THC Treatment Alters Glutamate Receptor Gene Expression in Human Stem Cell-Derived Neurons

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Abstract
Given the cognitive and behavioral effects following in utero Δ9-tetrahydrocannabinol (THC) exposure that have been reported in humans and rodents, it is critical to understand the precise consequences of THC on developing human neurons. Here, we utilize excitatory neurons derived from human-induced pluripotent stem cells (hiPSCs), and report that in vitro THC exposure reduced expression of glutamate receptor subunit genes (GRIA1, GRIA2, GRIN2A, and GRIN2B). By expanding these studies across hiPSC-derived neurons from individuals with a variety of genotypes, we believe that a hiPSC-based model will facilitate studies of the interaction of THC exposure and the genetic risk factors underlying neuropsychiatric disease vulnerability.

Introduction
Cannabis is the most prevalent illicit drug of abuse [1]. Although prenatal exposure is associated with negative effects on fetal brain development in humans [2–9], cannabis use is self-reported by over 25% of pregnant women [10]. The major psychoactive ingredient in cannabis is Δ9-tetrahydrocannabinol (THC), which acts primarily via the G-protein coupled cannabinoid receptor 1 (CB1R) [11], signaling via the activation of ERK kinases and/or the inhibition of adenyl cyclase (reviewed in [12, 13]).

CNR1 (CB1R) expression is detected in the human fetal cortex in the first trimester, increasing during development [14, 15] and ultimately found in both excitatory and inhibitory neurons and in glia cells in the adult brain (reviewed in [16]). Evidence from neuron subtype-specific deletions suggests that CB1R may have different functions and pharmacological properties in different cell types (reviewed in [17]) and that its subcellular localization may mediate distinct signaling [18, 19]. Endocannabinoids play critical roles during fetal brain development, being involved in neuronal differentiation, survival and the regulation of neurotransmitter systems [20–22].

Exposure to THC has been shown to alter neuronal excitability [21, 23, 24] via changes in expression of synaptic components, such as glutamate receptor subunits (GRIA1, GRIA2, GRIN1, GRIN2A, and GRIN2B) [21, 24, 25] as well as CNR1 [26, 27] and COX2 [21, 28], two important mediators of intracellular cannabinoid signaling. Resolving the consequences of prenatal THC on human fetal brain development is complicated by variable...
THC exposures (dose, timing, and duration) between pregnancies, confounding exposures to multiple illicit drugs [29], and genotype-dependent effects [30]. Because neural cells differentiated from human-induced pluripotent stem cells (hiPSCs) most resemble fetal brain tissue [31–34], they provide an unprecedented platform for studying the molecular, cellular, and functional results of fetal THC exposure to neural cells, across a variety of genetic backgrounds. In this study, we demonstrate that THC treatment of human excitatory neurons (whether generated by \textit{NGN2} induction from hiPSCs (\textit{NGN2}-hiPSC neurons) [35] or hiPSC-derived neural progenitor cells (\textit{NGN2}-NPC neurons) [36], or via directed differentiation from NPCs [37, 40] recapitulated several known molecular consequences of THC exposure, such as changes in glutamate receptor subunit expression, at least partially in a CB1R-dependent manner.

Methods

\textit{hiPSC Reprogramming and NPC Differentiation}\n
For cohort 1, human fibroblasts were obtained in collaboration with Judith Rapoport, MD (NIMH) as previously described [38] (NSB553, NSB2607, NSB690). Derivation of hiPSCs was done by Sendai virus reprogramming and then cultured on mEF plates using HUES media ((DMEM/F12 [Life Technologies], 20% Knockout Serum Replacement [Life Technologies], 1× Glutamax [Life Technologies], 1× NEAA [Life Technologies], 55 μM β-mercaptoethanol [Sigma], and 20 ng/mL FGF2 [Invitrogen]). hiPSCs were passaged approximately 1:3 every 5–7 days with 1 mg/mL Collagenase (Invitrogen) in DMEM (Life Technologies) and fed every day. Passage-matched hiPSCs were used for all experiments. Cohort 1 NPCs were derived from hiPSCs using dual-SMAD inhibition as previously described [39, 40]. NPCs were cultured on Matrigel (BD)-coated plates in NPC media (DMEM/F12, 1× N2 [Life Technologies], 1× B27-RA [Life Technologies], and 20 ng/mL FGF2), maintained at high density and passaged with Accutase (Millipore).

For cohort 2, human fibroblasts were obtained from ATCC (CRL-2522) and Coriell (GM03440, GM03651, GM04506, and AG09319). hiPSCs were reprogrammed using tetracycline-inducible lentiviral vectors and differentiated to NPCs as previously described [40]. Passage-matched NPCs were used for all experiments. All hiPSC and NPCs used were mycoplasma-free (Table 1).

\textit{Neural Induction/Differentiation}\n
\textit{NGN2}-hiPSC neurons: hiPSCs were washed with PBS, dissociated with Accutase (Millipore), and plated on Matrigel (BD)-coated plates in NPC media (DMEM/F12, 1× N2 [Life Technologies], 1× B27-RA [Life Technologies], and 20 ng/mL FGF2) with ROCK inhibitor (10 μM Y27631). Tetracycline-inducible lentivirus expressing \textit{NGN2} was constituted in MEF-conditioned HUES media and spinfected into dispersed hiPSCs (1,000 g, 1 h) after 24 h recovery (day –1). Media was changed at day 0 to neural differentiation media (DMEM/F12, 1× B27, 1× N2, 20 ng/mL BDNF, 20 ng/mL GDNF, 1 mM d-cAMP, 200 mM ascorbic acid, 1 μg/mL laminin, and 1 μg/mL doxycycline) to commence neural induction. Cells were selected with 0.2 μg/mL puromycin from days 2 to 4. Media was replaced at day 2; subsequently, only 50% of the media was changed every other day.

\textit{NGN2}-NPC neurons: NPCs were dissociated with Accutase, plated on Matrigel, spinfected with doxycycline-inducible \textit{NGN2} lentivirus, and selected with 0.2 μg/mL puromycin, as previously described [36]. Neurons were fed neural differentiation media every other day for 3 weeks after induction.

hiPSC forebrain neurons: NPCs were dissociated with Accutase, plated on polyornithine/laminin– (Fig. 3b–d) or Matrigel (Fig. 3e–g, Fig. 4) coated plates for 6 weeks as previously described [40]. Neurons were fed neural differentiation 1–2 times per week for 6 weeks.

\begin{table}
\centering
\caption{Case and control hiPSC and NPC lines}
\begin{tabular}{llll}
\hline
Cohort & Individual & Source & Sex & hiPSC/NPC line ID \\
\hline
1 & C1 & NIH & male & NSB553 hiPSC#2 / NSB553 hiPSC#S1 NPC#1 \\
1 & C2 & NIH & male & NSB2607 hiPSC#4 / hiPSC#4 NPC#1 \\
1 & C3 & NIH & male & NSB690 hiPSC#3 / NSB690 hiPSC#2 NPC#1 \\
1 & C4 & NIH & female & NSB3183 hiPSC#1 \\
1 & C5 & NIH & female & NSB3121 hiPSC#1 \\
2 & Ca & ATCC & male & BJ hiPSC#2 NPC#A \\
2 & Cb & Coriell & male & GM03440 hiPSC#5 NPC#1 \\
2 & Cc & Coriell & female & GM03651 hiPSC#A NPC#A \\
2 & Cd & Coriell & female & GM04506 hiPSC#B NPC#A \\
2 & Ce & Coriell & female & AG09319 hiPSC#2 NPC#A \\
2 & S1 & Coriell & male & GM01792 hiPSC#1 NPC#A, GM01792 hiPSC#1 NPC#E \\
2 & S2 & Coriell & male & GM02038 hiPSC#1 NPC#A, GM02038 hiPSC#1 NPC#B \\
2 & S3 & Coriell & female & GM01835 hiPSC#1 NPC#5 \\
2 & S4 & Coriell & female & GM02497 hiPSC#1 NPC#C \\
\hline
\end{tabular}
\end{table}
THC was dissolved in DMSO to 1 mg/mL and prepared as previously described [38]; in all experiments, an equivalent volume of DMSO was used as a vehicle control. THC treatment of NGN2-hiPSC and NGN2-NPC neurons occurred with each media change (every other day) for the final 7 days of maturation (5 nM every 48 h for 7 days); induced neurons were harvested for experiments on the 21st day. For forebrain neurons, cells were treated with either 5 nM THC every 48 h for 7 days or with acute THC exposure (1 μM THC for 24 h) and chronic THC treatment (50 μM THC daily for 7 days) immediately prior to harvest at 6 weeks. The CB1R antagonist SR141716A (RIM, Tocris) was dissolved in DMSO and used at a final concentration of 20 nM; an equivalent volume of DMSO was used as a vehicle control.

**Lentivirus Generation**

Lentivirus production was as previously described [36]: 12.2 μg lentiviral DNA, 8.1 μg MDL-gagpol, 3.1 μg Rev-RSV, and 4 μg CMV-VSVG were mixed together with 500 μL of Opti-MEM (Life Technologies) and 1 μg/μL polyethylenimine (Polysciences) 25kD linear and added per 15 cm plate of HEK 293T cells. Medium was changed 5 h later. Virus was harvested from media supernatant 48 h after transfection and again 48 h later. Virus was concentrated by ultracentrifugation at 13,000 g for 2 h, resuspended in DMEM, aliquoted, and stored at −80 °C.

**qRT-PCR**

Total RNA was extracted from cells using Trizol (Life Technologies). One-step qRT-PCR was performed from 500 ng RNA.
**Fig. 1.** THC-induced alteration in gene expression in NGN2-hiPSC neurons. **a** Schematic of NGN2-hiPSC neuron induction from hiPSCs. **b–d** Real-time PCR analysis of GRIA1, GRIA2, GRIN1, GRIN2A, GRIN2B, CNR1, and COX2 expression in NGN2-hiPSC neurons treated with DMSO (control) or THC (5 nM) for 7 days. C1–C5 indicate neurons induced from 5 different individuals (controls 1–5). Values are expressed as mean ± SEM, relative to DMSO-treated levels for C1. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

**Fig. 2.** THC-induced alteration in gene expression in NGN2-NPC neurons. **a** Schematic of NGN2-NPC neuron induction from hiPSC forebrain NPCs. **b–d** Real-time PCR analysis of GRIA1, GRIA2, GRIN1, GRIN2A, GRIN2B, CNR1, and COX2 expression in NGN2-NPC neurons treated with DMSO (control) or THC (5 nM) for 7 days. C1–C3 indicate neurons induced from 3 different individuals (controls 1–3). Values are expressed as mean ± SEM, relative to DMSO-treated levels for C1. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

(For figure see next page.)
and the results reported include at least 3 independent RNA preparations (qRT-PCR from cohort 2 for Fig. 3 was from an independent RNA preparation and required, following Trizol RNA purification, clean up with chloroform followed by ethanol precipitation with glycogen and conversion to cDNA using the high-capacity RNA-to-cDNA kit [Thermofisher]). Primers spanned intron/exon junctions (except for ACTIN and MAP2); primer specificity and the absence of genomic RNA contamination was confirmed by the melting curve of the products. The PCR cycling parameters were 94 °C for 2 min, 40 cycles at 94 °C for 15 s, 60°C for 20 s, and 72°C for 40 s. The comparative threshold cycle value (Ct) method was used for data analysis. mRNA values were normalized to both GAPDH and ACTIN by dividing the expression level (per technical replicate) by the average of the 2 housekeeping Ct values (per technical replicate). Primer sequences are listed in online supplementary Table 1.

Statistical Analysis
Statistical analyses were performed using GraphPad Prism version 7.0b for Mac OS X (GraphPad Software, San Diego, CA,
Results

THC Exposure Alters Gene Expression of Glutamate Receptor Subunits

We and others have previously demonstrated that NGN2-hiPSC neurons [35], NGN2-NPC neurons [36], and directed differentiation forebrain neurons [37, 40] have neuronal morphology (online suppl. Fig. 1, 2; see www.karger.com/doi/10.1159/000477762 for all online suppl. material), are positive for neuronal markers such as βIII-TUBULIN, the dendritic marker MAP2AB (online suppl. Fig. 2), the excitatory synaptic marker VGLUT1, and other synaptic proteins. Gene expression studies indicate that a variety of neuronal enzymes and synaptic proteins are expressed and that these cells are most similar to fetal forebrain tissue [31, 42]. All three populations of neurons undergo action potentials and show evidence of spontaneous neuronal activity [35–37].

In mice, THC is typically used to induce dose- and time-dependent alterations in gene expression in vivo at concentrations from 5 to 10 mg/kg body weight for 4 h to 7 days; it has been used on primary mouse neurons in vitro at doses ranging from 25 nM to 30 μM for 12–20 h (Table 2). Critically, because THC induced cell death in two human neuroblastoma cell lines (SK-N-SH and NUB-6) in treatments as low as 20 nM THC [43], we first treated hiPSC-derived neurons with a minimal dose of THC (5 nM for 7 days), increasing in later experiments (50 nM THC for 7 days and 1 μM THC for 24 h).

We tested the effect of 7-day 5-nM THC treatment, relative to DMSO-treated vehicle controls, on glutamate receptor gene expression across 3-week-old NGN2-hiPSC neurons from 5 independent control hiPSC lines (cohort 1, controls 1–5) as well as 3-week-old NGN2-NPC neurons from 3 independent control NPC lines (cohort 1, controls 1–3), in order to determine if THC effects are consistent in excitatory neurons derived from multiple control individuals via differing methodologies. Across 3 independent experimental replicates (triplicate biological samples within each experimental replicate) for both NGN2-hiPSC and NGN2-NPC neurons, THC treatment reduced GRIA1, GRIA2, GRIN2A, and GRIN2B expression (NGN2-hiPSC neurons: pGRIA1 = 0.0345, pGRIA2 = 0.0064, pGRIN1 = 0.0518, pGRIN2A < 0.0001, pGRIN2B < 0.0001, pCNR1 < 0.0001, pCOX2 < 0.0001; NGN2-NPC neurons: pGRIA1 = 0.0004, pGRIA2 = 0.0367, pGRIN1 = 0.6205, pGRIN2A < 0.0001, pGRIN2B < 0.0001, pCNR1 = 0.0054, pCOX2 < 0.0001; two-way ANOVA with Holm-Sidak test for multiple comparisons). Changes in GRIA1 were confirmed across 2 independent experimental replicates of western blot, from 3 controls each (cohort 1, controls 1–3), which demonstrated that GLUA1 (p = 0.0367) (online suppl. Fig. 3), but not MAP2 (p = 0.6342) (online suppl. Fig. 4), protein levels were decreased following 5-nM THC treatment of NGN2-hiPSC neurons. THC also increased CNR1 and COX2 expression in NGN2-hiPSC and NGN2-NPC neurons (Fig. 1, 2; Table 2).

To validate these findings, we further tested the effect of acute THC exposure (1 μM THC for 24 h), relative to DMSO-treated vehicle controls, of 6-week-old forebrain neurons generated via directed differentiation from 4 in-
dependent control NPC lines (cohort 2, controls a–d) (triplicate biological samples within 1 experimental replicate). Again, we observed that THC treatment reduced GRIA1, GRIA2, GRIN2A, and GRIN2B expression and increased CNR1 and COX2 expression ($p_{GRIA1/acute} = 0.1542$, $p_{GRIA1/chronic} = 0.0267$, $p_{GRIA2/acute} = 0.9590$, $p_{GRIA2/chronic} = 0.0417$, $p_{GRIN2A/acute} = 0.9701$, $p_{GRIN2A/chronic} = 0.02709$, $p_{GRIN2B/acute} = 0.9636$, $p_{GRIN2B/chronic} = 0.0106$, $p_{CNR1/acute} = 0.0512$, $p_{CNR1/chronic} = 0.012$, $p_{COX2/acute} = 0.1542$, $p_{COX2/chronic} = 0.0267$, nonparametric Friedman ANOVA with Dunn’s multiple comparison test) (Fig. 3b–d). Similar to the changes observed in controls, when this same THC treatment paradigm (1 μM THC treatment for 24 h) was applied to 6-week-old forebrain neurons derived from 3 cases with schizophrenia, we again observed reduced GRIA1 and GRIA2 and increased CNR1 expression in acute and chronic THC-treated neurons ($p_{GRIA1\,control\,vs.\,acute} < 0.0001; p_{GRIA1\,control\,vs.\,SZ-GRIA1\,control} < 0.0001; p_{GRIA1\,control\,vs.\,SZ-GRIA1\,acute} < 0.0001; p_{GRIA2\,control\,vs.\,acute} = 0.0017; p_{GRIA2\,control\,vs.\,SZ-GRIA2\,control} < 0.0001; p_{GRIA2\,control\,vs.\,SZ-GRIA2\,acute} < 0.0001; p_{CNR1\,control\,vs.\,acute} < 0.0001; p_{CNR1\,control\,vs.\,SZ-CNR1\,control} = 0.8261; p_{CNR1\,control\,vs.\,SZ-CNR1\,acute} = 0.028) (Fig. 3e–g).

Finally, when using a THC treatment paradigm consistent with that used for the NGN2 neurons (5 nM THC treatment for 7 days on 6-week-old forebrain neurons), we also observed reduced GRIA1 and GRIA2 and increased CNR1 ($p_{GRIA1} < 0.0001$, $p_{GRIA2} < 0.0001$, $p_{CNR1} < 0.0001$, $p_{COX2} < 0.0001$, nonparametric Friedman ANOVA with Dunn’s multiple comparison test) (Fig. 3h–j).
$p_{\text{GRIA2}} < 0.0001$, $p_{\text{SZ-GRIA2}} < 0.0001$, $p_{\text{CNR1}} < 0.0001$, $p_{\text{SZ-CNR1}} < 0.0003$ (Fig. 4).

Although these data suggest that neurons derived from cases with schizophrenia and controls respond similarly to THC, given the size of this small cohort, we caution that we are likely underpowered to conclude that there are (or are not) meaningful diagnosis-dependent differences in THC response.

Altered Gene Expression by THC Involves a CB1-Dependent Mechanism

Because CB1R is the predominant cannabinoid receptor expressed in the central nervous system, we tested whether THC-mediated gene expression changes were dependent on CB1R activity, by treating with SR141716A, a selective CB1R antagonist [44]. THC-induced changes in GRIA1 and CNR1 mRNA expression in 3-week-old...
NGN2-NPC neurons were blocked by concurrent 20-nM SR141716A treatment (triplicate biological samples within 2 experimental replicates), suggesting that THC-mediated effects on GRIA1 and CNR1 may be dependent on CB1R activity (GRIA1: $p_{THC} = 0.0312$, $p_{THC/SR141715A} = 0.9767$, $p_{SR141715A} = 0.9048$; CNR1: $p_{THC} = 0.0045$, $p_{THC/SR141715A} = 0.7052$, $p_{SR141715A} = 0.9963$, ordinary one-way ANOVA followed by Dunnett’s test for multiple comparisons) (Fig. 5).

Discussion

Cannabinoids result in decreased glutamate receptor subunit protein levels in mice and a subsequent functional impairment in glutamatergic transmission [21, 24]. Consistent with this, we demonstrate that THC induced similar effects on NGN2-hiPSC neurons, NGN2-NPC neurons, and forebrain neurons from control individuals, indicating that hiPSC-derived neurons may serve as a human cell-based platform for studying the molecular and cellular effects of THC on developing human neural cells across a variety of genetic backgrounds. Overall, our results are consistent with results from animal studies of THC effects (Table 2). Of course, functional studies, including an examination of synaptic density, size, and activity, will be necessary to confirm the synaptic effects predicted by our gene expression analyses.

Notably, we observed variation in expression levels of a number of the glutamatergic genes between control individuals, a finding that is consistent with postmortem analyses [45] and likely reflects both the genetic variation between individuals as well as technical differences that arise from both the reprogramming and neuronal differentiation/induction processes. For this reason, THC-treated neurons were compared to their isogenic vehicle-treated controls.

The established role of endogenous cannabinoids and CB1R in fine-tuning brain development during sensitive developmental periods suggests that overstimulation of cannabinoid signaling by THC may perturb critical physiological processes at their most vulnerable periods and result in deficits in cortical circuits. Indeed, prenatal cannabinoid exposure is associated with lower IQs and delinquent behaviors in children [2, 3]. Moreover, adolescent cannabis use is associated with an increased risk of developing schizophrenia and/or accelerated onset of symptoms [5, 46–49]. Emerging evidence suggests that cannabinoid exposure may have complex interactions with genetic factors associated with schizophrenia and other neuropsychiatric disorders (reviewed in [50, 51]), such as the link between CNR1 gene polymorphisms and cannabinoid exposure on both brain structure and clinical outcome in schizophrenia patients [30]. By expanding this work across a variety of hiPSC-derived neurons from individuals with different schizophrenia risk alleles, we hope that our hiPSC-based model will facilitate studies of the interaction of THC exposure and genetic risk factors underlying schizophrenia vulnerability.

Conclusions

In light of widespread cannabis use, our understanding of the molecular and cellular effects of THC on human neural cells must improve. Our study suggests that cannabinoid exposure results in changes in glutamate signaling in developing human neurons.

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Statement of Ethics

The participants provided written informed consent.

Disclosure Statement

The authors declare that no conflicts of interest exist.

Author Contributions

I.V.O. contributed to the experimental design as well as to the qPCR experiments and analysis. H.M. and K.S. also contributed to qPCR experiments and analysis. E.K.F. contributed confocal imaging. I.V.O. and K.J.B. designed the experiments and wrote the manuscript.
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