Endocannabinoid Signaling in Early Neurodevelopment: Effect of Gestational Δ^9-THC Exposure

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Abstract: Marijuana is the most commonly abused illicit drug by pregnant women in the world. Its psychoactive cannabinoid, Δ^9-tetrahydrocannabinol, crosses the placenta and accumulates in the fetus, potentially harming its development. In humans, marijuana use in early pregnancy is associated with an increased risk for miscarriage, anencephaly, as well as subtle neurodevelopmental defects in the offspring, including ADHD, psychiatric disorders, learning disabilities and memory impairment. Little is known about the mechanisms by which marijuana exert its detrimental effects on the developing embryo, although recent evidence points to the possibility that Δ^9-tetrahydrocannabinol might interfere with an endogenous endocannabinoid system present in the embryo during early stages of pregnancy. Here we review our current knowledge on evidence for an endocannabinoid system in early embryonic development and discuss a possible mechanism of action for Δ^9-tetrahydrocannabinol in early pregnancy.

Keywords: Marijuana, *Cannabis L. Sativa*, THC, Endocannabinoid system, CB_1 Receptor, Anencephaly, Neurogenesis, Brain development, Embryo, Marijuana legalization and rescheduling.

1. PREVALENCE OF CANNABIS USE IN PREGNANT WOMEN

Marijuana (*Cannabis L. Sativa*) is the most widely used psychoactive substance in the world since it is estimated to be consumed by 200-300 million people worldwide [1-3]. In the USA alone and within the year 2002, it was used by 10% of women aged 15-44 years [4], and 25.7% of women within the 18-25 age group [5]. Rates of newborns prenatally exposed to marijuana in 1990, were estimated at levels from 3 to 20%, which indicates that every year in the US alone, women give birth to between 125,370 and 835,800 children prenatally exposed to marijuana [6]. Its psychoactive constituent Δ^9-THC [7, 8] crosses the placental barrier and accumulates in foetal tissue and amnionic fluid, reaching its highest concentration in the foetal brain [9-11], and thus has the potential for harming embryonic development [12].

The potentially harmful effects of marijuana use during pregnancy are aggravated by the fact that the potency of marijuana preparations, in terms of Δ^9-THC content, has increased almost 8-fold since 1970, when the content of Δ^9-THC in marijuana was 1.25% [13]; Δ^9-THC content in marijuana now averages 8.12%, reaching up to 37.2% in marijuana preparations derived from dried flowering buds due to sophisticated cannabis cultivation methods [14] (Fig. **1A-C**). Similarly, Δ^9-THC content in hashish (dried cannabis resin and compressed flowering buds) currently averages 28.19%, compared to 2.3% in the 1970s (table 9 in [14]), with some hashish samples containing up to 66% Δ^9-THC [14]. In the last 25 years there has been an alarmingly steady increase in the availability of marijuana containing high Δ^9-THC content (9.0% or higher) versus low content (less than 3%) [14] (Fig. **1D**): In 1989, only 1.8% of marijuana samples seized in the U.S. contained high Δ^9-THC content (compared to 52.6% samples containing low Δ^9-THC content); By contrast, in 2004 and 2007, approximately 28% and 37% seized samples contained high Δ^9-THC respectively [14]. Furthermore, marijuana is now becoming the focus of intense biotechnological research, opening new avenues for biotechnological production of cannabinoids [3]: Initial steps in this direction have already been undertaken with the synthesis of Δ^1-THC through the use of yeast-based expression systems [15] and transgenic tobacco hairy roots [16] (Fig. **1E**); Δ^1-THC can be readily transformed into psychoactive Δ^9-THC through heat decarboxylation [17]. Most alarmingly, with the accessibility of the Internet, Cannabis is now readily available for seeding *via* internet [*e.g.* 18], cultivation methodology and production of marijuana and hashish through online [19-22] and/or on site courses in the U.S. and elsewhere [23].

2. Δ^9-THC AND THE ENDOCANNABINOID SYSTEM

In the adult central nervous system CNS, Δ^9-THC exerts its psychotropic effects by activating presynaptic $G_{i/o}$

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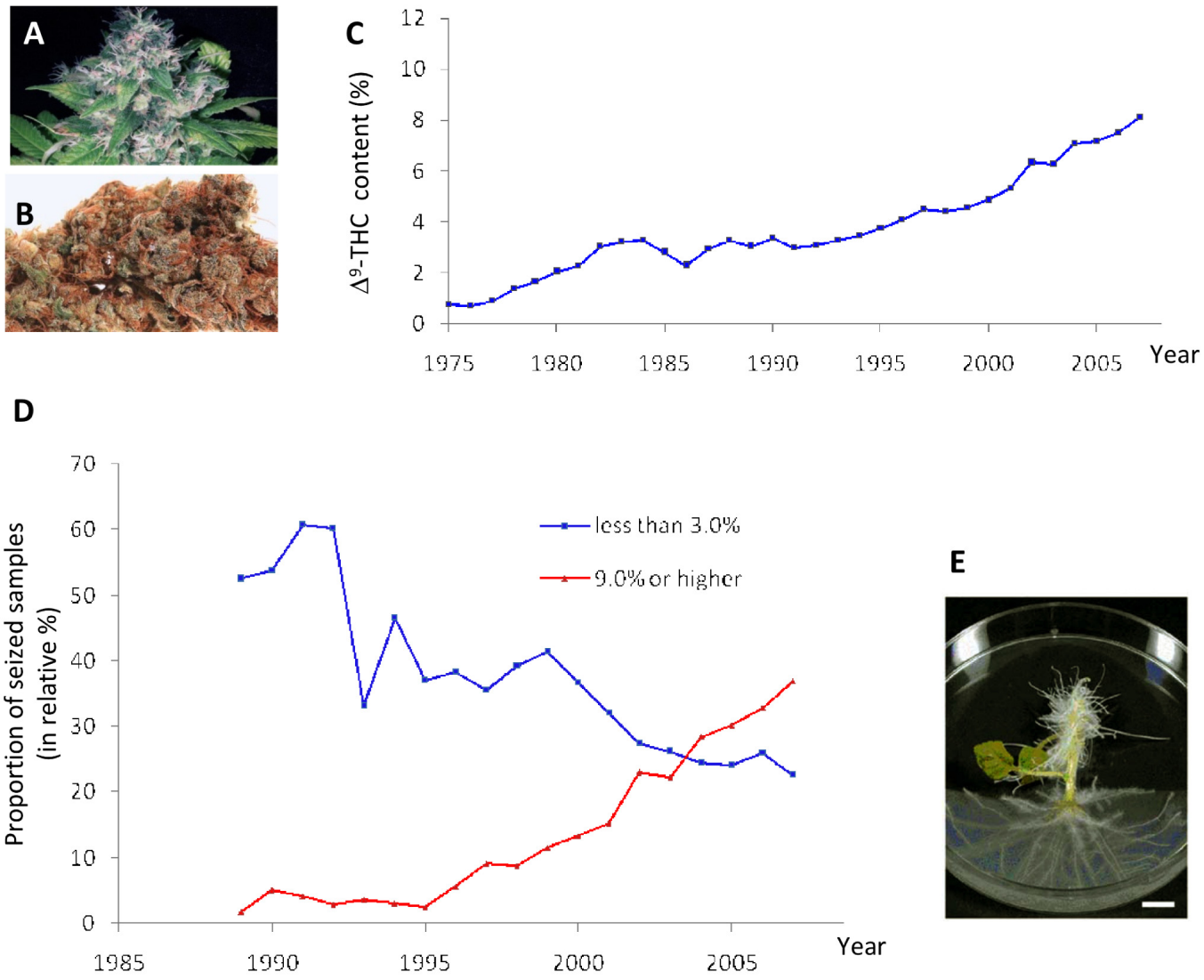

Fig. (1). Potency of marijuana: **A**, live Sour Diesel and **B**, dried flowering buds of NYC Diesel, some of the most potent current Cannabis varieties (source High Times Magazine archives [18]; courtesy of Danny Danko); **C**, Non-normalized average Δ^9-THC content per year seized (period 1975-2008) adapted with permission from Prof. ElSohly [table 2, fig. 1; 14]; **D**, Prevalence of high potency marijuana (period 1989-2007) adapted with permission from Prof. ElSohly (table 1 in [14]); (only seized samples with relative Δ^9-THC content less than 3% or higher than 9% are shown (samples with relative Δ^9-THC content 3-4.9% and 5-8.9% were omitted from the graph for simplicity); **E**, Synthesis of Δ^1-THC using transgenic tobacco hairy roots expressing *THCAS* [3]. Figures reproduced with permission from High Times Magazine Inc. (**A**, **B**), and Bentham Science Publishers Ltd (**E**).

protein–coupled CB_1 cannabinoid receptors (CB_1) either as an agonist or partial agonist [24]. The distribution of CB_1 in adult CNS accounts for the psychoactive properties of Δ^9-THC, since it encompasses regions implicated in the actions of Δ^9-THC, including basal ganglia, hippocampus, amygdala, cerebral cortex, tectum, and cerebellum; In addition to CNS, CB_1 is also expressed in the spinal cord, and in tissues involved with metabolism, such as adipose tissue, liver, and skeletal muscle. CB_1 is part of the endocannabinoid (eCB) system, a signaling network which encompasses, in addition to presynaptic CB_1 and CB_2 receptors, endogenous ligands AEA and 2-AG, proteins required for the synthesis (*via* DAGLα) and inactivation (*via* FAHH, MAGL) of endocannabinoids, reviewed in [25, 26]. The eCB system is extensively characterized in the adult CNS, where it functions to modulate neurotransmitter signaling during feeding, fear, anxiety, memory, cognition, perception and motor coordination, mainly by retrograde transmission: In this, endocannabinoids are released by post-synaptic neurones to suppress presynaptic neurotransmitter release *via* retrograde mechanisms [27-30].

Other mechanisms of action of eCB system in the adult include modulation of neuronal signaling pathways *via* modulation of synaptogenesis in adult cerebellar neurones [31] and regulation of neurogenesis: In the adult CNS, the subgranular zone of the hippocampal dentate gyrus and the cortex constitute the principal neuroproliferative zones in the adult brain. The hippocampal dentate gyrus contains neural stem/progenitor cells capable of generating new neurones. Evidence suggests that the eCB system controls neuronal progenitor cell proliferation and differentiation in both systems [32-34]. Downstream targets to CB_1 regulation of

proliferation and differentiation of neuronal progenitors include ERK1/2 [35] and p38-MAPK phosphorylation [36], possibly *via* PI-3K activation by CB_1 followed by Raf activation *via* phosphorylation, or *via* direct MAPK activation by CB_1 *via* its effects on cAMP [37]. The ability of Δ^9-THC to mimic the function of endocannabinoids, and thereby to interfere with eCB in signal transmission, neurogenesis and synaptogenesis in the adult CNS, is reviewed in [38-40].

The eCB also modulates non-neuronal signaling pathways in the adult brain, such as activation of immediate early gene expression of *c-fos* and *c-jun* in rat adult forebrain [41] and *Krox-24* (*via* phosphorylation and activation of ERK subtype of MAPK) in non-neuronal cell lines expressing CB_1 [42, 43]. Cannabinoids also promote oligodendrocyte progenitor survival in forebrain of newborn rats [44-46], as well as and astrocyte survival [47] *via* PI-3K activation. Other examples of eCB action include modulation of neuritogenesis in adult hippocampus and neuroblastoma cells *via* FRNK [48, 49]. Finally, cannabinoids can inhibit invasion in glioma and inhibit cell migration in glioma cell lines, *via* down-regulation of MMP-2 expression [50].

3. ENDOCANNABINOID SYSTEM DURING EARLY EMBRYONIC DEVELOPMENT

Besides its role in adult CNS, the eCB system is also functional during embryogenesis: So far, its functional role during implantation and neuronal development has been extensively examined: During implantation, a series of spatially and temporarily regulated events is required for uterine receptivity and implantation of the blastocyst [51], including an interplay between CB_1 expression in the embryo and AEA synthesis in the uterus [reviewed in 52, 53]. A role for the eCB system has also been demonstrated during neuronal development, where this system is required for the correct establishment of neuronal diversity and connectivity within the developing hippocampus and cortex; The eCB system is implicated in neurogenesis, neuronal migration, dendritogenesis, axon guidance, synaptogenesis, lineage specification and gliogenesis [reviewed in 54, 55]: During neuronal development, CB_1 receptors are expressed in early neural progenitors [56, 57], with receptor levels increasing throughout neuronal specification and synaptogenesis and CB_1 being progressively localized to developing axonal projections [58-61]; CB_1 receptors are also highly expressed in the rat hippocampus during initiation of gliogenesis [62]. In the developing hippocampus and cortex of 17 day rat embryos, endocannabinoids inhibit lineage commitment and differentiation program of neural progenitor cells into mature neurones, *via* attenuation of ERK pathway by CB_1, and promote astroglial differentiation [33, 56, 62-64].

Endocannabinoids also function as diffusible axon guidance cues to modulate neuronal migration, synaptogenesis and target selection in hippocampus and neocortex [31, 61, 65-67]: In the developing cortex, interneuron specification and migration is in part governed by epigenetic cues in neocortex including BDNF which act on TrkB receptors of interneurones [65]; Endocannabinoids are shown to control interneuron specification and migration by acting as chemoattractants which regulate BDNF/TrkB receptor signaling [65-66]. Finally, CB_1 signaling is required for FGF-dependent axonal growth of cerebellar neurones [68], as well as axonal growth and fasciculation in zebrafish [69] (see section 8), reviewed in [55, 57].

4. eCB SYSTEM PRIOR TO NEURONAL DEVELOPMENT

Besides a role for the eCB system in implantation and neuronal development, recent evidence suggests presence of this system in the period starting after implantation and ending before neuronal development *i.e.* during gastrulation, neurulation, formation of brain primordia and somitogenesis; It is during this developmental period, that the basic scaffold for the cerebral cortex, amygdala and hippocampus originate from a simple neuroepithelium, the neural plate (Fig. **2A**): This is the earliest recognizable form of the CNS and appears at mouse GD7 (equivalent to human day 15 of gestation). The neural plate is subdivided into presumptive territories for the different precursors of the forming CNS, the forebrain, midbrain and hindbrain (Fig. **2A**); A crease appears along the midline of the neural plate, and deepens until its sides arch over and fuse with each other to form the neural tube, the anterior segment of which will form the CNS. As the embryo develops, the anterior neural tube becomes divided into 3 vesicles, the forebrain, midbrain and hindbrain (Fig. **2A**), reviewed in [70]. At the start of neuronal development, the forebrain differentiates into telencephalon and diencephalon; The telencephalon will develop into the cerebral cortex, through the process of corticogenesis, a process for which the role of endocannabinoids is well characterized (section 3), as well as amygdala; The diencephalon will become the optic vesicles, thalamus, epithalamus, hypothalamus and hippocampus; The midbrain will differentiate into tectum, and the hindbrain will give rise to pons, cerebellum and medulla oblongata [71]. At early stages of neurodevelopment, chick, mouse and human embryos share the same developmental cascades, as well as similar basic morphology (Fig. **2B**, **C**).

In human, CB_1 is expressed from the earliest stages of neuronal differentiation at week 14 ([72]; earlier stages not yet investigated). At this stage, CB_1 is expressed at low levels (compared to adult) in a homogeneous pattern throughout the developing brain.

In animal models, CB_1 and other components of the eCB system are detectable prior to neurogenesis, indicating non-neuronal functions for CB_1: In rat, earliest expression of CB_1 is detectable at stage E11 (equivalent to human day 24; earlier stages not yet investigated) [73]: In those embryos, CB_1 mRNA expression is detected throughout the marginal layer of the neural tube and in somites (Fig. **3A**), suggesting a potential function for CB_1 in induction and patterning of CNS precursors into forebrain, midbrain and hindbrain, and in somitogenesis. At a later stage (E12; equivalent to human day 28), CB_1 mRNA is expressed in the telencephalon (neocortical neuroepithelium) of rat embryos [73].

In chick, CB_1 expression is visible at stage $HH11^-$ (corresponding to human day 23; earliest stage investigated in this study), although there is now evidence that CB_1 is expressed throughout earlier stages too (see below); In

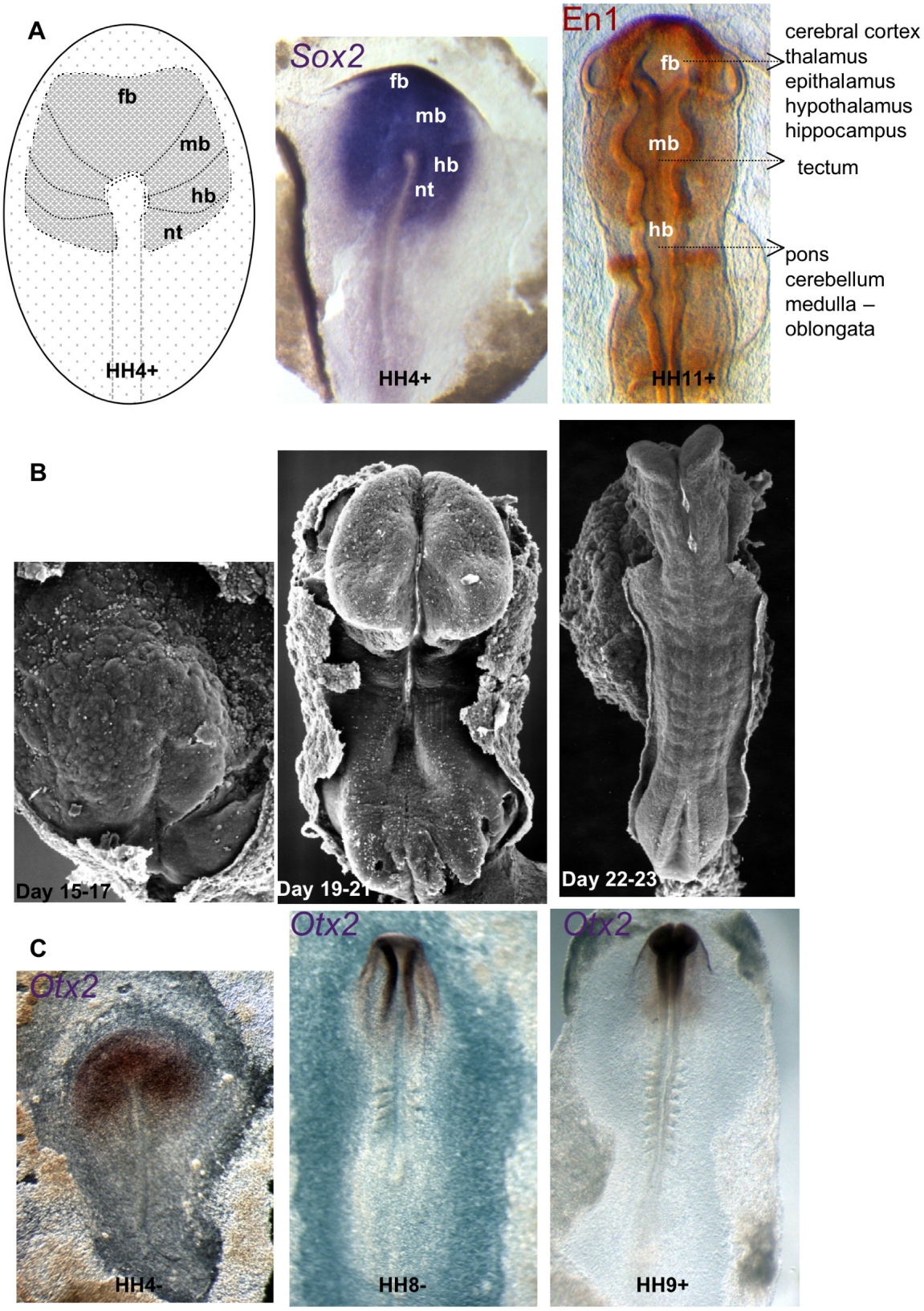

Fig. (2). Precursors for brain in the developing embryo: **A**, HH5 chick embryo (early neural plate stage): Dotted area, presumptive neural plate, subdivided into presumptive forebrain (fb), midbrain (mb), hindbrain (hb) and spinal cord (sc) territories (adapted from [71] visible at HH11^{+}; HH4^{+} embryo hybridized with neural plate marker *Sox2* (gift of Dr. Lovell Badge); HH11^{+} embryo processed with neural crest antibody Pax7 (gift of NIDHD; source JoVE]); **B**, human embryos days 15-23, courtesy of Dr. Kathleen Sulik; **C**, equivalent stages in chick embryos hybridized with forebrain/midbrain marker *Otx2* (probe gift from Dr. Bally-Cuif). (Fig. **2C** is reproduced with permission from John Wiley & Sons, Inc).

HH11$^-$ embryos, CB_1 expression is visible in the primordium of the ventral forebrain [74] (Fig. **3B**), a region which will give rise to the hippocampus and the cerebral cortex at later gestational stages; At slightly later stage (HH11; equivalent to human day 24), CB_1 mRNA expression is also visible in rhombomeres r4 and r6 of the hindbrain [74] (Fig. **3C**). In addition to its expression in developing CNS, CB_1 is also visible in the presomitic mesoderm (musculoskeletal precursors) [74] (Fig. **3D**); At a slightly later stage (HH12; equivalent to human day 26), CB_1 mRNA expression is visible in the differentiating interneurones of rhombomere 4 of the hindbrain [75]. Other studies find that CB_1 protein expression at HH12 is more widespread than the existing data on mRNA, encompassing moderate expression throughout the developing HH12 embryo, with intense labeling in the emerging neural crest cells (Fig. **3E**), neural tube, somites (Fig. **3F**) and developing brain [*in prep.*].

There is also evidence from chick, mouse [*in prep.*] and zebrafish [76] that CB_1 might be expressed at stages earlier than previously investigated: Earliest CB_1 expression is detectable at stage 3 somite in zebrafish embryos (equivalent to human days 21-23) [76]. This result is corroborated by findings in chick and mouse embryos, which show that CB_1 is also expressed at stages earlier than 3 somites, in fact from gastrulation onwards (stages HH3$^+$ to 10; equivalent to human days 15 to 23 after conception), and thus much earlier than previously thought (Fig. **4**) [*in prep.*]. These preliminary studies also indicate that other components of the eCB system (DAGLα and MGLL) are also present during this critical period of development (Fig. **4**; [*in prep.*]; FAAH not investigated so far). Together, the above results support a novel, so far uncharacterized role for the eCB system in early embryogenesis in gastrulation, neural induction, formation of brain primordia and somitogenesis, a role which would be clearly discernible from its function in neuronal development.

Adverse Outcome Following Gestational Marijuana Exposure

Research on the gestational effects of marijuana in human has associated its use with increase risks for spontaneous abortions/resorptions (section 5), growth retardation [77], gross-teratological malformations, such as FAS-like symptoms, VSDs, gastroschisis and anencephaly [77, 78] (section 7), and neurobehavioural deficiencies (section 8):

5. OCCURRENCE OF RESORPTIONS AND MISCARRIAGE FOLLOWING GESTATIONAL Δ^9-THC EXPOSURE

In human, there are two possible mechanisms for the increased risk of early miscarriage:

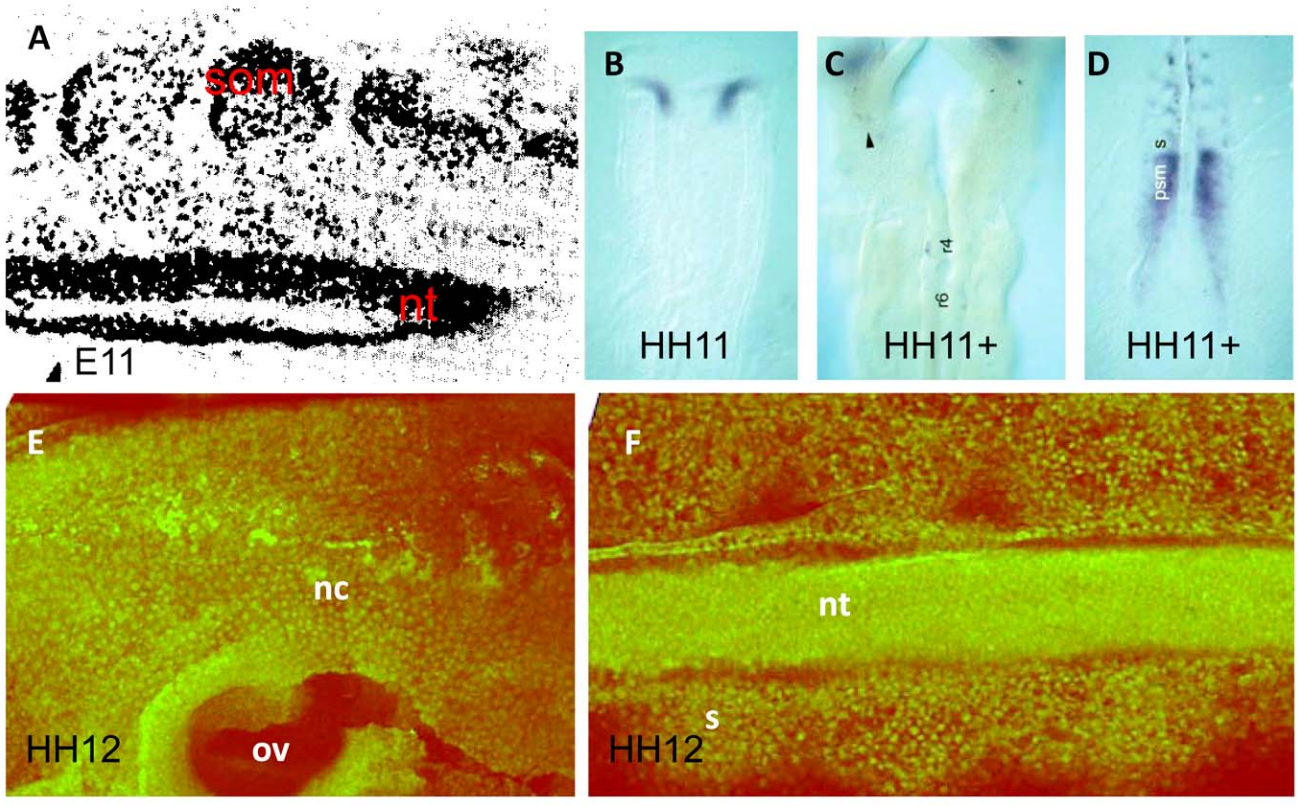

Fig. (3). CB_1 receptor expression in embryogenesis: **A**, *CB_1* receptor expression in E11 rat embryo [73]; *CB_1* receptor mRNA expression is detected throughout the marginal layer of the neural tube and in somites. nt, neural tube, som: somites; **B-D**, *CB_1* receptor expression in HH11 chick embryo [74]; *CB_1* receptor expression is visible in the primordium of the ventral forebrain (**C**), and, at slightly later stage in r4 and r6, as well as in presomitic mesoderm (**D**); **E**,**F**, expression of CB_1 receptor at stage HH12 chick embryo is homogenous throughout the embryo, with high levels in emerging neural crest (**E**), neural tube and somites (**F**) [*in prep.*]. Figures reproduced with permission from Elsevier Science Inc. (**A**), John Wiley & Sons, Inc. (**B-D**).

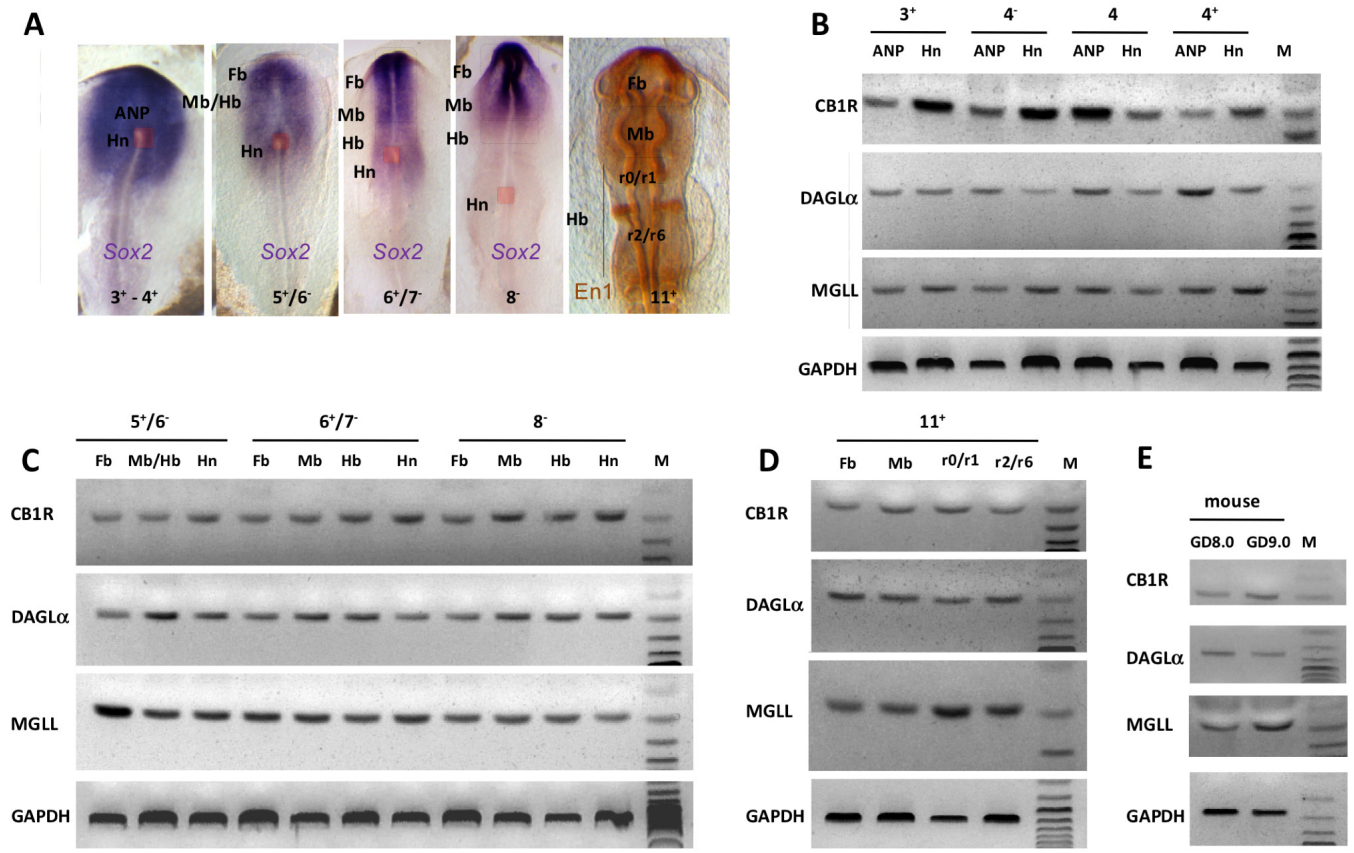

Fig. (4). Preliminary findings on endocannabinoid system in early chick and mouse embryos [*in prep.*]: **A**, top panel given only for reference; in situ hybridization with neural plate *Sox2* and immunochemistry with neural crest marker Pax7 to show Hensen's node (Hn) and neural precursor populations ANP (anterior neural plate), Fb (forebrain), Mb (midbrain), and Hb (hindbrain); **B-D**, Areas shown in A were dissected and processed for RT-PCR using chick specific primers for CB_1R (184 bp cDNA), DAGLα (185 bp), MGLL (185 bp), and GAPDH (579 bp); **E**, RT-PCR using mouse specific primers for CB_1R (280 bp), DAGLα (335 bp), MGLL (200 bp), and GAPDH (290 bp).

(1) Lack of blastocyst implantation due to a non-receptive endometrium [79], as demonstrated in rodent models [80]; For implantation to occur, endometrial levels of AEA have to be reduced at the presumptive site of implantation, which they are due to local FAAH activity [79]; If high levels of AEA are maintained, implantation will not occur and the embryo will abort [80]. High levels of Δ^9-THC in the endometrium of marijuana users might act in a manner similar to elevated AEA levels, thus preventing uterine receptivity and consequently implantation to occur, resulting in spontaneous abortion 7-12 days following conception.

(2) Severe embryonic malformations following embryonic exposure to Δ^9-THC, as in the case on animal models (see below [81]), would result in death of the embryo, and be equivalent to spontaneous abortion at days 19-24 following conception, a phenotype which could easily misinterpreted as lack of implantation in human or rodent models. In human studies, Δ^9-THC would be deemed devoid of any effects, except lack of implantation. The problem of some human studies is that subjects might be selected after pregnancy is confirmed, and therefore it is not possible to investigate the possibility that exposure to marijuana early in gestation is associated with lethality for severely malformed fetuses; We now know that CB_1 mRNA and other components of the eCB system are expressed during the stages following implantation and prior to neuronal development in animal models (*i.e.* human days 12-24). It is therefore possible that Δ^9-THC mediates its teratogenic effects in animals and perhaps human, *via* interference with an endocannabinoid system in the early embryo. Since neuronal development has not taken place yet, this would suggest that the eCB has an hitherto unknown function at these early stages. The function of the eCB at these early stages constitutes the focus of our current research [*in prep.*].

6. Δ^9-THC AND TEMPORAL PATTERN OF EMBRYOTOXICITY IN ANIMAL MODELS

Classical studies show that the developmental stage at which Δ^9-THC is administered is a critical factor in determining the degree of embryotoxicity of Δ^9-THC: The period of greatest susceptibility to the embryotoxic effects of Δ^9-THC (or window of sensitivity to Δ^9-THC) occurs during early organogenesis (GD6-GD8 in mouse). During this period, Δ^9-THC administration results in a high incidence of resorptions (embryo death) and congenital malformations including defects in CNS formation and patterning (including holoprosencephaly, anencephaly and exencephaly

[reviewed in 82] (section 7); By contrast, exposure to Δ^9-THC prior to organogenesis (peri-implantation stages GD1-GD6), results in 100% resorption before the embryo can reach organogenesis [83, 84], equivalent to spontaneous abortions in human at days 5-14 (section 5); Administration of Δ^9-THC after organogenesis (mouse GD9-GD14), no longer results in a high incidence of resorptions or congenital malformations [*e.g.* 85, 86]; Thus, there is a developmental window of susceptibility to the embryocidal and teratogenic effects of Δ^9-THC, a window which coincides with the period of early organogenesis (approx. GD6.5-GD8.5); In human, this period corresponds to gestation days 15-22, a time point during which most younger women are unaware of their pregnancy and of the risks of concomitant use of marijuana.

7. RISK OF ANENCEPHALY FOLLOWING GESTATIONAL Δ^9-THC EXPOSURE

Anencephaly is a typical teratological malformation of the CNS, in which the brain fails to form (Fig. **5A**). Previous reports on marijuana use and fetal developmental outcome did not report any case of anencephaly: these reports were analyzing cases prior to 1997 (period 1983 to 1994) [87-89], in other words a period during which the average Δ^9-THC content in marijuana was essentially below 3.1%, varying between 2.2% and 3.4% (calculated from data on table 2 in [14] Fig. **1D**). Yet, a recent report which analyzed data from the NBDPS, which recruited births in the period 1997 and 2003, found a clear correlation between gestational marijuana exposure and anencephaly [90]; A note to mention that in this 1997-2003 period, Δ^9-THC content in marijuana averaged 5.2% (varying between 4.5% and 6.4%) compared to 3.1% in the period 1983 to 1994, and 8.12% in 2007 (table 2 in [14]). In this study [90], which included 10,241 infants with major congenital malformations and 4,967 infants without major congenital malformations born between 1997 and 2003, it was determined that periconceptional cannabis use (first trimester) is associated with an increased risk of anencephaly (adjusted OR = 1.7; 95% CI = 0.9-3.4). Restricting the analysis to cannabis use in the first month after conception, during which the neural tube closes, confirmed this finding (adjusted OR = 2.5; 95% CI = 1.3–4.9). Cannabis use in the other months of the periconceptional period was not associated with an increased risk of anencephaly [90]. From these results, we can predict that the risk of infants born with anencephaly will increase in the coming years, considering that not only the number of childbearing women potentially exposed to marijuana has increased, but so has the Δ^9-THC content found in marijuana preparations. In animal models, classical studies on marijuana embryotoxicity describe cases of holoprosencephaly, partial anencephaly (in which the anterior neural tissue is partially closed) and exencephaly (an early stage of anencephaly in which the neural tissue gradually degenerates, leading to anencephaly phenotype) [*e.g.* 83, 91].

Recent experiments with chick embryo report anencephaly and other CNS malformations following exposure to a water soluble Δ^9-THC analogue O-2545 [81]: In embryos treated with low dose of O-2545, the neural folds fail to elevate and to fuse, most likely comparable to exencephaly in mammalian systems (fig. 1BE in [81]). In embryos treated with medium dose of O-2545, the brain is poorly segmented into forebrain, midbrain and hindbrain primordia (Fig. **5B**), a phenotype most likely comparable to anencephaly in mammalian systems. In those embryos, neurulation is severely disrupted since the neural plate fails to extend along the AP and ML axes of the embryo (fig. 5K, N in [81]). The results also reveal that brain development is most susceptible to the effects of O-2545 at stages $HH3^+$ to 4^+, stages at which anterior neuronal precursors migrate anteriorly to form the anterior neural plate. These stages correspond to 15-19 days after conception in human. At these early stages, most women are unaware of their pregnancy and of the risks of concomitant use of marijuana.

To summarize, Δ^9-THC exerts most of its teratogenic effects during the period after implantation and prior to neuronal differentiation; from section 4, we know that CB_1 is expressed during this period, and so are other components of the eCB system. This could suggest that Δ^9-THC mediates its teratogenic effects by interfering with an endogenous eCB system, present in the young embryo, a system required not only for neuronal development, but presumably also for earlier mechanisms, such as neural induction, patterning into brain primordia and somitogenesis. Because the basic molecular mechanisms that control early organogenesis are evolutionary conserved amongst species [92-94], the knowledge gained by analyzing the mechanism of Δ^9-THC mediated embryotoxicity in animal models is applicable to our understanding of human Δ^9-THC induced teratogenesis.

8. IS THERE A NEURAL BASIS FOR THE NEUROBEHAVIOURAL DEFICIENCIES IMPOSED BY GESTATIONAL Δ^9-THC EXPOSURE?

In human, gestational marijuana exposure is associated with neurobehavioural deficiencies including visual behavioural alterations [95] in neonates, lower mental test scores [96] and lower scores in verbal and memory domains [97] in 3 year olds; lower intelligence at age 6 [98]; lower IQ and lower intelligence at age 6 [98, 99]; decrease in learning abilities [100], ADHD [101], long-term language acquisition difficulties, neuropsychiatric disorders (depression, schizophrenia, anxiety, social behavioural disturbances [102-105]), as well as long-term abnormal cognitive and behavioural function in young adults [106], reviewed in [88, 107-109]. These neurobehavioural deficiencies stem from defects in cognitive and emotional centers of the cortex, hippocampus, amygdala and nucleus accumbens. In rat, sub-teratogenic doses of cannabinoids (Δ^9-THC or agonist WIN) during gestational period GD5.0-GD20 also induce deficits in memory, learning as well as emotional hyperactivity, anxiogenic-like profile and heroin seeking profiles in offspring [110-115].

We know that the eCB system is required for neuronal development and correct establishment of neuronal circuitry within both developing cortex and hippocampus (section 3) [66]. Gestational exposure to marijuana may interfere with the ontogeny of neurodevelopment, ultimately resulting in abnormal neuronal circuitry within the developing cortex, hippocampus, amygdala and nucleus accumbens; this in turn would lead to abnormal neurobehavioural outcome in the

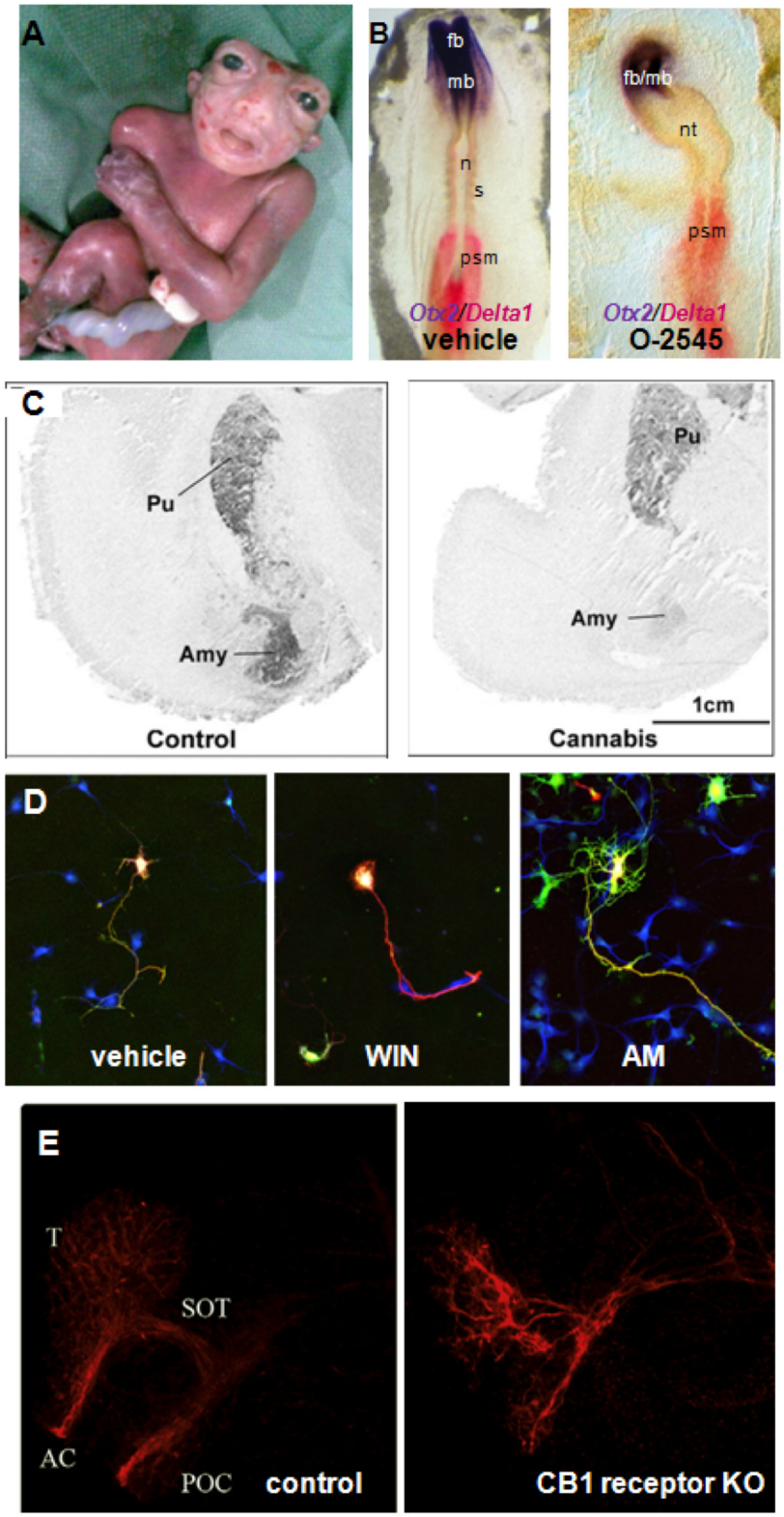

Fig. (5). Neural development following gestational exposure to cannabimimetics: **A**, typical anencephaly in neonate (source Wikipedia); It is not known whether this particular foetus was exposed to marijuana during gestation; This is only shown here to illustrate anencephaly in human offspring; **B**, O-2545 induces anencephaly in chick embryos, as evidenced by abnormal morphology and *Otx2* expression in forebrain and midbrain; *Otx2* and *Delta*1 probes are gifts from Drs. Bally-Cuif and Henrique); **C**, Levels of dopamine D2 mRNA expression are sharply reduced following gestational exposure to marijuana in human fetal amygdala (18-22 wks gestation) [123]; **D**, WIN inhibits dendritogenesis in cultured hippocampal neurones derived from E17 rat embryos, whereas AM281 exerts opposite effects [58], courtesy of Dr. Zsolt Lenkei; **E**, Both anterior and posterior commissures of the forebrain present tight fascicles in controls; By contrast, axons appear disorganized along the DV and ML axis in CB_1-morpholino treated embryos [69]. Figures reproduced with permission from Wikipedia Inc. (**A**), John Wiley & Sons, Inc (**B**), Elsevier (**C**, **E**), and Wiley-Blackwell (**D**).

offspring. There is evidence in both human and animal models that cannabimimetics comprimise neuronal development by interfering with (1) neurotransmitter synthesis and (2) morphogenesis within the developing CNS:

Interference with neurotransmitter synthesis: Gestational exposure to cannabimimetics in rat results in local modification of neurotransmitter synthesis, including dopamine, a neurotransmitter required for proper establishment of cognitive circuitry in the cortex and for development of emotional behavioural in the amygdala: Cannabimimetics interfere with the expression of tyrosine hydroxylase gene (the enzyme responsible for the dopamine synthesis), and the activity of this enzyme in catecholaminergic neurones of the midbrain during early rat fetal brain development [116-118]; This in turn might lead to abnormal neuronal circuitry involving dopamine and henceforth cognitive anomalies in the offspring. Similarly, analysis of amygdala obtained from mid-gestation human fetuses which were gestationally exposed to marijuana, shows a severely impaired dopamine mRNA expression [119] (Fig. **5C**). It is possible that defective GABA neurotransmitter in the amygdala following Δ^9-THC exposure might be in part responsible for abnormal emotional behavioural observed in offspring of marijuana users (such as neuropsychiatric disorders observed by [102-105]).

Gestational exposure to cannabimimetics also results in perturbations in the GABAergic, serotonergic and opioid systems during neuronal development and in the offspring [120-123]; Furthermore, gestational cannabimimetics are shown to perturb also both noradrenergic and glutamatergic systems during neuronal development; both these neurotransmitter systems are required for cognitive processes in cortex and hippocampus [124-126]: gestational cannabimimetics are able to modify the expression of components of both noradrenergic and glutamatergic systems, and to decrease levels of noradrenaline and glutamate in the offspring [58, 113]. Finally, evidence suggests that Δ^9-THC can inhibit proenkephalin mRNA expression in the nucleus accumbens during early neurodevelopment [115]. This is associated with long-lasting neurobiological impairments in neuronal systems linked with opioid/reward/stress limbic function in the offspring [115], suggesting that impairment of proenkephalin signaling during gestation (*via* exposure to Δ^9-THC) might result in deficient circuitry in nucleus accumbens, and henceforth aberrant limbic function in the offspring. Interestingly, proenkephalin is highly expressed in proliferating neuronal and glial progenitors in GD14 rat, its level of expression then decreases sharply and is hardly detectable until GD21, suggesting that this neurotransmitter might be responsible for proliferation and commitment of neuronal precursors within the developing cortex [127], a function which could also be potentially impeded following gestational Δ^9-THC exposure.

Interference with development of cortical and hippocampal neurones: In addition to their ability to interfere with neurotransmitter synthesis during neuronal development, cannabinoids can also impede with the formation of neuronal circuitry in the developing embryo: by using cultured hippocampal neurones derived from E17 embryos, WIN was shown to inhibit dendritogenesis, *via* reduction of both length and number of primary dendrites, while CB_1 antagonist AM281 exerted opposite effects [58] (Fig. **5D**). The same studies found that CB_1 was shown to translocate from the axonal ends to the somatic compartment of hippocampal neurones in E16.5 embryos which had received one single sub-teratogenic dose of Δ^9-THC analogue CP55,940 and which were sacrificed 12 hr later [58]. Similar results were observed for hippocampal interneurones in rat neonates which had been exposed to Δ^9-THC throughout gestation: Δ^9-THC was found to interfere with the specification and migration of interneurones in the developing hippocampus; in those embryos, postnatal interneurones had failed to migrate within the hippocampus and had remained within the strata radiatum, lacunosum-moleculare of the CA1–CA3 subfields [65, 66]. Finally, WIN was found to inhibit the neuronal outgrowth and branching in cultures derived from cerebral cortex of neonates which were gestationally exposed to WIN [110].

Together, the above data suggest that disturbance of neuronal development in cortex, hippocampus and possibly amygdala and nucleus accumbens, following gestational cannabinoid exposure, might in part result in disruptions in neurotransmitter signaling, as well as interference with neuronal morphogenesis and proper circuitry. These aberrations would in turn lead to subtle defects in cognitive, neurobehavioural and emotional processing in the offspring, which is the phenotype we observe in the offspring born to marijuana users.

Recent studies in zebrafish are of particular interest in illustrating this point: whereas previous studies focused on behavioural of neurones/axons at the earliest E17 in mouse slices following treatment with cannabimimetics, this study used 1 to 4 cell stage embryos, in other words a period corresponding to peri-implantation in human. In CB_1-morpholino treated zebrafish embryos reticulospinal neurones of the hindbrain (which correspond to reticulospinal and vestibulospinal pathways in human) show aberrant patterns of axonal growth at 72 hpf. In treated embryos, the medial longitudinal fascicule, which normally runs along the AP axis as segmented tight bundles of axons, appear clearly disorganized, spreading along the mediolateral axis of the embryo [69]; Furthermore, treated embryos present extensive crossings of axons along the AP midline [69], suggesting that they are receiving the wrong cues/or fail to receive cues upon CB_1 inactivation. Watson *et al.* also describe abnormal in the anterior and posterior commissures of the forebrain in CB_1 morpholino-treated embryos. In those embryos, both commissures fail to tight fascicles (organized bundles of axons); Instead, axons appear disorganized along the DV and ML axis [69] (Fig. **5E**), suggesting again that these axons receive the wrong cues/or fail to receive cues upon CB_1 inactivation. The anterior and posterior commissures of the forebrain are responsible for transferring information between the two cerebral hemispheres to coordinate localized functions in the adult, such as memory establishment [128] and visual discrimination [129], both functions which are impaired in offspring following gestational exposure to marijuana [58, 90, 95, 97, 100, 107].

9. CONCLUDING REMARKS

The argument that marijuana is a “harmless” drug is no longer valid: The recent advances in registry and statistical

evaluation of effects, which now take into account confounding variables, has enabled us to clearly affirm that marijuana is detrimental to pregnancy. This is enhanced by the recent discovery of an eCB system in the developing embryo, a system of which the function is impeded following maternal exposure to marijuana. Most alarmingly, Δ^9-THC content of marijuana has increased from 1.25% in the 1970s to an average content of 8.12% in modern preparations [14], with some preparations containing up to up to 37.2% Δ^9-THC [14]. Marijuana has regained its popularity from the 1970's, especially amongst teens/young adults, where it has regained its social and cultural status as the most popular drug of abuse; As a result, this poses not only a risk for the foetus of pregnant teen/young adults, but also for teens in general [130]. Clearly, additional awareness should be provided to teens and young adults in particular, concerning the health deficits caused by marijuana, especially given the current debates on rescheduling, legalization and decriminalization of marijuana based on its medical applications [131, 132].

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ABBREVIATIONS

Δ^9-THC	=	Δ^9-Tetrahydrocannabinolic acid
Δ^1-THC	=	Δ^1-Tetrahydrocannabinolic acid
AEA	=	*N*-Arachydonylethanolamide
2-AG	=	2-Arachidonoylglycerol
DAGLα	=	sn-1 specific Diacylglycerol Lipase, alpha
FAAH	=	Fatty Acid Amide Hydrolase
MAGL	=	Monoacylglycerol Lipase
MGLL	=	gene encoding MAGL
FRNK	=	Focal adhesion kinase-Related Non-Kinase
MMP-2	=	Metalloproteinase-2
BDNF	=	Brain-Derived Neurotrophic Factor
TrkB	=	neurotrophic Tyrosine Kinase, receptor, type 2
GD	=	Gestational Day
FGF	=	Fibroblast Growth Factor
HH	=	Hamburger and Hamilton stage
FAS	=	Fetal Alcohol Syndrome
NBDPS	=	National Birth Defects Prevention Study
AP	=	Anteroposterior
ML	=	Mediolateral
WIN	=	WIN55,212-2
CP	=	CP-55940
DV	=	Dorsoventral
THCAS	=	cDNA encoding tetrahydrocannabinolic synthase

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