A functional endocannabinoid system in human induced pluripotent stem cell-derived cortical cultures

A DISSERTATION

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Abstract

The endocannabinoid system is an increasingly popular therapeutic target in many neurological conditions, due in large part to its ability to protect neurons from damage caused by hyperactivity and excitotoxicity. Despite recent interest in cannabinoid-based treatments, the unavailability of human brain tissue and species differences between humans and animal models present obstacles to drug development. Human induced pluripotent-derived stem cells (hiPSCs), which can be obtained less invasively from skin samples and then reprogrammed into neurons and glia, are one possible solution to this problem. However, it is not clear whether hiPSC-derived neurons actually have a working endocannabinoid system to study. In this thesis I characterize the endocannabinoid system in a commercially available line of hiPSC-derived cortical neuron/astrocyte cultures using calcium imaging and a fluorescent cannabinoid indicator expressed in live neurons. hiPSC-derived cultures produced and metabolized endocannabinoids in addition to responding to exogenously applied cannabinoids, indicating that they do indeed possess a fully functional endocannabinoid system. I also show that endocannabinoid synthesis evoked by a muscarinic receptor agonist in hiPSC-derived cortical cultures is not calcium-dependent, and that an inhibitor of endocannabinoid metabolism produces less receptor desensitization than a cannabinoid receptor agonist with prolonged exposure. These studies demonstrate that hiPSC-derived neuron/astrocyte cultures are a powerful new tool for investigating open questions about the regulation of the human endocannabinoid system.

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List of Abbreviations

2-AG	2-Arachidonoylglycerol
2-BP	2-bromohexadecanoic acid
AAV	Adeno-associated virus
ABHD6/12	α/β-hydrolase domain 6/12
AD	Alzheimer's disease
AEA	Anandamide; *N*-arachidonoylethanolamine
AIDS	Acquired immunodeficiency syndrome
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CB_1R	Cannabinoid type 1 receptor
CB_2R	Cannabinoid type 2 receptor
CBD	Cannabidiol
Cch	Carbachol
CHPG	Chlorohydroxyphenylglycine
CNQX	Cyanquixaline (6-cyano-7-nitroquinoxaline-2,3-dione)
CNS	Central nervous system
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DHPG	(S)-3,5-Dihydroxyphenylglycine
DMEM	Dulbecco's modified Eagle's Medium
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-methyl-2-propanyl)oxy]carbonyl}piperazinyl){4-[(4-trifluoromethoxy)phenyl]-1H-1,2,3-triazol-1-yl}methanone
EAAT2	Excitatory amino acid transporter 2
EAE	Experimental autoimmune encephalitis

eCB	Endocannabinoid
ECS	Endocannabinoid system
EPSC	Excitatory postsynaptic current
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FAAH	Fatty acid amide hydrolase
FABP5	Fatty acid binding protein 5
FDA	United States Food and Drug Administration
GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
GFAP	Glial fibrillary acidic protein
GPCR	G protein-coupled receptor
GRAB	G protein-coupled receptor activation based sensor
GRK	G protein-coupled receptor kinase
hiPSC	Human induced pluripotent stem cell
HBSS	HEPES buffered Hanks' salt solution
HD	Huntington's disease
IP_3	Inositol 1,4,5-trisphosphate
JZL184	4-Nitrophenyl 4-[di(2H-1,3-benzodioxol-5-yl)(hydroxy)methyl]piperidine-1-carboxylate
MAGL	Monoacylglycerol lipase
$[Mg^{2+}]_o$	Extracellular Mg^{2+} concentration
mGluR	Metabotropic glutamate receptor
MK801	(+)-5-methyl-10,11-dihydroxy-5H-dibenzo(a,d)cyclohepten-5,10-imine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NAPE-PLD	*N*-acyl phosphatidylethanolamine phospholipase D
NESS 0327	N-piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo [6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide]
NMDA	*N*-Methyl-d-aspartic acid
PCR	Polymerase chain reaction

PD	Parkinson’s disease
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PLC-β	Phospholipase C-β
PPAR	Peroxisome proliferator-activated receptor
PSD95	Post-synaptic density protein 95
ROI	Region of interest
SD	Standard deviation
Syn	Synapsin
TBI	Traumatic brain injury
TeNT	Tetanus toxin
Thaps	Thapsigargin
THC	Δ^9-tetrahydrocannabinol
TRPV1	Transient receptor potential vanilloid 1
TTX	Tetrodotoxin
Win-2	Win 55,212-2; R-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone

Chapter One:

Introduction

I. Cannabis and cannabinoids

Plants of the genus *Cannabis* have a long history of human use, with the oldest archaeological evidence of *Cannabis* in human settlements dating back 10,000 years (Kobayashi et al., 2008). From its early origins as a fiber and oil seed plant, *Cannabis* has been extensively bred for its psychotropic effects and has been used as a recreational, medicinal, and ritualistic drug for at least 2,500 years (McPartland et al., 2019; Pisanti & Bifulco, 2019). Today marijuana is the most commonly used illicit drug in the United States by a wide margin, with an estimated 49.6 million American users in 2020 (Substance Abuse and Mental Health Services Administration, 2020). In addition, an increasing number of jurisdictions worldwide have decriminalized or legalized it, and 37 US states now allow its use either recreationally or medicinally (National Confernce of State Legislatures, 2022).

Over 100 active compounds, collectively termed phytocannabinoids, have been isolated from *Cannabis* (reviewed in Hanuš et al., 2016). The most well-known is Δ^9-tetrahydrocannabinol (THC), which is responsible for most of the psychoactive effects of marijuana (Gaoni & Mechoulam, 1964). Cannabidiol (CBD) is a popular medicinal choice which lacks the psychoactive effects of THC (Mechoulam et al., 2002). A large number of synthetic cannabinoids have also been produced for research and other uses, in addition to the plant-derived phytocannabinoids (Banister & Connor, 2018).

In the United States and Canada, 27% of people aged 16-65 report having tried medical cannabis at least once, most commonly for pain, mood disorders, trouble sleeping, and decreased appetite or nausea (J. Leung et al., 2022). Evidence-based cannabinoid drug development, meanwhile, has lagged behind this widespread public interest—only four cannabis-related drugs are approved by the FDA, and only for a limited range of indications: CBD for seizures associated with Lennox-Gastaut syndrome

or Dravet syndrome; dronabinol (synthetic THC) for nausea associated with cancer chemotherapy and AIDS-related weight loss; nabilone (a synthetic THC analog) for nausea associated with cancer chemotherapy, and nabiximols (a THC:CBD mixture) for spasticity associated with multiple sclerosis (FDA, 2020). There is therefore an acute need to deepen our understanding of the therapeutic uses of cannabinoids and to develop and rigorously test both plant-based and synthetic drugs.

II. The endocannabinoid system

Cannabinoids exert their effects via the endocannabinoid system, one of the major systems of neurotransmitters in the CNS. The endocannabinoid system consists of endocannabinoids, their receptors, and the enzymes which synthesize and degrade them, described below and summarized in Figure 1.1 (next page).

Endocannabinoids and endocannabinoid synthesis

The endogenous ligands of the endocannabinoid system are lipid-based signaling molecules known as endocannabinoids, which are synthesized on demand from components of the cell membrane. The major endocannabinoids in the brain are 2-arachidonoylglycerol (Mechoulam et al., 1995; Sugiura et al., 1995) and anandamide (Devane et al., 1992), although many other proven and proposed endocannabinoids exist (reviewed in Fezza et al., 2014).

2-arachidonoylglycerol (2-AG) is the most abundant endocannabinoid in the brain (Buczynski & Parsons, 2010), and one of the most well-studied. 2-AG is canonically synthesized from the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) via a two-step pathway: first, phospholipase C-β (PLC-β) cleaves PIP_2 to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Hashimotodani et al., 2005;

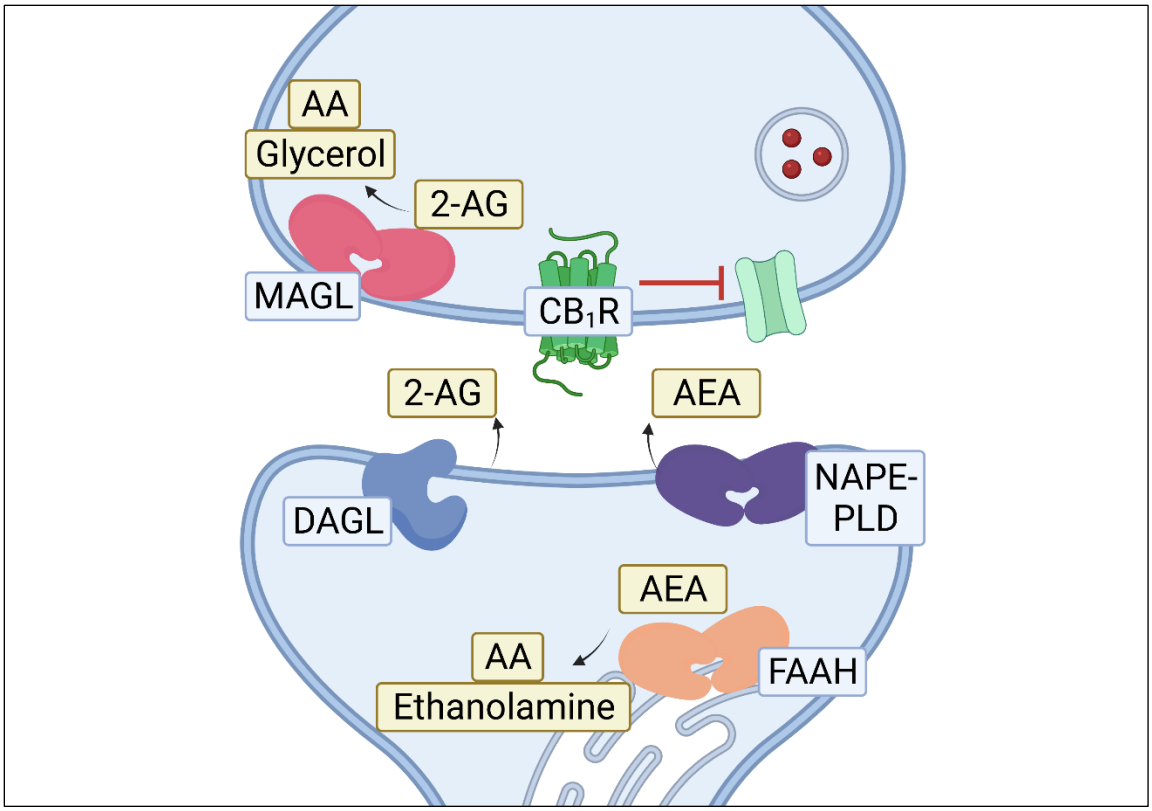

Figure 1.1. Key components of the endocannabinoid system in neurons. Endocannabinoids (2-AG and AEA) are produced in the postsynaptic cell (bottom) and activate receptors on the presynaptic cell (top), leading to inhibition of voltage gated calcium channels and reduced neurotransmitter release. After signaling, they are degraded by MAGL and FAAH, producing arachidonic acid. 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AEA, anandamide; CB_1R, cannabinoid type 1 receptor; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acyl phosphatidylethanolamine phospholipase D. Image created with Biorender.

Maejima et al., 2005); DAG lipase (DAGL) then cleaves DAG to produce 2-AG (Bisogno et al., 2003). The 2-AG synthesis enzyme DAGL is selectively expressed in the CNS in mice and the CNS and pancreas in humans (Bisogno et al., 2003) and is commonly localized to dendritic spines, where it mediates the synthesis of 2-AG by postsynaptic cells during retrograde signaling (Yoshida et al., 2006). 2-AG is also synthesized by astrocytes and microglia (Carrier et al., 2004; Schüle et al., 2021).

2-AG synthesis is triggered by excitation of the postsynaptic cell and can be elicited experimentally in two main ways: electrical depolarization (Ohno-Shosaku et al., 2001; Stella et al., 1997) and pharmacological activation of G_q-coupled receptors (Maejima et al., 2001; Martin et al., 2015). The two methods differ in how they activate PLC-β to produce DAG, the precursor to 2-AG. Depolarization-induced 2-AG synthesis is a calcium-dependent process in which PLC-β is activated by an influx of Ca^{2+} through voltage-gated calcium channels (Stella et al., 1997). G_q-induced 2-AG synthesis, on the other hand, is a calcium-independent process in which G_q activates PLC-β (Maejima et al., 2001). These two mechanisms of PLC-β activation synergize with each other, allowing PLC-β to function as a coincidence detector *in vitro* (Hashimotodani et al., 2005). The physiological relevance of each of these 2-AG synthesis pathways is still unclear, but one study in nucleus accumbens slices suggested that G_q-evoked 2-AG synthesis relies on a combination of G_q signaling and Ca^{2+} release from intracellular stores (Robbe et al., 2002). 2-AG synthesis is also modulated at the level of DAGL, which is differentially regulated by calcium/calmodulin dependent kinase II and protein kinase A (Shonesy et al., 2013, 2020). This integrates endocannabinoid signaling with other neurotransmitter systems.

Anandamide (*N*-arachidonoylethanolamine, AEA) was the first endocannabinoid discovered (Devane et al., 1992). AEA is synthesized from the membrane phospholipid

precursor *N*-arachidonoyl phosphatidylethanolamine (Devane et al., 1992). Canonical AEA synthesis is carried out by *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), although multiple variations on the synthesis pathway exist with different enzymes and intermediate steps (D. Leung et al., 2006).

Unlike many other neurotransmitters, endocannabinoids are not packaged into a pool of synaptic vesicles to be released, and the exact mechanism of endocannabinoid release and reuptake across the plasma membrane has been controversial. As lipid-based signaling molecules, endocannabinoids have long hydrophobic tails and diffuse readily within lipid bilayers (Tian et al., 2005), leading to the view that their release and uptake occurs by diffusion (Glaser et al., 2003). However, there is also evidence for a yet-unidentified membrane transporter for AEA (Chicca et al., 2012; Di Marzo et al., 2004; Nicolussi et al., 2014). Diffusion across the synaptic cleft may be aided by secreted carrier proteins (Haj-Dahmane et al., 2018).

Endocannabinoid receptors and downstream signaling pathways

The first cannabinoid receptor discovered (Matsuda et al., 1990) was CB_1R, a G-protein coupled receptor (GPCR) which mediates the psychoactive effects of THC (Ledent et al., 1999). CB_1Rs are present in tissues throughout the body (Haspula & Clark, 2020) with particularly high expression in the CNS—early quantitative studies found brain CB_1R content comparable to common ionotropic receptors (Herkenham et al., 1991), meaning that CB_1R is possibly the most abundant GPCR in the brain. CB_1R is expressed by diverse subtypes of neurons throughout the brain (Glass et al., 1997; Mailleux & Vanderhaeghen, 1992) as well as by astrocytes (Navarrete & Araque, 2008). On a subcellular level CB_1R is commonly located on presynaptic axon terminals (Katona et al., 1999), allowing for retrograde signaling from endocannabinoid-producing

postsynaptic cells to CB_1R-expressing presynaptic cells (Ohno-Shosaku et al., 2001; Shen et al., 1996), but it can also be found on dendrites (Maroso et al., 2016).

In classical retrograde endocannabinoid signaling, CB_1R on presynaptic terminals couples to $G_{i/o}$-containing G-proteins, promoting the dissociation of the $G\alpha_{i/o}$ and $G\beta\gamma$ subunits which act as intracellular messengers. $G\alpha_{i/o}$ inhibits adenosine cyclase and reduces intracellular cAMP, inhibiting protein kinase A (PKA) signaling (Mato et al., 2008). The $G\beta\gamma$ complex, meanwhile, acts on multiple targets, most notably inhibiting the voltage-gated calcium channels which are required for calcium-induced neurotransmitter release (Mackie et al., 1995; Twitchell et al., 1997); this is how CB_1R activation decreases neurotransmitter release from presynaptic terminals (Shen et al., 1996). Aside from retrograde signaling between pre- and post-synaptic cells, CB_1R activation modulates the integration of excitatory inputs in dendrites (Maroso et al., 2016) and mediates inhibitory autocrine signaling in some types of GABAergic neurons (Bacci et al., 2004). CB_1R signaling also influences multiple intracellular kinase pathways with diverse downstream effects (Blázquez et al., 2015; Bouaboula et al., 1995; Graham et al., 2006).

Like other GPCRs, CB_1R is regulated by G-protein receptor kinases (GRKs) and β-arrestin (X. Chen et al., 2014; Kouznetsova et al., 2002). GRK-mediated phosphorylation of the intracellular C-terminal domain of GPCRs recruits β-arrestin to associate with the receptor, where it contributes to inhibition of G-protein signaling, receptor desensitization and internalization, and ERK1/2 signaling (Rajagopal & Shenoy, 2018).

While the majority of CB_1R signaling under normal conditions is $G_{i/o}$-coupled, the receptor is also capable of coupling to G_s (Glass & Felder, 1997), which stimulates adenylyl cyclase activity to increase intracellular cAMP. The ratio of $G_{i/o}$ to G_s signaling, and therefore the overall effect of CB_1R activation on cAMP levels, is context-dependent and influenced by the level of receptor expression relative to the amount of available $G_{i/o}$

(Finlay et al., 2017). In astrocytes, CB_1R couples to $G_{q/11}$, which activates PLC-β signaling; CB_1R-mediated PLC-β activation leads to calcium release from intracellular stores and subsequent gliotransmitter release, allowing bidirectional communication between neurons and glia (Navarrete & Araque, 2008).

CB_2R, the second cannabinoid receptor, is another $G_{i/o}$-coupled GPCR (Munro et al., 1993) which is highly expressed in many types of immune cells, including microglia in the CNS, and plays an important role in modulating their activation (C Benito et al., 2008; Ehrhart et al., 2005; Howlett & Abood, 2017). CB_2R expression is highly context-dependent, with low or even undetectable levels at baseline and marked upregulation during disease states (Benito et al., 2005; Zhang et al., 2003). This makes CB_2R an attractive target for modulating disease without affecting unrelated tissues. Like CB_1R, CB_2R signals through $G_{i/o}$ to inhibit adenylyl cyclase (Bayewitch et al., 1995; Bouaboula et al., 1996) and also regulates intracellular kinase signaling (Cao et al., 2018). In microglia, this affects proliferation (Carrier et al., 2004), migration (Walter et al., 2003), and cytokine release (Puffenbarger et al., 2000), generally resulting in an anti-inflammatory phenotype. CB_2R is also expressed by neurons and may be involved in neuroprotective signaling (Onaivi et al., 2006; Q. Wu et al., 2020).

More recent research has uncovered a number of previously orphan GPCRs that interact with the various endocannabinoid ligands (Biringer, 2021). The most well-known of these receptors is GPR55, a $G_{12/13}$-coupled GPCR which is expressed widely in the brain, albeit at much lower levels than CB_1R (Ryberg et al., 2007). GPR55 activation regulates cytoskeletal rearrangement and leads to increases in intracellular calcium concentration (Lauckner et al., 2008; Ryberg et al., 2007).

Endocananbinoids also signal through ionotropic receptors, chief among them the transient receptor potential vanilloid 1 (TRPV1) receptor (Muller et al., 2019). TRPV1 is a

nonselective cation channel which is expressed on nociceptive neurons and activates in response to noxious heat, acid, and capsaicin (Vay et al., 2011). Cannabinoid agonists of TRPV1 include AEA (Zygmunt et al., 1999) and the phytocannabinoid CBD (Iannotti et al., 2014).

In addition to activating receptors on the cell surface, endocannabinoids (Bouaboula et al., 2005; Rockwell et al., 2006) and some of their metabolites (Raman et al., 2011) activate members of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors, thus regulating gene expression related to metabolism and inflammation (Iannotti & Vitale, 2021).

Endocannabinoid metabolism

Endocannabinoid signaling is tightly controlled by metabolic enzymes which degrade endocannabinoid ligands to terminate signaling. The primary metabolic enzyme for 2-AG is monoacylglycerol lipase (MAGL), which cleaves 2-AG into glycerol and arachidonic acid (Dinh et al., 2002). 2-AG can also be metabolized by the α/β-hydrolase domain 6/12 (ABHD6/12) enzymes (Blankman et al., 2007). 2-AG metabolism both terminates endocannabinoid signaling and contributes to pro-inflammatory downstream signaling pathways by providing arachidonic acid for prostaglandin synthesis (Nomura et al., 2011). In fact, the MAGL-mediated hydrolysis of 2-AG is the main source of arachidonic acid in the CNS (Nomura et al., 2011), making MAGL inhibitors powerful anti-neuroinflammatory drugs in addition to their cannabimimetic effects.

Anandamide, meanwhile, is primarily degraded by fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine (Cravatt et al., 1996). FAAH is located intracellularly on endoplasmic reticulum membranes, meaning that AEA must diffuse across the aqueous cytoplasm to be degraded; this diffusion is assisted by the carrier

protein FABP5 (fatty acid binding protein 5) (Kaczocha et al., 2009), and inhibition of FABP5 causes analgesia reminiscent of the effects of AEA (Peng et al., 2017).

In summary, the endocannabinoid system is a crucial modulator of a broad range of physiological functions in the CNS and beyond. In the classical endocannabinoid pathway, endocannabinoids are synthesized on demand by the postsynaptic cell, signal via CB_1R in a retrograde fashion to reduce neurotransmitter release from presynaptic axon terminals, and are then metabolized to terminate signaling. However, endocannabinoid signaling also involves many other components and is highly influenced by cellular context, and its true complexity is only beginning to be understood.

III. Cannabinoid drugs as therapeutic agents in neurological disease

Preclinical and clinical evidence

The ECS regulates a dizzying array of physiological functions; because of this, it is being investigated as a therapeutic target in an equally broad variety of diseases. A few examples of the main neurological conditions for which ECS drugs are approved or under investigation include seizures, multiple sclerosis, neuropathic pain, and several neurodegenerative diseases.

Seizures. The use of cannabinoids for seizure conditions is backed by a body of evidence in rodent models of epilepsy. Seizures and their long-term effects are ameliorated by exogenous application of cannabinoid agonists (Bhaskaran & Smith, 2010; Wallace et al., 2003), overexpression of CB_1R in excitatory neurons (Guggenhuber et al., 2010), and increasing 2-AG levels through MAGL inhibition (Terrone et al., 2018) or AEA levels through FAAH inhibition (Colangeli et al., 2017).

Conversely, seizures are exacerbated by inhibition or genetic deletion of CB_1R (Deshpande et al., 2007; Monory et al., 2006), CB_2R (Sugaya et al., 2016), or the 2-AG synthetic enzyme DAGLα (Sugaya et al., 2016). This suggests that the endocannabinoid system plays a role in endogenous seizure suppression in addition to mediating the effects of exogenously applied drugs. Interestingly, cannabinoid receptor expression is highly upregulated in the hippocampus in rodent models of temporal lobe epilepsy (Wallace et al., 2003), which may represent an endogenous compensatory mechanism based around the ECS.

In humans, CBD reduces seizure frequency in patients with Lennox-Gastaut and Dravet syndromes and is FDA-approved for this use (Devinsky et al., 2019; Patel et al., 2021; Thiele et al., 2018).

Multiple sclerosis (MS). A major symptom of multiple sclerosis is spasticity—that is, painful and debilitating muscle spasms and tremors which greatly reduce patient quality of life (Zettl et al., 2016). Cannabinoid agonism reduces spasticity in the mouse experimental immune encephalitis (EAE) model of multiple sclerosis. (Baker et al., 2000). Early clinical trials of THC:CBD (nabiximols) in humans with MS spasticity identified a subset of patients who responded to treatment (Collin et al., 2010); additional trials in this patient population demonstrated long-term efficacy (Novotna et al., 2011) and nabiximols are now FDA-approved for use in humans with MS-related spasticity.

THC:CBD for spasticity is a symptomatic treatment, but cannabinoids have also been investigated for slowing disease progression in MS. Cannabinoids suppress autoimmune activation and neuroinflammation in EAE mice, ultimately leading to less damage to axons and less severe symptoms (Maresz et al., 2007; Sánchez et al., 2006; Shao et al., 2014). However, oral dronabinol, although well-tolerated in patients, did not alter disease progression in a 36-month human trial (Zajicek et al., 2005).

Pain. Over half of people using medical marijuana in the United States and Canada report using it off-label for pain management (J. Leung et al., 2022). This use is backed by a large body of animal studies showing analgesic effects of ECS activation in models of both inflammatory (Elmes et al., 2005) and neuropathic pain (Bridges et al., 2001; Kinsey et al., 2009). A recent meta-analysis of clinical trials for chronic pain found that cannabinoids overall exhibit a small but still clinically relevant analgesic effect in humans, with the majority of studies examining neuropathic pain (Wong et al., 2020).

Neurodegeneration. Cannabinoid drugs can be neuroprotective and show promise for treating neurodegenerative diseases in preclinical animal and cell culture models. This is perhaps best studied in Huntington's disease (HD). The ECS is abnormally downregulated in HD, and augmenting cannabinoid signaling prevents neuron loss and partially rescues the motor phenotype in HD model mice (Ruiz-Calvo et al., 2019; Valdeolivas et al., 2017). However, a small-scale trial of THC:CBD (Sativex) in human HD patients failed to produce any effect on symptoms (López-Sendón Moreno et al., 2016). This could be due to dosage, the short length of treatment, or the fact that the trial was in relatively late disease stages.

Outside of HD, pilot human trials are ongoing for nonmotor symptoms of Parkinson's disease (Leehey et al., 2020; Peball et al., 2019) and agitation associated with Alzheimer's disease (Hillen et al., 2019).

Neuronal hyperexcitability as a shared target of cannabinoid drugs

Drugs targeting the ECS exert their therapeutic effects through a diverse array of cellular mechanisms, including but not limited to: anti-inflammatory signaling via CB_2R, PPARs, and downregulation of arachidonic acid synthesis (Iannotti & Vitale, 2021; Malfitano et al., 2013; Nomura et al., 2011; Sánchez et al., 2006; Shao et al., 2014; Terrone et al., 2018) modulation of the AKT/mTOR pathway regulating cell survival

(Blázquez et al., 2015; Cao et al., 2018; Q. Wu et al., 2020), rapid desensitization of TRPV1 in pain (Muller et al., 2019), and antioxidant effects (Hampson et al., 1998; Nazıroğlu et al., 2019). One major feature of cannabinoid drugs is their ability to curb neuronal hyperexcitability; this mechanism of action is shared by multiple drugs across a wide variety of diseases—including those with successful ECS-based human therapies—and is therefore the main focus of this thesis.

Neurons are vulnerable to excitotoxicity, a process by which excitable cells are damaged or destroyed by an uncontrolled rise in intracellular Ca^{2+} (Bano & Ankarcrona, 2018). Excitotoxicity can be triggered by neuronal hyperexcitability and the resulting excessive glutamate exposure, which leads to a positive feedback loop involving Ca^{2+} from NMDA receptors, mGuR-mediated IP_3 receptor activation, and voltage-gated calcium channels (X. Dong et al., 2009). Because mild chronic hyperexcitability prompts neurons to downregulate synapses as a compensatory mechanism (Green et al., 2018), excitotoxicity can cause both cell death and synapse loss.

Endocannabinoids, which can be synthesized on demand as a negative feedback mechanism in response to neural activity, are perfectly positioned to help restore calcium homeostasis during excitotoxicity. Their anti-excitotoxic and neuroprotective effects are mediated in part through the effects of CB_1R on ion channel activity, calcium signaling, and neurotransmitter release (Haghani et al., 2012; Zhuang et al., 2005). CB_1R-mediated neuroprotection has been observed in animal seizure models (Wallace et al., 2003) and *in vitro* models of hyperexcitability (Deshpande et al., 2007), and the control of spasticity in multiple sclerosis model mice is also mediated by CB_1R (Pryce & Baker, 2007). CB_2R activation protects against seizures as well, but it is still unclear whether this is due to direct effects on neuronal excitability or glia-mediated mechanisms (Shapiro et al., 2019).

Neuronal hyperexcitability is a central feature of seizures and spasticity, but these are not the only conditions in which it plays a role; chronic glutamate excitotoxicity is also implicated in many neurodegenerative diseases (Armada-Moreira et al., 2020). For example, Aβ protein triggers glutamate excitotoxicity in Alzheimer's disease (Mattson et al., 1992), and memantine, one of the few drugs to treat AD symptoms, is a low-affinity NMDA receptor antagonist which counteracts excitotoxicity (Sonkusare et al., 2005). The neuroprotective effects of cannabinoids in artificially induced excitotoxicity have been demonstrated repeatedly (Giovanni Marsicano et al., 2003; Rangel-López et al., 2015; Zhuang et al., 2005), although more research is needed to examine their potential for treating specific neurodegenerative diseases in this way. Notably, striatal injection of the excitotoxin quinolinic acid in rats causes a Huntington's disease-like phenotype which can be lessened by the cannabinoid agonist Win55,212-2 (Pintor et al., 2006).

In summary, cannabinoids and the endocannabinoid system show great promise for treating multiple neurological conditions, particularly those which involve hyperexcitability and neuroinflammation. Because neuronal hyperexcitability is a shared feature of so many conditions and can be regulated by classical endocannabinoid signaling, it is an important target for cannabinoid drug development.

IV. Limitations of current cannabinoid drug development

The endocannabinoid system offers many enticing therapeutic targets, but there are still plenty of obstacles to overcome when developing new ECS drugs. These include adverse effects, the risk of drug tolerance, and species differences between human patients and animal models.

Adverse effects

Given the effects of recreational THC, it is not surprising that CB_1R activation can lead to unwanted psychotropic effects in a therapeutic setting, including cognitive impairment and a subjective “high” (Bedi et al., 2013; Issa et al., 2014). THC thus has a very narrow therapeutic window, which hampers its widespread use and acceptance even though serious adverse effects are rare. Because the psychotropic effects of THC are mediated by CB_1R (Ledent et al., 1999), all newly developed CB_1R agonists must also contend with the possibility of on-target adverse effects.

Endocannabinoids themselves, meanwhile, can activate several receptors which may or may not have similar effects to CB_1R—notably, the heat- and capsaicin-activated TRPV1 channel is activated by endocannabinoids but can have opposite effects on pain compared to CB_1R (Maione et al., 2006). In addition, the enzymes which produce and degrade endocannabinoids act on other lipid substrates as well (Bisogno et al., 2003; Cravatt et al., 1996; Okamoto et al., 2004). Interfering with lipid metabolism can occasionally cause serious off-target adverse effects, by far the most dramatic of which was the tragic failure of FAAH inhibitor BIA 10-2474 in first-in-human safety trials, in which one participant was killed and several others severely injured by an unidentified non-AEA-mediated neurological syndrome (Kerbrat et al., 2016).

Receptor desensitization and drug tolerance

Many of the conditions which could benefit from endocannabinoid system drugs (multiple sclerosis, seizure disorders, chronic pain, neurodegenerative diseases) would require long-term drug treatment. Unfortunately, it may prove difficult to provide robust long-term drug effects. Prolonged or repeated THC exposure leads to drug tolerance and loss of cannabinoid receptors in humans, animals, and cultured cells (Breivogel et

al., 1999; Colizzi & Bhattacharyya, 2018; Kouznetsova et al., 2002; Lundberg et al., 2005), and this problem of tolerance could extend to other drugs in development.

Tolerance to CB_1R and other GPCRs is mediated by receptor desensitization and downregulation, two related processes in which ligand-activated GPCRs first lose their G-protein mediated response to ligand binding and are later internalized and/or degraded (Rajagopal & Shenoy, 2018). Both CB_1R (Daigle et al., 2008) and CB_2R (X. Chen et al., 2014) are rapidly phosphorylated by G-protein receptor kinases (GRKs) upon activation, allowing them to associate with β-arrestin. This early phase of desensitization uncouples the receptor from G-protein signaling, often replacing it with β-arrestin-mediated signaling; in the case of CB_1R, β-arrestin recruitment mediates signaling through ERK1/2 (Daigle et al., 2008). On longer time scales, persistent GPCR activation leads to downregulation, or the β-arrestin-mediated internalization and sometimes degradation of the receptor (Guo et al., 2015). This process depends on a number of factors, including which agonist activates the receptor and which of the two β-arrestin isoforms are recruited (Ahn et al., 2013).

One interesting strategy for minimizing the effects of CB_1R desensitization is to focus on enhancing endogenous endocannabinoid signaling, thus preserving the normal temporal and spatial pattern of CB_1R activation rather than using a CB_1R agonist which would presumably activate—and possibly desensitize—many CB_1Rs throughout the body. This can be accomplished by inhibiting MAGL and/or FAAH to increase endogenous endocannabinoid levels. Unfortunately, animal studies of CB_1R desensitization with metabolic inhibitors have yielded conflicting results even with the same dose and timing of drug application (Feliszek et al., 2016; Schlosburg et al., 2010), and whether this is a viable strategy remains to be determined.

Species differences

Even though the human and rodent CB_1Rs are 96-97% identical at the amino acid level (Zou & Kumar, 2018), the differences that do exist can affect their interactions with drugs. A single amino acid change, for example, is enough to prevent the antagonist 6-alkoxy-5-aryl-3-pyridinecarboxamide from binding and inhibiting rodent CB_1R despite its high affinity for the human CB_1R (Iyer et al., 2015). A number of cannabinoid agonists also show differences in affinity between human and rodent CB_1R—a meta-analysis of studies which determined K_d and K_i values found that the synthetic agonists CP55,940 and particularly Win55,212-2 had higher affinity for rat than human CB_1R, while affinity of THC for the human CB_1R was nearly twice that of rat (McPartland et al., 2007).

Even highly conserved proteins are subject to species differences in their distribution and regulation. In humans, CB_1R is abundantly expressed in cortical areas associated with higher cognition, even showing lateralization in language-associated areas, whereas rodent CB_1R expression is more uniform (Glass et al., 1997). This suggests a role for the endocannabinoid system in cognitive processes that may not be fully shared between species. In addition, the CB_1R mRNA is alternatively spliced into several variants with different 5' untranslated regions and amino terminal sequences, some of which are species-specific (Liu et al., 2019; Ryberg et al., 2005).

Beyond the cannabinoid receptors themselves, every other component of the endocannabinoid system is also a potential source of species differences and the complications that come with them. These differences can be difficult to predict from sequence similarity: for example, the MAG lipase inhibitor JZL184 has similar potency against human and mouse MAG lipase but is far less potent against rat MAG lipase, despite the fact that the rodent lipases are much more similar to each other than to the human enzyme (Long, Nomura, et al., 2009). The active sites of human and rat FAAH

also contain six non-conserved residues which affect the sensitivity of the enzymes to inhibitors without greatly impacting their ability to hydrolyze endogenous substrates (Mileni et al., 2008). Given the complexity of the endocannabinoid system, it would be nearly impossible to prevent or even anticipate all of the species differences which will affect any given experimental drug.

Finally, animal models of human disease are just that—artificial models created to resemble important aspects of the disease in question. Although animal models have unquestionably made great contributions to medical research, disease etiology is just as subject to species differences as any other aspect of biology, and there is no guarantee that all facets of a human disease will be captured by a given animal model. For example, most genetic mouse models of Alzheimer's disease are based on early-onset familial AD even though the vast majority of the patient population has late-onset sporadic forms of the disease (Oblak et al., 2020), and fewer than a dozen humans have ever contracted parkinsonism via exposure to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), the most well-studied model of Parkinson's disease in non-human primates (Langston, 2017). Disease modeling concerns like these are less of an obstacle in human research, where participants can be recruited directly from the patient populations which the investigators hope to cure.

Strategies for overcoming limitations of current cannabinoid drugs

The problems of adverse effects and drug tolerance might be solved by clever new approaches to targeting the endocannabinoid system. For example, drugs with low blood-brain barrier permeability can be designed for peripheral targets, thus avoiding psychoactive side effects in the CNS (Mulpuri et al., 2018). There is also considerable interest in developing biased CB_1R ligands which activate desired downstream pathways while minimizing adverse effects or desensitization (Leo & Abood, 2021). Some

strategies for avoiding CB_1R tolerance focus on enhancing endogenous neuroprotective CB_1R signaling rather than activating (and possibly desensitizing) every receptor in the body; these strategies include the MAGL and/or FAAH inhibitors discussed above, as well as newly developed positive allosteric modulators of CB_1R (Hryhorowicz et al., 2019).

This rich variety of strategies holds excellent promise for future drug development; however, a range of potential lead compounds is only one step towards success. Species differences present an obstacle to drug screening in animal models, while non-neuronal cells overexpressing individual components of the human endocannabinoid system do not capture the full breadth and complexity of potential targets and lack neuron-like functional readouts. This highlights the need for a complete model of the human endocannabinoid system in neurons.

V. Human iPSC-derived neurons as an *in vitro* model system

Introduction to human induced pluripotent stem cells (hiPSCs)

Although conducting research in human cells is an important part of overcoming species differences in drug development, primary human neuron cultures are impractical on a large scale due to the scarcity of human brain tissue for research. 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. These fibroblasts are then reprogrammed into pluripotent stem cells—capable of differentiating into any of the three embryonic germ layers and subsequent specific cell types—through exogenous expression of

transcription factors necessary for pluripotency (Takahashi et al., 2007; Takahashi & Yamanaka, 2006).

Once hiPSCs are obtained, they can be committed to a neural fate through one of several methods, the most popular of which are embryoid body differentiation and dual SMAD inhibition. The embryoid body method involves growing hiPSCs in aggregates and exposing them to a sequence of neural fate-inducing growth factors in defined media to mimic neural development (Okabe et al., 1996; Zeng et al., 2010), while dual SMAD inhibition uses small molecule inhibitors of the transforming growth factor-β and bone morphogenic protein pathways, both of which belong to the SMAD family of intracellular signaling molecules (Chambers et al., 2009). In either case, the resulting neural stem/progenitor cells are exposed to additional growth factors or genetic manipulations to bias their differentiation towards neuronal or glial subtypes of interest. Thus, cells resembling a variety of different human neuron subtypes, from dorsal or ventral telencephalic neurons (Li et al., 2009) to midbrain dopaminergic neurons (Swistowski et al., 2010) and spinal motor neurons (Li et al., 2005), have been generated and grown both as two-dimensional cultures and as brain organoids which recapitulate some of the three-dimensional architecture of the brain (Lancaster et al., 2013). hiPSCs can also be differentiated into astrocytes for neuron/glia cocultures (Shaltouki et al., 2013).

Advantages of hiPSC-derived neurons

hiPSCs can help avoid the problem of species differences in rodent primary neuron cultures. For example, hiPSC-derived neuron/astrocyte cocultures are more sensitive than primary rodent cultures to a wider range of drugs known to cause seizures in humans (Tukker et al., 2020). In addition, hiPSCs can be derived from patients with a

disease of interest rather than relying solely on animal models with experimentally induced diseases (Brennand et al., 2015).

iPSC-derived cultures also offer advantages over cell lines stably expressing human proteins of interest, mainly by providing a closer approximation of the cellular context in which the protein acts *in vivo*. This is particularly relevant to proteins like CB_1R, whose preference for coupling to $G_{i/o}$ over G_s is heavily influenced by receptor expression levels and the availability of different G-protein subtypes (Finlay et al., 2017).

A final advantage of hiPSC-derived cultures is the potential for high-throughput applications which would otherwise be impractical or impossible to do with primary human brain tissue. While research using biopsied or post-mortem human brain tissue is largely limited to case studies or small exploratory studies, hiPSCs can be used to produce large batches of cells which are amenable to extensive basic research and high-throughput pharmacological screens.

Limitations of hiPSC-derived neurons

Like any model system, hiPSC-derived cultures have their own limitations. The “mature” neurons in hiPSC-derived cultures tend to resemble young cells from fetal brain, which could interfere with modeling age-related illnesses (Handel et al., 2016). There can also be considerable variability between individual hiPSC lines, potentially impacting reproducibility (Ortmann & Vallier, 2017). While hiPSCs capture more of the complexity of the brain than non-neuronal stable cell lines, they are far from capturing all of it. Cell cultures cannot replace animal models for behavioral assays, and two-dimensional cultures like those used in this thesis also lack the normal architecture of the human cortex. In addition, hiPSC-derived cultures often only contain a handful of different cell types depending on the differentiation protocol. Notably, microglia come from a lineage of yolk sac precursors and cannot be generated from neural stem cells like neurons or

astrocytes (Gonzalez et al., 2017). In order to be studied in hiPSC-derived neural cultures, microglia must be derived separately and cocultured with other cell types, a costly and difficult process.

Finally, a major limitation of hiPSC-derived cultures is that, as a newer technology, they are less well characterized than more established models. For example, in order to study the effects of cannabinoids on hiPSC-derived neurons, we first must test whether they actually have a functional endocannabinoid system to study. This is one of the main goals of this thesis research.

Previous research on cannabinoids and the endocannabinoid system in hiPSC-derived cultures

Because cannabinoids can affect stem and progenitor cells themselves, much of the existing cannabinoid research in hiPSC-derived neurons focuses on development; however, there are an encouraging number of similarities between cannabinoid effects in developing hiPSC-derived neurons and developing animal brains.

The effects of the endocannabinoid system on essentially every stage of neural development are well-documented in animal and primary cell culture models. Both endocannabinoids and exogenously applied cannabinoid agonists promote the proliferation of neural progenitor cells via CB_1R (Aguado et al., 2005) and CB_2R (Palazuelos et al., 2006). Once cell fate is specified, cannabinoids enhance their differentiation into neurons (Compagnucci et al., 2013) astrocytes (Aguado et al., 2006), and oligodendrocytes (Gomez et al., 2010). Endocannabinoid signaling is required for normal neuroblast migration (Oudin et al., 2011) and also plays a critical role in axon guidance: endocannabinoids promote the outgrowth of long, unbranched pyramidal cell axons (Mulder et al., 2008) and repel the growth cones of interneuron axons to ensure correct axon patterning (Berghuis et al., 2007).

Many of these effects are also seen in hiPSC-derived neuron development, hinting at a possible endocannabinoid system in at least the early stages of these cultures. During generation of hiPSC-derived neurons, CB_1R expression is already detectable at the stem cell stage and gradually increases as the cells differentiate into neurons (Bobrov et al., 2017). THC is capable of causing gene expression changes in hiPSC-derived neurons (Guennewig et al., 2018), providing indirect evidence of cannabinoid receptor function. In developing hiPSC-derived neurons, which express CB_1R by immunofluorescence, both THC and 2-AG negatively regulate neurite outgrowth in a rimonabant-sensitive manner (Shum et al., 2020). These studies provide both expression and functional evidence of working CB_1Rs in hiPSC-derived neural cultures during development.

Endocannabinoid research in mature hiPSC-derived neurons is more scarce, but the studies that have been published are consistent with the hypothesis that hiPSC-derived cultures have an endocannabinoid system. In a recent study, Papariello et al. (2021) demonstrated protein expression of multiple key ECS components in cortical spheroid hiPSC cultures in late development, and found that CB_1R played a role in synaptogenesis in mature neurons. However, there was no functional characterization of components other than CB_1R. Stanslowsky et al. (2017) observed profound baseline electrophysiological differences in hiPSC-derived neurons which had been exposed to THC or AEA during differentiation compared to vehicle-treated neurons, but did not test the mature cells' response to acute cannabinoid application.

Taken together, these published studies strongly suggest that at least portions of the endocannabinoid system, particularly CB_1R, are present in hiPSC-derived neuron cultures in at least some stages of development. However, most do not investigate the expression of other components like synthetic and metabolic enzymes, and functional assays of the hiPSC endocannabinoid system are lacking. In order to use hiPSC-derived

neurons for high-throughput drug development, it must be shown that all major parts of an endocannabinoid system are both present and fully operational—that is, producing endocannabinoids which can then affect neural activity and be degraded to terminate signaling.

VI. Summary of introduction and rationale for thesis

In summary, the endocannabinoid system contains promising targets for treating neurological conditions with an excitotoxic component, but current efforts in cannabinoid drug development are hindered by a lack of human model systems and by species differences between human and animal endocannabinoid system targets. hiPSC-derived neurons may be a useful model to investigate these targets in a more high-throughput way; however, whether they contain a complete and functional endocannabinoid system at maturity is unknown. Once an *in vitro* model of the human endocannabinoid system is established, it could be used to investigate promising new strategies in endocannabinoid pharmacology.

In this thesis, I demonstrate that a commercially available line of hiPSC-derived cortical neurons and astrocytes contains a fully functional endocannabinoid system which could be used as a human *in vitro* model for basic research or drug discovery. hiPSC-derived cultures are amenable to calcium imaging and a genetically encoded cannabinoid indicator (A. Dong et al., 2021), both of which could be used in future high-throughput applications. They are also susceptible to a low-magnesium model of NMDA-mediated hyperexcitability (Walther et al., 1986), allowing for functional studies of cannabinoid-mediated inhibition using the effects on this activity as a readout. Using this model, I demonstrate that G_q receptor-evoked cannabinoid synthesis is not calcium-

dependent in hiPSC-derived neurons and that JZL184, an inhibitor of 2-AG metabolism, causes less drug tolerance than a CB_1R agonist with prolonged exposure. My findings establish hiPSC-derived neurons as a tool for studying the human endocannabinoid system in the context of human cells and demonstrate their utility for answering open questions about receptor signaling and regulation.

Chapter Two:

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

Content adapted from manuscript in preparation:

Asher MJ, McMullan HM, Dong A, Li Y, and Thayer SA (2022). “A complete endocannabinoid signaling system modulates synaptic transmission between human induced pluripotent stem cell-derived cortical neurons.” (In preparation).

Contributions: MJA and SAT designed the study. MJA and HMM conducted experiments and analyzed data. AD and YL contributed reagents. MJA, SAT, and HMM wrote the manuscript.

I. Introduction

The endocannabinoid (eCB) system (ECS) regulates processes ranging from appetite and emesis to mood and memory (Lowe et al., 2021; Lu & 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_1Rs are primarily expressed in neurons and are widely distributed throughout the CNS (M. Glass et al., 1997). The principal active ingredient in cannabis, Δ^9-tetrahydrocannabinol (THC), exerts its psychoactive effects via CB_1R (Matsuda et al., 1990). CB_1R 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 & Abood, 2021). Much of the preclinical work on CB_1R 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_1Rs differ in their distribution and their affinities to a range of ligands (J. M. McPartland et al., 2007).

in vitro models with a complete ECS are useful for understanding how ECS components are affected by CB1/2R 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 (Van Egmond et al., 2021). 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 CB_1R is expressed in hiPSCs and hiPSC-derived neurons (Bobrov et al., 2017) and that 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 CB_1Rs appear functional because rimonabant, an inverse agonist of CB_1Rs, 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 CB_1Rs 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 cortical 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_1R-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.

II. 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 ($CaCl_2$, catalog number: C3881), and WIN 55,212-2 (catalog number: 131543-23-2) were from Millipore Sigma (St. Louis, MO, USA); 2-arachidonoylglycerol (2-AG, catalog number: 62610), NESS 0327 (catalog number: 10004184), JZL184 (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); Δ^9-tetrahydrocannabinol (THC) was from the National Institute on Drug Abuse Drug Supply Program (Research Triangle Institute, Research Triangle Park, NC, USA).

Human iPSC-derived cortical cultures. Human iPSC-derived cortical 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% CO_2/95% 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.

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 (S. 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 $GRAB_{eCB2.0}$ plasmid was described previously (A. Dong et al., 2021); $AAV_{PHP.eB}$-hSYN-$GRAB_{eCB2.0}$ virus was used at a titer of 3.68 x 10^{11} genome copies/mL. The hSyn-eGFP plasmid was a gift from Dr. Bryan Roth (Addgene plasmid #50465); $AAV_{PHP.eB}$-hSYN-GFP virus was used at a titer of 1.30 x 10^{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). $AAV_{PHP.eB}$-GFAP-mCherry virus was used at a titer of 1.41 x 10^{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_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 $[Mg^{2+}]_o$) 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 μm^3). 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% CO_2 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) Mg^{2+}.

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 was 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 $\Delta F/F_0$ values, using the minimum value for each ROI as F_0. 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 $\Delta F/F_0$ 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 $GRAB_{eCB.2.0}$ 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 $[Mg^{2+}]_o$) 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 = 1.1525x1.1525x1 μm^3) 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 JZL184) 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 positive control epoch was considered when selecting ROIs; the investigator was blinded to treatment condition and to the appearance of the processes during the experimental 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 $\Delta F/F_0$ for each ROI in each frame, with the baseline epoch average used as F_0. Because brightness varied considerably between individual processes, data are reported as a percentage of the Win-2 epoch average. The average peak $\Delta F/F_0$ (normalized to Win-2) of all four 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 JZL184 (added with a half media change containing 2x drug during regular feeding) for 15 min (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-min 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 individual 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 $GRAB_{eCB2.0}$ 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. The hiPSC 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 the Brown-Forsythe test for homogeneity of variance. 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. For comparisons of groups with non-homogenous variance, Welch's t-test was used to compare two groups and the Brown-Forsythe ANOVA with Dunnet's T3 multiple comparisons test was used to compare multiple groups. Statistical significance was defined as $p<0.05$. No tests for outliers were performed.

III. Results

hiPSC-derived neurons form a glutamatergic signaling network *in vitro*

hiPSC-derived cortical 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 (Figure 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 cortical neurons we evoked synaptically driven $[Ca^{2+}]_i$ spiking activity. Previous studies of synaptic networks in primary cultures of rat neurons have shown that reducing the extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) will evoke paroxysmal epileptiform bursts of action potentials, producing $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ oscillated in a seizure-like synchronized pattern. In 0.1 mM $[Mg^{2+}]_o$ the $[Ca^{2+}]_i$ spiking frequency was 3 ± 0.4 events/min (Figure 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 $[Mg^{2+}]_o$-evoked repetitive $[Ca^{2+}]_i$ spiking represents synaptic activity between networked hiPSC-derived cortical neurons.

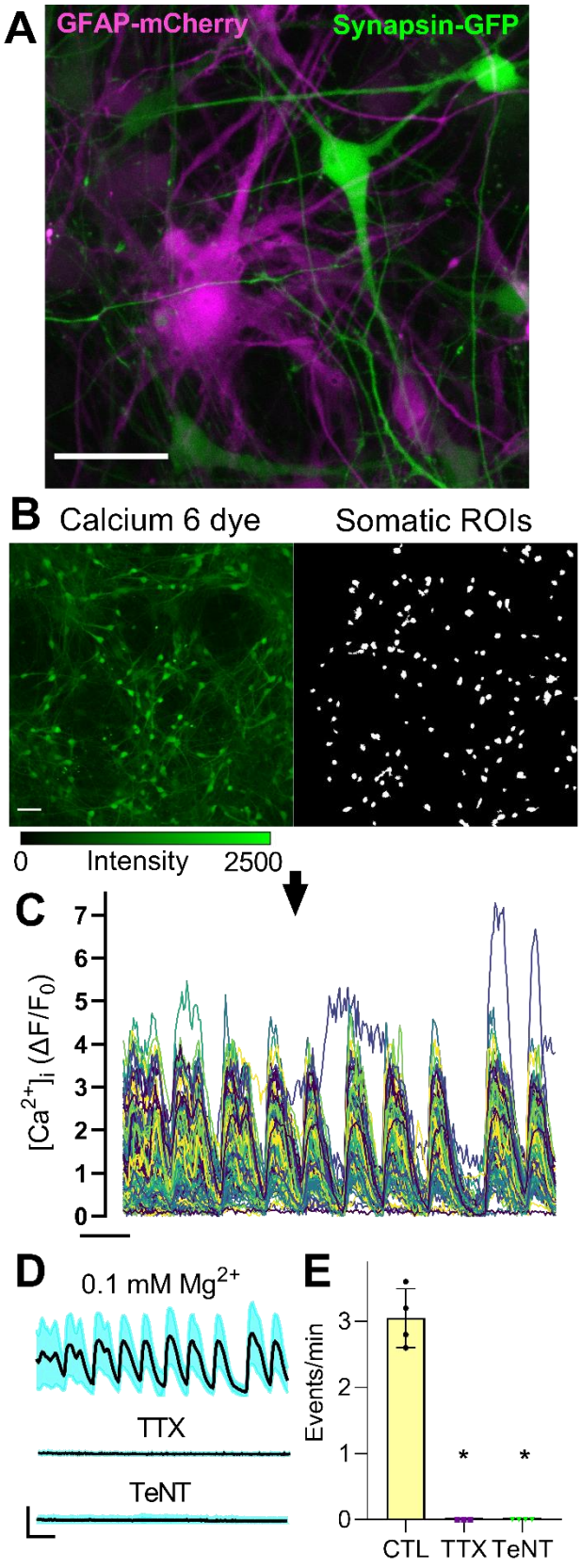

Figure 2.1. hiPSC-derived cortical cultures exhibit synaptically driven $[Ca^{2+}]_i$ spiking. (A) hiPSC-derived cortical cultures expressing mCherry from the GFAP promoter (magenta) and GFP from the synapsin promoter (green). Scale bar = 50 µm. **(B)** Representative image of hiPSC-derived cultures in Calcium 6 dye (left) and ROIs derived from this image using threshold-based segmentation in ImageJ (right). Scale bar = 50 µm. **(C)** Individual $[Ca^{2+}]_i$ traces representing $\Delta F/F_0$ for each ROI in (B). Scale bar = 30 s. **(D)** Representative traces show mean $[Ca^{2+}]_i$ (solid line) from a single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg^{2+} buffer containing vehicle (H_2O, 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 $\Delta F/F_0$. **(E)** Bar graph summarizes Ca^{2+} spiking activity (events per minute) in cultures treated with H_2O vehicle (CTL), TTX, or TeNT as in (D). *, $p<0.05$ relative to CTL. Kruskal-Wallis test with Dunn's correction, $p = 0.024$ for TTX and 0.013 for TeNT relative to vehicle, n=3-4 wells per treatment.

To study the pharmacology of this network activity the following treatment protocol was employed (Figure 2A). Cell culture media was replaced with HEPES buffered Hank's salt solution (HBSS) containing 0.1 mM $[Mg^{2+}]_o$ and the cultures were allowed to stabilize for 2 min. A 5 min baseline epoch was recorded which served as a 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 the cells were incubated in drug or vehicle 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 ($\Delta F/F_0$) 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 (Figure 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. 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 (Gulyaás-Kovaács et al., 2002). These results show that hiPSC-derived cortical cultures contain cells which resemble neurons morphologically and functionally, and which form a glutamatergic signaling network *in vitro* that could potentially be manipulated by cannabinoids.

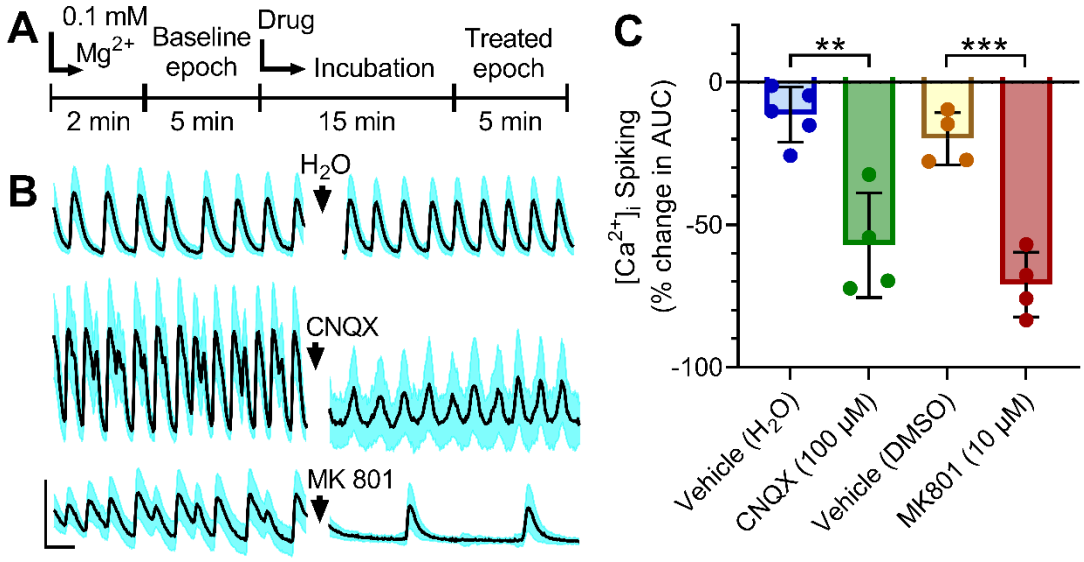

Figure 2.2. hiPSC-derived cortical cultures form a glutamatergic synaptic network. (A) Schematic shows drug treatments sequence for this and following figures. (B) Representative traces show mean $[Ca^{2+}]_i$ (solid line) for a single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg^{2+} buffer before and after the addition of vehicle (H_2O, top), 100 µM CNQX (middle), or 10 µM MK801 (bottom). Scale bars: horizontal = 30 s, vertical = 1.0 $\Delta F/F_0$. (C) Bar graph summarizes the change in $[Ca^{2+}]_i$ spiking activity (% change in AUC) before and after adding vehicle (H_2O 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 H_2O; $t = 7.0$ and $p = 0.0004$ for MK 801 relative to DMSO; n = 4-5 wells per condition.

hiPSC-derived neurons are sensitive to exogenous cannabinoids

We next tested whether the low $[Mg^{2+}]_o$-induced synaptic activity between hiPSC-derived cortical 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 (Figure 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 lower Win-2 potency in human relative to rodent cultures is in good agreement with a meta-analysis that compared cannabinoid binding affinity to human cortical tissue relative to rat cortical tissue (J. M. McPartland et al., 2007). The inhibition of $[Ca^{2+}]_i$ spiking by Win-2 was completely blocked by the selective CB_1R antagonist NESS 0327 (Ruiu et al., 2003) (Figure 2C & E), indicating that Win-2 acted on functional CB_1Rs in these neurons.

An endogenous ligand for CB_1Rs, 2-arachidonoylglycerol (2-AG), also inhibited 0.1 mM $[Mg^{2+}]_o$ evoked $[Ca^{2+}]_i$ spiking (Figure 2B & D). The IC_{50} for 2-AG-mediated inhibition of $[Ca^{2+}]_i$ spiking was 2.0 ± 0.6 µM. The lower potency relative to Win-2 is consistent with that previously described in rodent models (Straiker & Mackie, 2005). The potency of 2-AG against glutamatergic synaptic activity in hiPSC cultures is 5-fold lower than that described for inhibition of synaptic activity in rat hippocampal cultures (M. M. Wu & Thayer, 2020). Overall, these pharmacological studies indicate that hiPSC-derived cortical cultures respond to both synthetic cannabinoids and eCBs.

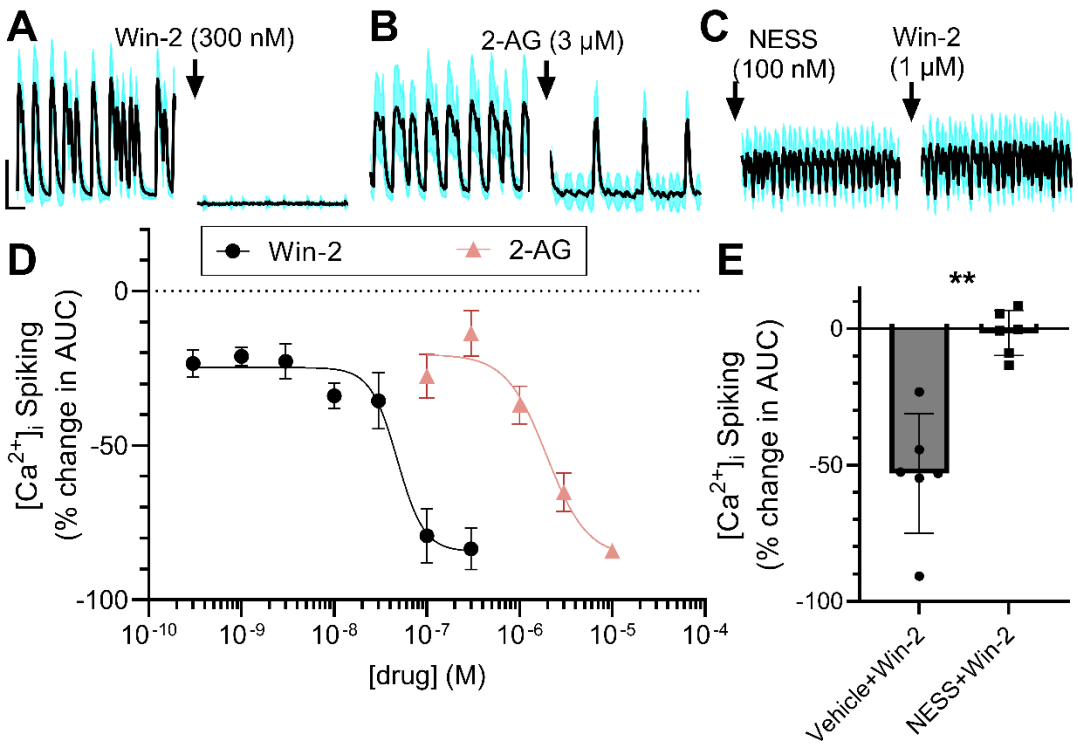

Figure 2.3. Cannabinoid agonists inhibit synaptic activity between hiPSC-derived cortical neurons via CB_1R. (A-C) Representative traces show mean (solid) $[Ca^{2+}]_i$ ($\Delta F/F_0$) of single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg^{2+} buffer before and after the addition of 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 $\Delta F/F_0$. **(D)** Concentration-response curves for Win-2 (circles) and 2-AG (triangles) were fit with a logistic equation of the form: % change in AUC = A_1 + [(A_2-A_1)/(1+$10^{(\log x_o - x)p}$)], where x_o = EC_{50}, x = log[drug], A_1 = % change at a maximally effective drug concentration, A_2 = % change in the absence of drug, and p = slope factor. The following values were calculated using a non-linear, least-squares curve fitting program: A_1 = -25% for Win-2 and -21% for 2-AG; A_2 = -84% for Win-2 and -86% for 2-AG; EC_{50} = 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 $[Ca^{2+}]_i$ 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.

THC acts as a partial agonist on CB_1R in hiPSC-derived neurons

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, THC acts as a partial agonist on both rodent and human $CB_{1/2}Rs$ (Govaerts et al., 2004; Roloff & Thayer, 2009). THC inhibited 0.1 mM $[Mg^{2+}]_o$ evoked $[Ca^{2+}]_i$ spiking in a manner consistent with action as a partial agonist (Figure 4). The IC_{50} of THC-mediated inhibition of $[Ca^{2+}]_i$ 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 THC elicited only 47 ± 14 % inhibition, consistent with its partial agonist properties, and in contrast to the full agonists Win-2 and 2-AG (Figure 3).

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, 3 µM THC antagonized the activity of 1 µM Win-2 when added to the cultures 15 min before Win-2 (Figure 4 C-D), confirming that THC acts as a partial CB_1R agonist in hiPSC-derived cortical cultures.

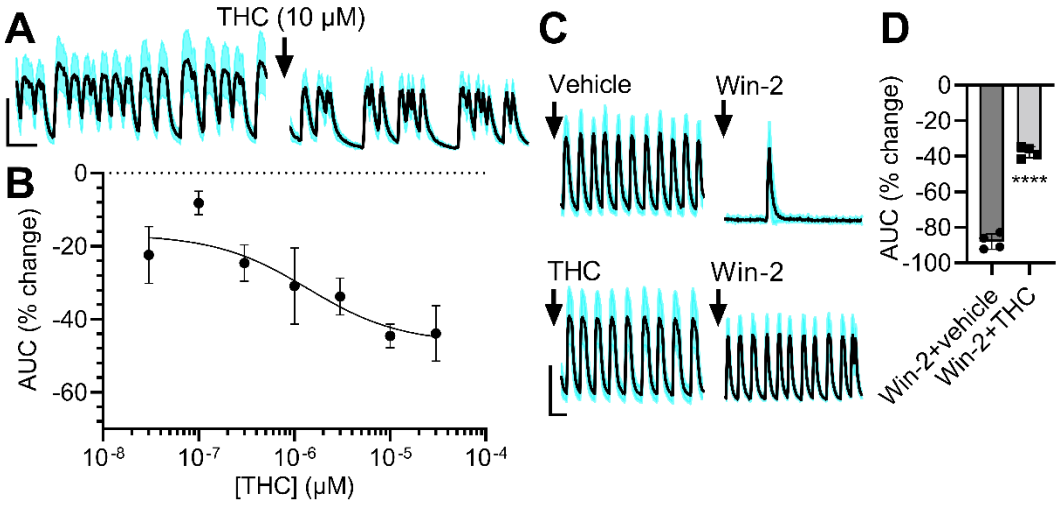

Figure 2.4. THC acts as a partial agonist to inhibit synaptic transmission in hiPSC-derived cortical cultures. (A) Representative traces show mean (solid line) $[Ca^{2+}]_i$ ($\Delta F/F_0$) of a single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg^{2+} buffer before and after the addition of 10 µM THC. Scale bars: horizontal = 30 s, vertical = 1.0 $\Delta F/F_0$. **(B)** Concentration-response curve for THC was fit with a logistic equation of the form: % change in AUC = $A_1 + [(A_2-A_1)/(1+10^{(\log x_o - x)p})]$, where $x_o = EC_{50}$, x = log[drug], A_1 = % change at a maximally effective drug concentration, A_2 = % change in the absence of drug, and p = slope factor. The following values were calculated using a non-linear, least-squares curve fitting program: A_1 = -17 ± 11%; A_2 = -47 ± 14%; EC_{50} = 1.4 ± 1.9 µM; p = -0.9 ± 1.2; n = 4-7 wells per concentration. **(C)** Representative traces show mean (solid line) $[Ca^{2+}]_i$ ($\Delta F/F_0$) 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 (0.095% ethanol, top) or 1 µM THC (bottom). Scale bars: horizontal = 30 s, vertical = 1.0 $\Delta F/F_0$. **(D)** Bar graph shows change in $[Ca^{2+}]_i$ spiking relative to baseline (AUC) after addition of 1 µM Win-2 in wells treated with vehicle or THC. Data are presented as mean ± SD. Student's t-test, $t_{(5.5)}$=18.8, p<0.0001, n = 5 wells per condition.

hiPSC-derived cortical cultures produce 2-AG via diacylglycerol lipase

We next investigated whether hiPSC-derived cortical cultures produce eCBs, in addition to responding to exogenously applied CBs. To detect eCB production, we used a GPCR-activation based eCB ($GRAB_{eCB2.0}$) sensor (A. Dong et al., 2021). The sensor consists of a modified CB_1R fused to circularly permutated GFP such that it fluoresces upon ligand binding. We infected the hiPSC-derived cortical cultures with an adeno-associated virus (AAV) vector that expressed $GRAB_{eCB2.0}$ under the control of the neuron-specific human synapsin promoter ($AAV_{PHP.eB}$-hSyn-$GRAB_{eCB2.0}$). Six days after infection, the cultures expressed functional $GRAB_{eCB2.0}$ sensor, as evidenced by a large increase in fluorescence upon the addition of Win-2 (Figure 5).

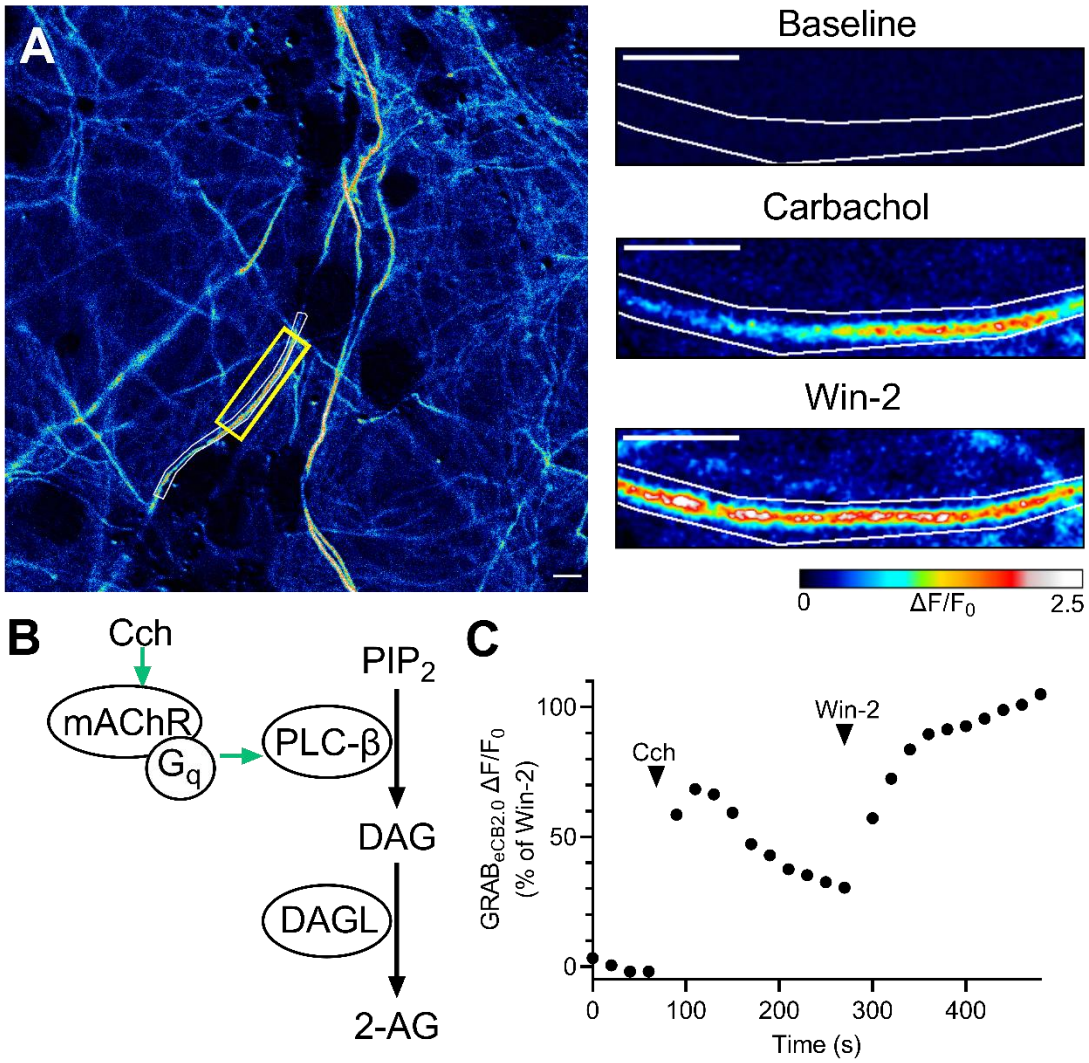

Figure 2.5. eCB GRAB sensor imaging in hiPSC-derived cortical cultures. (A) Left: $GRAB_{eCB2.0}$ fluorescence ($\Delta F/F_0$) in cells treated with 300 nM Win-2. Scale bar = 10 µm. Right: enlarged images of the boxed region at baseline (top) and after the addition of 1 µM carbachol (middle) and 300 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 ($\Delta F/F_0$) 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.

Metabotropic suppression of excitation (MSE) is a form of synaptic modulation mediated by eCBs produced following activation of G_q-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., 2001; Straiker & Mackie, 2007). MSE can be triggered by multiple G_q-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 cortical cultures expressing the $GRAB_{eCB2.0}$ sensor, fluorescence increased over 30-60 s to peak at 95 ± 43 % of the maximum response to Win-2 (Figure 5C and Figure 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 (Figure 6 A-B). This both confirms that the $GRAB_{eCB2.0}$ sensor is measuring eCB levels and suggests that 2-AG is the main eCB produced by carbachol stimulation of hiPSC-derived cortical cultures.

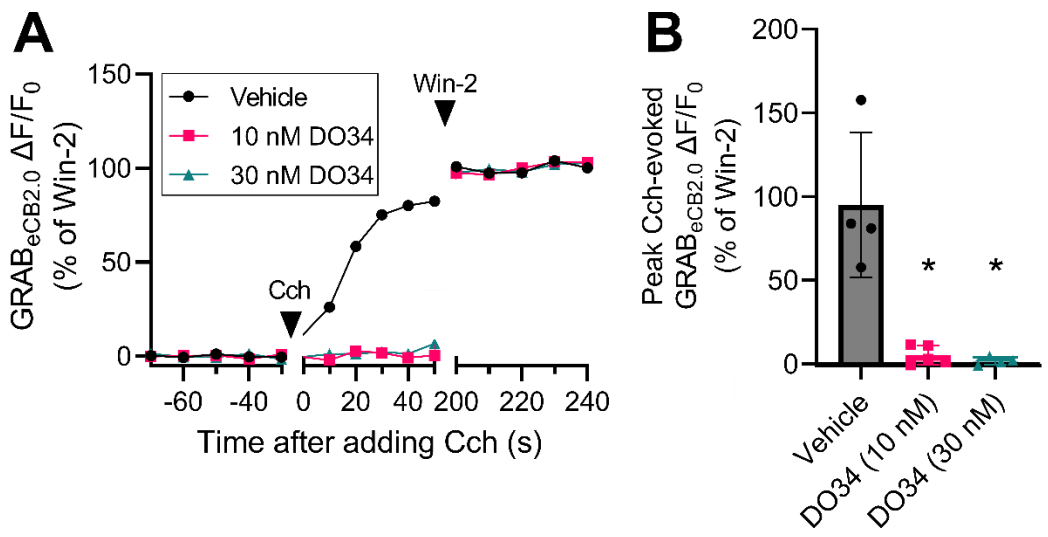

Figure 2.6. hiPSC-derived cortical cultures synthesize eCBs via DAG lipase. (A) Time courses showing mean $GRAB_{eCB2.0}$ fluorescence ($\Delta F/F_0$, expressed as a percentage of the Win-2 response) from representative wells pretreated for 1 h with vehicle (0.1% DMSO) or 10 or 30 nM DO34. Arrowheads 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 ($\Delta F/F_0$, 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.

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 $GRAB_{eCB2.0}$ expressing hiPSC-derived cortical 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 (Figure 7A). This decay was not due to photobleaching of the $GRAB_{eCB2.0}$ 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 (Figure 7A). When cultures were pretreated with JZL184, the decay of the carbachol-induced $GRAB_{eCB2.0}$ 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 (Figure 7B). The decay process was well fit by an exponential equation. The time constant for recovery increased significantly from $\tau = 82 \pm 20$ s in vehicle-treated cultures to $\tau = 108 \pm 17$ s in cultures treated with JZL184 (excluding one JZL184 recording in which the decay was too slow to calculate a time constant; Student's t-test, $t=2.4$, $p=0.04$). This indicates that MAG lipase is degrading 2-AG produced by stimulating hiPSC-derived cortical cultures with carbachol. Taken together, these results show that hiPSC-derived cortical cultures contain a complete and functional eCB system.

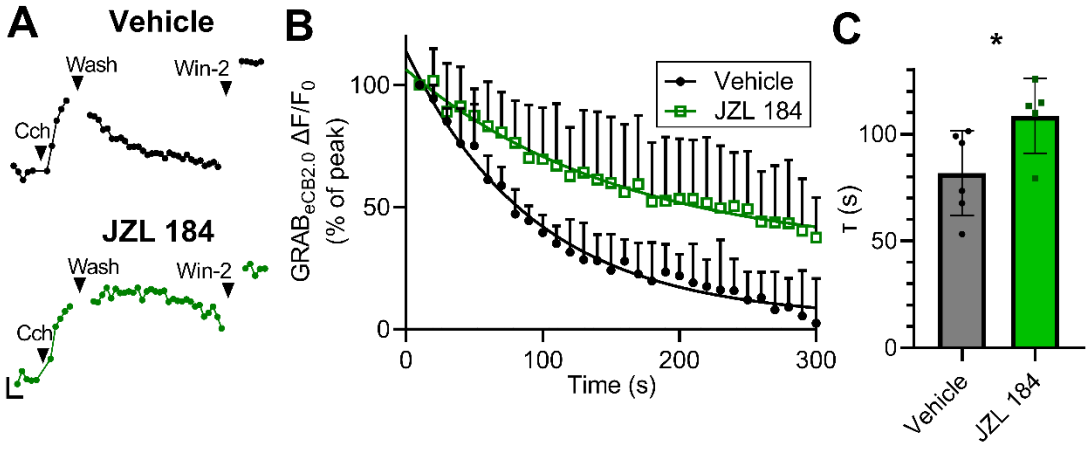

Figure 2.7. hiPSC-derived cortical cultures metabolize 2-AG via MAG lipase. (A) Traces showing average GRAB$_{eCB2.0}$ fluorescence from representative wells pretreated with vehicle or 1 µM JZL184. Arrowheads show addition of 1 µM carbachol (Cch), washout of carbachol (wash) and addition of 300 nM Win-2. Each trace is the average of 4 ROIs from a single well. Scale bars: horizontal = 30 s, vertical = 20% of saturating Win-2 response ($\Delta F/F_0$). **(B)** Time course showing the decay of carbachol-evoked GRAB$_{eCB2.0}$ fluorescence after washout of carbachol ($\Delta F/F_0$, expressed as a percentage of the peak fluorescence) in cells pretreated for 1 h with vehicle (0.1% DMSO, filled circles) or 1 µM JZL184 (open squares). n = 6 wells per treatment. Data are presented as mean ± SD. Curves were fit with an exponential decay function. **(C)** Bar graph shows time constants for GRAB$_{eCB2.0}$ fluorescence decay calculated from curves shown in (B) from cells pretreated with vehicle or JZL184. One JZL184 well decayed too slowly to calculate a time constant and is not shown in (C). Data are presented as mean ± SD. *, $p<0.05$. Student's t-test, $t_{(9)}=2.35$, $p=0.04$, n = 6 wells for vehicle and 5 wells for JZL184.

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

The enzymes responsible for 2-AG production, phospholipase C and DAG lipase (Figure 5B), are stimulated by increases in $[Ca^{2+}]_i$ (Hashimotodani et al., 2005, 2008). Here, we examined the Ca^{2+} sensitivity of 2-AG production in hiPSC-derived cortical cultures following activation of muscarinic receptors. Carbachol, acting via the muscarinic M1 receptor, stimulates phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and DAG. IP_3 releases Ca^{2+} from intracellular endoplasmic reticulum (ER) stores. Thus, it was not surprising that neither the carbachol-evoked increase in $[Ca^{2+}]_i$ nor the evoked increase in 2-AG required extracellular Ca^{2+} (Figure 8).

However, in cells pretreated for 30 min with 1 µM thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase that depletes ER Ca^{2+} stores, the carbachol-induced increase in $[Ca^{2+}]_i$ was completely blocked while the evoked production of 2-AG was not affected (Figure 8). Thus, Ca^{2+} mobilized from the ER does not appear to be required for 2-AG synthesis by this route. When cells were pretreated with thapsigargin in nominally Ca^{2+}-free buffer to eliminate store operated Ca^{2+} influx, carbachol caused no detectable increase in $[Ca^{2+}]_i$. However, carbachol-evoked 2-AG production was unaffected. Thus, a maximally effective concentration of carbachol triggers eCB production in a Ca^{2+}-independent manner in hiPSC-derived cortical cultures.

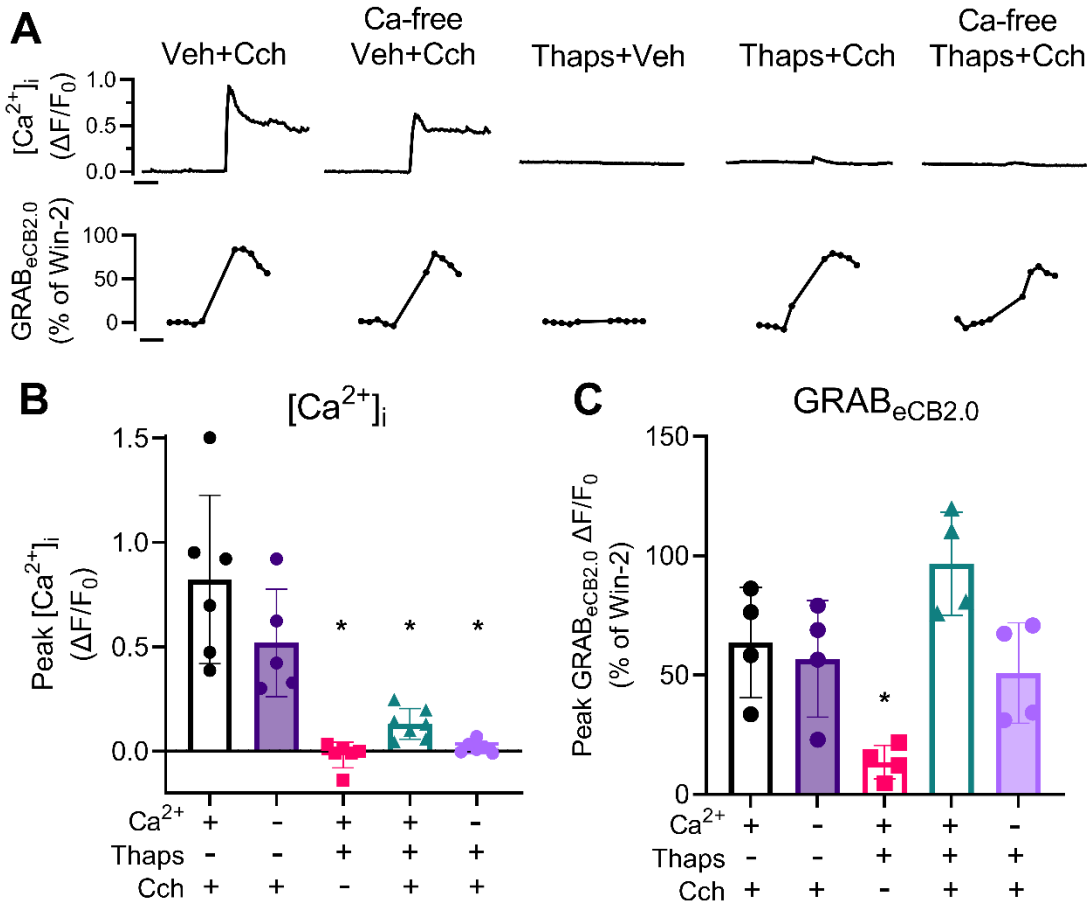

Figure 2.8. Carbachol-evoked 2-AG production is independent of $[Ca^{2+}]_i$. (A) Top: representative $[Ca^{2+}]_i$ traces from wells pretreated for 30 min with vehicle (0.1% DMSO) or 1 µM thapsigargin in the absence or presence of Ca^{2+} as indicated. Horizontal bar=30 s. Bottom: representative traces showing $GRAB_{eCB2.0}$ fluorescence in wells pretreated as indicated. Horizontal bar=30 s. **(B)** Bar graph summarizes peak carbachol-evoked $[Ca^{2+}]_i$ in cells treated with 1 µM Cch or 1 µM thapsigargin as indicated. Recordings were performed in the absence (nominally Ca^{2+}-free) or presence of 1.3 mM extracellular Ca^{2+} as indicated. n = 6 wells per condition. Data are presented as mean ± SD. *, $p<0.05$ relative to vehicle + Ca^{2+} + carbachol. Brown-Forsythe ANOVA with Dunnet's T3 multiple comparisons test, $F_{(4, 25)} = 5.0$, $p = 0.004$. **(C)** Peak carbachol-evoked $GRAB_{eCB2.0}$ 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. * $p<0.05$ relative to vehicle + Ca^{2+} + carbachol. Brown-Forsythe ANOVA with Dunnet's T3 multiple comparisons test, $F_{(4.0, 12.4)} = 8.4$, $p = 0.002$.

CB_1R desensitization is less pronounced with a metabolic inhibitor than a receptor agonist in hiPSC-derived cortical cultures

Although THC and other CB_1R 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 way to avoid this is to slow eCB metabolism instead, thus enhancing the endogenous spatial and temporal patterns of 2-AG signaling rather than activating all CB_1Rs for a prolonged period. We therefore tested whether the cannabinoid receptor agonist Win-2 and the MAG lipase inhibitor JZL184 desensitized CB_1R-mediated presynaptic inhibition in hiPSC-derived cortical cultures.

We used the 0.1 mM $[Mg^{2+}]_o$-evoked $[Ca^{2+}]_i$ 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 (Figure 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 JZL184 did not significantly affect the subsequent Win-2 test response. Thus, the wash protocol was sufficient to prevent any acute effects on CB_1R 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 (Figure 9B). This indicates that Win-2 desensitizes the CB_1R in hiPSC-derived cortical neurons. Exposing cultures to JZL184, on the other hand, caused less pronounced desensitization of the Win-2 response. A 1 d exposure to JZL184 significantly attenuated the test response but did not completely block the CB_1R-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.

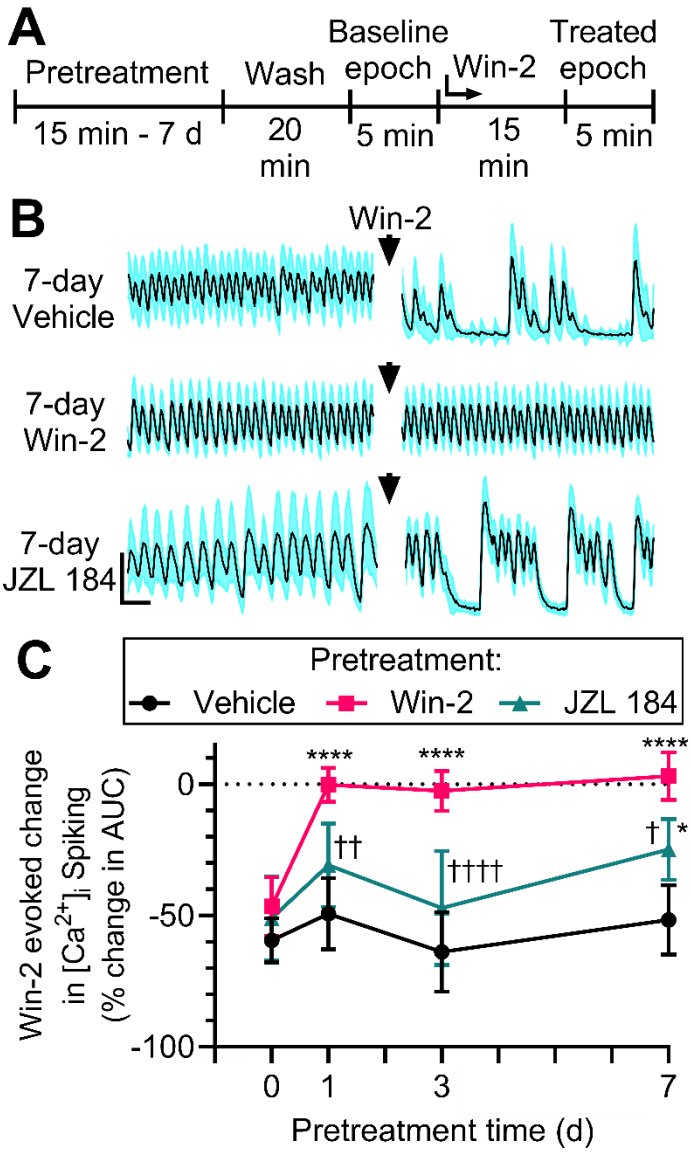

Figure 2.9. Win-2 and JZL184-induced desensitization of CB_1R-mediated inhibition of synaptic activity. (A) Timeline of pretreatment and imaging for desensitization experiments. **(B)** Representative traces show mean (solid line) $[Ca^{2+}]_i$ ($\Delta F/F_0$) of a single field of hiPSC-derived neuronal cells (SD denoted by blue shading) in 0.1 mM Mg^{2+} buffer before and after the addition of 1 µM Win-2 (arrowheads) in cells pretreated for 7 d with either vehicle (0.1% DMSO, top), 1 µM Win-2 (middle), or 1 µM JZL184 (bottom). Scale bars: horizontal = 30 s, vertical = 1.0 $\Delta F/F_0$. **(C)** Plot shows inhibition of $[Ca^{2+}]_i$ 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 JZL184 (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<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.

IV. 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 cortical 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. (2018) examined THC-induced gene expression changes in hiPSC-derived neurons, finding that genes affected by THC exposure overlap partially with those that are altered in neuropsychiatric conditions including schizophrenia. Because these cells responded to THC, they may express functional receptors although the signal transduction pathways were not characterized in this study. Our study showed for the first time that CB_1R 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 CB_1R agonists, which were less potent in hiPSC-derived cortical cultures than in primary rat hippocampal cultures previously studied by our laboratory (Figs. 3-4; Shen et al., 1996; M. M. Wu & Thayer, 2020). This may result from species differences in receptor sensitivity, in agreement with

previous studies showing that Win-2 acted on human CB_1Rs 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 CB_1Rs and synthesizes and metabolizes 2-AG. Our study focused on pharmacological characterization and evaluation of ECS function. This compliments another recent study which demonstrated expression of the ECS components we assessed functionally in an hiPSC-derived cortical spheroid model. Using immunofluorescence and quantitative reverse transcription PCR, Papariello et al., (2021) found that CB_1R, DAG lipase, and MAG lipase were expressed in cortical spheroids. Together with this study, our calcium 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. 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 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 cortical cultures also produced eCBs in response to carbachol. 2-AG is likely the main carbachol-evoked eCB in this system, based on the fact that 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.

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., 2001) 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 cortical neurons, based on the fact that nominally Ca^{2+}-free buffer combined with thapsigargin abolished the carbachol-evoked $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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_1Rs 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 THC tolerance and dependence in humans (Colizzi & Bhattacharyya, 2018) and rodents (González et al., 2005), and CB_1R is downregulated in postmortem human brain tissue from frequent cannabis users relative to non-users (Villares, 2007). Previous

studies have investigated JZL184-induced CB_1R desensitization in rodents, with mixed results. Schlosburg et al. (2010) found that a 6-day treatment with JZL184 led to tolerance in mice as measured by antinociceptive effects, and CB_1R binding sites were reduced in several brain regions including cortex. Conversely, Feliszek et al. (2016) saw only mild receptor desensitization in the hippocampus after a 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 cortical cultures support the hypothesis that JZL184 causes slower and/or less pronounced desensitization than a CB_1R 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 & 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 understanding neurodevelopmental disorders (Papariello et al., 2021).

Here, we focused on the effects of cannabinoids on a glutamatergic network; however, CB_1R is expressed at very high levels in GABAergic interneurons (Marsicano & Lutz, 2006) and at lower levels in astrocytes (Navarrete & 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, Nomura, 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 & 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 cortical cultures recapitulate a diverse suite of eCB functions, providing a minimally invasive and potentially high-throughput human platform for eCB research and drug discovery.

Chapter Three:

Concluding Remarks

I. Summary of present study

The study described in this dissertation establishes that hiPSC-derived cortical cultures contain a functional endocannabinoid system which not only responds to endocannabinoids but also produces and metabolizes them. Although this has been hinted at by previous research (Papariello et al., 2021; Stanslowsky et al., 2017), the present study is the first to demonstrate complete and functional endocannabinoid signaling in mature, differentiated hiPSC-derived cultures. I was able to evoke endocannabinoid production in hiPSC-derived cultures using a G_q-coupled receptor and found that this endocannabinoid production was independent of intracellular calcium levels. I also demonstrated the utility of hiPSC-derived cultures for longer-term drug tolerance assays, showing that activating CB_1R directly with Win-2 caused more pronounced desensitization than inhibiting 2-AG metabolism.

These results show that hiPSC-derived neuron/astrocyte cultures are a viable platform for studying the human endocannabinoid system in its native human cellular context, paving the way for more nuanced investigation than has been possible with existing animal and cell culture models.

II. Advantages and limitations

My dissertation research is among the first studies to use the $GRAB_{eCB2.0}$ sensor (A. Dong et al., 2021) for detecting endocannabinoids in living cells. Endocannabinoids are difficult to measure in live cell cultures, forcing many previous studies to rely on more indirect measures of their activity or simply report the protein expression of synthetic or metabolic enzymes; here, I was able to directly measure the production and metabolism of endocannabinoids via straightforward confocal microscopy without destroying the

cells, providing important functional data to complement previous expression studies. The $GRAB_{eCB2.0}$ sensor was especially useful for dissociating endocannabinoid production from calcium activity in order to test the calcium dependence of carbachol-evoked 2-AG synthesis. The overlapping excitation/emission spectra of the Calcium 6 dye and $GRAB_{eCB2.0}$ sensor prevented me from imaging calcium activity and $GRAB_{eCB2.0}$ fluorescence in the same cells; future studies could investigate both simultaneously using a red calcium indicator.

Mature hiPSC-derived cultures can be maintained for months without significant loss of viability, allowing for studies of long-term drug exposure. In this study Win-2 caused robust desensitization within 24 hours, and a week was sufficient to detect a small amount of desensitization from JZL184. Longer incubation times could determine whether JZL184-mediated desensitization ever reaches the level caused by Win-2.

I used Win-2 as a positive control for desensitization in this study because our lab has previously demonstrated rapid and robust Win-2 desensitization in rodent cultures (Lundberg et al., 2005), and I used JZL184 because it is widely used in research applications—including the existing rodent literature on drug tolerance to MAGL inhibitors (Feliszek et al., 2016; Schlosburg et al., 2010)—and is especially potent against the human MAGL (Long, Nomura, et al., 2009). However, there are caveats to using both these drugs. JZL184 covalently binds to the active site of MAGL and is therefore irreversible (Long, Li, et al., 2009), making true drug washout impossible. This is somewhat mitigated by the 15-minute washout control, which shows no significant difference between acute JZL184 and vehicle (Figure 9); however, there is still a possibility that endogenous 2-AG accumulates in JZL184-treated cultures over the course of imaging, complicating the interpretation of results.

In the case of Win-2, a very recent study found that it, but not other CB_1R agonists, can disrupt the Golgi apparatus in a CB_1R-independent manner, leading to broad inhibition of protein expression at high concentrations (Lott et al., 2022). This means that a portion of the “desensitization” I observed may be due to overall lower protein expression. The concentration of Win-2 I used for desensitization (1 µM) is lower than the concentration that caused severe effects in Lott et al. (5 µM), and it is unlikely that a nonspecific effect on protein expression could eliminate all detectible CB_1R activity without also having profound effects on cell health and survival via other proteins, especially after 7 days of exposure. However, further experiments using other CB_1R agonists could clarify how much of the desensitization effect is actually due to CB_1R agonism.

The study described here focused on the effects of cannabinoids in a model of neuronal hyperexcitability, as hyperexcitability is both a major feature of endocannabinoid neuroprotection and can be elicited easily and consistently in neuron/astrocyte cultures by manipulating extracellular ion concentrations. The low-Mg^{2+} model of neuronal hyperactivity both ensures high baseline activity against which to test the inhibitory effects of cannabinoids and is relevant to NMDA-mediated excitotoxicity in disease states. On the other hand, the anti-inflammatory effects of cannabinoids are also an important mechanism of action in many neurological conditions. Microglia play a pivotal role in neuroinflammation, and CB_2R activation in microglia is likely a major mechanism of endocannabinoid-mediated neuroprotection. The cultures used in this study lack microglia and therefore are not a suitable model for studying neuroinflammation.

A significant caveat of the present study is that all experiments were conducted in cultures derived from a single hiPSC cell line, making my conclusions vulnerable to the

variability that can occur between lines (Ortmann & Vallier, 2017). The agreement between this study and a recent study which found protein expression of ECS components in neurons derived from a different hiPSC line through a different differentiation protocol (Papariello et al., 2021) is highly encouraging in this regard; however, my data will need to be replicated in additional hiPSC-derived neuron lines as well, and efforts to do this are currently underway in our laboratory.

III. Future directions

This dissertation research provides an initial proof of concept for hiPSC-derived cultures as a human *in vitro* model in endocannabinoid research and drug development. The long-term goal of this line of investigation is to establish as complete a platform as possible for high-throughput cannabinoid drug screening in models of human disease. Several key next steps can be taken to achieve this.

First, although I have described the minimal components of a functional endocannabinoid system based around 2-AG, the full ECS is extremely complex and involves many receptors, ligands, and enzymes. Functional characterization of these other components in hiPSC-derived cultures will unlock the full potential of this model system to recapitulate as much of the human ECS as possible *in vitro*. This will ultimately need to involve more cell types than the neuron/astrocyte cultures described here. As techniques for producing microglia from hiPSCs are refined and optimized, hiPSC-derived neuron/astrocycte/microglia cocultures will likely emerge as a useful model for studying the ECS in neuroinflammation.

There are also technical improvements that could be made to increase the efficiency of this system. I used semi-automated image acquisition and analysis in the present

study, but the methods could be adapted for more high-throughput use, for example by using an automated liquid handler for drug addition and creating fully automated imaging and analysis pipelines.

I used $GRAB_{eCB2.0}$ fluorescence and inhibition of calcium activity as readouts for endocannabinoid system activity, but future studies could use other readouts to investigate more of the diverse effects of the endocannabinoid system, particularly in disease states. As an example, our lab previously developed an automated imaging-based assay for quantifying synapse number and cell death in live cell cultures over time (Green et al., 2019); both measurements are highly relevant to neurodegenerative disease and could be used in combination with measurements of neural activity to test whether cannabinoid drugs can prevent neurodegeneration by reducing neuronal excitability.

Finally, neuron/glia cultures for disease research and drug discovery can be derived from patient hiPSCs or from cell lines edited to express human disease genes on a human background (Brennand et al., 2015). Combined with the techniques for ECS research described here, such cultures would be a valuable tool for ECS drug discovery which avoids many of the limitations of animal disease models and stably expressing cell lines.

In conclusion, the research described in this thesis represents the first step towards developing a powerful platform for ECS research and drug development, which will hopefully aid in understanding and navigating the full complexity of the human endocannabinoid system.

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