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Dose-Dependent Teratogenicity of the Synthetic Cannabinoid CP-55,940 in Mice

Marcoita T. Gilbert[1], **Kathleen K. Sulik**[1,2,3], **Eric W. Fish**[1], **Lorinda K. Baker**[1], **Deborah B. Dehart**[1], and **Scott E. Parnell**[1,2,3]

[1]Bowles Center for Alcohol Studies, University of North Carolina, Chapel Hill, North Carolina, 27599-7178

[2]Carolina Institute for Developmental Disabilities, University of North Carolina, Chapel Hill, North Carolina, 27599-7255

[3]Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, North Carolina, 27599-7545

1. Introduction

In recent years, a new generation of potent compounds, synthetically-derived cannabinoids, has ranked among the most widely abused illicit substances in the United States and around the world, second only to natural cannabis (Gunderson, 2013; Gunderson et al., 2014). Synthetic cannabinoids (SCBs), the major active constituents of herbal “Spice” preparations, are a structurally diverse family of laboratory-derived chemicals that were originally created for investigating the mechanisms of cannabinoid receptor binding and signaling (Mechoulam and Carlini, 1978). In an effort by manufacturers of recreational SCB’s to increase potency and evade detection, new structural derivatives are emerging at a staggering pace, making it exceedingly challenging for law enforcement to monitor or control their distribution (Underwood, 2015).

Dangerous health effects are frequently reported among SCB users (McGuinness and Newell, 2012). Due to the demonstrated toxicity of SCBs in adults, prenatal exposures are also expected to be harmful. Despite accumulating epidemiological evidence, cannabinoids remain the most commonly abused illicit substances by pregnant women worldwide (Brown and Graves, 2013; Calvigioni et al., 2014; Holbrook and Rayburn, 2014), and international trends of illicit drug use over the last few decades reflect an increasing popularity of cannabinoids (both natural and synthetic) among women of reproductive age (Papaseit et al., 2014; Schneir et al., 2011). This usage, combined with the fact that approximately one half of all pregnancies, worldwide, are unintentional/unplanned; (Finer and Zolna, 2014; Singh et al., 2010) means that the likelihood of inadvertent early prenatal exposure is high. Taking

Corresponding Author: Scott E. Parnell, Bowles Center for Alcohol Studies, CB# 7178, University of North Carolina, Chapel Hill, North Carolina 27599-7178. sparnell@med.unc.edu.

this into consideration, study of the developmental toxicity/teratogenic potential of SCBs requires particular attention.

The majority of our current understanding of prenatal cannabinoid effects stems from earlier studies conducted in humans and animal models with marijuana or its psychoactive constituents. The results of human cannabinoid developmental toxicology/teratogenicity studies, most of which were published in the 1980s and 90s, show an association between marijuana use and growth retardation, distal limb defects, craniofacial abnormalities comparable to those in fetal alcohol syndrome, behavioral abnormalities, and occasionally, perinatal death (Gibson et al., 1983; Hingson et al., 1982). First trimester marijuana use has been noted to be particularly damaging (Gibson et al., 1983). Animal-based teratogenicity studies involving crude marijuana extracts have yielded confounding results. In part, this has been the result of interstudy variability in drug concentrations and developmental stage at the time of exposure. Among the teratogenicity-positive results are those from a study of rats that were administered cannabis at an intraperitoneal dosage of 4.2 mg/kg body weight between gestational days 1–6. Along with an increased incidence of fetal resorption and decreased fetal weight and size, a high incidence of syndactyly, encephalocele, phocomelia, and abdominal viscera eventration were reported (Persaud and Ellington, 1967). Similar congenital malformations were reported in guinea pigs and rabbits that were subcutaneously administered cannabis extract at dosages of 250 or 500 mg/kg during early gestation (Geber and Schramm, 1969).

The isolation and elucidation of Δ^9-THC as a main psychoactive ingredient of cannabis (Mechoulam and Gaoni, 1967) and administration of its pure form allowed greater insight into the potential teratogenicity of cannabinoids. Importantly, a study involving multiple strains of mice and single intravenous (10–20 mg/kg), subcutaneous (6.25–150 mg/kg), or intragastric (100–400 mg/kg) treatments of Δ^9-THC showed that while low doses were not overtly teratogenic, a consistent, reproducible array of gross morphological abnormalities occurred following administration of relatively high doses of Δ^9-THC at distinct critical stages of development. These endpoints occurred irrespective of drug administration route (Joneja, 1976). It was later determined that, as a lipid soluble compound, Δ^9-THC readily crosses the placenta and is recoverable from fetal animal compartments following documented exposure, with maternal and fetal plasma containing equivalent concentrations of Δ^9-THC 3 hours following maternal drug administration (Bailey et al., 1987). While these findings raise considerable human health concerns, a number of investigations have led to the opposing conclusion that neither Δ^9-THC nor cannabis is teratogenic (Banerjee et al., 1975; Borgen et al., 1971). It has been noted that the dosages of marijuana and/or Δ^9-THC extracts used to cause congenital malformations in many of these early teratology studies were above those reported in human users (Hunault et al., 2008). However, given that average Δ^9-THC concentrations in cannabis have increased greatly in the time since many of these experiments were performed, this conclusion may not be appropriate. Over the past 15 years, forms of cannabis have been developed that contain four to five times as much Δ^9-THC as in the past (Downey and Verster, 2014; Hall and Degenhardt, 2015). Recent changes to legislation, as well as changing attitudes towards cannabinoid consumption bring even more reason for concern (Hill and Reed, 2013; Metz and Stickrath, 2015).

In vitro binding and molecular modeling studies have shown that many SCBs found in herbal products possess mechanisms of action similar to Δ^9-THC (Pertwee et al., 2010; Reggio, 2005; Wiley et al., 2014). However, several important differences exist between the SCBs and Δ^9-THC, many of which contribute to an even greater likelihood that SCBs produce dangerous adverse physiological effects *in vivo*. When compared to Δ^9-THC, a relatively weak, partial agonist to cannabinoid receptors 1 and 2 (CB_1 and CB_2, (Breivogel and Childers, 2000; Sim et al., 1996; Tao and Abood, 1998), SCBs commonly found in herbal Spice preparations (e.g. JWH-018, HU-210, CP-47,497, and CP-55,940), display full cannabinoid receptor agonism. This, in turn, results in pharmacological efficacies, binding affinities, and potencies that are several orders of magnitude higher than those of Δ^9-THC. Of concern is that many of the potent synthetic cannabinoid analogs found in herbal preparations are also lipophilic, which increases the likelihood that these compounds cross the placenta (Thomas et al., 1990). Furthermore, upon metabolism, many synthetic cannabinoids are known to produce an array of secondary bioactive metabolites that also display full cannabinoid receptor efficacy (Brents et al., 2012; Fantegrossi et al., 2014; Rajasekaran et al., 2013). Taken together, SCBs possess pharmacological properties that contribute to health hazards distinct from, or greater than, Δ^9-THC, and as such, warrant further toxicity testing and characterization.

It is becoming increasingly clear that the endogenous cannabinoid system emerges much earlier in vertebrate neurodevelopment than has been commonly recognized. For example, CB_1 receptor mRNA, functional CB_1 receptors, the endocannabinoids AEA, and 2-AG, as well as the enzymes involved in their metabolism (NAPE-PLD, DAGLα, DAGLβ MAGL and FAAH) were identified in chick embryos as early as Hamburger and Hamilton (HH) stage 3 (Psychoyos et al., 2012). Also showing the presence of CB_1 receptor expression in early chick brain development (HH stage 11+) is work by Begbie et al. (2004) that identified the receptor in the primordia of the ventral forebrain, as well as in rhombomeres 4 and 6. Extending this work to mammalian systems, and following up on earlier studies of rat embryos by Buckley et al. (1998), Psychoyos et al (2012) found a progressive increase in the relative levels of CB_1 receptor mRNA when comparing expression in samples derived from the anterior neural plate of gestational day (GD) 7.5 embryos to the presumptive forebrain of GD 8.5, 9.5 and 10.5 C57BL/6J mouse embryos. In addition, this group found that *ex ovo* treatment of gastrulation-stage embryos with the water-soluble cannabinoid O-2545 interferes with the initial steps of head process and neural plate formation and causes abnormal CNS, heart, and somite formation (Psychoyos et al., 2008). These data strongly suggest, that as in chick embryos, these early stages of mammalian development will be sensitive to SCB-induced birth defects.

The present investigation was designed to test, in a mouse model, the teratogenic potential of the SCB agonist, CP-55,940. This compound is a constituent of several Spice varieties (Berkovitz et al., 2011), and is one of the most well-characterized and commonly used reference compounds in basic cannabinoid research (Tai and Fantegrossi, 2014). For this study, GD 8, a known critical period for induction of anterior neural tube defects, ocular, and craniofacial malformations, was selected as the time of CP-55,940 exposure. The results of this investigation clearly show the mammalian dose-dependent teratogenicity of CP-55,940

and strongly support the need for examination of additional critical periods, other SCBs, and in-depth mechanistic studies.

2. Materials and Methods

2.1 Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill, where studies were conducted in accordance with NIH and AAALAC guidelines. C57BL/6J mice obtained from The Jackson Laboratory (Bar Harbor, ME) were used as subjects in these experiments. The mice were maintained on a 12:12 light/dark cycle, and housed under controlled temperature and humidity, with free access to water and standard irradiated chow (Prolab Isopro RMH 3000, LabDiet, St. Louis, MO). For mating, two nulliparous females were placed with a single C57BL/6J male for a maximum of two hours. GD 0.0 was defined as the beginning of the breeding period in which a copulation plug was found.

2.2 Immunohistochemistry

For immunohistochemistry, GD 8 embryos were harvested in the uterus and immersion fixed in 4% paraformaldehyde. After fixation, intact embryos within their decidua were carefully dissected from the uterus, processed using a Leica brand tissue processor and then embedded in paraffin. Embryos and decidua were serially sectioned in the sagittal plane at 10 μm, followed by deparaffination, rehydration, and quenching with H_2O_2. Steam antigen retrieval was performed in combination with an antigen retrieval citra solution (Biogenex, Fremont, CA). Rabbit anti-CB_1, 1:200 (ABcam, Cambridge, MA) was the primary antibody employed. Following incubation with anti-rabbit secondary antibody and avidin/biotin-immunoperoxidase reactions (Vector Laboratories, Burlingame, CA), antigen was detected using diaminobenzidine as a substrate (Innovex Biosciences, Richmond, CA). Photographs of sections were taken using an Olympus BX60 light microscope.

2.3 Drug Treatment

On their 8^{th} day of pregnancy, mice were administered CP-55,940 suspended in a vehicle of (1:1:18) CP-55,940 in ethanol: Alkamuls El 620 (Rhodia, Cranberry, NJ): lactated Ringer's solution as a single intraperitoneal (i.p.) injection. Control animals at the same stage of pregnancy were vehicle treated with a comparable solution that was lacking CP-55,940. Injections were administered at a volume of 0.015 ml of solution per gram of maternal body weight. Maternal plasma ethanol concentrations were measured in a separate group of mice which were treated with vehicle; mean peak plasma ethanol concentrations measuring 43.8 mg/dl. For drug-response analyses at least 10 litters at each dosage were collected from dams treated with 0.0625, 0.125, 0.25, 0.5, 1.0, or 2.0 mg/kg CP-55,940. Single i.p. injections of CP-55,940 within this dosage range have previously been shown to evoke the characteristic tetrad of cannabinoid-induced behaviors (hypolocomotion, antinociception, decrease in rectal temperature, and catalepsy) in mice (Fride et al., 2006; Hamamoto et al., 2007). The cannabinoid tetrad is considered one of the most widely used assays for characterizing overt cannabinoid-induced behaviors in animals, and results are thought to closely model physiological responses to cannabinoid receptor activation in humans (Tai and

Fantegrossi, 2014). Janoyan and colleagues demonstrated that acute, single i.p. injections of CP-55,940 at dosages of 0.5–2.5 mg/kg reverse cannabinoid antagonist-induced head-twitch and ear-scratch responses in mice (Janoyan et al., 2002). In their study, the potency profile of CP-55,940 in reversing the cannabinoid antagonist-induced behaviors was consistent with its published potency for producing the tetrad of behaviors in mice (Compton et al., 1992), as well as its published binding affinities for the CB_1 and CB_2 receptors (Pertwee, 1997). In another study by Kow and colleagues, acute i.p. administration of 0.3 mg/kg CP-55,940 evoked the characteristic behavioral tetrad of effects in wildtype C57Bl/6 mice, but failed to do so in mice lacking CB_1 receptors (Kow et al., 2014). In the current study, CP-55,940-induced hypothermia was noted in dams dosed with 2.0 mg/kg, but was not observed at the lower dosages tested. To control for hypothermia in the high dose group, immediately following drug injection, the cages containing the pregnant mice were placed on a temperature-regulated surface to keep body temperatures of the mice at normal levels until they were capable of maintaining normothermia on their own (typically four hours). Temperature measurements were made using a rectal probe (Braintree Scientific, Braintree, MA).

2.4 Fetal Collection and Gross Morphological Analyses

On their 17^{th} day of pregnancy, vehicle and CP-55,940-treated mice were euthanized by brief CO_2 asphyxiation, followed by cervical dislocation. Gravid uteri were removed, and the numbers of live, dead, and resorbed fetuses were recorded. Live fetuses were weighed, crown-rump measurements taken, and specimens were collected in individual wells of chilled phosphate buffered saline (PBS) followed by examination at the dissecting microscopic level for gross morphological abnormalities. Fetuses were then euthanized by gradual reduction in body temperature using chilled PBS. Images of each whole fetus and of each eye were subsequently captured using a Nikon SMZ—U Stereoscopic Zoom dissecting microscope (Nikon Corporation, Melville, NY) and QCapture Suite software (QImaging, British Columbia, Canada). Fetuses were then immersion fixed in either 10% formalin or Bouin's solution.

2.5 Histological Analyses

Based on gross externally visible craniofacial malformations, subsets of fetuses were selected for routine histological analyses. For this, heads of Bouin's fixed specimens were cleared with 70% ethanol, then auto-processed for paraffin embedding. Serial 10 μm coronal sections were cut using a rotary microtome, mounted onto glass slides, and stained with hematoxylin and eosin (H&E). Stained sections were examined using an Olympus BX60 light microscope. Sections from the level of the middle of the eyes and from the level of the anterior commissure of the brain were selected for comparison of control and treated specimens. Images illustrating secondary palatal defects and a range of ocular and brain defects were captured using a MicroPublisher 5.0 digital camera with Real-Time Viewing and QCapture Suite software (QImaging, British Columbia, Canada).

2.6 Ocular Dysmorphology and Dose-Response Analyses

Ocular abnormalities at GD 17 were quantitatively assessed following maternal treatment on GD 8. Ocular development is highly interrelated with the development and patterning of the

prospective forebrain during the gestational period that includes neurulation (corresponding to the embryonic stage of CP-55,940 insult in the present investigation), in that the tissues that comprise both organs derive from the same region of the anterior neural plate (see Fig. 1, and (Cook and Sulik, 1986). Ocular defects induced at these early developmental stages, thus, typically reflect concurrent insult to the brain. With the most commonly found defects in this study involving the eyes, the incidence and severity of readily identifiable ocular malformations were utilized as a basis for dose-response analyses. An ocular defect rating paradigm modified from Parnell et al., (2006, 2010) was employed. Visual assessments of high-resolution digital images of both eyes of each fetus in this study were made by a single evaluator who was blinded to treatment. For each eye, a dysmorphology score was assigned with categories based on the following criteria: (1) normal globe size, normally shaped pupil; (2) apparently normal globe size with abnormally-shaped (but non-colobomatous) pupil; (3) mildly microphthalmic with small, abnormally-shaped (but non-colobomatous) pupil; (4) moderately microphthalmic with iridio-retinal coloboma; (5) severely microphthalmic with iridio-retinal coloboma; (6) severely microphthalmic, absent pupil; (7) apparently anophthalmic. Representative images of eyes falling into each of these 7 categories are shown in Fig. 2. For each fetus the score that reflected the more dysmorphic eye was used for dose-response analyses. Control and treatment groups were compared statistically as described below.

2.7 Statistics

For each treatment and control group, mean litter sizes, fetal weights, crown-rump lengths, and ocular dysmorphology scores were analyzed with GraphPad (version 5.0) using a one-way analysis of variance (ANOVA) with post-hoc Bonferroni-corrected comparisons, when appropriate. Statistical contingency analyses were conducted with SigmaPlot (Version 11.0) using Pearson Chi-Square analyses, and single linear regression analyses. For all analyses, significance was set at $p < 0.05$.

3. Results

3.1 CB_1 Receptors Are Present in GD 8 Mouse Embryos

As shown in a parasagittal section through a GD 8 mouse embryo (Fig. 1), immunohistochemical staining revealed CB_1 receptor expression at this early stage in embryogenesis. Staining was particularly intense in the pseudostratified epithelium of the developing anterior neural plate; tissue that will form the brain and eyes. Staining was also evident in cell populations that are progenitors for the developing heart and gut.

3.2 CP-55,940 Induces Craniofacial and Ocular Abnormalities in GD 17 Fetuses Following Treatment on GD 8

Presented in Table 1 are litter characteristics for this study. No significant differences were found between the GD 17 vehicle-treated control litters and those from all tested CP-55,940 dosages with respect to average litter size, fetal weight, or crown rump length.

At all CP-55,940 dosages tested, malformations involving the craniofacies and/or eyes were induced. Among the externally visible craniofacial defects were micrognathia, facial clefts,

facies consistent with holoprosencephaly (median facial deficiency), and exencephaly. The range of ocular defects observed include microphthalmia, iridial coloboma, and apparent anophthalmia. As shown in Table 1, the above-noted non-ocular craniofacial defects were observed in a relatively small number of the CP-55,940-exposed fetuses. The incidence and severity of these defects were greater in fetuses exposed to CP-55,940 dosages higher than 0.5 mg/kg. None of the vehicle-treated control fetuses presented with non-ocular malformations. All of the fetuses that had craniofacial defects also had ocular abnormalities. Details regarding the incidence and severity of the ocular defects for all of the fetuses in this study are shown in Table 2 and are also presented in the dose-response analyses section, below.

3.3 Histological Analyses Illustrate CP-55,940-Induced Palatal Clefting and Central Nervous System Abnormalities

Examination of coronal histological sections of selected CP-55,940-treated GD 17 fetuses, all of which had craniofacial malformations, allowed identification of concurrent brain defects (Fig. 3). Varying degrees of holoprosencephaly (Fig. 3D, F) and median forebrain tissue abnormality were observed (Fig. 3D, F, H). In addition, clefting of the secondary palate was noted in a CP-55,940-treated fetus that had a median facial cleft, and in another that had an oblique facial cleft. Also readily apparent in the histological sections of these fetuses were a range of ocular defects, some of them being very severe in the CP-55,940-treated specimens. Highlighted in Fig. 4 is severe cortical dysplasia, which was observed in a fetus whose dam had been treated with 2.0 mg/kg CP-55,940.

3.4 CP-55,940 Teratogenesis is Dosage-Dependent

The most commonly found gross dysmorphology in this study involved the eyes, with defects ranging from mild microphthalmia with or without colobomata to apparent anophthalmia. Employing a 7-point ranking system (with score values increasing as the degree of ocular dysmorphology increased; Fig. 2) and visual inspection of high-quality digital images by an independent evaluator blinded to treatment, each eye of each control and treated fetus was assigned a dysmorphology score. A score greater than one was considered abnormal. In the control group, 14.9% of the fetuses had at least one eye that was dysmorphic; a finding consistent with the previously-reported C57BL/6J mouse predisposition for this type of eye defect (Cook and Sulik, 1986; Parnell et al., 2006; Smith et al., 1994b; Tyndall and Cook, 1990). Chi-square analyses revealed that the incidence of abnormal ocular morphology increased significantly and in a direct dose-dependent manner following exposure to all dosages of CP-55,940 tested; 36.9% at 0.0625 mg/kg CP-55,940, ($X^2(1, N = 220) = 13.66$, $p = 0.0002$), 46.4% at 0.125 mg/kg CP-55,940, ($X^2(1, N = 259) = 30.83$, $p < 0.0001$), 49.4% at 0.25 mg/kg CP-55,940, ($X^2(1, N = 230) = 31.62$, $p < 0.0001$), 59.3% at 0.5 mg/kg CP-55,940 ($X^2(1, N = 238) = 50.92$, $p < 0.0001$), 69.8% at 1.0 mg/kg CP-55,940 ($X^2(1, N = 233) = 71.44$, $p < 0.0001$), and 81.5% at 2.0 mg/kg CP-55,940 ($X^2(1, N = 268) = 63.53$, $p < 0.0001$; see Fig. 5). In order to estimate a dose of CP-55,940 that caused an eye defect in 50% of the drug-treated fetuses relative to the spontaneous eye defects, a linear regression was performed between the dosages of 0.0625 and 2.0 mg/kg. Using the equation $Y = 20.97X + 28.56$, the ED_{50} was estimated to be 1.0 mg/kg. A positive correlation was found between these two variables, with gestational CP-55,940 treatment

accounting for 90% of the variability in ocular dysmorphology observed in the sampled population ($r^2 = 0.90$).

As summarized in Table 2, the severity of ocular dysmorphology was also dose-dependent. In the vehicle control group, the ocular dysmorphology score never exceeded 3, while the more severe defects (ocular dysmorphology score range of 4–7) occurred only in drug-treated animals. Mean ocular dysmorphology scores for each treatment group were analyzed using a 1-way ANOVA, which revealed significant increases in ocular severity in cannabinoid-treated versus vehicle control groups [$F(6, 679) = 18.23$; $p < 0.0001$, see Table 2]. Subsequent Bonferroni- corrected analyses revealed that these significant differences in ocular severity lie between vehicle-treated fetuses and fetuses exposed to all doses ≥ 0.125 mg/kg. A linear regression analysis was conducted to further define the correlation between mean ocular severity and gestational CP-55,940 exposure. A significant positive correlation was found between these two variables, with gestational CP-55,940 treatment, accounting for 92% of the variability in ocular dysmorphology observed in the sampled population ($r^2 = 0.92$).

4. Discussion

The results of this study clearly show that within the range of dosages tested, the synthetic cannabinoid agonist CP-55,940 is a mammalian teratogen. The drug-induced defects observed involved the craniofacies, eyes, and brain; results that are predictable based on insult on GD 8 in mice, when the cranial neural plate and associated facial and ocular tissues are beginning to form. This is also the critical period for induction by other teratogens including alcohol and retinoic acid of abnormalities comparable to those caused by CP-55,940 (Johnston and Bronsky, 1991; Kotch and Sulik, 1992; Padmanabhan and Ahmed, 1997). The ability of a variety of environmental agents, including commonly abused drugs, to cause similar birth defects can be clinically problematic. In helping to determine teratogenic mechanisms, however, this type of overlap can provide important clues. As with alcohol (Dunty et al., 2001; Kotch and Sulik, 1992) and retinoic acid (Alles and Sulik, 1992), excessive cell death in early neuroepithelial or neural crest cell populations as the pathogenic basis for CP-55,940-induced birth defects is a possibility that requires further investigation. Importantly, cannabinoids are known inducers of p53-regulated apoptosis through activation of CB_1 receptors (Downer et al., 2007; Maccarrone et al., 2014); and p53-dependent apoptosis in the developing forebrain of GD 8 mouse embryos has been shown to result in defective ocular and craniofacial development (Wubah et al., 1996).

While sensitivity to CP-55,940-induced teratogenesis following exposure on GD 8 may or may not be cannabinoid receptor-dependent, our illustration of CB_1 receptor expression in a relevant embryonic cell populations (i.e. the anterior neural plate) at the time of treatment in this study supports the possibility of receptor involvement. To confirm this, additional mechanism-directed studies, including investigation of the potential of cannabinoid receptor antagonism to modify CP-55,940's teratogenicity, are needed.

Of interest, recent studies by others are suggestive of a direct (non-receptor-dependent) teratogenic mechanism involving cannabinoid-induced disruption of cholesterol

modification of Sonic Hedgehog (Shh) or perturbation of the downstream Shh pathway proteins (Khaliullina et al., 2015; Li et al., 2007). Shh signaling is critical for early brain and facial development and alterations in this pathway yield defects comparable to those seen in this study (Echelard et al., 1993). During normal Shh signaling, Shh binds to the transmembrane protein Patched (Ptc), thereby alleviating Ptc-mediated suppression of the signal transducer Smoothened (Smo). Smo activation then triggers a series of intracellular events that regulate cellular survival, growth, differentiation, and morphogenesis during embryonic development (Alcedo et al., 1996; Chen et al., 2002). Khaliullina and colleagues (Khaliullina et al., 2015) have demonstrated that in *Drosophila*, circulating endogenous cannabinoids directly inhibit Shh pathway activation by acting as negative allosteric modulators of Smo.

Considering that the processes that regulate mammalian embryonic development are relatively conserved across species, along with increasing SCB usage by women of child-bearing age, and the emergence of increasingly potent SCB analogs, the findings from this study raise substantial public health concerns. Importantly, the results of the current investigation have shown CP-55,940 teratogenicity in mice following maternal exposure to dosage levels that appear to be pharmacologically relevant in humans (FDA, 2005). While studies directed toward defining the drug disposition and pharmacological profile resulting from the CP-55,940 treatment paradigm used in this investigation are needed, the drug-induced maternal behavioral and physiological changes noted are consistent with those shown by others to follow CB_1 receptor agonism in mice. These changes include analgesia, catalepsy, decreased rectal temperature, and locomotor suppression and are referred to as the cannabinoid tetrad (Janoyan et al., 2002; Smith et al., 1994a). The CP-55,940 dosages used in the present study are also within the range of those employed in previously published rodent drug discrimination studies (Wiley et al., 1995).

In summary, the renewed interest in SCBs as recreational drugs of abuse has raised considerable public health concern, especially in regard to women who are or may become pregnant. Emergency rooms and poison control centers are encountering cases of SCB toxicity more frequently than ever before; however, there is a paucity of published data regarding effects of SCBs on pregnant women and of the potential negative impact of SCBs on the developing fetus. Overall, the results reported herein provide novel insight into the teratogenic potential of SCBs. Importantly, they indicate that analyses of additional developmental periods are warranted, with the expectation that other developmental stage-dependent, CP-55,940-inducible birth defects will also be identified. This work should serve as a catalyst for additional teratology studies evaluating other potent cannabimimetics that are being used either alone or in combination with other drugs by women of child-bearing age. Basic research, such as that detailed in this study, is clearly needed to address the void in knowledge regarding the teratogenicity of SCBs.

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Highlights

- CP-99,540, a synthetic cannabinoid, administered to mice at doses as low as
- 0.0625 mg/kg during early pregnancy is teratogenic.
- CP-99,540 dosages at the low end of those found to be teratogenic are within
- the realm of potential human exposure.
- Maternal CP-99,540 administration at neurulation stages of development result in
- craniofacial, ocular and brain dysmorphology.
- The developmental stages at which CP-99,540 was shown to be teratogenic
- occur prior to typical pregnancy recognition in humans.
- These results strongly support the need for more research and commensurate prevention-directed public health approaches.

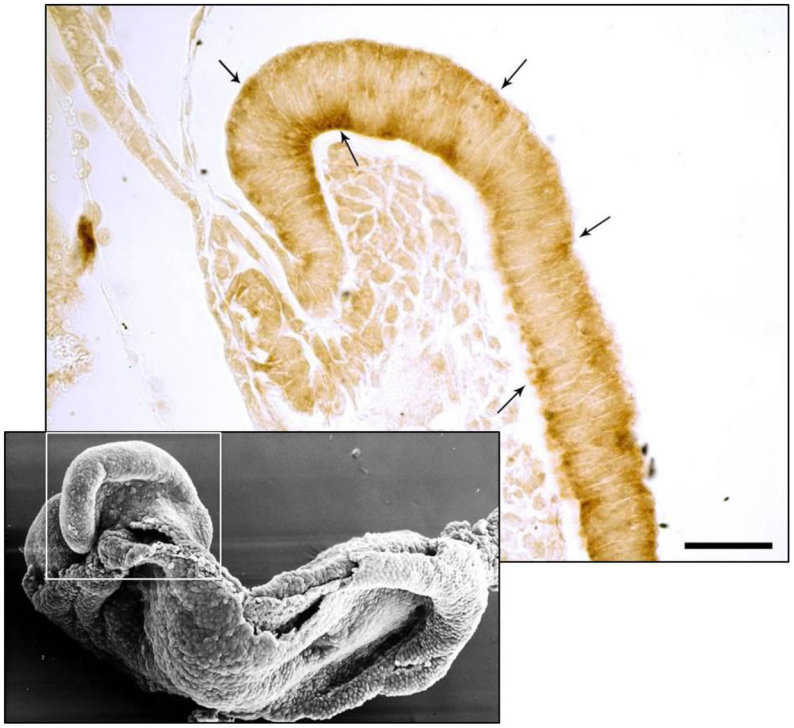

Figure 1.

Anti-CB_1 receptor immunohistochemistry. Illustrated is a sagittal section through a GD 8 embryo that was stained with an antibody specific to the CB_1 receptor. The arrows indicate CB_1 expression which appears somewhat segmented in pattern in the neuroepithelium of the developing neural plate (precursor of the eyes and brain). The inset is a scanning electron micrograph of a GD 8 mouse embryo, with the boxed area showing the sectioned region. Scale bar = 100 μm.

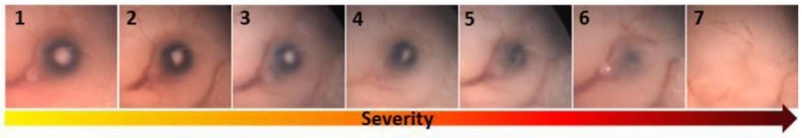

Figure 2.

Illustrated is a normal eye (1) and a range of CP-55,940-induced dysmorphic eyes (2–7), that form the basis for the 7-point scoring system employed in ranking the severity of ocular abnormality. For each eye, a dysmorphology score was assigned with categories based on the following criteria (1) normal globe size, normally shaped pupil; (2) apparently normal globe size with abnormally-shaped (but non-colobomatous) pupil; (3) mildly microphthalmic with small, abnormally-shaped (but non-colobomatous) pupil; (4) moderately microphthalmic with iridio-retinal coloboma; (5) severely microphthalmic with iridio-retinal coloboma; (6) severely microphthalmic, absent pupil. A score of 7 represents anophthalmia; thus no eye is present in the last image. Assignment of scores is based on visual assessment by an experienced dysmorphologist, with discrimination between groups dependent on overall ocular size as well as pupil size and shape.

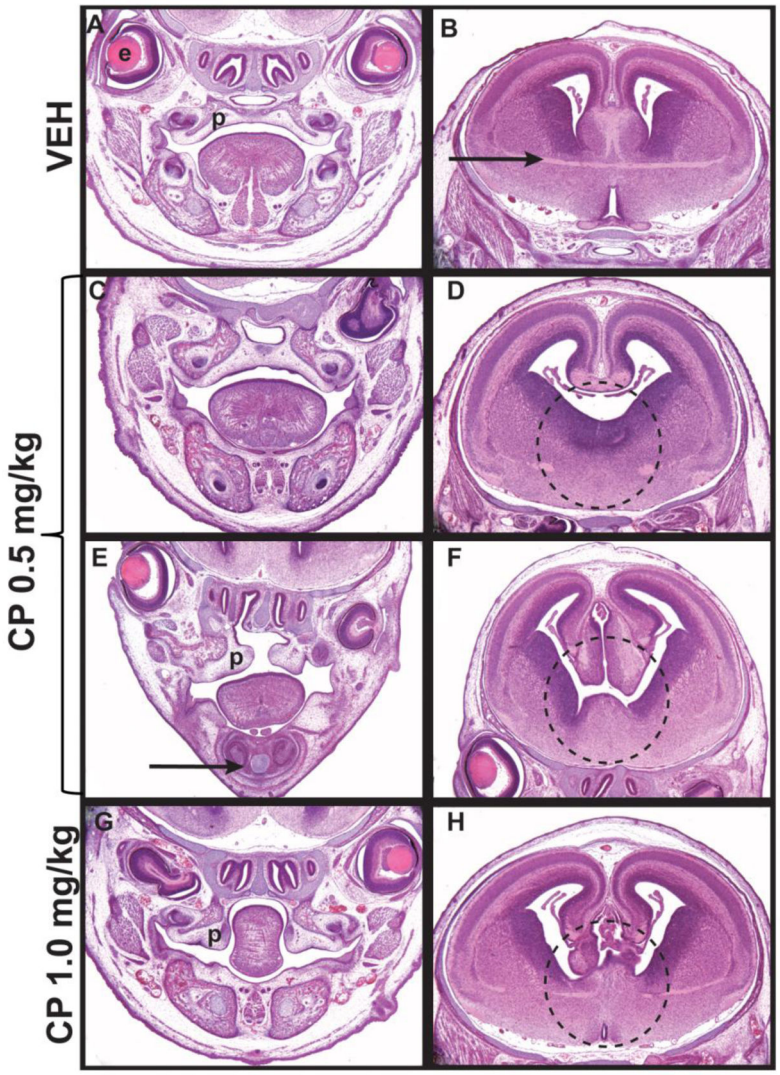

Figure 3.

Histological sections of control (A, B), and CP 55,940-treated GD 17 fetal mice illustrate that maternal dosages of 0.5 mg/kg (C–F) and 1.0 mg/kg (G, H) administered on GD 8 yield abnormalities of the brain, eyes, palate, and mandible. Figures in the left column are from sections made through the center of the eyes (e), while those in the right column are through the brain at the level of the anterior commissure (arrow in B). Notable are varying degrees of ocular abnormalities involving the right and/or left eyes of the treated animals. Additionally, the fetuses shown in E and G each have secondary palatal clefts (compare to the closed palate [p] in the control specimen). This is accompanied by a small mandible in the fetus shown in E (arrow). The brain abnormalities in the fetuses shown in D–F are most evident in the ventral midline (dashed circles) and include septal region defects.

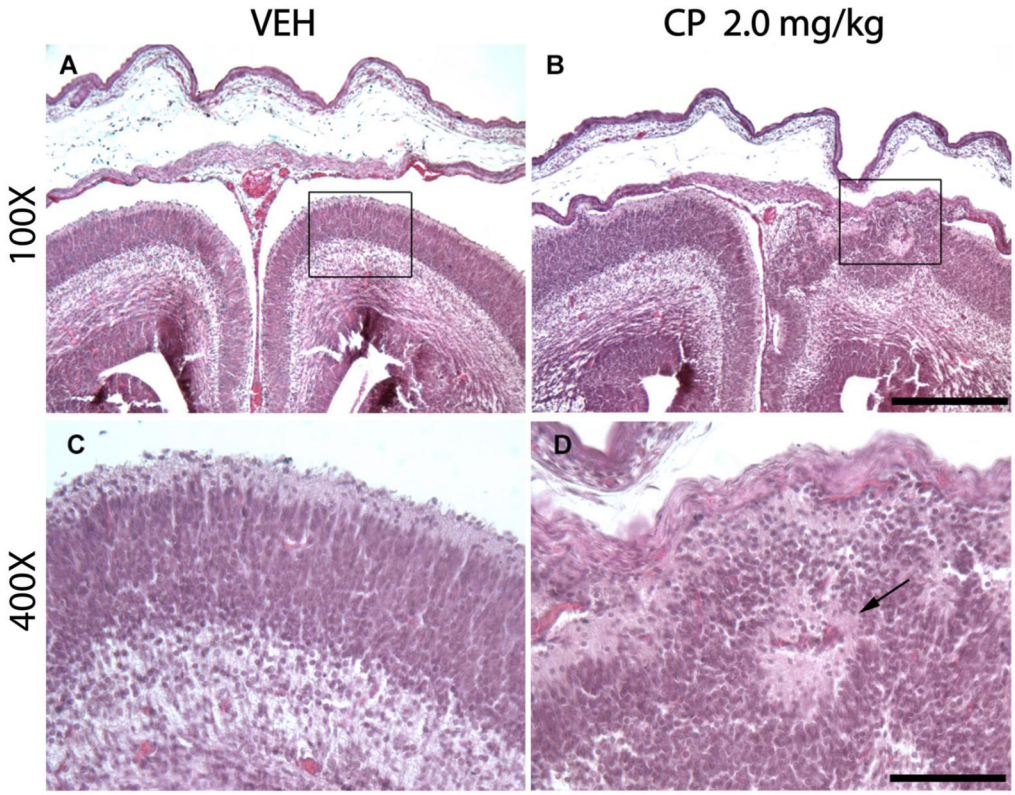

Figure 4.

Shown are coronal histological sections from a control GD 17 mouse fetus (A, C), and a fetus whose mother was treated with 2.0 mg/kg CP 55,940 on GD 8 (B, D). The boxed areas shown in A and B are the cerebrocortical regions shown at higher magnification in C and D, respectively. Readily apparent at both low and high magnification is severe dysplasia involving the left cerebral hemisphere of the cannabinoid-exposed fetus. The arrow in D is directed toward a region of cerebrocortical dysplasia. Scale bar in B = 500μm, and in D = 200μm.

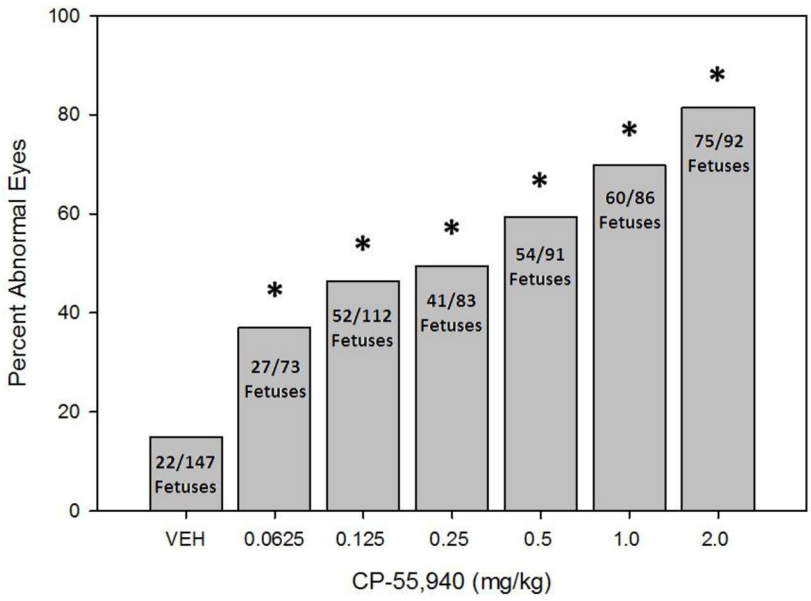

Figure 5.

CP-55,940 dose response analyses illustrate teratogenicity at maternal dosages ranging from 0.0625 to 2.0 mg/kg. Scoring for ocular defects is based on the severity scale described in Fig. 2. Consistent with previous studies, 14.9% of the vehicle-treated mice present with spontaneous eye defects, most of which are subtle. As compared to control, CP-55,940 induced a significantly greater number of eye defects in a dose-dependent manner, with the 1.0 mg/kg exposure group presenting with ocular defects above vehicle treated levels in 50% of the drug-treated animals (p 0.0002).

Table 1

Gross Morphological Analyses Following CP-55,940 Exposure on GD 8.

	Vehicle	CP-55,940-treated					
		0.0625 mg/kg	0.125 mg/kg	0.25 mg/kg	0.50 mg/kg	1 mg/kg	2 mg/kg
Total Number of Litters	20	10	15	10	13	11	12
Mean Litter Size ± SEM	7.35 ± 0.54	7.30 ± 0.78	7.47 ± 0.31	8.30 ± 0.44	7.00 ± 0.63	7.82 ± 0.62	7.67 ± 0.50
Total Resorptions	4	3	3	2	4	2	3
Mean Fetal Weight (g ± SEM)	0.79 ± 0.006	0.79 ± 0.008	0.78 ± 0.007	0.79 ± 0.007	0.81 ± 0 .008	0.80 ± 0.008	0.80 ± 0.008
Mean Crown-Rump Length (mm ± SEM)	16.9 ± 0.05	17.0 ± 0.07	16.9 ± 0.06	16.9 ± 0.06	17.1 ± 0.07	17.0 ± 0.07	16.9 ± 0.07
Number of Fetuses with Non-ocular Craniofacial Malformations	0	1[1]	0	1[1]	6[1(3),2(1),3(2)]	4[1(2), 3(2)]	10[1(4), 3(4), 4(1),5(1)]

[1] = Micrognathia;

[2] = Oblique facial cleft;

[3] = Holoprosencephaly;

[4] = Median facial cleft;

[5] = Exencephaly (#)= number of fetuses with each of the indicated defects

Table 2

Distribution of Fetal Ocular Dysmorphology Scores Across CP-55,940 Treatment Groups.

Eye Score	Vehicle	CP-55,940-treated					
		0.0625 mg/kg	0.125 mg/kg	0.25 mg/kg	0.5 mg/kg	1.0 mg/kg	2.0 mg/kg
1	125	46	60	42	37	26	17
2	20	24	42	31	46	48	52
3	2	1	6	7	3	8	15
4	0	0	4	2	1	2	4
5	0	2	0	0	1	1	3
6	0	0	0	0	2	1	1
7	0	0	0	1	1	0	0
Total Number of Fetuses	147	73	112	83	91	86	92
Mean Ocular Dysmorphology Score ± SEM	1.16 ± 0.03	1.47 ± 0.09	1.59 ± 0.07	1.69 ± 0.10	1.82 ± 0.11	1.92 ± 0.10	2.21 ± 0.10