 (0 nM vs 30 nM: p > 0.001, 0 nM vs 300 nM: p > 0.001). The significant decrease of gray value intensity indicates decreased RhoA activation in the dose groups when compared to the control group. Additionally, an increase in RhoA activity was seen in the 300 nM group (25–75% range: 0.465–1.11 a.u., median: 0.728 a.u. n = 57,577) when compared to the 30 nM group (25–75% range: 0.417–1.03 a.u., median: 0.670, n = 48,899) (30 nM vs 300 nM: p > 0.001). Significance determined by Kruskal–Wallis test with significance defined as p < 0.05. Control group (0 nM SR) had n = 43,862 VGLUT1-positive areas analyzed. 3 independent sets of 3 cortical spheroids were analyzed per treatment group for a total of 4–6 cortical spheroids analyzed. Normalized mean gray values are reported as relative intensity with arbitrary units (a.u.).
in excitatory synapses using STORM microscopy. Our data supports cortical spheroids as a model of the fetal ECS system.

**Treatment with CB₁ antagonist SR141716A increases the number and total area of excitatory synapses.** Having established the expression and presynaptic localization of CB₁ within our system, we sought to determine how ECS disruption impacts synaptogenesis. In order to selectively perturb CB₁ during synaptogenesis, we allowed cortical spheroids to develop for 90 days, so as to not disrupt neural differentiation and migration preceding synaptogenesis. At 90 days old, our cortical spheroids model the mid-gestational fetal human brain, a critical window of development during which the brain undergoes rapid synaptic proliferation. Disruptions to the spatial and temporal regulation of synaptogenesis during this critical window is thought to drive developmental disorders such as ASD. At 90 days of development, we have previously demonstrated that our cortical spheroids exhibit both excitatory and inhibitory synapses. Furthermore, these synaptic connections exhibit a high level of plasticity, and are readily altered by acute perturbations to either the intracellular cytoskeleton or extracellular matrix. Thus, we have established a window to selectively observe how CB₁ signaling contributes to the initial formation of synaptic connections and subsequent development of synaptic activity. In order to selectively disrupt the process of synaptogenesis, we acutely treated 90-day-old cortical spheroids with selective CB₁ antagonist SR141716A (SR) for 24 h and observed the resulting effects on excitatory and inhibitory synapses.

Using confocal image analysis (Fig. 2a), we determined the effects of SR treatment on excitatory and inhibitory synaptogenesis by independently measuring pre- and post-synaptic marker area. Cortical spheroids were stained with antibodies against excitatory synaptic markers [vesicular glutamate transporter 1 (VGLUT-1) and postsynaptic density protein 95 (PSD-95)] or inhibitory synaptic markers [vesicular GABA transporter (VGAT) and gephyrin (Gephyrin)]. We defined the area of overlap between presynaptic marker (VGLUT-1 or VGAT) and their respective postsynaptic marker (PSD-95 and Gephyrin) as a “synapse”. We determined the effect of SR on the number of synapses and size of synapses in the outer 100 µm of the spheroid using this method. Example confocal images used for analysis are given in Fig. 2b, c. Under basal conditions, our cortical spheroids have more excitatory synapses than inhibitory synapses. However, we found that SR treatment impacts both excitatory and inhibitory synapses. SR treatment increased expression of excitatory synapses markers in a dose-dependent fashion, whereas increased inhibitory synapses were only observed at the lower dose of 30 nM SR. To compare the area of synaptic markers across treatment groups, we normalized the area of the synaptic marker to the endogenous β-actin-GFP expression in our cortical spheroids.

The area of excitatory presynaptic marker VGLUT-1 significantly increased from 32.4 ± 4.6% in the vehicle control to 76.7 ± 6.7% and 74.0 ± 7.8% in the 30 nM and 300 nM SR dose groups, respectively (0 vs 30: p < 0.001, 0 vs 300: p = 0.001) (Fig. 2d). The area of the excitatory postsynaptic scaffold PSD-95 also significantly increased from 14.9 ± 1.4% in the vehicle control to 27.8 ± 2.4% and 49.5 ± 3.0% in the 30 nM and 300 nM SR dose groups, respectively (0 vs 30: p < 0.001, 0 vs 300: p < 0.001) (Fig. 2d). Additionally, there was a significant, dose dependent relationship between the low and high doses of SR on PSD-95 expression (30 vs 300: p < 0.001) (Fig. 2d). Using the colocalization of excitatory presynaptic marker VGLUT-1 and postsynaptic marker PSD-95, we determined that SR treatment significantly increased both the total area (30 vs 300: p < 0.001) (Fig. 2f) and number (30 vs 300: p = 0.003) (Fig. 2g) of excitatory synapses in a dose dependent manner. Excitatory synapse area significantly increased from 1.6 ± 0.3% in the vehicle control to 5.7 ± 0.6% and 10.7 ± 1.2% in the 30 nM and 300 nM SR dose groups, respectively (0 vs 30: p < 0.001, 0 vs 300: p < 0.001) (Fig. 2f). Additionally, the number of excitatory synapses per area of actin significantly increased from 0.03 ± 0.005 synapses/µm² in the vehicle control group to 0.05 ± 0.005 synapses/µm² and 0.09 ± 0.007 synapses/µm² in the 30 nM and 300 nM SR dose groups, respectively (0 vs 30: p = 0.009, 0 vs 300: p < 0.001) (Fig. 2g). The size of individual excitatory synapses trended towards an increase but was found not significant by Kolmogorov–Smirnov test, despite a rightward shift in the cumulative distribution plot (Fig. 2i). Having observed that CB₁ antagonism increases excitatory synaptogenesis, we also sought to determine whether synaptic CB₁ distribution was altered in response to SR treatment. We therefore examined CB₁ localization to excitatory and inhibitory synapses. CB₁ area as a percent of total excitatory synapse area significantly increased from 25.6 ± 1.5% of total excitatory synapses to 37.5 ± 1.6% of total excitatory synapses after application of 30 nM SR (0 vs 30: p < 0.001) (Fig. 2h). Surprisingly, the percent of CB₁-positive excitatory synapses returned to a value similar to the vehicle control after application of 30 nM SR (28.4 ± 2.6% of total excitatory synapses) (0 vs 300: p = 0.719, 30 vs 300: p = 0.014) (Fig. 2h). Thus, the 30 nM SR treatment captures a window of dynamic ECS alterations at excitatory synapses.

Interestingly, the effect of SR on inhibitory synapses was greatest at the lower, 30 nM dose of SR. The area of presynaptic inhibitory marker VGAT significantly increased from 23.2 ± 4.6% in the vehicle control to 34.8 ± 5.7% in the 30 nM SR dose group (0 vs 30: p = 0.003) (Fig. 2e). A decrease in VGAT was observed in the high dose group (19.4 ± 1.5%) when compared to the low dose group (30 vs 300: p < 0.001) (Fig. 2e). When compared to the controls, the area of the postsynaptic inhibitory scaffold, gephyrin, was not significantly altered, however, there was a decrease between low and high doses (30 vs 300: p = 0.011) (Fig. 2e). There was a significant increase in the area (0 vs 30: p = 0.012) (Fig. 2f) and number (0 vs 30: p = 0.001) (Fig. 2g) of inhibitory synapses in the low dose SR group compared to the control group. The area of inhibitory synapses trended from 0.015 ± 0.002 synapses/µm² in the control group to 0.035 ± 0.005 synapses/µm² and 0.021 ± 0.003 synapses/µm² in the low and high dose groups, respectively (0 vs 30: p = 0.012, 0 vs 300: p = 0.030) (Fig. 2f). The number of inhibitory synapses per area of actin changed from 0.015 ± 0.002 synapses/µm² in the control group to 0.035 ± 0.005 synapses/µm² and 0.021 ± 0.003 synapses/µm² in the low and high dose groups, respectively (0 vs 30: p = 0.001, 30 vs 300: p = 0.047) (Fig. 2g). Unlike the results we observed earlier where there was a redistribution of synaptic CB₁ at excitatory synapses, we did not observe
SR141716A increased variability of synaptic activity as measured by microelectrode array (MEA). The effects of cannabinoid modulation on neural activity are complex due to CB₁ localization at both glutamatergic and GABAergic synapses. To address whether the complex changes in synaptogenesis altered the development of spontaneous activity in neural circuits, we used MEA to measure the extracellular field potential which corresponds to action potential. After 90 days of development, we dissociated cortical spheroids directly onto microelectrodes (Fig. 4a, b). In order to observe consistent activity in our control spheroids, we allow neurons to re-establish connections for an additional month after dissociation, resulting in reproducible activity measurements. We then measure spontaneous neural activity with or without SR treatment. Spontaneous extracellular activity caused by multiple, local action potentials is measured by the electrodes in units called “spikes” (Fig. 4c). Thus, a spike represents an increase in activity across a small area of multiple cells. Multiple spikes of 5 or more in quick succession (<100 ms between spikes) are defined as “bursts” and represent rapid communication between populations of cells. Synchronous bursting between multiple electrodes is characteristic of mature communication patterns.

We determined that our vehicle decreased the weighted mean firing rate (WMFR) of dissociated cortical spheroids over a period of 24 h. Immediately after dosing, the mean WMFR was 7.64 ± 7.0% above the pre-treatment average, however, after 3 h of treatment the WMFR started to decrease (-9.1 ± 6.95% of pre-treatment) and continued to decrease. The WMFR of the vehicle control group significantly decreased from the initial recording after 15 h of vehicle treatment (0 h vs 15 h: p = 0.0423, 0 h vs 18 h: p = 0.0131, 0 h vs 21 h p = 0.0094, 0 h vs 24 h p = 0.0099). There was no significant change to WMFR over 24 h of SR treatment. (e) Bursting frequency was significantly reduced after 6 h of vehicle treatment (0 h vs 6 h: p = 0.0442). (f) The average WMFR IQR of the control group over 24 h was 39.3 ± 4.7 compared to 61 ± 4.6 in the 3 nM group, 124 ± 7.3 in the 30 nM group, and 87 ± 7.3 in the 300 nM group. SR treatment significantly increased the WMFR IQR when compared to vehicle treated controls (VEH vs 3 nM: p = 0.026, VEH vs 30 nM: p < 0.001, VEH vs 300 nM: p = 0.001). (f) The variability of the 30 nM group was significantly higher than the 3 nM group (3 nM vs 30 nM: p < 0.0001) but the variability of the 300 nM group was significantly lower than the 30 nM group (30 nM vs 300 nM: p = 0.0166). (g) Bursting frequency IQR within the control group over 24 h was 32.5 ± 3.5. SR increased the mean IQR to 110 ± 20 in the 3 nM group, 647 ± 88 in the 30 nM group, and 137 ± 27 in the 300 nM group. SR significantly increased bursting frequency variability (VEH vs 3 nM: p = 0.0221, VEH vs 30 nM: p = 0.0005, VEH vs 300 nM: p = 0.0267). Variability was greatest at 30 nM (3 nM vs 30 nM: p = 0.0008, 30 nM vs 300 nM: p = 0.0009). Effects in panels (d) and (e) were compared using one-way ANOVA with multiple comparisons. Effects in panels (f) and (g) were compared using Dunnett’s T3 multiple comparisons test. Data represented as mean ± SEM. Significance (*) defined by p < 0.05. Schematics in panel (a) and (c) were created with BioRender (https://biorender.com/).
**Figure 1.** Stepwise generation of MEA cultures from hiPSCs.

**a**. Schematic representation of the process.

- **hiPSC culture (10-14 days)**: hiPSCs are cultured to reach a sufficient population.
- **Cortical spheroid culture (>90 days)**: hiPSCs are differentiated into neural cells and organized into spheroids.
- **Papain dissociation**: The spheroids are dissociated into single cells.
- **MEA plating**: The dissociated cells are plated onto an MEA substrate.

**b**. Image of a MEA recording setup.

**c**. MEA recording on an Axion Biosystems Maestro.

**d**. Weighted mean firing rate (WMFR) analysis with vehicle and 3nM SR treatments.

- **Vehicle** and **3nM SR** treatments show changes in WMFR over time, with significant differences indicated.

**Graphs**:

- **Vehicle**
  - % change from pre-treatment WMFR vs. hours post treatment for different concentrations.
- **3nM SR**
  - Similarly, % change from pre-treatment WMFR vs. hours post treatment for different concentrations.

**Legend**:
- **SPIKES**: Spikes in activity.
- **BURST (in red)**: Burst activity highlighted in red.

**Notes**:
- The graphs illustrate the effects of different treatments on the WMFR over time, with statistical significance marked by asterisks (*).
Figure 4. (continued)
vs 12 h \(p < 0.0001\), 0 h vs 15 h: \(p < 0.0001\), 0 h vs 18 h: \(p = 0.0010\), 0 h vs 21 h: \(p < 0.0000\), 0 h vs 24 h: \(p = 0.0001\) (Fig. 4e). While the low dose of SR showed a significant decrease in bursting frequency after 18 h (0 h vs 18 h: \(p = 0.0440\), 0 h vs 21 h: \(p = 0.0308\)), this decrease took a longer time to manifest when compared to the vehicle-treated group (Fig. 4e). Treatment with 30 nM of SR increased bursting frequency by over 200% for every time point, but this increase did not vary significantly across time. The high dose of 300 nM SR also increased overall bursting frequency by about 75% but there was no significant effect over time.

While we did not observe significant increases in WMFR and bursting frequency, we did observe a high degree of variability across our SR dose groups. We measured the effects of variability by utilizing the interquartile ranges (IQR) of each timepoint within dose groups and then compared dose groups. The average WMFR IQR of the control group over 24 h was 39.3 ± 4.7 compared to 61 ± 4.6 in the 3 nM group, 124 ± 7.3 in the 30 nM group, and 87 ± 7.7 in the 300 nM group. SR treatment significantly increased the WMFR IQR when compared to vehicle treated controls (VEH vs 3 nM: \(p = 0.026\), VEH vs 30 nM: \(p < 0.001\), VEH vs 300 nM: \(p = 0.0001\) (Fig. 4f). The variability of the 30 nM group was significantly higher than the 3 nM group (3 nM vs 30 nM: \(p < 0.0001\)) but the variability of the 300 nM group was significantly lower than the 30 nM group (30 nM vs 300 nM: \(p = 0.0166\)) (Fig. 4f). Our bursting frequency results parallel the results of the WMFR, where SR caused significantly more variability compared to the vehicle control (VEH vs 3 nM: \(p = 0.0221\), VEH vs 30 nM: \(p = 0.0005\), VEH vs 300 nM: \(p = 0.0267\) (Fig. 4g). Bursting frequency IQR within the control group over 24 h was 32.5 ± 3.5. SR increased the mean IQR to 110 ± 20 in the 3 nM group, 647 ± 88 in the 30 nM group, and 137 ± 27 in the 300 nM group. Similar to WMFR measurements, we observed a biphasic dose response that displayed significantly more IQR variability in our 30 nM group than in our 3 nM group (3 nM vs 30 nM: \(p = 0.001\)) and less variability in our 300 nM group when compared to the 30 nM group (30 nM vs 300 nM: \(p = 0.001\)) (Fig. 4g). Increased variability of synaptic activity, particularly at the 30 nM dose of SR, parallels the complex and differential changes to excitatory and inhibitory synapse formation we observed in our confocal analysis of synaptic area.

### Discussion

Human iPSC-derived cortical spheroids represent a powerful model to explore the effects of genetic and pharmacological manipulation on developing neural circuits which resemble human fetal brain development. The ECS is expressed in human iPSC-derived neurons38, mouse iPSC-derived brain organoids46, and human iPSC-derived forebrain organoids47. Concordantly, we observed ECS expression in both iPSC-derived neurons (Sup. Fig. S3) and cortical spheroids (Fig. 1). Our iPSC-derived neurons displayed a biphasic response to CB1 antagonism, where CB1 antagonist treatment with 3–300 nM SR abolished WIN-induced neurite length reduction (Supplemental Fig. S4). This indicates that CB1 receptors located on neurons which have been differentiated for only 24 h are functional through their response to exogenous cannabinoid modulation. Similarly, recent studies have demonstrated the mutability of the ECS in human-derived brain organoids, specifically showing that 1 μM THC treatment reduces neuronal activity as measured by mean firing rate58. Additionally, chronic treatment have demonstrated the mutability of the ECS in human-derived brain organoids, specifically showing that 1 μM THC treatment reduces neuronal activity as measured by mean firing rate58. Additionally, chronic treatment of dissociated brain organoids with cannabinoid modulators has been shown to produce profound impacts on the process of neuronal differentiation and maturation52. With expanding legalization and resultant increased recreational use of cannabis by pregnant women67, it is necessary to evaluate the effects of cannabis on fetal neurodevelopment. Importantly, distinctions must be made between acute and chronic maternal use as well as acute and chronic effects of cannabis on the fetal brain. In this research, we sought to characterize the expression and synaptic localization of ECS components in developing neural circuits and to analyze the functional consequences of acute CB1 antagonism on synaptic development.

We first wanted to determine if constituents of the ECS were present in our cortical spheroid model and consequently found that CB1 mRNA was expressed in our cortical spheroids. Notably, CB1 is the predominant cannabinoid receptor in the CNS and one of the most abundant G-protein coupled receptors in the brain28,29. CB1 mRNA was not detected in the cortical spheroids, however, this differential expression of cannabinoid receptors is in line with our model which does not express microglial cells35, the main host of CB1 in the brain47. We additionally observed differential regulation of DAGLα and MAGL mRNA expression in our cortical spheroids derived from autistic patient iPSCs. Specifically, there was a significant increase in both DAGLα and MAGL mRNA expression. Interestingly, these changes mirror those observed in the mouse model of Fragile X syndrome, where FMR1 knockout increased striatal DAGLα and MAGL expression60. The expression of DAGLα, MAGL, and FAAH dramatically increase in post-natal development, coinciding with a period of synaptic refinement and maturation61. Thus, increased ECS enzyme levels earlier in development could represent accelerated maturation of neural circuit development, a phenotype which has been observed in neurons derived from autism patients46. Pharmaceuticals which alter CB1 activation and 2-AG metabolism may be useful treatment options for specific ASD symptoms linked to stress and anxiety, however, the consequences of endocannabinoid manipulation during brain development are unclear and may have unintended results. For example, while the inhibition of DAGL in patients with Fragile X syndrome appears reasonable due to increased DAGL expression in mouse models46, reduced 2-AG synthesis is associated with increases in stress63, impaired neuroinflammation, and disrupted synaptogenesis62. Additionally, MAGL inhibition, while linked to anxiolytic and nociceptive effects65, is also associated with impaired learning and memory66. Thus, an evaluation of the endocannabinoid system as a primary cause of synaptic dysfunction or as a compensatory mechanism in response to other synaptic changes at the patient level is warranted. We suggest that ECS disruptions may drive the pathophysiology of neurodevelopmental disorders, which would likely disturb the spatial and temporal regulation of homeostatic synapse selection in the developing brain44. Additionally, while we believe neurodevelopmental disorders such as ASD are caused by a constellation of deficits that culminate at the synaptic level15, we also believe that the disruption of the ECS greatly impacts synaptogenesis and perpetuates synaptic deficits during development.
To determine how antagonism of ECS signaling impacts neural circuit development, we used SR141716A to acutely and selectively disrupt CB1 activity during the period of cortical spheroid development coinciding with synaptogenesis. At 30 nM SR, CB1 antagonism increased both glutamatergic and GABAergic synaptogenesis. However, at 300 nM, SR continued to increase glutamatergic synaptogenesis but GABAergic synaptogenesis did not significantly differ from the controls. This biphasic effect observed at inhibitory synapses, but not excitatory synapses, is interesting and may be explained by a combination of variables. Firstly, we observe distinct basal expression of CB1 at inhibitory and excitatory synapses, with CB1 favoring inhibitory synapses (excitatory synapses with CB1 = 25%, inhibitory synapses with CB1 = 39%, Fig. 2h). This ratio of excitatory to inhibitory CB1 expression is comparable to fetal murine models6,68, but is notably different from adult CB1 expression in the neocortex, where CB1 primarily localizes to interneurons68. Secondly, we found that CB1 receptor count scaled up with both excitatory and inhibitory postsynaptic marker count (Sup Fig. S2), indicating that the number of CB1 receptors at the presynapse was a function of synapse size, regardless of type. Our observation of dynamic CB1 expression at excitatory synapses during fetal synaptogenesis is consistent with previous literature which describes CB1 regulation of glutamatergic neurons from the start of their migration79,80. Thirdly, our model system does not have a 1:1 ratio of excitatory to inhibitory synapses in the zone of active synaptogenesis, but rather expresses significantly less inhibitory synapses77. Thus, the biphasic effect only observed at inhibitory synapses may due to extraordinarily sensitive CB1-positive inhibitory synapses. However, while CB1 expressing inhibitory synapses may be more sensitive to the antagonist treatment, we did not observe a decrease in overall activity which would be predicted if CB1 antagonism at inhibitory synapses was dominant over CB1 antagonism at excitatory synapses. Due to cell-specific and synaptic location-specific effects, it is not fully reliable to characterize ECS regulatory strength based upon the count or density of CB1 at excitatory and inhibitory synapses71. For example, sparsely expressed CB1 on glutamatergic neurons of adult mice plays an outsized role in controlling neural activity in the hippocampus82. The disparity between excitatory and inhibitory synapse response to SR141716A in our system may also be explained by the differential expression of glutamatergic and GABAergic neurons across time during development, whereby glutamatergic projection neuron generation and migration occurs prior to interneuron generation81.

Complex changes in synaptogenesis mediated by SR141716A were reflected in the variability of neural activity. Under basal culture conditions, spiking and bursting variability decreases across time, mirroring the emergence of synchronized neural networks in the developing brain81. Synchronization is a large-scale network process observed in maturing neurons, whereby neuronal spiking activity becomes less variable and larger groups of neurons participate in simultaneous action potentials82. In contrast, SR141716A increased the variability of firing activity, indicative of disruption to developing neural networks. Notably, asynchronous activity is observed in neuropsychological disorders such as ASD83 and schizophrenia84. The disruption of neural synchronicity (i.e. increased variability) was most prominent at 30 nM SR141716A, the dose at which we observed significant increases in both inhibitory and excitatory synapses (Fig. 2f, g). This increase, along with the greater expression of CB1-positive excitatory synapses at 30 nM (Fig. 2h) may explain the greater variability of activity. Interestingly, GABA receptor agonists in organoid models decrease synchronicity85. Additionally, CCK + interneurons play a role in determining the firing threshold of pyramidal cells in the hippocampus86 and may have a similar effect in the cortex. This fact may help to explain why our model experienced greater variability, as CB1 antagonism at GABAergic synapses would likely increase GABAergic signaling through the disruption of endocannabinoid-mediated presynaptic inhibition. However, not all inhibitory synapses are the same, and these results are further complicated by the differential impact of CB1 at GABAergic perisomatic synapses versus axodendritic synapses84. Thus, we report that 30 nM SR141716A results in dynamic ECS alterations that impact synaptogenesis and the resulting neural activity. These complex changes are consistent with the variable and state-dependent response of rat cortical neurons to CB1 antagonism87. Together, these results demonstrate that ECS signaling critically modulates developing neural circuits by coordinating the proper development and synchronizing of excitatory and inhibitory synapses.

The ECS impacts synaptogenesis not only by modulating synaptic strength at mature synapses, but also by influencing axon targeting through autocrine 2-AG release around the growth cone. Prior to synaptogenesis, autocrine 2-AG signaling prevents premature synapse creation by thwarting presynaptic vesicular exocytosis and inducing repulsive, cytoskeletal motility34,39. In the presence of SR141716A, this inhibitory process is released and we observed significant increases to excitatory synapse area and count (Fig. 2), indicating an increase in finding in ASD20 and increased glutamatergic synaptic spine density has been observed in post-mortem brains complicated by the differential impact of CB1 at GABAergic perisomatic synapses versus axodendritic synapses71. The ECS impacts synaptogenesis not only by modulating synaptic strength at mature synapses, but also by influencing axon targeting through autocrine 2-AG release around the growth cone. Prior to synaptogenesis, autocrine 2-AG signaling prevents premature synapse creation by thwarting presynaptic vesicular exocytosis and inducing repulsive, cytoskeletal motility34,39. In the presence of SR141716A, this inhibitory process is released and we observed significant increases to excitatory synapse area and count (Fig. 2), indicating an increase in finding in ASD20 and increased glutamatergic synaptic spine density has been observed in post-mortem brains complicated by the differential impact of CB1 at GABAergic perisomatic synapses versus axodendritic synapses71. The ECS impacts synaptogenesis not only by modulating synaptic strength at mature synapses, but also by influencing axon targeting through autocrine 2-AG release around the growth cone. Prior to synaptogenesis, autocrine 2-AG signaling prevents premature synapse creation by thwarting presynaptic vesicular exocytosis and inducing repulsive, cytoskeletal motility34,39. In the presence of SR141716A, this inhibitory process is released and we observed significant increases to excitatory synapse area and count (Fig. 2), indicating an increase in finding in ASD20 and increased glutamatergic synaptic spine density has been observed in post-mortem brains complicated by the differential impact of CB1 at GABAergic perisomatic synapses versus axodendritic synapses71. The ECS impacts synaptogenesis not only by modulating synaptic strength at mature synapses, but also by influencing axon targeting through autocrine 2-AG release around the growth cone. Prior to synaptogenesis, autocrine 2-AG signaling prevents premature synapse creation by thwarting presynaptic vesicular exocytosis and inducing repulsive, cytoskeletal motility34,39. In the presence of SR141716A, this inhibitory process is released and we observed significant increases to excitatory synapse area and count (Fig. 2), indicating an increase in finding in ASD20 and increased glutamatergic synaptic spine density has been observed in post-mortem brains complicated by the differential impact of CB1 at GABAergic perisomatic synapses versus axodendritic synapses71.
the ECS plays an outsized role due to the global expression of CB1. In this research, we have established that cortical spheroids are an appropriate model for exploring the ECS in the context of fetal brain development and childhood neuropsychiatric disorders. Additionally, we have demonstrated that CB1 antagonism produces disruptions to excitatory and inhibitory synaptic balance in cortical spheroids. Our results further confirm the role of the ECS in synaptic pathology and we propose the utilization of CB1 as a targetable receptor for therapeutics in neurodevelopmental disorders.

Received: 3 December 2020; Accepted: 9 April 2021
Published online: 30 April 2021

References

1. Di Marzo, V. Endocannabinoid signaling in the brain: biosynthetic mechanisms in the limelight. Nat. Neurosci. 14, 9–15 (2011).
2. Biegon, A. & Kerman, I. A. Autoradiographic study of pre- and postnatal distribution of cannabinoid receptors in human brain. Neuroimage 14, 1463–1468 (2001).
3. Mato, S., Del Olmo, E. & Pazos, A. Ontogenetic development of cannabinoid receptor expression and signal transduction functionality in the human brain. Eur. J. Neurosci. 17, 1747–1754 (2003).
4. Oudin, M. J. et al. Endocannabinoids regulate the migration of subventricular zone-derived neuroblasts in the postnatal brain. J. Neurosci. 31, 4000–4011 (2011).
5. Harkany, T., Keimpema, E., Barabás, K. & Mulder, J. Endocannabinoid functions controlling neuronal specification during brain development. Mol. Cell. Neurosci. 38, 89–97 (2008).
6. Gaffuri, A.-L.L., Ladarre, D. & Lenkei, Z. Type-1 cannabinoid receptor signaling in neuronal development. Pharmacology 90, 19–39 (2012).
7. Watson, S., Chambers, D., Hobbs, C., Doherty, P. & Graham, A. The endocannabinoid receptor, CB1, is required for normal axonal growth and fasciculation. Mol. Cell. Neurosci. 38, 89–97 (2008).
8. Gaffuri, A.-L.L., Ladarre, D. & Lenkei, Z. Type-1 cannabinoid receptor signaling in neuronal development. Pharmacology 90, 19–39 (2012).
9. Oudin, M. J., Hobbs, C. & Doherty, P. DAGL-dependent endocannabinoid signalling: roles in axonal pathfinding, synaptic plasticity and adult neurogenesis. Eur. J. Neurosci. 34, 1634–1646 (2011).
10. Garner, C. C., Zhai, R. G., Gundelfinger, E. D. & Ziv, N. E. Molecular mechanisms of CNS synaptogenesis. Trends Neurosci. 25, 243–250 (2002).
11. Silbereis, J. C. et al. Phenotypic differences in hiPSC NPs derived from patients with schizophrenia. Mol. Psychiatry 20, 361–368 (2015).
12. Kim, H. & Holtzman, D. M. Human neurons derived from induced pluripotent stem cells as a new platform for preclinical drug screening and development. JAMA Neurol. 71, 1475 (2014).
13. Papariello, A. & Newell-Litwa, K. Human-derived brain models: windows into neuropsychiatric disorders and drug therapies. Annu. Drug Dev. Technol. https://doi.org/10.1089/adt.2019.922 (2020).
14. Wilson, E., Knudson, W. & Newell-Litwa, K. Hyaluronan regulates synapse formation and function in developing neural networks. Sci. Rep. 10, 16459 (2020).
15. Howlett, A. C. International union of pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol. Rev. https://doi.org/10.1124/pr.54.2.161 (2002).
16. Soderstrom, K., Leid, M., Moore, F. L. & Murray, T. F. Behavioral, pharmacological, and molecular characterization of an amphibian cannabinoid receptor. J. Neurochem. https://doi.org/10.1046/j.1471-4159.2000.0750413.x (2000).
17. Piet, R. et al. State-dependent, bidirectional modulation of neural network activity by endocannabinoids. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.4297-11.2011 (2011).
18. Blankman, J. L., Simmon, G. M. & Cravatt, B. F. A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. Chem. Biol. 14, 1347–1356 (2007).
19. Kitsimpila, E. et al. Differential subcellular recruitment of monoacylglycerol lipase generates spatial specificity of 2-arachidonoylglycerol signaling during axonal pathfinding. J. Neurosci. 30, 13992–14007 (2010).
20. Keimpema, E. et al. Diacylglycerol lipase a manipulation reveals developmental roles for intercellular endocannabinoid signaling. Sci. Rep. 3, 1–9 (2013).
21. Macconnell, M., Guzman, M., Mackie, K., Doherty, P. & Harkany, T. Programming of neural cells by (endo)cannabinoids from physiological rules to emerging therapies. Nat. Publ. Gr. https://doi.org/10.1038/nm3846 (2014).
35. Berghuis, P. et al. Endocannabinoids regulate interneuron migration and morphogenesis by transactivating the TrkB receptor. Proc. Natl. Acad. Sci. U. S. A. https://doi.org/10.1073/pnas.0509494102 (2005).
36. Berghuis, P. et al. Hardwiring the brain: endocannabinoids shape neuronal connectivity. Science 316, 1212–1216 (2007).
37. Mulder, J. et al. Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning. Proc. Natl. Acad. Sci. 105, 8760–8765 (2008).
38. Shun, C. et al. δ9-tetrahydrocannabinol and 2-AG decreases neurite outgrowth and differentially affects ERK1/2 and Akt signaling in hiPSC-derived cortical neurons. Mol. Cell. Neurosci. 103, 103-463 (2020).
39. Roland, A. B. et al. Cannabinoid-induced actomyosin contractility shapes neuronal morphology and growth. Elife 3, e03159 (2014).
40. Tortorillo, G. et al. Miswiring the brain: δ9-tetrahydrocannabinol disrupts cortical development by inducing an SGC10/stathmin-2 degradation pathway. EMBO J. 33, 668–685 (2014).
41. Zuccarini, G. et al. Interference with the cannabinoid receptor CB1R results in miswiring of GnRH3 and AgRP1 axons in zebrafish embryos. Int. J. Mol. Sci. 21, 168 (2020).
42. Roberts, B. et al. Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. Mol. Biol. Cell 28, 2854–2874 (2017).
43. Soderstrom, K. & Tian, Q. CB1 cannabinoid receptor activation dose dependently modulates neuronal activity within caudal but not rostral song control regions of adult zebra finch telencephalon. Psychopharmacology https://doi.org/10.1007/s00213-008-1190-z (2008).
44. Holland, T. L. & Soderstrom, K. Chronic CB1 cannabinoid receptor antagonism persistently increases dendritic spine densities in brain regions important to zebra finch vocal learning and production in an antidepressant-sensitive manner. Brain Res. https://doi.org/10.1016/j.brainres.2017.07.015 (2017).
45. Soderstrom, K., Tian, Q., Valenti, M. & Di Marzo, V. Endocannabinoids link feeding state and auditory perception-related gene expression. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.3298-04.2004 (2004).
46. Howlett, A. C. & Abood, M. E. CB1 and CB2 receptor pharmacology. Adv. Pharmacol. https://doi.org/10.1016/bs.apha.2017.03.007 (2017).
47. Cabral, G. A., Raborn, E. S., Griffin, L., Dennis, J. & Marciano-Cabral, F. CB2 receptors in the brain: role in central immune function. Br. J. Pharmacol. 153, 240–251 (2008).
48. Pertwee, R. G. The pharmacology of cannabinoid receptors and their ligands: an overview. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.0372-06.2006 (2006).
49. Kotton, I. et al. Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.19-11-04544.1999 (1999).
50. London, M., Segov, I., Magee, J. C. & Cook, E. F. Synaptic scaling in vitro and in vivo (multiple letters). Nat. Neurosci. 4, 853–855 (2001).
51. Fagioli, M. & Leblanc, J. J. Autism: a critical period disorder?. Neuropsychopharmacology 12, 837–847 (2015).
52. Cristino, L., Imperatore, R. & Di Marzo, V. Techniques for the cellular and subcellular localization of endocannabinoid receptors and enzymes in the mammalian brain. Methods Enzymol. 593, 61–98 (2017).
53. Domenici, M. R. et al. Cannabinoid receptor type 1 located on presynaptic terminals of principal neurons in the forebrain controls glutamatergic synaptic transmission. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.0372-06.2006 (2006).
54. Katona, I. et al. Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.19-11-04544.1999 (1999).
55. London, M., Segov, I., Magee, J. C. & Cook, E. F. Synaptic scaling in vitro and in vivo (multiple letters). Nat. Neurosci. 4, 853–855 (2001).
56. Fagioli, M. & Leblanc, J. J. Autism: a critical period disorder?. Neural Plast. https://doi.org/10.1155/2011/921680 (2011).
57. Paraiso-Luna, J. et al. Endocannabinoid signalling in stem cells and cerebral organoids drives differentiation to deep layer projection neurons via CB1 receptors. Development 147, dev192161 (2020).
58. Guimas Almeida, C. et al. hiPSC-based model of prenatal exposure to cannabinoids: effect on neuronal differentiation. Front. Mol. Neurosci. 13, 70–81 (2020).
59. Z., et al. One-stop microfluidic assembly of human brain organoids to model prenatal cannabis exposure. Anal. Chem. 92, 4630–4638 (2020).
60. Skelton, K. R., Hecht, A. A. & Benjamin-Neelon, S. E. Recreational cannabis legalization in the US and maternal use during the preconception, prenatal, and postpartum periods. Int. J. Environ. Res. Public Health 17, 1009 (2020).
61. Maccarrone, M. et al. Abnormal mGlu 5 receptor/endocannabinoid coupling in mice lacking FMRP and BC1 RNA. Neuropsychopharmacology 35, 1500–1509 (2010).
62. Kh, I. et al. Spatio-temporal transcriptome of the human brain. Nature https://doi.org/10.1038/nature10523 (2011).
63. Johnson, M. B. et al. Functional and evolutionary insights into human brain development through global transcriptome analysis. Neuron https://doi.org/10.1016/neuron.2009.03.027 (2009).
64. Schafer, S. T. et al. Pathological priming causes developmental gene network heterochronicity in autistic subject-derived neurons. Nat. Neurosci. https://doi.org/10.1038/n41593-018-0295-x (2019).
65. Jenniches, I. et al. Anxiety, stress, and fear response in mice with reduced endocannabinoid levels. Biol. Psychiatry 79, 858–868 (2016).
66. Ogawara, D. et al. Rapid and profound rewiring of brain lipid signaling networks by acute diaeyglycerol lipase inhibition. Proc. Natl. Acad. Sci. U. S. A. https://doi.org/10.1073/pnas.1523546111 (2016).
67. Bedse, G., Hill, M. N. & Patel, S. Review 2-arachidonoylglycerol modulation of anxiety and stress adaptation: from grass roots to novel therapeutics. Biol. Psychiatry https://doi.org/10.1016/j.biopsych.2020.01.015 (2020).
68. Griebel, G. et al. Selective blockade of the hydrolysis of the endocannabinoid δ2-arachidonoylglycerol impairs learning and memory performance while producing antinociceptive activity in rodents. Sci. Rep. https://doi.org/10.1038/srep07642 (2015).
69. Hill, E. L. et al. Functional CB1 receptors are broadly expressed in neocortical GABAergic and glutamatergic neurons. J. Neurophysiol. 97, 2580–2589 (2007).
70. Lafourcade, M., Elezgarai, I., Mato, S., Bakiri, Y. & Grandes, P. Molecular components and functions of the endocannabinoid system in mouse prefrontal cortex. PLoS ONE 2, 709 (2007).
71. Sacer, T. M. M., Aronne, M. P., Cattana, L. & Brusco, A. H. Prenatal exposure to the CB1 and CB2 cannabinoid receptor agonist WIN 55,212-2 alters migration of early-born glutamatergic neurons and GABAergic interneurons in the rat cerebral cortex. J. Neurochem. 129, 637–648 (2014).
72. Lee, S. H., Foley, C. & Soltész, I. Distinct endocannabinoid control of GABA release at perisomatic and dendritic synapses in the hippocampus. J. Neurosci. 30, 7993–8000 (2010).
73. Uhlaas, P. J. & Singer, W. Abnormal neural oscillations and synchrony in schizophrenia. Nat. Rev. Neurosci. 11, 100–113 (2010).
74. Dinstei, I. et al. Disrupted neural synchronization in toddlers with autism. Neurom. 70, 1218–1225 (2009).
75. Klausberger, T. GABAergic interneurons targeting dendrites of pyramidal cells in the CA1 area of the hippocampus. Eur. J. Neurosci. 30, 947–957 (2009).
76. Hütter, J. J. & Zhang, H. Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders. Brain Res. https://doi.org/10.1016/j.brainres.2009.09.120 (2010).
77. Njoo, C., Agarwal, N., Lutz, B. & Kuner, R. The cannabinoid receptor CB1 interacts with the WAVE1 complex and plays a role in actin dynamics and structural plasticity in neurons. PLoS Biol. 13, 1–36 (2015).
77. Katona, I. & Freund, T. F. Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. Nat. Med. https://doi.org/10.1038/nm.1869 (2008).

Acknowledgements
This study was funded by ECU. Special thanks to Joani Zary Oswald for her histology expertise.

Author contributions
A.P., K.S. and K.L. conceptualized and designed experiments. A.P. collected data, analyzed data, and wrote the manuscript. K.S and K.L. are the primary investigators and supervisors of this study. D.T. aided in project development and funding.

Funding
Support from ECU Department of Pharmacology & Toxicology, ECU Department of Anatomy & Cell Biology. Additional support was awarded to K.S. and K.L. from ECU Department of Research, Economic Development, and Engagement in the form of an Interdisciplinary Research Award.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-88750-2.

Correspondence and requests for materials should be addressed to K.S. or K.L.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021