2 Physiology of the Endocannabinoid System During Development

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Abstract

The endocannabinoid (eCB) system comprises endogenously produced cannabinoids (CBs), enzymes of their production and degradation, and CB-sensing receptors and transporters. The eCB system plays a critical role in virtually all stages of animal development. Studies on eCB system components and their physiological role have gained increasing attention with the rising legalization and medical use of marijuana products. The latter represent exogenous interventions that target the eCB system. This chapter summarizes knowledge in the field of CB contribution to gametogenesis, fertilization, embryo implantation, fetal development, birth, and adolescence-equivalent periods of ontogenesis. The material is complemented by the overview of data from our laboratory documenting the functional presence of the eCB system within cerebral arteries of baboons at different stages of development.

Keywords

Cannabis · Cannabinoid · Baboon · Nonhuman primate · Fetal artery · Cerebral artery

Abbreviations

ABHD4	α/β-hydrolase domain 4
AEA	anandamide
CB	cannabinoid
COX-2	cyclooxygenase-2
CP55,940	(-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol
DAGL	diacylglycerol lipase
eCB	endocannabinoid (system)
ERK	extracellular-signal-regulated kinase
FAAH	fatty acid amide hydrolase
GABA	gamma aminobutyric acid
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MAGL	monoacylglycerol lipase
NAE	N-acylethanolamine
NAPE	N-acylphosphatidylethanolamine
NAPE-PLD	N-acylphosphatidylethanolamine-specific phospholipase D
PCR	polymerase chain reaction
THC	Δ^9-tetrahydrocannabinol
TRP	transient receptor potential (protein, channel)
VGAT	vesicular GABA transporter
2-AG	2-arachidonoylglycerol.

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2.1 Introduction

Humans have been consuming cannabis in the form of herbs since ancient times [1]. Although adverse reactions and high doses of cannabinoid preparations may trigger dysphoria [2], low-to-moderate consumption confers analgesic, anxiolytic, and antiemetic properties [3–6]. The modern understanding of mechanisms that stand behind physiological effects of cannabis consumption started to emerge in the middle of twentieth century with the isolation and characterization of the main psychoactive substance in *Cannabis sativa* plant – Δ^9-tetrahydrocannabinol (THC) [1]. These findings were followed by the discovery of the first cannabinoid receptor (CB1), also by structure elucidation and isolation of endogenously produced cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [1]. Although cannabis preparations currently are used primarily for recreational purposes, they are also consumed for medical reasons: to reduce nausea and vomiting, relieve symptoms associated with neurological disorders, reduce intraocular pressure in glaucoma patients, and as an analgesic remedy [1, 7]. Moreover, CB-based preparations have been increasingly recognized as having therapeutic potential for treatment of numerous pathological conditions, including depression, epilepsy, anxiety-related disorders, and obesity [8–12].

The endocannabinoid (eCB) system is comprised of endogenously produced CBs, their receptors, as well as eCB synthesis, degradation enzymes, and transporting molecules [13]. The eCB system plays a crucial role at all stages of human early development, stemming from the influence over gametogenesis and embryo implantation, spreading into control of nervous system development, peripheral organogenesis, and finishing with postnatal development [14]. Thus, studies of the eCB system help to delineate fundamentals of development and pinpoint potential sites of pharmacological interventions against prevalent developmental disorders. In addition, the understanding of mechanisms that govern eCB-mediated control over ontogenesis will help to expand our knowledge on the consequences of prenatal exposure to marijuana. The latter is reported to affect 3.9–7% of pregnancies [15], reaching even higher numbers (13% of meconium samples) within high-risk populations [16].

Remarkably, there is no acute toxicity to the major psychoactive substance in marijuana – THC [2]. Yet, THC consumption leads to a plethora of physiological and psychological effects [2, 17, 18], reflecting the complexity of the eCB system and its susceptibility to exogenous interventions. Widening legalization of marijuana use, development of synthetic approaches to obtain a "transgenic pot," and the growing number of pharmaceutical agents that target eCB signaling are reasons that call for comprehensive understanding of the side effects and risks associated with modifications of eCB function.

This chapter summarizes knowledge in the field of CB contribution into gametogenesis, fertilization, embryo implantation, fetal development, birth, and adolescence-equivalent periods of ontogenesis. The literature overview in each section starts with the data on rodents and then gradually shifts to humans. The material is complemented by data from our laboratory documenting the functional presence of the eCB system within cerebral arteries of baboons at different stages of development.

2.2 Brief Overview of eCB System: From Genes to Products

Upon demand, eCBs are synthesized *de novo* using hydrolyzed lipid precursors from cellular membrane [14]. The two most widely studied endogenously produced CBs are AEA and 2-AG. AEA belongs to the N-acylethanolamine (NAE) family of lipid mediators that represent CB-related compounds. For instance, N-palmitoylethanolamine is able to activate cannabinoid receptors [19, 20]. The physiological role of NAEs is being actively investigated [21–23]. AEA synthesis originates from the rate-limiting step of the N-acylation of phosphatidylethanolamine rendering N-acylphosphatidylethanolamine (NAPE) [24]. One of the major pathways in AEA synthesis is

mediated by N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) that hydrolyses NAPE into AEA [24]. Other pathways engage phosphatase PTPN22 [25], α,β-hydrolase domain 4 (ABHD4) [26], and glycerophosphodiesterase (GDE1) [27].

The major synthetic pathway of 2-AG is presented as a two-step reaction. The first step is initiated upon phospholipase C activation and results in the generation of diacylglycerol from phosphatidylinositol. The second step, resulting in 2-AG release, is carried out by a membrane-bound diacylglycerol lipase (DAGLα/β) [24, 28].

Cell types that are most actively involved with eCB production are still under investigation, but likely represent quite an exhaustive catalog [29]. Upon production and release, eCBs are quickly degraded by hydrolytic enzymes. Fatty acid amide hydrolase (FAAH) largely enables AEA degradation, with additional contribution from other enzymes such as cyclooxygenase-2 (COX-2) [30–33]. 2-AG is largely processed by the monoacylglycerol lipase (MAGL) with additional contribution from α,β-hydrolases 6 and 12 [34, 35]. The relative contribution of these enzymes into 2-AG catabolism is tissue/cell specific [34, 35].

CB receptors are presented by canonical receptors of type 1 (CB1) and 2 (CB2) that are Gi/o protein-coupled receptors exhibiting constitutive activity [36–41].

CB1 receptors are widely expressed in numerous neuronal populations [42], within glia [43, 44], and are also found in peripheral organs such as adrenal gland, heart, lung, prostate, uterus, ovary, testis, bone marrow, thymus, and tonsils [45, 46]. Presence of a "pre-nervous" eCB system operating via transient receptor potential (TRP) protein orthologs has been proposed in invertebrate echinoderms (sea urchin, starfish) [47]. However, mammalian CB1 receptor ortholog-coding genes are absent in commonly studied nonvertebrate species such as *Drosophila melanogaster* and *Caenorhabditis elegans* [48]. To some extent, the lack of a CB1 receptor ortholog in invertebrate species poses limitations on the choice of animal models for CB-related studies. However, human CB1 ortholog-coding genes are described in a variety of vertebrates including fish, amphibians, avian species, and nonhuman primates [48–50].

Although CB2 receptors have been detected in glia [51, 52] and in specific areas of the brain [52–55], they are also attributed as CB receptors of predominantly peripheral tissue locations that play a central role in immunity [42, 45, 46, 56].

Stimulation of CB receptors results in a number of signaling events, including inhibition of adenylate cyclase, activation of mitogen-activated protein kinase (MAPK) signaling, and modulation of ion channels' activity [36, 37, 57–65]. For instance, CBs target calcium, potassium, and TRP channel families [66, 67], either directly or via secondary signaling events. Besides canonical CB receptors, G protein-coupled receptors GPR55 and GPR119 are advanced as eCB sensors [68].

One must recognize that the eCB system is under constant control from various physiological stimuli, such as hormonal levels. For example, progesterone and estrogen regulate AEA level and expression of FAAH [69]. Also, FAAH in human lymphocytes is upregulated by progesterone [70]. Neither AEA nor THC modifies the level of follicle stimulating hormone; however, AEA decreases while THC increases growth hormone production [71]. In rats, prolonged glucocorticoid treatment decreases CB1 receptor density in the hippocampus [72].

ECB controls a wide range of physiological processes that include, but are not limited to, energy metabolism, inflammation, cardiovascular function, etc. [11, 12]. Thus, it comes as no surprise that a dysfunctional eCB system frequently underlies common human pathologies. The search for mechanisms of prevalent disorders, and studies of novel medications, both feed interest in eCB system functions, including its role in ontogenesis.

2.3 Cannabinoids in Fertility and Gametogenesis

ECB system components are present in virtually any reproductive tissue/organ [13, 73]. Early work described the ability of AEA to diminish sperm fertilizing capacity in sea urchins by inhib-

iting the acrosome reaction [74]. The CB2 receptor is detected in mouse Sertoli cells [75]. In human oocytes, both CB1 and CB2 receptors have been detected by means of reverse transcription polymerase chain reaction (PCR) and Western blotting [76].

The selective activation of the CB2 receptor induces progression of spermatogonia towards meiosis, and thus plays a critical role in spermatogenesis [77]. Notably, a mouse study reports a high amount of 2-AG in the caput (head) of the epididymis [78]. In this compartment, sperm cells are immobile or do not possess consistent motility. However, in the epididymis tail, 2-AG amount is lower, and such a gradient in 2-AG levels is believed to empower caudal spermatozoa with physiologically necessary motility via a CB1 receptor-mediated mechanism that involves the CB1 receptor on the sperm cell membrane [78]. In humans, reduction in viability characteristics and acrosome reaction of mature sperm is documented in response to AEA and is mediated by the CB1 receptor [69, 79].

Plasma levels of AEA fluctuate with the female cycle. The highest levels are observed during ovulation and follicular phase, while the lowest are reported during luteal phase [80–82]. ECB activity exerts an effect on hormonal production, as reported for AEA- and THC-driven downregulation of luteinizing hormone and prolactin levels in ovariectomized rats [71]. THC, however, has been found to inhibit ovulation by suppressing the plasma levels of follicle-stimulating hormone and luteinizing hormone in rats when animals were exposed to THC on the day of proestrus [83].

Clinical observations in human population concur on the risks of CB exposure during periconception [84, 85]. In particular, heavy use of marijuana and cannabis-derived psychoactive products is associated with decreased female fertility, loss of pregnancy, and embryotoxicity [86, 87]. Chronic marijuana use also decreases male fertility in humans and in animal models due to reduced testosterone production, sperm mobility and viability [88–90]. Linkage between the phenomenology of CB's effect on fertility and molecular players that enable such action represents an area of active investigation [84, 85].

2.4 Embryonic Development

Zebrafish *Danio rerio* has been emerging as an important model for studying the role of eCB and consequences of environmental CB exposure in development [91, 92]. Use of this model allows tracing of eCB system gene expression using qPCR in zebrafish embryos throughout 1–120 h post-fertilization. Analysis reveals diverse patterns of eCB system gene expression [91]. For example, low levels of *cnr1, gpr55a,* and *abhd6* expression are detected throughout development [91]. In contrast, expression levels of *cnr2, cnrip1a* and *dagl* family are relatively high [91]. Expression levels of *naaa1a* and *abhd12* are progressively decreased, while expression of *ptgs2a* and *mgll* is increased throughout development [91].

A whole-mount *in situ* hybridization study on chick embryos detects the presence of CB1 gene expression within the first-appeared neurons of the central nervous system (within hindbrain, as early as stage 10 in the chick), followed by appearance within the peripheral nervous system (ophthalmic trigeminal placode) at stage 11 [93]. After these early milestones, CB1 expression is detected in other neuronal populations, such as within vestibuloacoustic, epibranchial ganglions and dorsal root ganglia. Notably, CB1 expression is not uniquely present in neurons. For example, CB1 expression is detected in the ventral forebrain that does not produce neurons in early development [93]. In addition, CB1 expression is detected in the mesoderm. Although neurons are showing CB1 expression very early on, the expression disappears at later stages of chick development [93]. Expression of CB2, TRPV1 and GPR55 is not apparent in chick embryonic central nervous system during development [94]. At early stages of nervous system establishment, eCB system is believed to play a critical role in axonal growth and formation of synaptic connections. Indeed, treatment of chick embryo central nervous system explants with CB1 receptor antagonist AM251 results in defective axonal growth and fasciculation [94]. With regards to other component of the eCB system, DAGL α and β isoforms are widely expressed throughout

the embryonic chicken brain, while MAGL is only expressed at later stages [94].

A combination of qPCR, mass spectroscopy, immunohistochemistry and Western blotting detects the presence of eCB system components in chick and mouse embryos at stages 9–11 and E8.75, respectively [41]. These time points correspond to the pre-neuronal phase of early development [95] and represent post-coital days 22–26 of human gestation [41]. An additional study utilizing an agonist-stimulated [^{35}S]GTPγS binding assay documents G protein activation in the brain primordium of chick embryos at stages 9 to 11 in presence of CB1 receptor agonist CP-55,940 [41]. This activation vanishes following pretreatment of samples with the inverse agonist of CB1 receptor SR141716A [41].

In mammals, a balanced eCB system is crucial for successful early pregnancy. At this stage, synchronization of embryo development with uterine receptivity for implantation is central for maintaining viable pregnancy. The eCB system has been shown to play a critical role at all stages of such synchronization, starting from the development of the preimplantation embryo, its movement through oviduct, throughout implantation and placenta development. AEA synthesis and degradation enzymes NAPE-PLD and FAAH, respectively, are expressed at the two-cell stage of embryonic development [69, 96]. While the CB1 receptor is detected from the four-cell stage, CB2 is found as early as the one-cell stage of embryonic development [69, 96]. Systematic studies point at the CB1 receptor and CB1-mediated signaling as critical players in early development. In the mouse model, only CB1 receptor is present in the maternal oviduct and uterus, while both CB1 and CB2 receptors are detected in preimplantation embryo [86, 87, 97]. Cross-talk between maternal and embryonic CB systems is at the center of successful early pregnancy and development. Indeed, CB1, CB2, or double knock-out mice show asynchronous embryo development during early pregnancy [86, 87, 97]. Remarkably, the development is rescued when knock-out females are mated with wild-type males, pointing at the ability of heterozygous embryos to navigate the proper timing of development and disregard the abnormal maternal CB knock-out environment [86, 87, 97].

Movement of the preimplantation embryo through the maternal oviduct ensures a path to implantation. CB1 knock-out mice show 40% pregnancy loss at this stage [86, 87, 97]. Unlike embryo development, movement along the oviduct cannot be restored by mating CB1 knock-out females with wild type males [86, 87, 97]. Thus, at this stage, maternal factors gain critical weight over embryonic characteristics of the CB system. It is noted that embryos that fail to move to implantation site, still retain their quality, as they could be implanted into pseudopregnant recipient uteri [86, 87, 97]. Implantation of normal embryos into pseudopregnant recipient uteri of CB1 knock-out mice also renders non-developing pregnancies [86, 87, 97]. This finding provides independent verification of the critical role of *maternal* CB characteristics in controlling embryo movement through the oviduct.

Mechanistic studies reveal a closely coordinated cross-talk between CB1-mediated and adrenergic signaling in control of oviduct motility. Loss of CB1 function increases noradrenaline release from adrenergic nerve terminals and increases smooth muscle contractility via alpha-adrenergic receptor (α-AR) preventing embryo movement. In contrast, CB1 receptor stimulation relaxes oviduct muscle and promotes embryo movement [97]. Consistent with observations in animal models, reduced CB1 expression in Fallopian tubes is detected during ectopic pregnancy in humans [98].

Last but not least, CB tone is central for embryo implantation. This process is tightly controlled by hormonal release of estrogens and progesterone. While progesterone primes the uterus for implantation, estradiol and its metabolite 4-hydroxy-17β-estradiol, produced in the uterus, mediate blastocyst activation for implantation [87, 99]. It has been well established that lower levels of CB1 receptors in the blastocyst and a decrease in uterine AEA levels are crucial for sustaining the "window" of uterine receptivity [86, 87, 97–100]. In the blastocyst, CB1 expression is lower during the activated state when compared to the dormant blastocyst [101]. In

uterus, higher levels of AEA, NAPE-PLD activity, and NAPE-PLD mRNA are detected in nonreceptive uterine states compared to receptive uteri [102, 103]. Of note, AEA levels in the uterus, similar to other organs (see below), are dramatically lower than those of 2-AG [13].

Studies in animal models are closely correlated with findings in humans: elevated plasma AEA levels are detected in women with nonviable pregnancies, while lower AEA is associated with positive pregnancy outcome [82, 104–106]. Moreover, decreased FAAH activity is detected in peripheral lymphocytes of women with pregnancy failure [82, 104, 105]. Low activity of placental FAAH, together with elevated CB1 levels, characterize human miscarriages, while higher FAAH activity is detected in placentas during normal pregnancies [107].

Notably, NAPE-PLD has been shown to play a critical role in maintaining uterine AEA levels [103] despite the fact that NAPE-PLD knock-out mice are characterized by near-normal AEA levels [108]. In the uterus, the implanting blastocyst exerts an inhibitory effect on NAPE-PLD activity [103], ensuring coordination in CB signaling between embryonic and maternal sites. NAPE-PLD has also been identified in human placental tissue and is believed to mediate AEA production in the placenta [105].

Further on in the process, successful implantation relies on the differentiation and invasion of a trophoblast originating from the blastocyst trophectoderm during early stages of pregnancy [109]. Low levels of AEA promote trophoblast growth, while elevated AEA inhibits the development of embryos [14]. Exploration of potential mechanisms that underlie the dual role of AEA in early pregnancy suggests two distinct pathways. In the murine model, low concentrations of AEA activate blastocyst extracellular-signal-regulated kinase (ERK) signaling and promote implantation [87, 99]. In the murine model and sheep pregnancy, higher levels of AEA inhibit calcium mobilization, induce cell apoptosis, and inhibit blastocyst cell proliferation thereby precluding successful implantation [87, 99, 110]. In a rat model, CB1 receptor activation results in ceramide release and p38 MAPK-mediated mitochondrial stress, leading to production of reactive oxygen species and apoptosis of uterine decidual cells [111]. MAPK triggers the COX-2 oxidative metabolism of AEA. Such metabolic pathways result in the formation of prostaglandin-like compounds termed “prostamides” [112]. The COX-2 metabolic pathway for AEA is competing with the conventional FAAH pathway [113]. In addition to COX-2-mediated oxidative metabolism resulting in oxidative stress, AEA-induced apoptotic effect is also associated with NF-kB activation [112].

When compared to CB1, the role of the CB2 receptor in early stages of pregnancy is less established. However, CB2 receptor transcript has been identified in both placenta and trophoblasts [114]. During hematopoietic differentiation of murine embryonic stem cell-derived embryoid bodies, CB1 and CB2 antagonists (AM251 and AM630, respectively) induce stem cell death and inhibition of cannabinoid agonist-induced chemotaxis [115].

A study on rat species utilizing RT-PCR, Western blot, and immunohistochemistry documents the presence of CB1 and CB2 receptors, TRPV1 transcripts, and protein products of corresponding encoding genes in rat mesometrial decidua [116]. While transcripts and protein products for CB1, CB2, and TRPV1 decrease throughout pregnancy overall, the CB1 protein amount shows a remarkable spike during day 12 of rat pregnancy. The spike is not detected in the CB1 transcript [116]. This fact underscores the importance of post-transcriptional regulatory mechanisms in maintaining optimal levels of key players within the eCB system.

In addition to direct targeting of eCB receptors within embryonic and maternal tissues, the eCB system has been proposed as exerting a modulatory effect on early pregnancy outcome via immunity [24]. Indeed, eCB system components are present in immune cells [117]; interestingly, immune response has been put forth as an important player in pregnancy initiation and maintenance [118, 119]. Possible cross-talk between the eCB system, immune cells, and reproductive success resulting in a formation of an “endocannabinoid-immune-reproductive axis” requires multifaceted experimental validation [24].

2.5 Midgestation

AEA and 2-AG are produced throughout the prenatal period, but their amounts are not constant [43, 120]. AEA levels in rat brain remain low throughout perinatal period, but then gradually increase as animals reach adulthood [120]. In contrast, changes in 2-AG level show a different time course. They remain relatively constant with the exception of a significant spike at day 21 of rat gestation [43, 120]. This is a full-term pregnancy in rats and in terms of brain development milestones, corresponds to approximately 23 weeks of pregnancy in humans [121].

The CB1 receptor is critical for placental development, as *Cnr* knock-out mice have smaller placentas and higher resorption rate at mid gestation when compared to their wild-type (CB1 receptor-containing) counterparts [122]. With regards to fetal tissue, CB1 receptor transcript is detected in rat embryo neural tube structures at E11 [114]. CB2 receptor messenger RNA is detected in rat embryonic liver as early as at E13 [114].

The occurrence and functional characterization of the eCB system has been actively studied within the developing nervous system [55, 123]. Establishment of critical structural components and connectivity within neuronal networks relies on several critical events, such as neuronal progenitor cell proliferation and differentiation, neuronal migration to target regions, and formation of synapses. All aforementioned events are connected with the functionality of eCB system components at various levels of resolution as shown in different experimental models [124].

Immunofluorescence labeling of rat fetuses detects the presence of CB1 receptors in E12.5-16.5 in migrating post-mitotic neurons during corticogenesis [125, 126]. Moreover, prenatal exposure to CB1 receptor agonist WIN 55,212-2 (0.75 mg/kg) via daily subcutaneous (s.c.) delivery to pregnant dams results in significant increases in the number of post-mitotic neurons [126]. However, this increase is not accompanied by a corresponding increase in *gamma*-aminobutyric acid (GABA)-positive immunostaining. In contrast, WIN 55,212-2 exposure increases immunofluorescence associated with T-box transcription factor Tbr2 that is characteristic of progenitor cells destined for glutamatergic development [126]. In contrast, the marker of post-mitotic glutamatergic neurons Tbr1 responds with transient decreases at E12.5 and E14.5 in brain samples from WIN 55,212-2-exposed fetuses [126]. Thus, the CB1 receptor plays a critical role in neuronal migration and corticogenesis. Variations in fetal CB1 receptor activity and functioning of the eCB system result in a complex reshaping of neuronal development, thus affecting formation of neuronal networks [127]. Studies in knock-out mouse lines demonstrate that lack of CB1 and CB2 receptors leads to impairment of neural progenitor cell proliferation [128, 129]. Mice lacking the CB1 receptor are characterized by diminished cortical progenitor cell proliferation and astrogliogenesis [128, 130]. Notably, work on cultured cell lines indicates that CB1 receptor activation can induce either neurite growth or retraction, depending on the CB1 receptor activation-triggered downstream signaling pathway [55, 131, 132]. Despite the fact that experimental probing of the CB1 receptor impact on the direction of neuronal development renders somewhat conflicting results, the overall picture seems to support positive correlations between CB1 receptor activation and neuronal cell proliferation and migration [124]. Yet, diminished CB1 receptor activity would likely favor cell differentiation, formation of a neuronal phenotype, and synaptogenesis [124].

Analysis of the CB1 receptor expression pattern in human fetuses during midgestation (17–22 weeks) reveals region-specific presence of CB1 receptor mRNA: while CB1 receptor expression is high in limbic structures, only moderate levels are detected in cerebral cortex, thalamus, medial/ventral striatum, and subventricular zone [133]. This expression profile remains unaltered by cannabis exposure *in utero* [133]. However, maternal cannabis use is associated with a significant decrease in dopamine receptor subtype 2 (D2) mRNA in the amygdala of male, but not female fetuses [133, 134]. D2 mRNA decrease is also detected in striatum; however,

dopamine receptor subtype 1 (D1) expression remains unchanged [133, 134]. In addition, prenatal rat THC exposure leads to fluctuations in brain mRNA levels of the enzyme tyrosine hydroxylase that represents a rate-limiting step in dopamine synthesis [135]. This effect exhibits marked sexual dimorphism, the latter being characteristic of alterations in dopaminergic system by prenatal exposure to cannabinoids [43].

With regards to the opioid system, prenatal cannabis exposure is associated with increased mu opioid receptor expression in amygdala [133]. Yet, proenkephalin RNA levels are decreased in fetal striatum from cannabis-using mothers, while prodynorphin levels remain unchanged [133].

In addition to alterations in dopaminergic and opioid systems, prenatal and postnatal exposure to THC in a rat model (from gestational day 5 to postnatal day 20) is reported to result in a decreased immunoreactivity against the GluR1 subunit in Bergmann glial cells and the GluR2/3 subunit in Purkinje neurons when evaluated at postnatal day 20 [136]. These changes persist after THC withdrawal at postnatal days 30 and 70 [136]. Moreover, the expression of glial (GLAST) and neuronal (EAAC1) glutamate transporters in astroglial cells and Purkinje neurons, respectively, is decreased in THC-exposed rat offspring compared to saline-treated controls [137].

The eCB system in the developing brain also represents a target for a drug of abuse other than cannabis itself – alcohol [138, 139]. Data from our laboratory show alcohol-induced decreases in blood velocity in the fetal middle cerebral artery during baboon maternal alcohol intoxication during second trimester-equivalent of human pregnancy [50, 140]. This drop is consistent with fetal cerebral artery dilation, and the latter is replicated using *in vitro* pressurized arteries from fetal baboons [50]. Notably, alcohol-induced (63 mg/dL ethyl alcohol) dilation of fetal cerebral arteries is blocked in the presence of AM251 in a mixture with AM630 (Fig. 2.1) [50]. The fact that an alcohol effect *in vitro* is sensitive to CB receptor block, and is mimicking the *in vivo* scenario, strongly suggests an active eCB system within fetal cerebral arteries of nonhuman primates.

In human brain, the CB1 receptor is immune-detected in the cortical plate as early as gestational week 9 [141]. It is notable that in the case of brain malformation, CB1 receptors are still present in dysplastic neurons [141]. By the second trimester (20 weeks of human gestation), the CB1 receptor mRNA is spiked within hippocampal CA region and basal nuclear group of the amygdaloid complex [142]. Notably, the adult brain, cerebral cortex, caudate nucleus, putamen and cerebellar cortex are also characterized by high mRNA levels of CB1, in addition to high levels in hippocampus and amygdala [142]. Thus, CB1 receptor occurrence is brain region-specific.

With regards to the relative distribution of CB1 and CB2 receptors across cell types, a differential expression pattern is documented at early stages of development. For example, while CB1 receptors are traced to astrocytes, CB2 receptors are present in microglia [141].

Prenatal marijuana use in humans does not alter fetal growth rates, evident from the analysis of human fetuses aborted at midgestation (17–22 weeks of pregnancy) [143]. However, there is a significant reduction in fetal foot length and body weight in the group of marijuana-exposed fetuses when compared to controls. Moreover, fetal foot length growth is inversely correlated with the amount and frequency of marijuana use reported by the mothers [143]. The consequences of maternal marijuana use are long-lasting. For instance, prenatal marijuana exposure significantly correlates with the age of onset and frequency of marijuana use among 14-year-old teens [144]. Overall, the early appearance, wide distribution, differential expression pattern, and physiological function of eCB system components support the hypothesis of a critical role of eCB signaling in physiology and pathology during midgestation.

2.6 Neonatal and Postnatal Development

At birth, eCB system components are widely distributed in maternal and fetal tissues [14, 49, 145]. While AEA remains low during normal

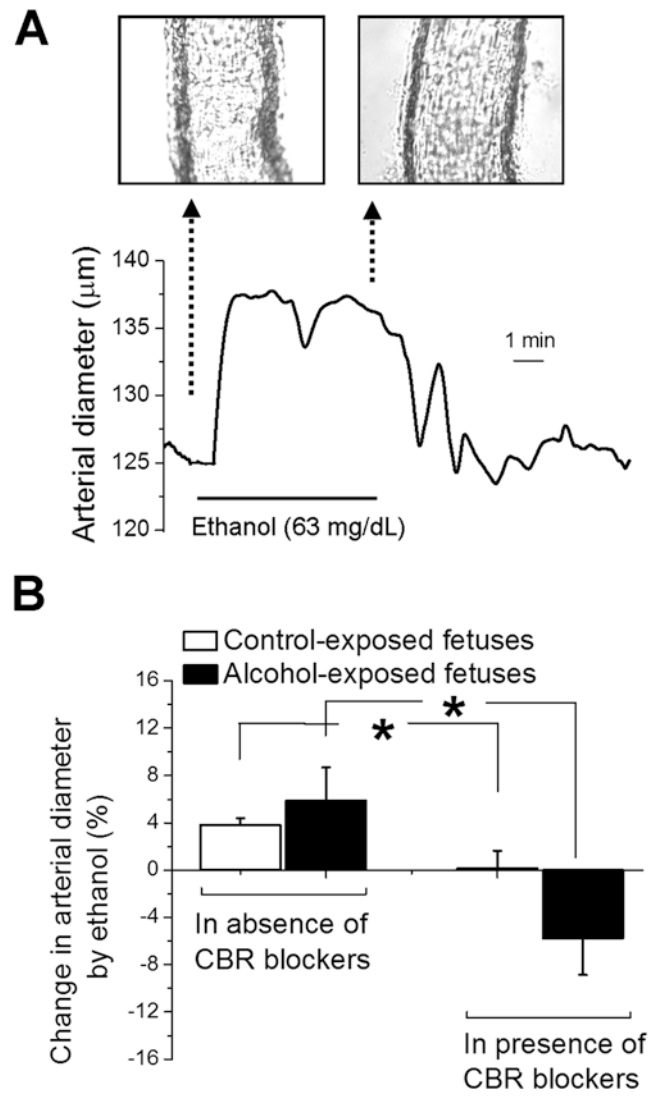

Fig. 2.1 Ethanol-induced dilation of fetal cerebral arteries is mediated by CB receptors. (**a**) Original diameter trace showing fetal cerebral artery dilation in response to 63 mg/dL ethanol. (**b**) Averaged data showing diminished ethanol-induced dilation of fetal cerebral arteries in presence of CB1 and CB2 receptor blockers AM251 and AM630, respectively. ∗Statistically significant difference ($P < 0.05$ by t-test). With modifications from [50]

pregnancy [82], its level increases dramatically as labor approaches [14]. Upon delivery, eCBs continue to play a central role in maternal-fetal interaction, as 2-AG is present in maternal milk [13]. 2-AG in milk exceeds that of AEA by 100–1000-fold and serves as a critical contributor into the initiation of milk suckling [146, 147]. Indeed, injections of CB1 receptor antagonist SR141716 (5–20 mg/kg s.c.) into newborn but not older mouse pups drastically reduces milk ingestion and pup growth [146]. This effect is not specific to the particular antagonist, as it has been replicated by another CB receptor antagonist VCHSR [13]. Interestingly, when CB receptor antagonist-treated mouse pups are introduced to a dish with a milk/cream mixture, they are able to lick and ingest this food [13]. Thus, CB1 receptor block is specifically altering suckling behavior, presumably via alterations in synaptogenesis required for neuromuscular coupling within tongue tissue [13].

Around early postnatal development (postnatal day 5), a peak in rat brain 2-AG level is observed when compared to prenatal and adult 2-AG content [13, 148]. In contrast to the bell-shaped ontogeny of 2-AG, AEA levels in rat brain progressively increase from birth into adulthood [13, 148]. Levels of 2-AG detected in

rat brain are much higher than those of AEA in rat brain: 2000–8000 pmol/g of tissue versus 3–6 pmol/g of tissue, respectively [43, 120]. However, regional patterns of 2-AG and AEA ontogeny do not always follow net levels, this variability being further complicated by a gender-specific component [42].

With regard to CB receptors, rodent and human brain CB1 receptor levels in fetal and juvenile tissues are generally higher than in adulthood [42, 120, 149, 150]. Yet, CB1 receptor distribution also shows region-specific variability with the predominant location within fetal white matter [148, 149], while CB1 expression in adulthood is predominantly located within grey matter [42, 148].

In a study utilizing C57BL/6 mouse strain, it was shown that the amount of CB1 receptor and its co-localization with GABA and glutamatergic synapses in the visual cortex is modulated by developmental plasticity and by visual input [151]. In particular, immunostaining against CB1 receptor reveals differential distribution of this protein across various layers of mouse visual cortex. The highest intensity of anti CB1 receptor staining is detected in layers II/III and VI. Moreover, CB1 receptor co-localization with presynaptic GABA transporter is detected by vesicular GABA transporter (VGAT)-positive staining and with vesicular glutamate transporter (VGluT)-positive staining. The former is attributed to localization of CB1 receptor within nerve terminals of inhibitory neurons, while the latter is associated with excitatory neurotransmission [151]. Dark rearing of mouse pups up to P30 results in the overall decrease of CB1 receptor-associated staining, decrease in co-localization of CB1 receptor with VGluT in deep layer of visual cortex, but produces an increase in co-localization of CB1 receptor with VGAT in this layer [151]. Notably, naturally uneven distribution of immunostaining signal among visual cortex layers remains unaltered by rearing conditions. Moreover, dark rearing until P50 does not modify the overall level of CB1 receptors, suggesting that visual input only exerts a modulatory role in the developmentally-programmed trajectory of CB1 receptor amounts in the deep layer of visual cortex [151]. At postnatal day 100, the overall amount of CB1 receptor in mouse primary visual cortex as detected by Western blotting, is significantly higher than the amount at an earlier postnatal age (P20) [151].

Targeting of the eCB system by administration of five daily AEA injections (20 mg/kg s.c.) to newborn mice from day 6 of age does not result in any effects on open field performance of the progeny until 4 weeks of age [152]. However, from 40 days of age, the offsprings from AEA-treated dams are characterized by a decrease in open field activity, catalepsy, and hypothermia [152]. It is noteworthy that a fully functional eCB system does not seem to emerge until adulthood, as acute challenge of mouse pups with AEA (20 mg/kg i.p.) does not produce anticipated analgesia and motor depression [152]. This outcome could be explained by the fact that the effects of CB challenge require complex interplay between several components of the eCB system that are only reaching their final levels and patterns of distribution in adulthood [42, 43].

THC exposure during the perinatal period (2.5–5 mg/kg per os, from gestational day 15 to postnatal day 9) results in an increased rate of vocalization in 12-day-old rat pups [153, 154]. However, acute treatment of 11–13-day-old rat pup with CB receptor agonist (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP 55,940) shows a dose-dependent decrease in ultrasonic vocalizations, with a 1000-μg/kg CP55,940 causing an almost complete shutdown of vocalized calls [155]. CB receptor antagonist SR 141716A (20 mg/kg) reverses this effect [155]. Thus, there are critical windows of vulnerability to eCB system targeting that enable differential outcomes of eCB challenge on physiology/behavior.

In addition to nearly immediate effects, targeting of eCB system during *in utero* development has long-lasting consequences on developmental trajectories [156–159]. For example, treatment of rats with cannabinoid receptor agonist HU-210 (25 μg/kg throughout gestation and lactation) results in decreased corticosterone levels in adult male progeny [160].

Altered gene expression in progeny born to THC-treated rat dams has been described for 112 brain genes [161], including elevated pre-proenkephalin mRNA expression in the nucleus accumbens and central and medial amygdala [162], as well as modifications of cortical genes related to glutamatergic system and also to noradrenergic transmission [163]. Alterations in gene expression are associated with a decrease in the cortical extracellular levels of both neurotransmitters [163]. Within the serotoninergic system, perinatal THC exposure (5 mg/kg body weight from gestational day 5 through postnatal day 24) leads to a reduced serotonin level in rat brain samples [164].

Prenatal exposure to CB1 receptor agonist WIN55,212-2 (0.5 mg/kg s.c.) disrupts memory retention in 40- and 80-day-old rat progeny [165]. This memory impairment is correlated with alterations of hippocampal long-term potentiation (LTP) and glutamate release. LTP in hippocampal CA3-CA1 synapses decays faster in brain slices of progeny that was prenatally exposed to WIN55,212-2 when compared to a control group [165]. The effect is specific to a particular parameter of LTP, as post-tetanic and short-term potentiation is similar in WIN55,212-2-exposed and control groups [165]. *In vivo* microdialysis studies detect a decrease in basal and potassium-induced glutamate levels in the cerebral cortex of adolescent and adult rats born to WIN55,212-2-treated dams (0.5 mg/kg s.c.) when compared to a vehicle-treated group [166]. This decrease is reported to be associated with a WIN55,212-2-triggered increase in glutamate uptake through overexpression of GLT1 and EAAC1 glutamate transporter subtypes, as demonstrated in rat frontal cerebral cortex [167]. Moreover, while WIN55,212-2 treatment (0.1 mg/kg i.p.) increases dialysate glutamate levels in adult rats, blockade of the CB1 receptors with the selective antagonist SR141716A only ablates the WIN55,212-2-induced increase of glutamate in a control group of rats, but not in rats that were prenatally exposed to WIN55,212-2 [166]. It is noteworthy that, while prenatal stimulation of eCB system does not generally result in severe fetal malformations or exert apparent toxicity, prenatal exposure to WIN55,212-2 is reported to be associated with impaired neuronal growth/neurite branching [168]. In synthesis, *in utero* exposure to exogenous cannabinoids reshapes the eCB system and its communication with major neurotransmission systems [163, 168, 169].

Outside the central nervous system, consequences of exposure to cannabinoids during the perinatal period include long-term alterations in cytochrome P-450 levels [170], enkephalin and norepinephrine sensitivity in vas deferens [171], and neurochemical response to stress [43, 172].

Behavioral consequences of prenatal THC exposure may last into adulthood. Following THC exposure (2 mg/kg twice daily, s.c. gestational days 1-22, postnatal days 2–10), adult (90-day-old) male progeny exhibits decreased time in the inner part of the open field and increased investigation time in the test of social interaction [173]. Moreover, rats that were exposed to THC (0.15 mg/kg, from gestational day 5 to postnatal day 2) exhibit higher heroin-seeking activity at postnatal day 62 [162]. Likewise, daily oral THC administration (5 mg/kg, starting from gestational day 5 throughout postnatal day 24 at weaning) modifies rat brain mu opioid receptor density in a region- and gender-specific manner and facilitates morphine self-administration behavior [162, 174].

Alterations in the eCB system during neonatal and perinatal periods can be achieved not only by exposure to cannabinoids, but also by common stressors such as maternal deprivation. For example, maternal deprivation of neonatal rat pups leads to increased 2-AG content in male hippocampus [175]. Maternal deprivation for 24 h at postnatal day 9 induces a significant increase in DAGLα but not DAGLβ levels upon immunostaining of hippocampi from rat male and female progeny (13-day-old pups) [176]. Maternal deprivation also decreases CB1 receptor expression while CB2 receptor levels are increased [177]. The former phenomenon (decreased CB1 receptor expression and lack of CB1 receptor function) in turn increases progeny's susceptibility to stress [178]. MAGL protein and mRNA levels are decreased in deprived males [176]. A similar paradigm of maternal deprivation (24 h,

postnatal days 9–10) renders increased expression of eCB system component-coding genes in frontal cortex and hippocampus of adolescent (postnatal day 46) male and female rat progeny, respectively [179]. Sexual dimorphisms observed within eCB system distribution and function has been extensively discussed in recent literature [180–184]. Based on studies showing a link between stress and the eCB system, it has been proposed that cannabinoids serve as modulators of the hypothalamic-pituitary-adrenal axis; this modulatory effect may be critical in shaping brain maturation during development [185].

The role of nutrition as another environmental factor that shapes eCB system development has been studied in a model of variable postnatal nutrition in cross-fostering mouse dams [186]. In this study, mouse pups are assigned to new mothers upon birth, and different pup-to-mother ratios are used to regulate nutritional intake (3, 6, or 10 pups per mother). Conceivably, groups with 3 pups per mother show higher growth measures when compared to 6 and 10 pups/mother groups [186]. ECB system components evaluated at postnatal day 50 show progressive decreases in FAAH and MAGL gene expression in liver as pup-to-mother ratio was increased [186]. Visceral adipose tissue does not render significant changes in FAAH gene expression level as a function of early postnatal nutrition, yet MAGL expression level is decreased with increased pup-to-mother ratio. Moreover, expression of NAPE-PLD and DAGLα in visceral adipose tissue is also progressively decreased as pup-to-mother ratio is increased [186]. This study promotes the peripheral eCB system as a sensor of early postnatal nutrition. Conceivably, maternal high-fat diet (≈29% of calories from fat) in a rat model results in profound modification of the eCB system protein levels of progeny [187, 188]. In particular, male offspring of mothers subjected to high-fat diet exhibit significant increase in hypothalamus CB1 receptor protein level, while females show increased CB2 receptor protein level in this brain region when evaluated at birth [188]. In brown fat tissue, a maternal high-fat diet results in increased FAAH level in male and increased CB2 receptor protein level in female progeny, respectively [188]. However, unlike the hypothalamic CB1 receptor, the brown fat tissue CB1 receptor is decreased in male progeny from mothers fed a high-fat diet [188]. These findings reiterate the tissue- and gender-specific nature of the eCB system, showing sensitivity to modulation by exogenous interventions.

In addition to nutrition, alcohol exposure in rodent models emerges as another critical modulator of eCB system function during perinatal/early postnatal development. Indeed, ethyl alcohol treatment of C57BL/6J mice at postnatal day 7 (2.5 g/kg s.c. twice) increases AEA levels [189]. Unlike AEA, 2-AG level remains unchanged due to alcohol-induced up-regulation of both DAGLβ and MAGL activities [190]. Alcohol treatment also results in up-regulated CB1 receptor protein expression in the cortex and hippocampus [189]. Moreover, such alcohol treatment triggers neurodegeneration that is absent in CB1 receptor knock-out mice [189]. These findings reinforce the concept of a cross-talk between the eCB system and the molecular targets of another drug of abuse, alcohol [138, 139].

Data from nonhuman primates are consistent with reports on predominant abundance of 2-AG over AEA: mass spectroscopy analyses of baboon samples from our laboratory show ≈30 times higher 2-AG levels when compared to AEA in the blood circulation of near-term fetuses and their corresponding mothers (Fig. 2.2). Similarly, the relative abundance of 2-AG in baboon cerebral arteries is higher than AEA in both mothers and near-term fetuses (Fig. 2.2). Data from our laboratory also present evidence of dynamic changes in CB1 receptor function within baboon cerebral arteries during development. In particular, application of AM251 (1 μM) to *in vitro*-pressurized branches of middle cerebral arteries, harvested from fetal baboons at the end of second trimester equivalent of human pregnancy, renders artery constriction (Fig. 2.3) [50]. However, identical pharmacological probing results in artery dilation in near-term fetal and their maternal cerebral artery segments (Fig. 2.3).

With regards to other nutritional interventions, maternal high-fat diet (12% fat) during preg-

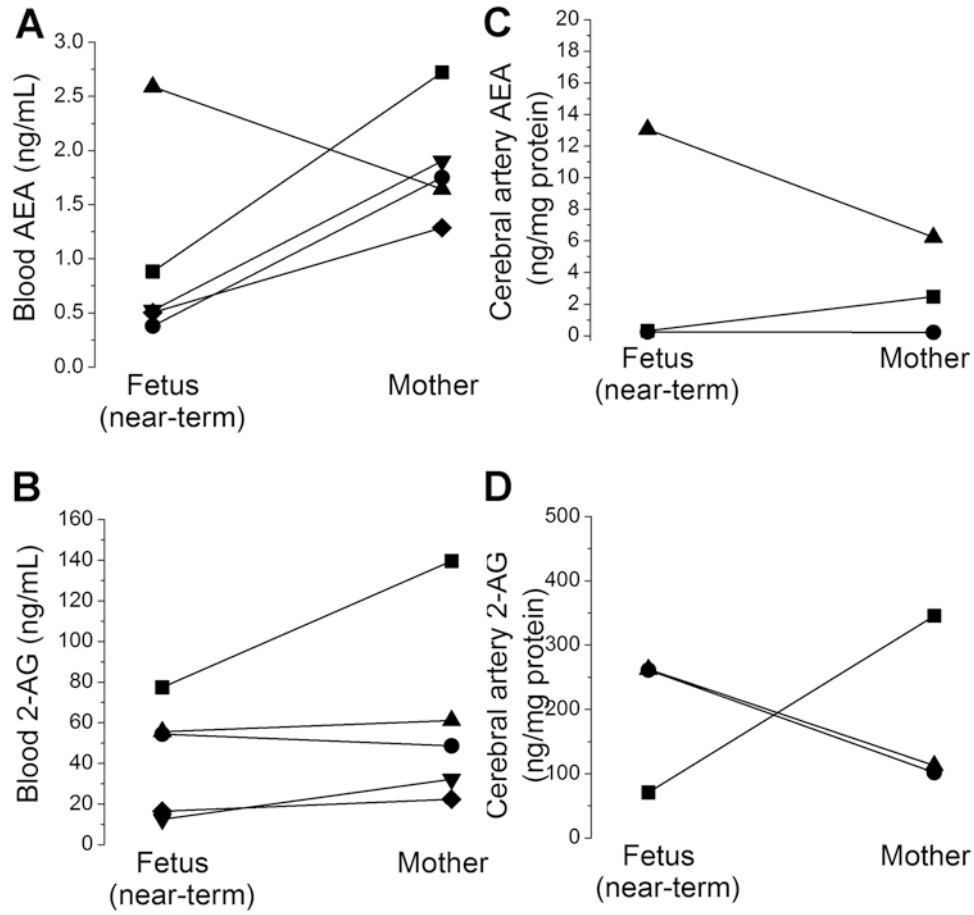

Fig. 2.2 Blood and tissue eCB levels in baboon (*Papio spp.*). (**a**) AEA levels in circulating blood of near-term fetal baboons and their corresponding mothers. Here and in B-D, data from a given fetus-mother pair are connected by a solid line. Different symbols correspond to data-points from separate fetus-mother pairs. (**b**) Circulating 2-AG levels in near-term fetal baboons and their corresponding mothers. (**c**) AEA levels in cerebral artery lysates of near-term fetal baboons and their corresponding mothers. Here and in D, the eCB reading within each sample was normalized to protein amount. (**d**) 2-AG levels in cerebral artery lysates of near-term fetal baboons and their corresponding mothers

nancy leads to decreased fetal baboon circulating 2-AG levels near–term, independent of fetal gender [191]. Interestingly, maternal baboon circulating 2-AG levels are increased by high-fat diet. In addition to modification of fetal circulating 2-AG level, fetal hepatic CB2 receptor, FAAH, and COX-2 expression values are lower in fetuses of both genders from the high-fat group. Within this group, DAGLα expression is selectively decreased in male fetuses [191].

In humans, a qPCR study on post-mortem samples from the middle frontal gyrus area of the donors between 39 days and 49 years of age unveils complex developmental trajectories for critical players within the eCB system [192]. In particular, expression of the CB1 receptor is progressively decreasing from infancy into adulthood with a slight local peak at toddler age. A similar profile is followed by MAGL [192]. In sharp contrast, NAPE-PLD, FAAH, and ABHD6 are progressively increased from infancy into

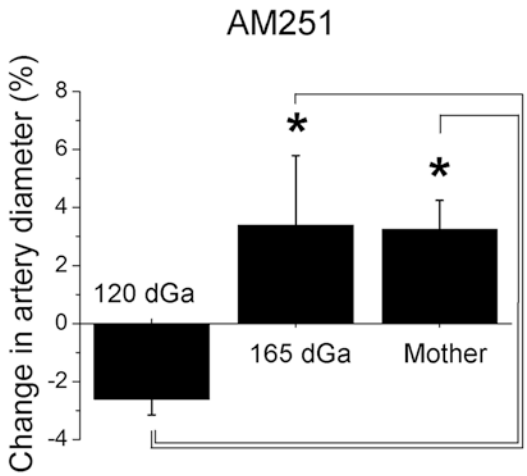

Fig. 2.3 Averaged Changes in cerebral artery diameter were assessed by probing of *in vitro* pressurized branches of fetal and maternal middle cerebral arteries harvested from baboons (*Papio spp.*). Effect of AM251 (1 μM) is presented as a percent change in artery diameter from pre-AM251 level. ∗Statistically significant difference ($P < 0.05$ by one-way ANOVA with Tukey post-test). dGa: days of gestational age

adulthood. Finally, DAGLα mRNA shows a bell-shaped pattern with the peak around school age [192].

Consequences of *in utero* cannabis exposure on developmental trajectories in humans have been studied in several longitudinal cohort studies, and are found to be long-lasting. Several early reports describe increased tremors, startle response, and poor visual responsiveness of cannabis-exposed infants [193]. These characteristics are apparent in the absence of effect on morphometric (growth) parameters, such as weight and head circumference [194]. Notably, characteristics of infants born to cannabis users disappear by 30 days of age [193], demonstrating a remarkable plasticity that allows the development of compensatory measures in response to cannabis-driven alterations in physiological processes. Later in childhood, however, prenatal cannabis exposure negatively reflects on attention processes [195] and cognitive performance within executive function [154, 196]. *In utero* CB exposure leads to more aggressive behavior and attention problems in 18-month-old girls [17]. Moreover, maternal light-to-moderate marijuana use during pregnancy is associated with deficits in Wide Range Achievement Test-Revised reading and spelling scores and a lower rating on the teachers' evaluations of the children's performance in 10-year-olds [197]. Functional magnetic resonance imaging on eighteen-to-twenty two-year-old adults that were prenatally exposed to cannabis demonstrates alteration in visuospatial working memory processing [198]. Increased impulsive behavior has also been reported as a consequence of prenatal cannabis exposure [133, 199]. Yet, maternal marijuana use does not affect growth parameters of the progeny in puberty [200].

2.7 Adolescence

Although exact age limits of the adolescent period are poorly defined, adolescence usually refers to as a period of pubertal maturation [201]. Adolescence is a period of active brain development, representing the transition between childhood and adulthood [201, 202]. It is also often characterized by cannabis use [202, 203]. Considering that eCB system controls several fundamental processes of neuronal and glial development, such as cell proliferation, migration, and differentiation [1, 204, 205], alterations in the eCB system during adolescence are expected to impact neuronal maturation.

Studies in rat species describe a peak of CB1 receptor expression in prefrontal cortex, limbic, striatal, and midbrain areas during adolescence (postnatal days 25–29 in rats), this peak later declines to adult levels [185, 206, 207]. Consistent with this peak in CB1 receptor expression, studies in rodent models show that the adolescent brain is particularly vulnerable to CB stimulation when compared to adults. Rats that were exposed to THC (1.5 mg/kg i.p. every third day) at postnatal days 28 (early adolescence) to 49 (late adolescence) show profound alterations in the endocannabinoid levels in prefrontal cortex and nucleus accumbens regions [208]. Adolescent rats that were repeatedly exposed to THC (5 mg/kg i.p. starting from postnatal day 28) show less vocalization during the THC administration pro-

cedure when compared to adults (starting from postnatal day 60) [209]. This result suggests that THC is less aversive to adolescent rats. Also, after THC withdrawal, THC-exposed adolescent rats exhibit impaired object recognition memory. Proteomics analysis of hippocampal samples detects significant changes in 27 proteins following THC exposure in adolescence, compared to only 10 proteins in adults. The former are represented by oxidative stress/mitochondrial and cytoskeletal targets [209]. This finding confirms the greater vulnerability of the adolescent brain to cannabis exposure compared to the adult brain. Similarly, synthetic cannabinoid agonist WIN 55,212-2 (1.2 mg/kg i.p.) treatment of pubertal rats results in poorer recognition memory when compared to identical treatment of adult rats [210]. Also, working memory impairment and a significant decrease in social interaction is reported in female rats in response to CB receptor agonist CP 55,940 administered daily for 21 days at 150, 200, and 300 μg/kg i.p. for 3, 8 and 10 days, respectively [211]. In addition, in a study on rats using eCB system stimulation with CP 55,940, it is concluded that chronic CB exposure leads to long-term memory impairments and increased anxiety, irrespective of the age at which drug exposure occurs (either at the perinatal period, adolescence, or young adulthood) [212].

In a different experimental paradigm, adolescent male rats were administered AEA hydrolysis inhibitor URB597 (0, 0.1, or 0.5 mg/kg/day at postnatal days 38–43) [213]. Following this treatment, a decrease in CB1 receptor is detected in caudate-putamen, nucleus accumbens, ventral tegmental area, and hippocampus, while an opposite effect is observed in the locus coeruleus [213]. Similar treatment with FAAH inhibitor URB597 (0.3 mg/kg i.p.) reverts depressive-like symptoms induced by adolescent exposure to THC in female rats [214]. Moreover, MAGL inhibitor JZL 184 ameliorates a deficit in presynaptic long-term plasticity triggered by exposure of adolescent mice to WIN 55,212-2 [215]. These findings demonstrate the possibility of persistent attenuation in AEA and 2-AG levels as an underlying cause of neuronal deficits associated with adolescent THC exposure.

In addition to alterations within the eCB system, adolescent exposure to CB stimulation interferes with sensitivity to other drugs of abuse. For example, exposure of adolescent rats to THC (1.5 mg/kg i.p., every third day during postnatal days 28–49) results in increased sensitivity to opiates and heroin self-administration in adulthood (postnatal days 57 and 102) [216]. Mu opioid receptor GTP-coupling is potentiated in mesolimbic and nigrostriatal brainstem regions in THC-exposed animals, with mu opioid receptor function in the nucleus accumbens shell being specifically correlated with heroin intake [216]. Thus, the consequences of eCB alteration during adolescence are likely region-specific.

There is also a gender-specific component in responses of the adolescent brain to eCB stimulation. THC administration to rats twice a day (2.5 mg/kg at postnatal days 35–37, 5 mg/kg at postnatal days 38–41, and 10 mg/kg at postnatal days 42–45, i.p) results in significant decreases of CB1 receptor level and CB1/G-protein coupling in the amygdala, ventral tegmental area, and nucleus accumbens in females [217]. However, males only exhibit these alterations in the amygdala and hippocampus. Additional neuronal consequences of adolescence THC exposure include dendritic atrophy and decreases in markers of neuroplasticity [218, 219]. At the behavioral level, females present behavioral despair in a forced swim test, which is accompanied by anhedonia in a sucrose preference test [217]. Males only present anhedonia [217].

Ontogeny of CB1 receptor expression in humans somewhat differs from that of rats. In particular, a gradual increase in CB1 expression in the human brain towards adulthood is reported [149]. The function of these receptors is successfully assessed by [^{35}S]GTPγS autoradiography. Moreover, high levels of CB receptors are detected during prenatal development in fiber-enriched areas, these areas being devoid of CB receptor signal in adulthood [149]. Several other reports on human dorsolateral prefrontal cortex samples also fail to detect a rodent-characteristic peak in brain CB1 receptor level in adolescence [192, 220]. While species-specific expression pattern of the CB1 receptor and, perhaps, eCB

system function, should be considered as a primary cause for such discrepancy, it has been proposed that such inconsistency between reports might reflect overall instability of the developing eCB system [42].

Computer-assisted attention testing that addresses visual scanning, alertness, divided attention, flexibility, and working memory in humans detects a significant impairment in visual scanning reaction times in early-onset (before age 16) cannabis users but not in late-onset (after age 16) users [221]. This outcome suggests that the brain in early adolescence is particularly vulnerable to alterations in the eCB system upon exposure to exogenous cannabis. Adolescent cannabis use has also been suggested to exert a modulatory effect over anxiety-related behaviors and depression [154]. In the latter case, a link between adolescent cannabinoid exposure and serotonergic hypoactivity has been proposed [222].

2.8 Concluding Remarks

Ample data from invertebrate and vertebrate species, including humans, document the complex roles of the eCB system in development. Gender, timing, and pharmacological routes of eCB challenge are all-important in establishing the final trajectory of eCB ontogenesis and its role in physiology and pathology.

Continuous growth in proposed CB-based pharmacological remedies and increasing THC content in recreational cannabis preparations [223, 224] call for concerns over incomplete understanding of eCB function. Despite the fact that the eCB system represents an attractive therapeutics target for various conditions that represent developmental pathology, the major difficulty in developing eCB-targeting pharmacotherapy arises from the complexity of the eCB system. We are far from finalizing complete characterization of all eCB components, therefore the process of fully characterizing the eCB system continues.

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