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Research article

Effects of prenatal synthetic cannabinoid exposure on the cerebellum of adolescent rat offspring

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ABSTRACT

Cannabis is the most commonly used illicit drug worldwide. Recently, cannabis use among young pregnant women has greatly increased. However, prenatal cannabinoid exposure leads to long-lasting cognitive, motor, and behavioral deficits in the offspring and alterations in neural circuitry through various mechanisms. Although these effects have been studied in the hippocampus, the effects of prenatal cannabinoid exposure on the cerebellum are not well elucidated. The cerebellum plays an important role in balance and motor control, as well as cognitive functions such as attention, language, and procedural memories. The aim of this study was to investigate the effects of prenatal cannabinoid exposure on the cerebellum of adolescent offspring. Pregnant rats were treated with synthetic cannabinoid agonist WIN55,212-2, and the offspring were evaluated for various cerebellar markers of oxidative stress, mitochondrial function, and apoptosis. Additionally, signaling proteins associated with glutamate dependent synaptic plasticity were examined. Administration of WIN55,212-2 during pregnancy altered markers of oxidative stress by significantly reducing oxidative stress and nitrite content. Mitochondrial Complex I and Complex IV activities were also enhanced following prenatal cannabinoid exposure. With regard to apoptosis, pP38 levels were significantly increased, and proapoptotic factor caspase-3 activity, pERK, and pJNK levels were significantly decreased. CB1R and GluA1 levels remained unchanged; however, GluN2A was significantly reduced. There was a significant decrease in MAO activity although tyrosine hydroxylase activity was unaltered. Our study indicates that the effects of prenatal cannabinoid exposure on the cerebellum are unique compared to other brain regions by enhancing mitochondrial function and promoting neuronal survival. Further studies are required to evaluate the mechanisms by which prenatal cannabinoid exposure alters cerebellar processes and the impact of these alterations on behavior.

1. Introduction

Historically, derivatives of the plant *Cannabis sativa* have been used for recreational, medical, and veterinary purposes (ElSohly et al., 2016). *Cannabis* is currently one of the most commonly used recreational drugs in the world. In the United States of America, as more and more states are legalizing the use of marijuana, the rate of marijuana abuse has increased (Center for Behavioral Health Statistics, 2020). The use of synthetic cannabinoids in young adults is also increasing, and synthetic cannabinoids are currently the 3rd most commonly used drug in this population ("Monitoring the Future 2019 Survey Results: Overall Findings | National Institute on Drug Abuse (NIDA)," 2019). Synthetic cannabinoids are inexpensive and can be easily acquired from drug paraphernalia shops, novelty stores, gas stations, and online ("Synthetic Cannabinoids (K2/Spice) DrugFacts | National Institute on Drug Abuse (NIDA)," 2020). Both natural and synthetic cannabinoids can cross the blood placental barrier and affect fetal growth and development (Bailey et al., 1987; Dong et al., 2019a; Sun and Dey, 2014).

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Studies have demonstrated a strong correlation between maternal use of cannabinoids and adverse cognitive outcomes in the offspring, particularly in younger children. Animal studies have confirmed that prenatal cannabinoid exposure results in learning and memory deficits that are consistent with the cognitive deficits observed in children who were prenatally exposed to cannabinoids (Campolongo et al., 2011). Impaired short-term memory, verbal reasoning, and attention and increased impulsivity and hyperactivity in early childhood as well as impairment in visual reasoning, problem-solving, sustained attention, and visual-motor coordination are reported (Sobrian, 2016). Studies on children of various ages suggest that prenatal exposure to high doses of marijuana can affect the learning of tasks as well as emotional outcomes (Day et al., 2006; Goldschmidt et al., 2000; Gray et al., 2005). These neurobehavioral changes are long lasting and might be associated with alterations in various neurotransmitter systems (Pinky et al., 2019).

There are two types of cannabinoid receptors - cannabinoid receptor type 1 (CB1R) (Matsuda et al., 1990) and cannabinoid receptor type (CB2R) (Munro et al., 1993). The density of CB1R in the central nervous system (CNS) is particularly high, and its distribution is widespread in the basal ganglia, cerebellum, cerebral cortex, and hippocampus (Felder et al., 1995). The cerebellum is involved in motor coordination, the implementation of associative learning, and the processing of temporal operations (Stella, 2013). In the cerebellum, the vast majority of CB1Rs are found at the presynaptic terminals received by Purkinje cells (Takahashi and Linden, 2000). CB1R is moderately high in the molecular layer and low in the granule cell layer of the cerebellum. Chronic cannabinoid exposure can cause motor incoordination and abnormal eyeblink reflex conditioning in mice, leading to impairment in cerebellar-associated learning (Cutando et al., 2013). Altered time perception and self-paced behaviors due to THC intoxication have also been reported, which were linked to an altered internal clock (Mathew et al., 1998; O'Leary et al., 2003).

Although there are several studies that examined the effects of prenatal alcohol, nicotine, and other substance use in the offspring (Bhattacharya et al., 2018; Bookstein et al., 2006; Koning et al., 2017; Luo, 2015), to date, there is limited data regarding the role of cannabinoid exposure on the cerebellum during the developmental period. Previously, substance abuse during pregnancy was shown to alter various behavioral parameters and motor development in the offspring (de Salas-Quiroga et al., 2015 de Salas-Quiroga, A., Díaz-Alonso, J., García-Rincón, D., Remmers, F., Vega, D., Gómez-Cañas, M., Lutz, B., Guzmán, M., Galve-Roperh, I., 2015; Farah Naquiah et al., 2016; Kelly et al., 2000; Saberi Moghadam et al., 2013; Sobrain, 1977; Wu et al., 2011). Therefore, we first determined whether prenatal cannabinoid exposure induced alterations in common behavioral parameters. Additionally, we have evaluated how cerebellar mitochondrial function is altered as a result of prenatal cannabinoid exposure, since CB1Rs are found in mitochondria where they modulate neuronal energy homeostasis (Bénard et al., 2012). The generation of reactive oxygen species (ROS) triggers oxidative stress and induces irreversible oxidation of lipids and proteins, resulting in cell death. We investigated ROS, nitrite, and lipid peroxidation content to determine whether prenatal cannabinoid exposure alters these parameters in the cerebellum. Since cannabinoid induces apoptosis in cerebellar granular cells (Pozzoli et al., 2006), we also investigated whether prenatal cannabinoid exposure initiates apoptosis in the cerebellum during the developmental period via measuring caspase-1 and caspase-3 activity. The study further evaluated the effect of prenatal cannabinoid exposure on the key signaling molecules which are important for maintenance of synaptic plasticity, cerebellar mitochondrial function, and apoptosis.

2. Materials and methods

2.1. Animals

Time-pregnant Sprague Dawley rats were purchased from a commercial vendor. The procedures were performed in accordance with NIH guidelines and approved by Auburn University Animal Care and Use Committee (IACUC). Rats were anaesthetized on gestational day 3 under isoflurane anesthesia for subcutaneous implantation of an osmotic mini pump (Alzet 2004). The pump delivered either vehicle (NMP) or the synthetic cannabinoid (WIN55,212-2, 2 mg/kg body weight/day) at a rate of 0.25ul/hour until the pups were born. The dose of cannabis was based on previous studies and corresponds to low to moderate cannabinoid exposure (Campolongo and Trezza, 2012; French et al., 1997; Hampson and Deadwyler, 2000; Tortoriello et al., 2014). Osmotic pumps were removed from the dams after delivery. Pups were weaned at postnatal day 21. Animals were housed in a vivarium maintained on a 12 h:12 h light: dark cycle (lights on at 6:00 am) and at a temperature of 22–24 °C according to the approved IACUC protocol. Pups were housed in groups of three per cage after weaning. At postnatal day 42, pups were euthanized via CO_2, and cerebellar tissue was extracted. There was no significant difference in the number of pups per litter, body weight, or weight of the cerebellum and whole brain in the prenatally cannabinoid exposed group compared to the control.

2.2. Chemicals

All the chemicals and antibodies used in the current study were purchased from Sigma (St. Louis, MO) and Cell Signaling Technology (Danvers, Massachusetts), respectively, unless otherwise specified.

2.3. Behavioral studies

Animals were observed for various physical and behavioral parameters including tremor, straub tail, seizures, hyperactivity (excessive jumping), hind limb abduction, head twitching, hair coat erection, fighting, drooling, diarrhea, and ataxia.

Straub tail was measured as the elevation of the rat's tail to more than 45° with a curvature of the tail. The results are expressed as straub tail phenomenon was detected (Y) or not detected (N). Tremor was assessed based on the observation of absence of tremor (N) or the observation of occasional muscle twitches, and/or slight to moderate, intense body/head shake (Weihmuller et al., 1988). Hind limb abduction was scored based on the limb movement away from the median position of the rats. The observation was reported as presence (Y) or absence of the hind limb extended away from the median position (Muralikrishnan and Mohanakumar, 1998). Seizure was observed based on the well-established racine score in a scale of 0–5 (0 = No Response, 1 = Staring/Reduced Locomotion, 2 = Rigidity/Extension of Legs, 3 = Repetitive Head & Leg Movement, 4 = Clonus, 5 = Tonic/Clonic). Observation for hyperactivity, allergic reactions (redness of the skin or eye) and anaphylactic shock/death were based on the following symptoms or sign: rubbing and/or scratching in the face (mostly near the nose), ear and head, puffiness around eyes and mouth, watery stool (diarrhea), pillar erect, reduced activity, and/or decreased activity with increased respiratory rate, wheezing, labored respiration, and cyanosis around mouth and tail, no activity after prodding or tremor and convulsion, mortality. All the observations were performed by blinded reviewers.

2.4. Biochemical studies

Animals were sacrificed in the morning to avoid diurnal variations of endogenous amines, enzymes, and other antioxidant molecules. The cerebellum was dissected, flash frozen in liquid nitrogen, and stored at − 80 °C. The cerebellar homogenate for the biochemical tests was prepared by homogenizing the tissue in 0.1 M phosphate buffer (pH 7.8), using a glass-teflon homogenizer, followed by centrifugation at 10,000g for 60 min at 4 °C, and the supernatant was collected for analysis (Ahuja et al., 2017).

2.5. Generation of reactive oxygen species (ROS)

ROS content in the cerebellar samples was measured by spectrofluorometric method. Cell-permeable non-fluorescent dye, 2′,7′-Dichlorofluorescin Diacetate (DCF, Calbiochem, 287810, 0.5%) dye reacts with ROS to form a fluorescent product. The fluorescent product formed was measured using a plate reader at the excitation wavelength of 492 nm and emission wavelength of 527nm. ROS formed and was normalized to total protein content (relative fluorescence unit/mg protein). Results were expressed as (%) change as compared to the control (Alhowail et al., 2019; Katz et al., 2018; Majrashi et al., 2018).

2.6. Assessment of lipid peroxidation

Tissues were homogenized with PBS and lysis buffer. The samples were sonicated (Qsonica) for 2–3 min. Then, samples were centrifuged at 12,000 x g for 20 min at 4 °C. The supernatant was transferred into new centrifuge tubes. The proteins were quantified by Bradford Protein Assay before running the lipid peroxidation assay. A spectrophotometric (colorimetric) method using thiobarbituric acid (Sigma, T5500, 1%) was used to assess lipid peroxidation. Lipid peroxide in the tissue reacted with the thiobarbituric acid to form thiobarbituric acid-reactive substances (TBARS). The index of lipid peroxidation was estimated by the formation of TBARS at 532 nm. TBARS was normalized to total protein content as TBARS formed/mg protein (Alhowail et al., 2019; Majrashi et al., 2018).

2.7. Nitrite content

Nitrite content was measured spectrophotometrically using the commercially available Griess reagent (Promega). The Griess method relies on nitrite reaction with sulfanilamide under acidic condition resulting in the production of diazonium ion. The diazonium ion then combines with N-(1-naphthyl) ethylenediamine to form chromophoric azo product which can be measured spectrophotometrically at 545 nm. A sodium nitrite standard curve was prepared from commercially acquired sodium nitrite. Results were expressed as (%) change as compared to the control (Giustarini et al., 2008; Green et al., 1982; Majrashi et al., 2018).

2.8. Mitochondrial Complex I activity

Samples were homogenized with phosphate-buffered saline (PBS) and lysis buffer and centrifuged at 14000 x g and 2–4 °C for 15 min. The supernatant was transferred into new, clean 1.5 mL microcentrifuge tubes and frozen at -80 °C until use. The proteins from each sample were quantified by Bradford Protein Assay before starting assessment of mitochondrial Complex I activity. Mitochondrial Complex-I activity is a measure of NADH oxidation. Oxidation of NADH (VWR, 0384, 1mM) by NADH dehydrogenase was measured spectrophotometrically at 340 nm (Thrash-Williams et al., 2016). The Complex I activity refers to NADH oxidized/mg protein.

2.9. Mitochondrial Complex-IV activity

Mitochondrial Complex-IV activity is a measure of cytochrome-C oxidation. Cytochrome C oxidation is catalyzed by mitochondrial Complex-IV (Cytochrome C oxidase, Sigma C7752, 2.5mM). Cytochrome C oxidation was spectrophotometrically measured in the control and WIN55,212-2 treated animals at 550 nm. Complex-IV activity refers to cytochrome c oxidized/mg protein (Thrash-Williams et al., 2016).

2.10. Caspase-1 activity

Caspase-1 was measured by spectrofluorometric method using Ac-Tyr-Val-Ala-Asp-7-amino-4- Trifluoromethlcoumarin (Ac-YVAD-AMC) as a substrate. Cleavage of 7-amino-4-Methylcoumarin (AMC, 100uM, Enzo-260-024-M005) by Caspase-1 generates fluorescent product-AMC that is fluorometrically measured at excitation of 340–350nm and emission of 440–460nm. The Caspase-1 activity is measured as free AMC formed/mg total protein. Results are expressed as (%) change as compared to the control (Majrashi et al., 2018; Usha et al., 2000).

2.11. Caspase -3 activity

Caspase-3 activity was measured spectrofluorometrically using the non-fluorescent Caspase-3 substrate, N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-Methylcoumarin (Ac-DEVD-AMC, 1000uM Enzo-260-031-M005). Cleavage of AMC by Caspase-3 generates fluorescent product-AMC that is fluorometrically measured at excitation of 340–350nm and emission of 440–460nm. The Caspase-3 activity refers to free AMC formed/mg total protein. Results are expressed as (%) change as compared to the control (Bhattacharya et al., 2018; Usha et al., 2000).

2.12. Monoamine oxidase (MAO) activity

Kynuramine (stable monoamine, non-fluorescent, Santa Cruz, 207782A, 1mM), is a substrate of monoamine oxidase. Kynuramine is oxidized to form 4-hydroxyquinoline (fluorescent product) by monoamine oxidase (MAO). A 4-hydroxyquinoline standard curve was obtained. Spectrofluorometric method using kynuramine as a substrate was used to measure MAO activity (excitation at 315 nm and emission at 380 nm). MAO activity refers to 4-hydroxy quinolone (μM)/mg protein (Thrash-Williams et al., 2016).

2.13. Tyrosine hydroxylase activity

Tyrosine (substrate, 2mM, sigma T3754) was added to the samples and samples were then incubated for 30 min to induce the formation of L-dopa by tyrosine hydroxylase present in the sample. Following the reaction, sodium periodate (Beantown chemicals) was added. The formed L-dopa reacts with sodium periodate to form dopaquinone. Dopaquinone can be measured spectrophotometrically at 475nm. The L-Dopa standard curve was prepared from commercially acquired L-Dopa. Results are expressed as (%) change as compared to the control (Vermeer et al., 2013).

2.14. Western blot analysis

Total protein was isolated using cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) containing protease inhibitor cocktail (P8340, Sigma, St. Louis, MO) and phosphatase inhibitors (P 5726, Sigma, St. Louis, MO). Protein concentration was measured using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, ThermoFischer Scientific). Western blot analysis was performed as previously described (Alhowail et al., 2019). Each sample was denatured at 95 °C for 5 min before loading onto freshly prepared 10% SDS-PAGE gel for protein separation. Separated proteins on SDS-PAGE were transferred onto PVDF membranes (Immobilon-p Millipore, Germany). Non-specific binding sites on the membranes were blocked with 5% non-fat milk in Tris-buffered saline plus 0.1% Tween-20 (TBST) at pH 7.4. The membranes were then incubated overnight at 4 °C with specific antibody constituted in 5% BSA in TBST. Primary antibodies used in this study included: anti-GluA1, anti-GluN2A, anti- ILK, anti-AKT, anti- JNK, anti-GSK3β, anti ERK1/2, anti JNK, anti P38 MAPK, anti-phospho AKT, anti-phospho JNK, anti-phospho GSK3β, anti-phospho ERK1/2, anti-phospho JNK, anti-phospho p38 MAPK (Cell signaling, Denver) and anti CB1 (Abcam). All the primary antibodies were used at a 1:1000 dilution except CB1 that was used at 1:350 dilution. Membranes were then washed with TBST (3X, each for 10 min) and incubated with anti-rabbit or anti-mouse conjugated secondary antibodies (1:3000) for 60–90 min at room temperature. Membranes were again washed three times for 10 min with TBST. After washing, membranes were imaged in FluorChemQ® system Imaging. Protein band intensity was quantified by

AlphaView software band densities for each sample were normalized to their respective β-actin (1:1000, Cell Signaling) or GAPDH (1:10000, EMD Millipore) signal and reported as percentage change from control.

2.15. Statistical analysis

Data analysis was performed using GraphPad PRISM 8. Statistical analysis consisted of Two tailed Student's T Test (Control vs. prenatal cannabinoid exposure). Results were presented as mean ± SEM, and differences between groups were considered statistically significant at $p \leq 0.05$. 5 to 6 rats per group were examined.

3. Results

3.1. Prenatal cannabinoid exposure does not alter behavioral parameters

In prior studies, cannabis use during pregnancy has led to alterations in behavioral parameters and motor development in the offspring (De Salas-Quiroga et al., 2015; Farah Naquiah et al., 2016; Kelly et al., 2000; Saberi Moghadam et al., 2013; Sobrain, 1977; Wu et al., 2011). The dose used in the current study did not induce alterations in common behavioral parameters (No changes in behavioral parameters were observed in the prenatally WIN55,212-2 exposed offspring at a dose of 2 mg/kg/day as described in Table 1.

3.2. Prenatal cannabinoid exposure reduces ROS, lipid peroxidation, and nitrite content in the cerebellum

ROS triggers oxidative stress and induces irreversible oxidation of lipids and proteins, resulting in cell death. We investigated the ROS content and the TBARS content to determine whether prenatal cannabinoid exposure exerts a neurotoxic effect on cerebellum. In the DCF based ROS assay, prenatal cannabinoid exposure led to a statistically significant reduction in ROS generation compared to control ($T_{(8)} = 2.234$, $p = .05$; Figure 1A) along with a significant reduction in lipid peroxidation ($T_{(8)} = 2.374$, $p = .04$; Figure 1B) and nitrite content ($T_{(6)} = 3.061$, $p = .02$; Figure 1C) which serve as additional markers of oxidative stress.

3.3. Prenatal cannabinoid exposure improves mitochondrial complex activity

To investigate how prenatal cannabinoid exposure affects the mitochondrial system, we measured Complex I and Complex IV activity. In the prenatally cannabinoid exposed offspring, there was an increase in both complex I and complex IV activity. Prenatal cannabinoid exposure notably improved mitochondrial bioenergetics, as demonstrated by significant increase in Complex I ($T_{(8)}t = 2.736$, $p = .03$; Figure 2A) and in Complex IV ($T_{(8)} = 2.692$, $p = .03$; Figure 2B).

Table 1. Effect of prenatal cannabinoid exposure on various physical and behavioral parameters.

Behavioral parameters	Control	Win55,212-2 (Prenatal Cannabinoid Exposure)
Tremor	N	N
Straub tail	N	N
Seizure	N	N
Mortality observed	N	N
Hyperactivity (Excessive Jumping)	N	N
Hind limb abduction	N	N
Head twitching	N	N
Hair coat erection	N	N
Fighting (Aggressive Behavior)	N	N
Drooling	N	N
Diarrhea	N	N
Ataxic behaviors	N	N
Anaphylactic shock/Death	N	N
Allergic reaction (redness of the Skin or Eye)	N	N

N = not observed.

3.4. Prenatal cannabinoid exposure reduces caspase-3 activity, ERK and JNK phosphorylation

Caspases are a family of endoproteases which is crucial for regulating inflammation and cell death (McIlwain et al., 2013). To determine whether cannabinoid exposure can initiate an apoptotic cascade in the cerebellum during the developmental period, we investigated caspase 1 and caspase 3 activity (Figures 3A, 3B). Prenatal cannabinoid exposure did not result in any changes in caspase-1 ($T_{(6)} = 1.827$, $p = 0.11$, Figure 3A). However, caspase -3 was significantly reduced in the WIN55, 212-2 group ($T_{(6)} = 2.645$, $p = 0.03$; Figure 3B). Following this, we also observed a significant reduction in ERK ($T_{(6)} = 3.117$, $p = 0.01$; Figure 3C) and JNK phosphorylation ($T_{(6)} = 3.115$, $p = 0.02$; Figure 3D) accompanied by an increase in P38 phosphorylation ($T_{(6)} = 2.505$, $p = 0.04$; Figure 3B) in the cerebellum of the prenatally cannabinoid exposed group. Since these protein molecules play an essential role in the inhibition of apoptosis, our results suggest that prenatal cannabinoid exposure might play an anti-apoptotic role in the cerebellum.

3.5. Prenatal cannabinoid exposure modulates cannabinoid and glutamatergic receptors

The drug WIN55,212-2 is a full agonist at CB1R. Since WIN55,212-2 exert its effects through CB1R we measured whether CB1R levels were altered in the cerebellum of prenatally cannabinoid exposed offspring. Western blot analyses revealed that prenatal cannabinoid exposure did not alter CB1R levels in the cerebellum (Figure 4A). We also investigated whether prenatal cannabinoid exposure altered glutamatergic receptor expression by examining major glutamate receptor subtypes AMPA-GluA1 and NMDA-GluN2A. While GluA1 is mainly recognized for its role in synaptic plasticity, GluN2A is associated with cytotoxic effects. In this study, prenatal cannabinoid exposure had no effect on GluA1 level (Figure 4B) in the cerebellum of the offspring, but a significant reduction in GluN2A level ($t_{(4)} = 2.82$, $p = 0.04$) was observed in these animals compared to control animals.

3.6. Prenatal cannabinoid exposure modulates dopaminergic activity

Since MAO is known to play a 'morphogenetic' role during development and aging process, we investigated the total MAO activity in the cerebellum of these animals (McIlwain et al., 2013). MAO activity was significantly reduced in the cerebellum of prenatally WIN55,212-2 exposed animals ($T_{(6)} = 2.866$, $p = 0.02$; Figure 5A). Tyrosine hydroxylase activity was also measured to evaluate the effect of prenatal cannabinoid exposure on cerebellar catecholamine content. There was no significant difference in the TH activity in the two groups ($T_{(6)} = 0.5886$, $p = 0.57$; Figure 5B).

3.7. Prenatal cannabinoid exposure and markers of excitotoxicity and synaptic plasticity

Next, we investigated the effect of PCE on the key signaling molecules such as AKT, GSK3β and ILK, which are important for maintenance of synaptic plasticity and cerebellar mitochondrial function. Prenatal cannabinoid exposure did not alter pAKT (Figure 6A, $p > .05$), pGSK3β (Figure 6B, $p > .05$), or ILK levels (Figure 6C, $p > .05$).

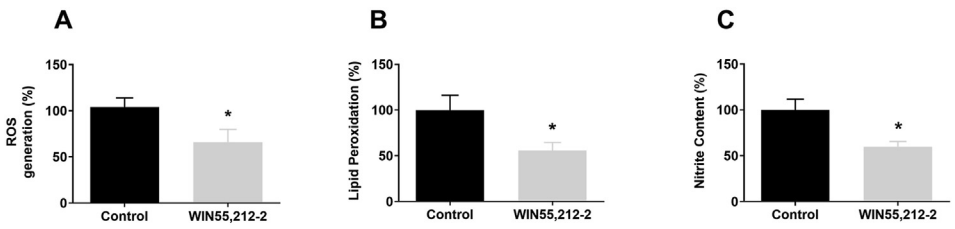

Figure 1. Effect of prenatal cannabinoid exposure on ROS, lipid peroxide, and nitrite content in the cerebellum: (A) Significant reduction in the oxidative stress level measured by ROS generation ($p = .05$) (B) significant reduction in cerebellar lipid peroxide content in prenatally cannabinoid exposed offspring ($p = .04$) (C) Nitrite content was significantly reduced in prenatally cannabinoid exposed group ($p = .02$). Results are expressed as Mean ± SEM, $n = 4–5$ rats per group. * indicates a significant difference when $p \leq .05$. Two tailed student's T test.

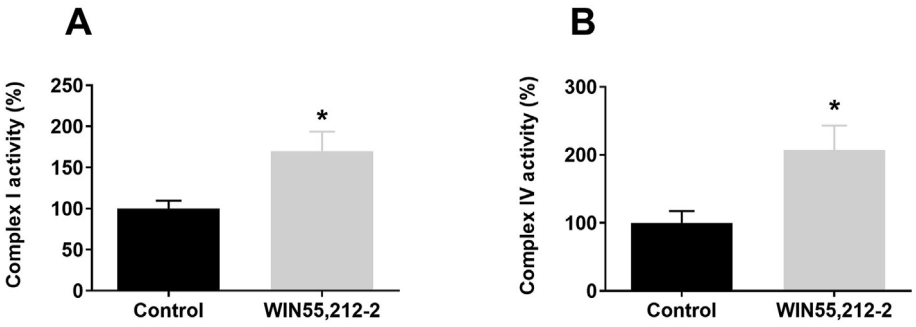

Figure 2. Effect of prenatal cannabinoid exposure on complex I and complex IV activity: (A) significant increase in complex I activity in prenatally WIN55,212-2 exposed group ($p = .03$). (B) Complex IV activity has also increased in WIN55,212-2 exposed group ($p = .03$). Results are expressed as Mean ± SEM, $n = 4$ rats per group. * indicates a significant difference when $p \leq .05$. Two tailed student's T test.

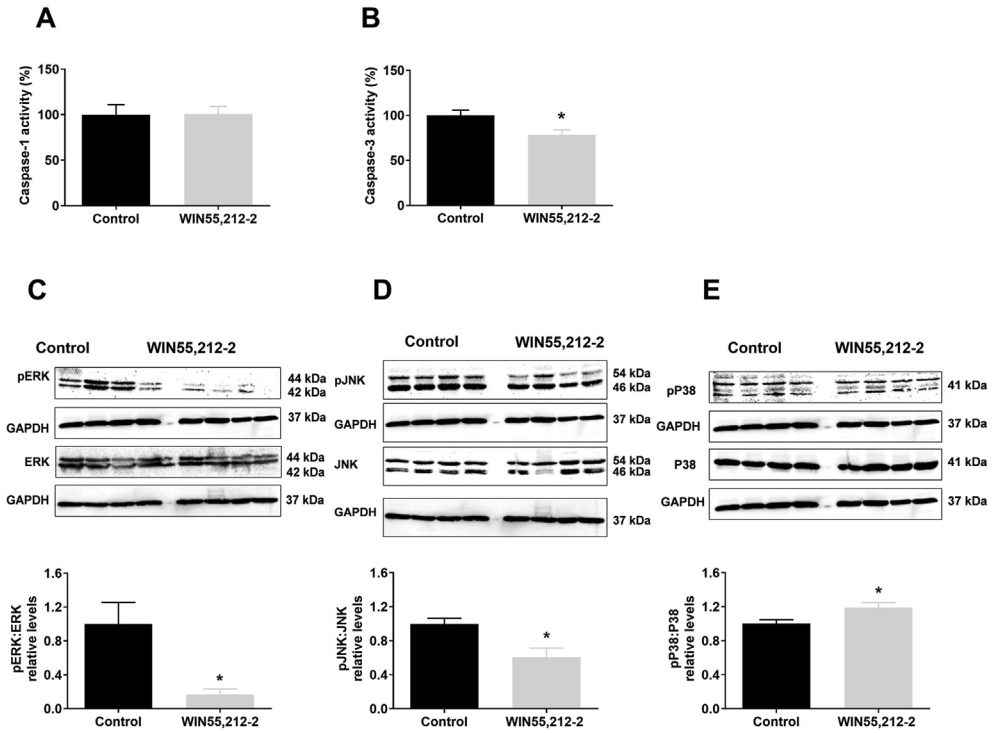

Figure 3. Effect of prenatal cannabinoid exposure on apoptotic markers: (A) Prenatal cannabinoid exposure did not cause any alteration in the caspase 1 activity ($p > .05$). (B) Caspase-3 activity was significantly reduced in the WIN55,212-2 exposed group. Representative immunoblots showing (C) pERK/ERK ($p = .01$), (D) pJNK/JNK ($p = .02$), (E) pP38/P38 ($p = .05$). relative densities. Results are expressed as Mean ± SEM, $n = 3–4$ rats per group. * indicates a significant difference when $p \leq .05$. Two tailed student's T test. Refer Supplementary material Figure 3.

4. Discussion

In light of the growing popularity of commercially available synthetic cannabinoid products, abuse of highly potent synthetic cannabinoids is a major public health concern due to intoxication and emergency room visits (Dong et al., 2019b). While the harmful effects of natural marijuana are somewhat well established, the effects of synthetic cannabinoids are yet to be explored. Despite federal ban on the use of synthetic cannabinoids, people continue using it for feeling a new 'high' like that produced by marijuana while escaping the standard routine drug test ("Synthetic Cannabinoids (K2/Spice) DrugFacts | National Institute on Drug Abuse (NIDA)," 2020). However, synthetic cannabinoids are more potent than natural cannabinoids and can cross the blood brain barrier readily (Hess et al., 2016).

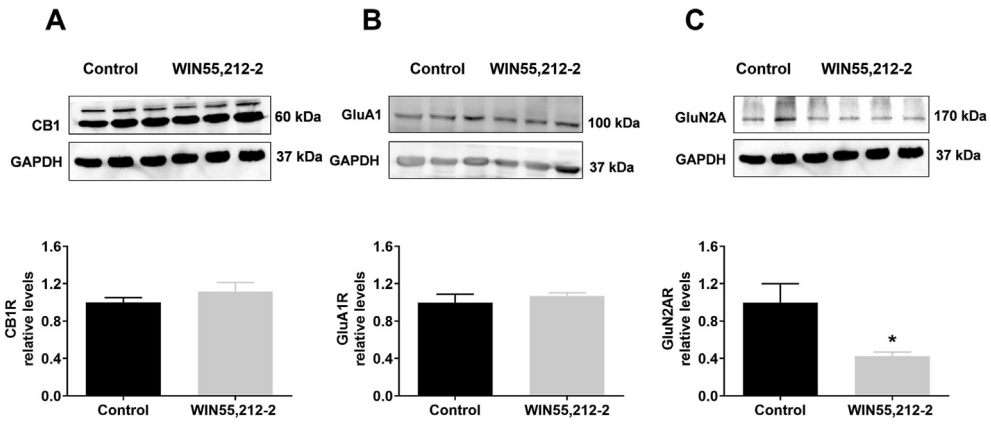

Figure 4. Effect of prenatal cannabinoid exposure on cerebellar signaling molecules associated with cannabinoid and glutamatergic neurotransmission: Representative immunoblots showing (A) CB1/GAPDH ($p > .05$), (B) GluA1/GAPDH ($p > .05$) and (C) GluN2A/GAPDH relative expression ($p = .04$) Results are expressed as Mean ± SEM, $n = 3–4$ rats per group. * indicates a significant difference when $p \leq 0.05$. Two tailed student's T test. Refer Supplementary material Figure 4.

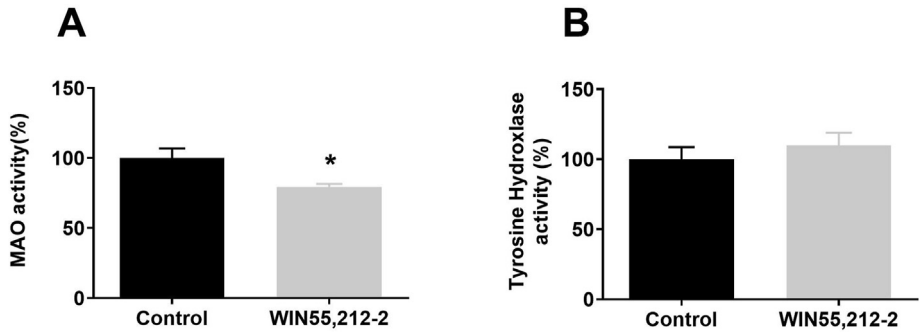

Figure 5. Effect of prenatal cannabinoid exposure on MAO and tyrosine hydroxylase activity: (A) MAO activity was significantly reduced in prenatally cannabinoid exposed group ($p = 0.02$). (B) No significant change in the tyrosine hydroxylase content in between the groups ($p > .05$). Results are expressed as (%) change as Mean ± SEM. $n = 4$ rats per group. * indicates a significant difference when $p \leq .05$. Two tailed student's T test.

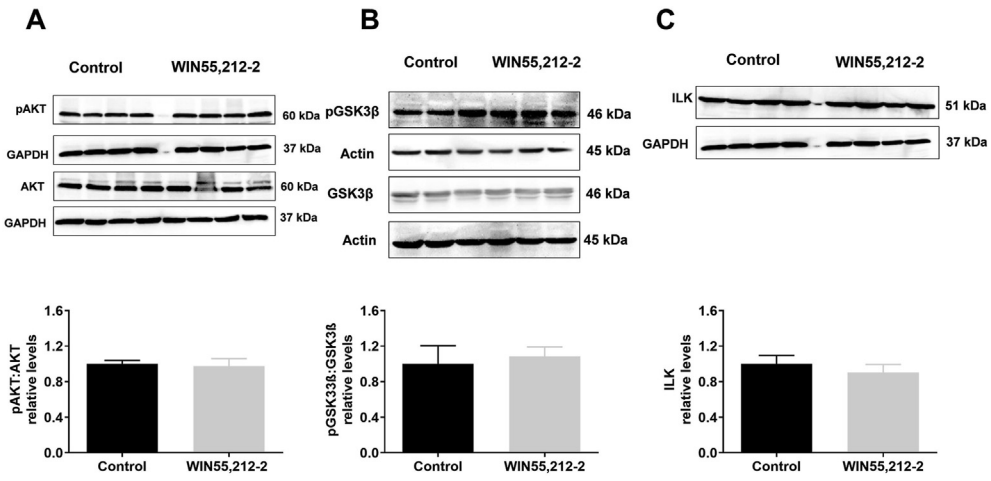

Figure 6. Effect of prenatal cannabinoid exposure on cerebellar signaling molecules associated with markers of excitotoxicity and synaptic plasticity: Representative immunoblots showing pAKT/AKT, pGSK3β/GSK3β, ILK/Actin/GAPDH, relative expression. (A) No change in the phosphorylation of AKT in response to prenatal cannabinoid exposure ($p > .05$) (B) No change in the phosphorylation of GSK3β at Serine-9 between the two groups ($p > .05$). (C) No change in ILK expression in the cerebellum in response to prenatal cannabinoid exposure ($p > .05$). Results are expressed as Mean ± SEM. $n = 3–4$ rats per group. * indicates a significant difference when $p \leq .05$. Two tailed student's T test. Refer Supplementary material Figure 6.

The dose of WIN55,212-2 (2 mg/kg body weight/day) used here corresponds to a low to moderate cannabis exposure in humans after correction for differences in body surface area (Campolongo and Trezza, 2012; French et al., 1997; Hampson and Deadwyler, 2000; Tortoriello et al., 2014). Based on this, we chose a moderate dose as the potency in confiscated marijuana as measured by THC content is approximately 300% higher than it was in the 1980s and this continues to rise (Volkow et al., 2014). Recent data also shows that the amount of marijuana consumed has been increased, in part due to the rising popularity of joints or pipes (Mariani et al., 2011) as well as relaxation of marijuana policies in various states (Jones et al., 2015). Therefore, we chose this dose of cannabinoid to reflect the increasing potency and level of marijuana to which pregnant women are likely to be exposed. In addition, this dose was not associated with maternal or fetal abnormalities, including alterations in maternal weight, fetal weight, litter size, gestation time, or pup mortality in the current study.

We have opted to examine the consequences of prenatal cannabinoid exposure on the periadolescence period (PND 38–46), because most

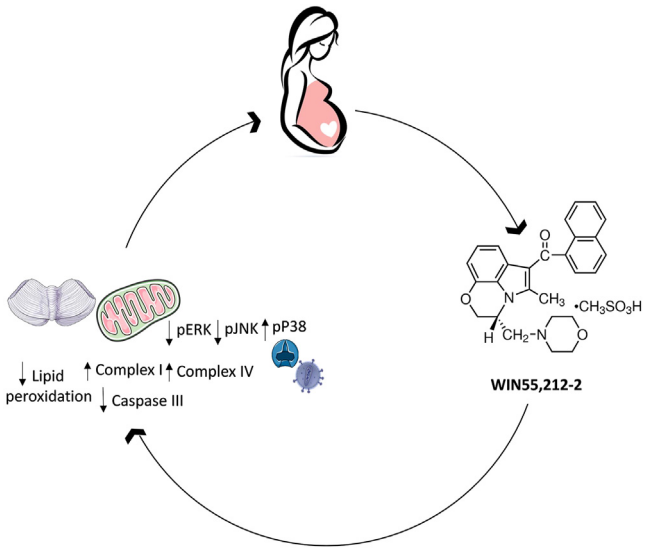

Figure 7. Effect of prenatal cannabinoid exposure on the developing cerebellum: Prenatal cannabinoid exposure results in alteration of phosphorylation of several molecules i.e. ERK, JNK, P38. It can also increase complex I and complex IV activity accompanied with reduction in caspase 3 activity and lipid peroxidation content demonstrating altered mitochondrial function. This figure was produced using Servier Medical Art (https://smart.servier.com/) and Library of science and medical Illustrations (http://www.somersault1824.com/science-illustrations/).

studies examining the consequences of prenatal cannabinoid exposure in humans have focused on this period, thereby allowing for comparison of our results to those obtained in humans. Several clinical studies have demonstrated prenatal cannabinoid exposure results in the impairments in memory, analysis, and attention as well as other cognitive impairments, during the adolescent period (Fried et al., 2003; Fried and Watkinson, 2001). Moreover, adolescent success is highly predictive of adulthood outcomes (Kansky et al., 2016; Seiffge-Krenke et al., 2014) so deficits during this period are likely to produce long-lasting consequences even if neurological alterations associated with prenatal cannabinoid exposure do not persist into adulthood.

Prenatal cannabinoid exposure has a range of long lasting and enduring effect on the fetus and offspring. Gestational exposure to cannabinoid can cause miscarriage in the first trimester (Bloch et al., 1979), preterm birth (Klebanoff et al., 2020), increase admission in neonatal intensive care (Gunn et al., 2016; Warshak et al., 2015) as well as result in several behavioral and cognitive deficits (Day et al., 2011; Fried, 1995; Kooijman et al., 2016). Not only this prenatal cannabinoid exposure also profoundly suppress immune function via T cell dysfunction and epigenetic modulation (Zumbrun et al., 2015) leading to decreased responsiveness to various viral antigens in the postnatal period (Lombard et al., 2011). In the current study, we investigated the effects of prenatal exposure to the synthetic cannabinoid WIN55,212-2 on the cerebellum of the adolescent offspring. Although we did not observe any gross motor abnormality of the offsprings in our study, gestational cannabinoid exposure during brain development can result in motor abnormality (Breit et al., 2019a, b). However, it depends on many variables such as dose, the period of exposure, age of the pups during testing, etc. (Pinky et al., 2019). To evaluate the motor abnormalities further, specific behavioral test such as rotarod test, pole test or open field test can be performed in future studies. Moreover, prenatal cannabinoid exposure has been shown to alter fine motor performance through alterations in corticospinal connectivity (de Salas-Quiroga et al., 2015). A dose dependent effect has also been observed in case of adult exposure to cannabis as manifested by increased latency of forelimb removal on the bar task (Scullion et al., 2016). Although not examined, based on this evidence, an alteration in the fine motor control may exist in the offspring in this study as well.

We report that prenatal cannabinoid exposure can reduce certain markers of oxidative stress i.e. ROS, lipid peroxidation content, nitrite content, and increase mitochondrial function in the adolescent offspring accompanied by alterations in key regulators of apoptosis and synaptic function (See Figure 7). We noted a reduction in caspase-3 activity with prenatal cannabinoid exposure indicating that caspase-3-dependent apoptosis does not occur in the cerebellum. This is in line with other studies which showed absence of volumetric reductions in the cerebellum (Cousijn et al., 2012). Furthermore, recent studies suggest that caspase-3 activation does not always lead to apoptotic cell death, and there are several instances where cells recover following caspase-3 activation (D'Amelio et al., 2010; McComb et al., 2010). Chronic THC intake is associated with altered cerebellar dependent learning in adult rodents (Cutando et al., 2013). In adolescent humans, prenatal cannabinoid exposure is linked to reduced left cerebellar activity (Smith et al., 2004). Although there are studies that explored the effect of cannabinoids on oxidative stress (Booz, 2011), inflammation, and apoptosis (Pozzoli et al., 2006), to date, few studies have examined the effects of prenatal cannabinoid exposure on the above mentioned parameters in the cerebellum. However, it is important to understand how prenatal cannabinoid exposure alters cerebellar functions since the cerebellum plays an essential role in learning and motor function.

We measured oxidative stress and lipid peroxidation in response to prenatal cannabinoid exposure as an indicator of neurotoxicity. Oxidative stress and lipid peroxidation occur as a result of dysregulated redox homeostasis leading to the accumulation of highly reactive molecules resulting in cellular and neuronal injury (Gallelli et al., 2018). Our finding is congruent with findings in adult animals where cannabinoid exposure reduces ROS and lipid peroxidation content in cortical neurons (Rangel-López et al., 2015), cerebellar granular cells, and hippocampal neuronal cells (Marsicano et al., 2002). CB1R is found in mitochondrial membranes, controlling cellular respiration and energy production (Bénard et al., 2012). ROS and lipid peroxidation products are proapoptotic factors and can activate apoptosis via mitochondrial dependent pathways (Wójcik et al., 2020). Since we observed an alteration in ROS and lipid content, we also expected an alteration in mitochondrial function. Although previous studies utilizing acute exposure of cannabinoids in adult rodents demonstrated reduced complex I and complex IV activity (Singh et al., 2015), we observed a significant increase in both Complex I and Complex IV activity. This suggests cannabinoids might regulate the mitochondrial respiratory chain function differentially with acute versus chronic exposure as well as in adult versus developmental exposure. We also observed a reduction in the nitrite content following prenatal cannabinoid exposure. This could be due to increased utilization of Nitric oxide (NO) or increased conversion of nitrite to NO (Rassaf et al., 2014), possibly indicating a neuroprotective effect. Nitrite is an inhibitor of complex I (Shiva, 2010), so the increased complex I activity observed in this study may be due to the reduction in nitrite following prenatal cannabinoid exposure.

Cannabinoids can also affect various brain monoamines levels i.e. dopamine, norepinephrine, and serotonin. A significant reduction in total MAO activity in the cerebellum of prenatally cannabinoid exposed offspring in our study aligns with previous findings in the adult pig brain cortex (Fišar, 2010). It is an interesting finding since MAO inhibition has been linked to alcohol and nicotine addiction previously (Amsterdam et al., 2006) and prenatal cannabinoid exposure indeed can contribute to cannabis use in early adolescent age (Day et al., 2006).

Prenatal cannabinoid exposure also alters tyrosine hydroxylase (TH) activity in the hippocampus (Castaño et al., 1995) and other brain regions (Bonnin et al, 1995, 1996). However, we did not observe any change in TH activity in our study, which is in contrast with previous reports in other brain regions (Bonnin et al, 1995, 1996; Rodríguez De Fonseca et al., 1992). We also did not observe any changes in CB1R level

in the cerebellum, activation of which leads to alterations in TH levels (Frau et al., 2019; Ginovart et al., 2012; Hernández et al., 2000). It is possible that the dose used here is unable to elicit the changes in the cerebellum, as other studies have found alterations in CB1R levels along with changes in TH level in other brain regions following prenatal cannabinoid exposure (Bonnin et al, 1995, 1996; Castaño et al., 1995).

Since the cerebellum has a high density of CB1Rs, and glutamatergic neurotransmission is altered by CB1R activation, we wanted to examine whether prenatal cannabinoid exposure also alters glutamatergic receptor subunits in the cerebellum. We observed a significant reduction in GluN2A level with no changes in GluA1 in the prenatally cannabinoid exposed offspring. Overactivation of NMDA receptors such as GluN2A shuts off the pro-survival signaling while molecular knockdown of GluN2A attenuates NMDAR-mediated neuronal death in cortical neurons (Zhou et al., 2013a). Because GluN2A can be considered excitotoxic (Deep et al., 2019; Zhou et al., 2013b), a decrease in GluN2A may be neuroprotective against calcium mediated cellular excitotoxicity. Although NMDA dysfunction is associated with motor discoordination, it is usually due to combined disruption of both GluN2A and GluN2C, but not from single disruption of the GluN2A in the cerebellum (Kadotani et al., 1996). This might explain why we did not observe any changes in the observed behavioral parameters.

To investigate whether prenatal cannabinoid exposure affects apoptosis, we examined caspase-1 and caspase-3 activity. Activation of inflammatory caspases like caspase-1 and caspase-3 can increase the production of various pro-inflammatory cytokines initiating cell death and apoptosis (McIlwain et al., 2013). We did not observe any change in caspase 1, an inflammatory marker, but we observed a significant decrease in caspase 3, an apoptotic marker. This indicates that prenatal cannabinoid exposure might lead to a reduction in apoptotic events in the cerebellum. There are three major mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-JUN N-terminal kinase (JNK), and P38 those are known regulators of apoptosis (Chuang et al., 2000). Although ERK activation is traditionally thought to promote cell viability, activation of ERK in response to DNA damage from insults can indeed result in proteasomal degradation and inhibition of apoptosis (Mebratu and Tesfaigzi, 2009). JNK induces brain-region specific apoptosis during early developmental period and helps in proper brain development (Kuan et al., 1999), while regulating neuronal migration, dendrite formation, and axon maintenance during later brain development (Björkblom et al., 2005; Chang et al., 2003). We observed reduced JNK phosphorylation along with a reduction in ERK phosphorylation in the cerebellum of prenatally cannabinoid exposed offspring. An increase in the phosphorylation of ERK and JNK has been associated with cerebellar neuronal death (Zahir et al., 2012). Hence, reduction in the phosphorylation of these proteins along with reduced oxidative stress markers may indicate a possible protective effect of cannabinoid exposure on cerebellar neuronal growth and survival. Inhibition of P38 also elicits an apoptotic event in certain tumor cells (Phong et al., 2010; Refaat et al., 2015). Since we found a decrease in anti-apoptotic marker caspase - 3 activity (Chuang et al., 2000; Herrera et al., 2005), we hypothesize that the increase in pP38 indicates an anti-apoptotic role.

The serine/threonine kinase AKT (also known as protein kinase B) plays a critical role in mediating diverse cellular functions including metabolism, growth, proliferation, survival, transcription and protein synthesis, and AKT dysregulation has been implicated in various disease as well as learning and memory (Hers et al., 2011). Increased oxidative stress and mitochondrial dysfunction can lead to AKT activation resulting in premature cell cycle arrest and apoptosis (Nogueira et al., 2008). AKT also serves as a regulator of GSK3β, a critical protein for synaptic plasticity. Acute cannabinoid administration can increase AKT and GSK3β phosphorylation in the hippocampus of adult rats, in a CB1R dependent manner (Ozaita et al., 2007). In our study, we did not observe any change in AKT or GSK3β phosphorylation, and no changes were observed in cerebellar CB1R levels. Thus, the changes in AKT and GSK3β phosphorylation might be observed in acute dosing regimens but not with developmental exposure (Trazzi et al., 2010). We also did not observe any change in cerebellar ILK level. ILK regulates various cellular processes, including migration, differentiation, survival as well as neurite outgrowth & dendritic morphogenesis (Kelly et al., 2003; Xu et al., 2015). Although we did not observe changes in the ILK levels or phosphorylation of AKT and GSK3β, more studies are needed to identify whether there are any alterations in neuronal morphology or synaptic plasticity in the cerebellum through other signaling pathways due to prenatal cannabinoid exposure (Bhattacharya et al., 2015).

5. Conclusion

Given the prevalence of synthetic cannabinoid consumption, there is an urgent need to better understand the pharmacology and toxicology of synthetic cannabinoids. A critical future direction is to determine whether THC produces similar effects to those observed with the synthetic cannabinoid WIN55,212-2. Our results demonstrate a unique effect of prenatal cannabinoid exposure in the cerebellum of adolescent offspring suggesting a possible neuroprotective effect on the cerebellum during early phases of brain development. Future studies should evaluate the in-depth mechanisms by which prenatal cannabinoid exposure alters cerebellar function and the impact of these alterations on behavior.

Declarations

Author contribution statement

Priyanka D Pinky: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mohammed Majrashi; Ayaka Fujihashi; Jenna Bloemer; Manoj Govindarajulu; Sindhu Ramesh: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Miranda Reed: Conceived and designed the experiments; Wrote the paper.

Timothy Moore: Analyzed and interpreted the data; Wrote the paper.

Vishnu Suppiramaniam; Muralikrishnan Dhanasekaran: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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