



# Cannabis and synaptic reprogramming of the developing brain

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**Abstract** | Recent years have been transformational in regard to the perception of the health risks and benefits of cannabis with increased acceptance of use. This has unintended neurodevelopmental implications given the increased use of cannabis and the potent levels of  $\Delta^9$ -tetrahydrocannabinol today being consumed by pregnant women, young mothers and teens. In this Review, we provide an overview of the neurobiological effects of cannabinoid exposure during prenatal/perinatal and adolescent periods, in which the endogenous cannabinoid system plays a fundamental role in neurodevelopmental processes. We highlight impaired synaptic plasticity as characteristic of developmental exposure and the important contribution of epigenetic reprogramming that maintains the long-term impact into adulthood and across generations. Such epigenetic influence by its very nature being highly responsive to the environment also provides the potential to diminish neural perturbations associated with developmental cannabis exposure.

There has been a large societal shift in recent years in the perception of harm from cannabis use coinciding with its widespread legalization for recreational and/or medicinal use in most states in the USA and many countries worldwide. Despite important societal outcomes of these changes, such as the decriminalization of cannabis use, the potential for harm particularly for vulnerable populations has raised concern as more teens and pregnant women use cannabis today. This is also pertinent given the exponential increase over the years in the concentration of  $\Delta^9$ -tetrahydrocannabinol (THC; the main psychoactive intoxicant) in the cannabis used recreationally and sold in dispensaries (BOX 1). Brain development is a dynamic process that extends well beyond the prenatal period, where full maturity is not achieved until early adulthood. The endocannabinoid (eCB) system (ECS) (BOX 1), which mediates the actions of THC, plays a critical regulatory role throughout all developmental stages, from the determination of cell fate and neuronal migration to the regulation of signalling pathways and synaptic transmission in the mature central nervous system (CNS). Therefore, the supraphysiological impact on the ECS by cannabis exposure during critical periods of development could change the normal trajectory of cellular processing and neurocircuitry, leading to behavioural disturbances later in life. In this Review, we provide a general overview of the ECS (which has been extensively reviewed) and describe the neurodevelopmental effects of cannabis/THC on the basis of animal and human studies relevant to neuropsychiatric risk.

While evident discrepancies exist in the literature and not all studies observe adverse cannabis outcomes, we focus on animal findings pertinent to human cannabis studies reporting neurobehavioural disorders in order to gain neurobiological insights germane to risk. We highlight epigenetic mechanisms as a critical link underlying the molecular processes that maintain protracted effects of developmental cannabis exposure, not just during one's lifetime but across generations.

## The endocannabinoid system

The ECS plays a broad and critical role in numerous developmental processes. Briefly, it consists of cannabinoid receptors (CBRs; CB<sub>1</sub>R and CB<sub>2</sub>R) and endogenous ligands (eCBs), including the most studied 2-arachidonoylglycerol (2-AG) and anandamide (AEA), as well as the proteins responsible for their transport, synthesis (diacylglycerol lipase (DAGL) and *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD)) and degradation (monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH)). eCBs, which are lipidic neuromodulators, can interact with other receptors, including transient receptor potential vanilloid 1 (TRPV1), peroxisome proliferator-activated receptor (PPAR) and G-protein-coupled receptor 55 (GPR55), to regulate synaptic transmission (for a review, see REF.<sup>1</sup>).

A substantial literature highlights fundamental roles of eCBs and CBRs in key developmental processes, including neurogenesis, glial formation, neuronal

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## Box 1 | The mutable cannabis plant

The cannabis plant has been used for thousands of years for its psychoactive and medicinal properties<sup>152</sup>, and in 1964,  $\Delta^9$ -tetrahydrocannabinol (THC) was discovered to be the primary psychoactive component contributing to its euphoric effects<sup>84,153</sup>. Today, more than 120 cannabinoids (for example, THC, cannabidiol (CBD) and cannabinol) have been identified in the cannabis plant, with more than 440 additional compounds, including terpenoids, that make up the complex, synergistic 'entourage' effects of the plant<sup>154</sup>. The two major types of cannabis plants are *Cannabis sativa* (most abundant subspecies and generic term often used to denote cannabis) and *Cannabis indica*, which were typically characterized by high THC and low CBD (non-intoxicating cannabinoid) levels and low THC and high CBD levels, respectively. In recent years, thousands of cannabis strains have been bred with many hybrids, resulting in varied cannabinoid constituents. Thus the names *C. sativa* and *C. indica* no longer reflect their initial content and related psychotropic effects. Additionally, THC concentrations in *C. sativa*, the most commonly used recreational plant, which ranges from 1.5% to 4% in the 1980s and 1990s<sup>155</sup>, have increased substantially in recent years, with some strains having as much as 29% THC<sup>156</sup>. Moreover, new routes of concentrated cannabis products such as 'dabs' which contain upwards of 76% THC<sup>157,158</sup> have been introduced to increase the speed and intensity of the 'high'.

migration, axonal elongation, fasciculation (axonal bundling), synaptogenesis and synaptic pruning<sup>2-4</sup>. While considerable gaps in knowledge remain regarding granular mechanisms by which the ECS regulates some of these processes, significant insights have been gained into specific expression patterns linked to the functional and structural maturation of the CNS (FIG. 1) relevant to cannabis exposure during these periods.

CB<sub>1</sub>R and CB<sub>2</sub>R are the primary targets of THC, with CB<sub>1</sub>R having a prominent role in CNS development given its abundant expression in the developing brain in contrast to CB<sub>2</sub>R, the role of which is aligned mainly with cells of microglial/macrophage lineage<sup>5,6</sup>. CB<sub>1</sub>Rs are present and functional from at least the ninth gestational week in humans, a period that overlaps with the initiation of cortical development, and from gestational day 11 in rodents<sup>5-7</sup>. In both rodents and humans, there is a transient presence of CB<sub>1</sub>R on white matter neuronal fibres during embryonic stages<sup>8-10</sup>, potentially reflecting CB<sub>1</sub>R on axons as they grow and migrate to their final site in establishing neuronal pathways, or their presence on non-neuronal cells (astrocytes and oligodendrocytes) that guide neuronal migration and axonal elongation. CB<sub>1</sub>R, expressed by diverse pluripotent cells, regulates cell differentiation and proliferation<sup>3,11</sup>, with expression correlated with neural differentiation<sup>12</sup>. Rodent studies have clearly demonstrated that CB<sub>1</sub>R expression and eCB signalling in postmitotic neurons play critical roles in neuronal migration and differentiation of glutamate and GABAergic cortical cells, cholinergic basal forebrain neurons, GABAergic cerebellar cells and hypothalamic neurons<sup>13</sup>. Moreover, the expression of CB<sub>1</sub>R by progenitor cells can control the neuron-to-glia ratio in the brain, and alterations in CB<sub>1</sub>R expression during fetal development can modify the connectivity between brain regions such as the cerebral cortex and hippocampus<sup>2,3</sup>. The developmental expression of CB<sub>1</sub>R is dynamic postnatally into adolescence before high levels are established in early adulthood, where CB<sub>1</sub>R is ubiquitously expressed and becomes the most abundant G-protein-coupled receptor<sup>14-16</sup>. CB<sub>1</sub>R in adulthood are primarily enriched in the cerebral cortex, basal ganglia,

hippocampus and cerebellum<sup>14</sup> and are predominantly localized to the synapse on presynaptic terminals<sup>17</sup> of both glutamatergic and GABAergic cells<sup>18</sup>.

eCB ligands also have distinct developmental patterns, with divergent ontogenic bioavailability of the two prominent ligands — AEA and 2-AG. AEA has been shown to be critical during early stages of pregnancy for embryo implantation in the uterus, whereas increasing 2-AG levels during embryonic development correlate with cell differentiation and axonal elongation in the CNS<sup>3</sup>. After reaching a peak at postnatal day 1 (PND1), 2-AG levels remain steady until adolescence when they fluctuate (high in both early and late adolescence) before stabilizing in adulthood<sup>8,19</sup>. In contrast, AEA concentrations

**Fig. 1 | Neurodevelopmental pattern of the endocannabinoid system in humans and rodents.** Top panel:

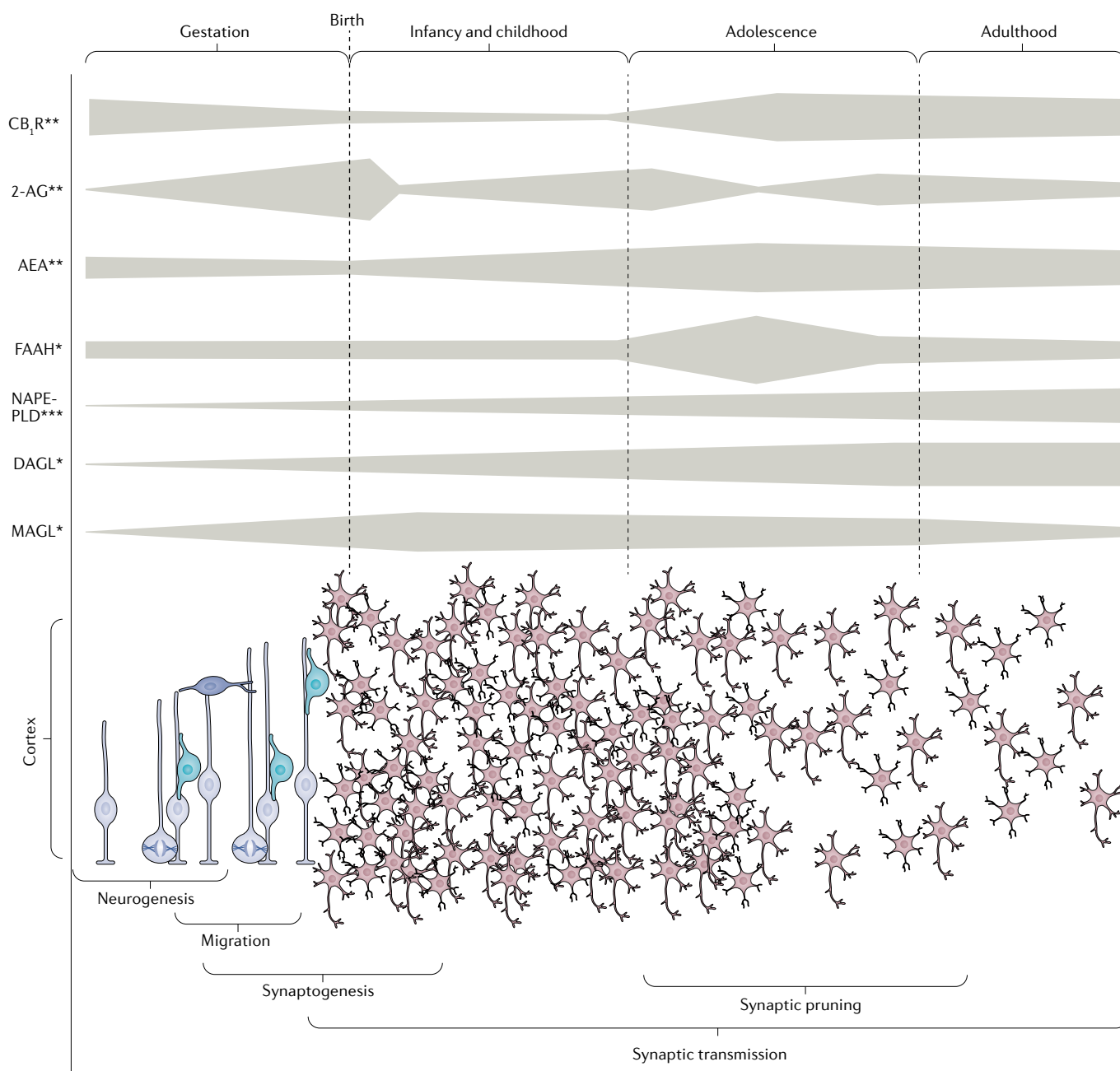
Schematic overview of the developmental pattern of the expression of cannabinoid receptor type 1 (CB<sub>1</sub>R), endocannabinoid (eCB) ligands (2-arachidonylglycerol (2-AG) and anandamide (AEA)), synthesis enzymes (diacylglycerol lipase (DAGL) and *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD)) and degradative enzymes (monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH)) detected in the brain (single asterisk, human; two asterisks, rodent; three asterisks, human and rodent). In rodents, the *Cnr1* gene, encoding CB<sub>1</sub>R, exhibits its highest expression during early development and then expression decreases until adulthood<sup>8</sup>. Consistently, CB<sub>1</sub>R binding progressively increases from early gestation to reach maximal densities during adolescence in various brain regions (striatum, limbic forebrain and ventral mesencephalon) before decreasing to sustained high levels in the adult brain<sup>149</sup>. In humans, CB<sub>1</sub>R appears to have a similar developmental pattern from mid-to-late gestation to adulthood in homologous brain areas<sup>9</sup> with an extremely high density in adulthood<sup>9,16,150</sup>. The eCB ligands also have distinct ontogenic patterns. While AEA expression exhibits a progressive increase from late gestation in rodents to reach maximal levels during adolescence, 2-AG expression peaks just after birth and fluctuates in early and late adolescence before reaching its adult levels<sup>8,19,20</sup>. In rodents, the AEA-synthesizing enzyme NAPE-PLD is first expressed in late gestation<sup>2</sup>, and human studies show that NAPE-PLD mRNA expression in the prefrontal cortex increases steadily over the lifespan, while expression of FAAH, the enzyme that degrades AEA, peaks during adolescence before maintaining stable levels into adulthood<sup>151</sup>. The ontogenic presence of 2-AG requires active DAGL and MAGL. While the expression profiles throughout development for MAGL and DAGL have not been well studied in rodents, the human prefrontal cortex exhibits a progressive increase of DAGL mRNA levels until early adulthood, whereas MAGL expression reaches its peak in early childhood before declining in early adulthood<sup>151</sup>. Bottom panel: Illustration of several neurodevelopment events which occur from gestation to early adulthood that are regulated by eCB signalling (see the top panel). The gestation period is characterized by neuronal generation and migration followed in a later stage by synaptogenesis and synaptic transmission that continues into infancy, childhood and adulthood. The adolescent period is characterized by synaptic pruning (elimination of synapses) that shapes the mature brain architecture with dynamic fluctuations of components of the eCB system, including CB<sub>1</sub>R expression, eCB ligands and FAAH activity during early and late adolescence.

gradually increase from gestational day 21 and reach maximal levels during adolescence in most brain regions that have been studied<sup>8,19,20</sup>. In the mature brain, the ECS has been mainly described for its role in neurotransmission modulation and synaptic plasticity<sup>21</sup>. eCBs are retrograde messengers synthesized in the postsynaptic neuron in response to specific events. The biosynthesis of AEA is triggered by increased intracellular calcium or cAMP levels in the postsynaptic neuron, whereas 2-AG is produced after postsynaptic depolarization and the increase of calcium influx following the activation of voltage-gated Ca<sup>2+</sup> channels. Once released from the postsynaptic site, eCBs are transported across the synaptic cleft to activate their main target, presynaptic CB<sub>1</sub>Rs. As a G<sub>i/o</sub>-protein-coupled receptor, CB<sub>1</sub>R activation by eCBs inhibits synaptic transmission by decreasing the probability of neurotransmitter release. Therefore, the activation

of CB<sub>1</sub>Rs inhibits Ca<sup>2+</sup> influx through voltage-gated Ca<sup>2+</sup> channels and activates presynaptic K<sup>+</sup> channels and cAMP/PKA signalling, resulting in depression of synaptic transmission<sup>22</sup>. The modulation of these ion channels by cannabinoid agonists regulates synaptic strength of excitatory and inhibitory neurons. Through this feedback modulation of synaptic transmission, eCBs display a powerful regulatory role of synaptic function in numerous brain areas controlling synaptic excitability on different timescales so as to regulate a wide range of behaviours, including cognitive processes, motor function, emotions, reward, memory and food intake<sup>23</sup>.

**Cannabis exposure during gestation**

Prenatal THC exposure is often associated with reduced *Cnr1* mRNA expression during fetal development<sup>11,24</sup>, which would have significant downstream



consequences considering the major role of the ECS in cell proliferation, migration, differentiation and in maturation of neurons relevant to the development of various neural systems. Indeed, prenatal exposure of rodent models (corresponding to the first and second trimesters in humans; gestational day 5 to gestational day 20 in rodents) to CBR agonists has repeatedly been shown to alter glutamatergic, GABAergic, dopaminergic, opioidergic and serotonergic systems<sup>25</sup>. Importantly, a number of the impairments observed with THC exposure in animal models are also evident in the brains of human fetuses with in utero cannabis exposure, such as decreased mRNA expression of dopamine D<sub>2</sub> receptor in the nucleus accumbens (NAc) and amygdala<sup>26,27</sup> as well as altered opioid receptor and opioid neuropeptide mRNA expression in the amygdala, mediodorsal thalamus and striatum<sup>28–30</sup>. These and other findings emphasize that several cannabis-associated effects evident on the human developing brain can be causally ascribed to THC despite the many challenges of human studies.

Synaptic plasticity is critically dependent on the precision of synaptic connections and structural organization to express its full functional working range later in life. In utero, cannabis has been shown to compromise cytoskeletal stability that can lead to erroneous neurite growth and corticofugal development. For instance, prenatal THC exposure reduces the hippocampal and striatal expression of SCG10 (also known as stathmin 2), a microtubule-binding protein in axons, an alteration also detected in cannabis-exposed human fetuses<sup>24</sup>. Disturbingly, the reduction of SCG10 expression is associated with reorganization of the fetal cortical circuitry, deficits that last into adulthood causing rearrangements in the localization of CB<sub>1</sub>Rs and reduced long-term depression (LTD) in the hippocampus<sup>24</sup>.

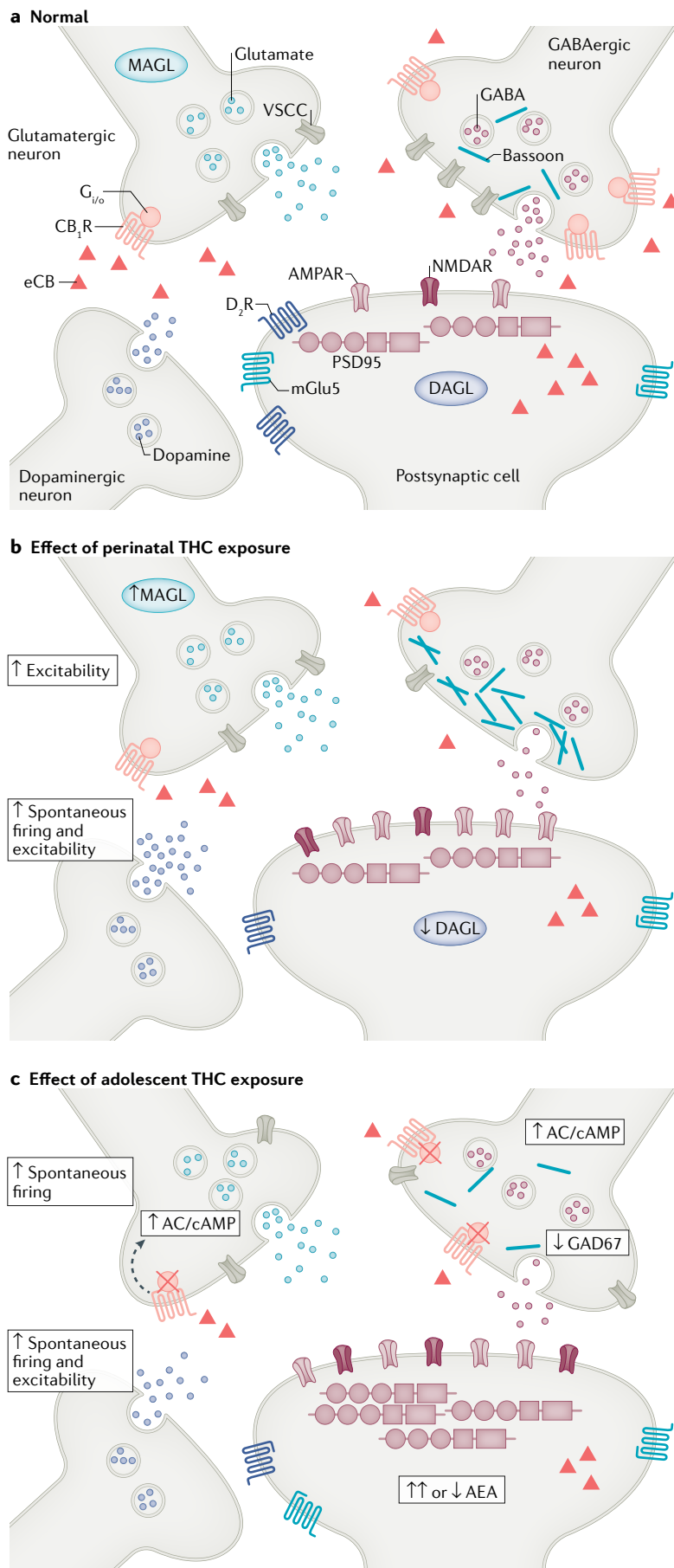
In addition to biochemical and molecular evidence, morphological experiments have also confirmed that maternal exposure to cannabinoids affects proliferation, neurite branching<sup>31–33</sup> and migration and differentiation of GABAergic and glutamatergic neurons<sup>34</sup>. Nanoscale insights are now also being revealed by the recent use of super-resolution imaging of the synapse, which demonstrates that THC exposure during gestation causes sex-specific molecular crowding at the pre-synaptic active zone of GABAergic terminals in the ventral tegmental area (VTA) of male rats, leading to decreased neurotransmitter release<sup>35</sup>. This further aligns with a decrease in the ratio of voltage-gated Ca<sup>2+</sup> channels to CB<sub>1</sub>Rs detected at inhibitory synaptic terminals after maternal THC exposure<sup>35</sup>.

The functional consequences of prenatal cannabinoid exposure are also reflected in the efficiency, activity and expression of receptors, channels, enzymes and other molecules essential for the expression of synaptic plasticity (FIG. 2). Given the crucial role of glutamatergic neurotransmission in synaptic plasticity, most studies have focused on this transmitter system in relation to fetal cannabinoid exposure. Such investigations provide evidence that baseline and evoked levels of glutamate are reduced in vivo in the hippocampus<sup>36</sup> and frontal cortex<sup>37</sup> in young and adult rats prenatally exposed to a synthetic high-affinity CBR agonist, WIN-55,212-2.

Similar results are evident in cortical cell cultures from PND1 pups exposed to WIN-55,212-2 in utero, accompanied by a reduction in the function of NMDA receptors<sup>33</sup>. Glutamate receptors are necessary for the expression of long-term potentiation (LTP) and LTD, and synaptic strength is mediated by the addition or removal of synaptic AMPA receptors<sup>38</sup>. Recently, adult male rats, but not adult female rats, with prenatal THC exposure were shown to have an increased AMPA/NMDA ratio associated with changes in the composition of receptor subunits in the VTA<sup>35</sup> and reduced mRNA expression of prefrontal cortex (PFC) perisynaptic mGlu5 receptors, crucial effectors for the expression of the eCB-mediated LTD<sup>39</sup>. The various synaptic disturbances identified in the hippocampus<sup>11,40,41</sup>, PFC<sup>39</sup> and VTA<sup>35</sup> due to prenatal cannabinoid exposure range from short-term (depolarization-induced suppression of inhibition, DSI) to long-term (NMDA-mediated LTP, NMDA-mediated LTD and eCB-mediated LTD) forms of synaptic plasticity.

Disruption in the gain and loss of synaptic efficiency induced in different cell types and neurotransmitter systems is relevant to the behavioural outcomes associated with in utero cannabinoid exposure. Interestingly, Frau and collaborators demonstrated sex-specific hyperexcitability of dopaminergic neurons due to an excitatory–inhibitory imbalance and reduced synaptic inhibition onto VTA dopaminergic neurons, highlighting a switch from LTD to LTP in juvenile males prenatally exposed to THC<sup>35</sup>. Such changes in dopaminergic neurons that project from the VTA to the NAc could explain the increased vulnerability for enhanced drug intake seen in adult male rats exposed to THC during gestation<sup>26,28</sup>. Plasticity deficits such as the alterations in hippocampal LTP<sup>36</sup> and LTD<sup>24</sup> and inhibition of DSI<sup>41</sup> as well as sex-dimorphic impaired hippocampal oscillations resulting in aberrant microcircuit function in males due to a decrease in the population of inhibitory (CCK-positive) interneurons<sup>40</sup> would be predictive of altered cognitive functions such as memory, learning and social interaction. Indeed, there is evidence that the PFC of adult male rats with prenatal THC exposure is characterized by an abolition of the eCB-mediated LTD and increased excitability in pyramidal neurons correlated with deficits in socialization<sup>39</sup>.

Dysfunction apparent in synaptic circuits within mesocorticolimbic pathways linked to prenatal cannabinoid exposure in preclinical models could be relevant to behavioural outcomes evident in human studies of cannabis exposure during fetal life. For instance, findings in preclinical studies echo observations of deficits in learning, memory, attention and aggressive behaviour assessed in children and adolescents exposed to maternal cannabis use<sup>42</sup>. Neuroimaging studies have also documented altered neuronal functioning in cortical regions of young adults with prenatal cannabis exposure in relation to working memory<sup>43</sup>. Moreover, alterations of the dopaminergic system revealed by animal models could reflect the increase in the expression of depressive symptoms<sup>44</sup>, anxiety-like behaviours<sup>45</sup> and drug use observed during childhood, adolescence<sup>46</sup> and young adulthood<sup>47</sup>. TABLE 1 provides a summary



**Fig. 2 | Impact of developmental THC exposure on synaptic plasticity in adulthood.** Schematic overview of the synapse under normal conditions or after perinatal or adolescent  $\Delta^9$ -tetrahydrocannabinol (THC) exposure based on rodent models. **a** | During normal conditions, in the absence of THC exposure, endocannabinoid (eCBs) synthesized postsynaptically are released into the synaptic cleft and influence GABAergic and glutamatergic synapses by binding to cannabinoid receptor type 1 ( $CB_1R$ ) expressed presynaptically. In this way, the eCB system tightly regulates these synapses as well as other neurotransmitter systems such as the dopamine system (for example, indirectly regulated by cannabinoid receptor on inhibitory GABA terminals that synapse onto dopaminergic neurons; not shown in figure). **b** | After prenatal THC exposure, GABAergic mechanisms are significantly abrogated due to a decrease in voltage-sensitive calcium channel (VSCC) density, synaptic ‘crowding’ by bassoon scaffolding proteins and reduced GABA release. In contrast, glutamatergic and dopaminergic inputs are sensitized (as measured by increased firing and excitability), which is paired with shifts in postsynaptic receptor expression, including reduced dopamine  $D_2$  receptor ( $D_2R$ ) and metabotropic glutamate receptor 5 (mGlu5) levels and increased AMPA/NMDA ratios, hallmarks of compensatory downregulation. Moreover, prenatal THC exposure impacts the expression of eCB system enzymes (increased monoacylglycerol lipase (MAGL) and decreased diacylglycerol lipase (DAGL) expression), resulting in a reduction of eCB bioavailability (represented by the red triangles). **c** | Similarly, adolescent THC exposure results in significant reductions in GABAergic tone via reductions in the expression of the critical GABA synthesis enzyme glutamate decarboxylase 67 (GAD67) as well as greater firing and excitability of presynaptic glutamatergic neurons and dopaminergic inputs. In the postsynaptic cell, expression of AMPA and NMDA receptors is increased in animals with adolescent THC exposure. Although the same biological markers are not always studied in perinatal and adolescent models, adolescent THC exposure also alters the architecture of the synapse, as evident in increased expression of postsynaptic density protein 95 (PSD95) at glutamatergic synapses. In addition, adolescent THC exposure leads to disruptions of key eCB system components, including reduced expression of  $CB_1R$ s, impaired  $CB_1R$  G protein functionality (as depicted by red crosses) associated with increased expression of second messengers such as adenylyl cyclase (AC) and cyclic adenosine monophosphate (cAMP) and abnormal levels of anandamide (AEA). AMPAR, AMPA receptor; NMDAR, NMDA receptor.

of the molecular and behavioural effects described for gestational cannabinoid exposure in humans and animal models.

**Cannabis exposure during lactation**

In the USA, it is estimated that 15% of breastfeeding women use cannabis<sup>48</sup>. Despite this high incidence, the effects of cannabis during lactation on children have not been well studied. As lipidic molecules, cannabinoids such as THC can easily be transferred through the maternal milk during breastfeeding<sup>49</sup>. A recent study reported that ~2.5% of maternal THC is transferred to the infant<sup>50</sup> depending on the chronicity and the concentration of THC in the cannabis used. Such exposure may have significant consequences given the

**Table 1 | Effects associated with exposure to cannabis or cannabinoid agonists during gestation and/or lactation in humans and animal models in relation to behavioural and neurobiological outcomes during exposure and later in life**

Gestation (0–40 weeks for humans, GD0–GD22 for rodents)	Infancy and childhood (0–11 years for humans, PND1–PND25 for rodents)	Adolescence (12–18 years for humans, PND28–PND65 for rodents)	Adulthood (>18 years for humans, >PND70 for rodents)
<b>Humans</b>			
↓ D <sub>2</sub> R mRNA (NAc and amygdala) <sup>36</sup>	↑ Sleep disturbances <sup>146,147</sup>	↑ Delinquency <sup>148</sup>	↑ Drug use <sup>47</sup>
↓ SCG10 (also known as stathmin 2) mRNA (hippocampus and striatum) <sup>24</sup>	↓ Cognition (verbal reasoning, attention, memory) <sup>42</sup>	↑ Drug-seeking behaviours <sup>46</sup>	↓ Executive functions <sup>43</sup>
↓ CB <sub>1</sub> R mRNA (cerebrum) <sup>24</sup>	↑ Aggression and impulsivity <sup>149,150</sup>	↓ Visual memory and integration <sup>151</sup>	↑ Neuronal functioning in cortical regions during a visuospatial task <sup>43</sup>
↓ DAGL mRNA (cerebrum) <sup>24</sup>	↑ Depressive and anxious behaviours <sup>44,45</sup>	–	–
↑ MAGL mRNA (cerebrum) <sup>24</sup>	–	–	–
<b>Rodents</b>			
↓ CB <sub>1</sub> R mRNA and function in cortical neurons <sup>11</sup>	Change in social communication <sup>33*,61,66*</sup>	↓ Memory retention <sup>36*</sup>	↓ Social interaction <sup>39,41,60</sup>
↓ D <sub>2</sub> R mRNA in NAc <sup>26</sup>	↑ Locomotor activity <sup>66*</sup>	↑ Social anxiety <sup>152</sup>	↑ Social discrimination <sup>60</sup>
–	GABA switch delayed in PFC <sup>61</sup>	↑ Sensitivity to THC challenge <sup>35</sup>	↑ Opioid sensitivity <sup>29,57</sup>
–	↓ NMDAR functions <sup>33*</sup>	↓ Glutamate levels in PFC and hippocampus <sup>31,36*,37*,55</sup>	↓ Memory <sup>33*,36*,40,55</sup>
–	↓ CB <sub>1</sub> R density in hippocampus <sup>40</sup>	↓ NMDA-mediated LTP in hippocampus <sup>36*</sup>	Change in motor functions <sup>41,36*</sup>
–	–	↑ Excitability of dopaminergic neurons in VTA <sup>35</sup>	↓ LTD and LTP in PFC and hippocampus <sup>35,36*,39,60</sup>
–	–	↓ GABA release in the VTA <sup>35</sup>	↓ DSI in hippocampus <sup>41*</sup>
–	–	↑ AMPA/NMDA ratio in VTA <sup>35</sup>	Change in pyramidal neuronal excitability of PFC <sup>39,60</sup>
–	–	↓ Glutamatergic inputs (number and/or strength) onto dopaminergic neurons in VTA <sup>35</sup>	↓ mGlu5, TRPV1 and DAGL mRNA in PFC <sup>39*,60</sup>
–	–	Change in long-term plasticity polarity <sup>35</sup>	↓ D <sub>2</sub> R mRNA in NAc <sup>26</sup>
–	–	–	↑ DOPAC/DA ratio in NAc and VTA <sup>58</sup>
–	–	–	↓ Glutamate levels (basal and evoked) <sup>36*,55</sup>
–	–	–	Change in CB <sub>1</sub> R density in cortex and hippocampus <sup>39</sup>
–	–	–	↓ CB <sub>1</sub> R function in striatum and hippocampus <sup>63,153*</sup>
–	–	–	↓ GABA levels (basal and evoked) in hippocampus <sup>63</sup>

The Δ<sup>9</sup>-tetrahydrocannabinol (THC) animal studies cited used low to moderate THC doses. CB<sub>1</sub>R, cannabinoid receptor type 1; DA, dopamine; DAGL, diacylglycerol lipase; DOPAC, dihydroxyphenylacetic acid; D<sub>2</sub>R, dopamine D<sub>2</sub> receptor; DSI, depolarization-induced suppression of inhibition; GABA, γ-aminobutyric acid; GD, gestational day; LTP, long-term potentiation; LTD, long-term depression; MAGL, monoacylglycerol lipase; mGlu5, metabotropic glutamate receptor 5, NAc, nucleus accumbens; NMDAR, NMDA receptor; PFC, prefrontal cortex; PND, postnatal day; TRPV1, transient receptor potential vanilloid 1; VTA, ventral tegmental area.

\*The cannabinoid receptor agonist WIN-55,212-2 was used.

early postnatal burst in synaptogenesis that occurs during this period when women breastfeed their children. Synaptogenesis, which begins during gestation, peaks in midchildhood, followed by activity-dependent pruning of excessive synapses later in childhood that continues through adolescence and into early adulthood<sup>51</sup>. Brain development during childhood that is marked by the emergence of increased neural connectivity is highly sensitive to environmental stimuli and activity-dependent experiences such as drug exposure.

To our knowledge, only two small studies have investigated the consequences of cannabis use during breastfeeding, with conflicting results on motor and mental performance in 1-year old children<sup>52,53</sup>. Those studies were conducted more than 30 years ago, when the concentrations of THC in available products were likely much lower than today (BOX 1). Given the evident challenges with human studies, particularly for this developmental epoch, animal models are critical to provide neurobiological insights. Most animal models

though have used ‘perinatal’ protocols that include both the prenatal period and the postnatal period, considering that the first 10 postnatal days in rodents correspond to the third trimester of pregnancy in humans<sup>54</sup>. Such studies have observed results similar to those from strictly prenatal models with alterations of glutamatergic<sup>31,55,56</sup>, opioidergic<sup>57</sup>, dopaminergic<sup>58</sup> and cannabinoid<sup>59</sup> function and expression in mesocorticolimbic brain areas with cannabinoid exposure extended to postnatal days (gestational day 5 to PND24). Moreover, the perinatal exposure also induces higher morphine self-administration<sup>57</sup> as well as deficits in memory, social recognition, olfactory and inhibitory avoidance tasks<sup>55</sup>. The similar long-lasting disturbances caused by these prenatal and perinatal models strongly suggest that the early trimesters of pregnancy are crucial windows of cannabinoid sensitivity for many long-term outcomes documented to date. However, while the deficits in CB<sub>1</sub>R mRNA expression after a prenatal THC exposure are mainly evident during fetal life<sup>11,24</sup>, perinatal THC exposure leads to long-lasting alterations in different brain areas in adulthood<sup>59</sup>. To differentiate the effects of THC exposure restricted to the late trimester and early infancy periods of development, a few studies have begun to strictly examine exposure during lactation in rodent models<sup>60,61</sup>. One line of these studies has focused on the GABAergic system since GABA signalling is critical for normal spontaneous network activity and for maturation and refinement of neuronal networks<sup>62</sup>. Moreover, perinatal THC exposure has been shown to reduce basal and stimulated GABA release in the hippocampus of adult rats<sup>63</sup>, whereas prenatal exposure to a CBR agonist increases the number of migrating GABAergic interneurons during early gestation<sup>34</sup>. Importantly, although GABA is known as the main inhibitory neurotransmitter in the CNS, it starts its developmental journey being excitatory in the immature brain. The GABA switch from excitatory to inhibitory transmission relies on a developmentally and genetically regulated expression of the sodium–potassium–chloride transporter (NKCC1) and potassium–chloride co-transporter 2 (KCC2). This shift from depolarization to hyperpolarization is due to a reduction of the intracellular levels of chloride during the second week of postnatal development in rodents<sup>64,65</sup>. Recent studies now provide evidence that exposure to THC during the lactation period (PND1 to PND10) delays the GABA switch in the PFC<sup>60</sup>. This reprogramming of the GABAergic trajectory correlates with retarded KCC2 gene upregulation necessary for the GABA excitatory to inhibitory switch. Moreover, the delay in GABA maturation is associated with aberrant ultrasonic vocalizations, reflective of impaired early social communication and aversive affective state, in the pups. Abnormality in this first form of cognition and emotional distress in rodents is also evident after prenatal cannabinoid exposure<sup>33,66</sup>. Lactation-delivered THC also induced electrophysiological disturbances that persist into adulthood, where a hypoexcitability of pyramidal neurons (layers 5 and 6) is evident and is associated with impairment of several forms of synaptic plasticity in the PFC, including a total abolishment of NMDA-mediated LTP and eCB-mediated LTD, with

augmentation in the magnitude of mGlu2/3-mediated LTD<sup>60,61</sup>. Both LTP and eCB-mediated LTD are restored by inhibition of MAGL, the degradative enzyme for 2-AG, suggesting a deficit in 2-AG bioavailability as contributing to the long-term effects of THC exposure during lactation.

While there remains a large gap in knowledge regarding the specific contribution of cannabis exposure during infancy, particularly with breastfeeding, initial preclinical lactation models appear to suggest an impact on neural network assembly, disturbance of the physiological trajectory of major neurotransmitter systems, dysregulation of the balance between excitation–inhibition balance leading to dysfunction in synaptic plasticity and behavioural disruptions as in adults.

### Second-hand cannabis exposure

Another aspect of postnatal cannabis exposure that deserves attention is second-hand exposure during the juvenile period. The evidence that second-hand cannabis smoke has effects on development is extremely limited compared with the evidence for second-hand tobacco smoke. However, it has been documented that there are significant concentrations of THC and other THC metabolites in oral fluid, blood and/or urine samples of children exposed to second-hand cannabis smoke<sup>67</sup>. Cannabis use among parents with children at home in the USA increased from 4.9% to 6.8% between 2002 and 2015, with an increase to 17.4% among cigarette-smoking parents<sup>68</sup>. Moreover, air particle monitoring in almost 300 homes of families with at least one child younger than 14 years showed that 15.1% had documented indoor cannabis smoking<sup>69</sup>. These trends are disconcerting given that toddlers exposed to second-hand cannabis smoke display cognitive and emotional problems<sup>70</sup>. While these and other studies suggest significant effects of second-hand cannabis exposure on children, there continues to be a lag in preclinical studies to address gaps in knowledge regarding potential short-term and long-term neurobiological effects and that can circumvent the multiple challenging variables of human developmental studies.

### Cannabis exposure during adolescence

Adolescence is a distinct period of maturation, wherein many of the juvenile neurobiological processes and behaviours recede, but a significant amount of refinement occurs, specifically in areas relevant for cognitive function, reward and emotionality. Indeed, due to processes nearing final maturation within the PFC, striatum and amygdala, adolescence is considered a critical period especially susceptible to reward and affective processes. For clinical studies that document an increased association between adolescent cannabis use and psychiatric risk, such conditions include anxiety, depression, addiction and psychosis disorders<sup>71,72</sup>. Neurobiologically, even under conditions with a lack of outward differences in volumetric or white matter perturbations, structural morphology has been shown to correlate with cannabis use patterns such as age of onset<sup>73</sup>. Furthermore, activation differences are evident in several brain regions, including the PFC, striatum and amygdala<sup>73</sup>. Animal studies

in which complex factors (for example, genetics, environment and THC concentration) can be partly controlled, as compared with human investigations, demonstrate that adolescent THC exposure can impact normal neurodevelopmental processes causally linked to protracted behavioural effects relevant to humans.

As depicted in FIG. 1, the ECS undergoes significant changes during adolescence, with a critical involvement in synaptic pruning, a process characteristic of this developmental period needed to establish the ultimate architecture of the mature brain. THC exposure during adolescence is well documented to change the normal ontogenic profile of the ECS. For example, the normal correlations seen between AEA and 2-AG levels throughout the adolescent period in the PFC and NAc are discordant in THC-exposed animals<sup>19</sup>. Such differences may relate to findings that adolescent THC exposure leads to a premature peaking of AEA levels in the NAc in male rats in midadolescence, achieving levels comparable to those in adulthood<sup>19</sup>. Similarly, AEA levels that are normally increased in the PFC in late adolescence are reduced by escalating, high-dose THC exposure in female animals<sup>74</sup>. Unlike AEA, neither moderate nor high THC exposure alters 2-AG levels<sup>19,74</sup>, suggesting unique sensitivity of AEA signalling in the PFC and NAc to adolescent THC exposure. On a physiological level, adolescent THC exposure inhibits the normal eCB-mediated LTD seen in medial PFC layer 2/3, an effect reversed by increased AEA bioavailability through the chronic administration of an FAAH inhibitor<sup>75</sup>.

Adolescent THC exposure also significantly impacts the CB<sub>1</sub>R, with important contributions of dose and sex on CB<sub>1</sub>R expression and functionality. For example, whereas moderate dosing elevates CB<sub>1</sub>R levels in the amygdala of THC-exposed rats<sup>76</sup>, escalating, very high doses decrease CB<sub>1</sub>R levels in this region as well as in multiple brain regions, including the PFC, striatum, hippocampus, thalamus, substantia nigra, VTA and cerebellum<sup>74,77,78</sup> in adulthood regardless of sex. These latter results are concomitant with the functional reductions in CB<sub>1</sub>R G protein activity (as measured by [<sup>35</sup>S]GTPγS) in these regions<sup>77</sup>. When both sexes are studied together, the strongest changes in CB<sub>1</sub>R functionality are seen in females<sup>77</sup>. Female rats are also more sensitive than male rats to adolescent THC exposure, showing pronounced depression-like behaviour, social avoidance and memory deficits<sup>78–81</sup>, whereas male rats exhibit working memory deficits and greater sensitivity to THC's rewarding effects<sup>78,82</sup>. Notably, male rats show reduced CB<sub>1</sub>R levels in glutamatergic terminals in the VTA, which may contribute to their greater reward responsivity<sup>78</sup>. In contrast, female rats have elevated CB<sub>1</sub>R levels in the amygdala after moderate doses but reduced levels after higher THC exposure<sup>77</sup>, a key structure in emotional regulation. Female rats not only exhibit overall greater changes in the ECS after adolescent exposure, but also in THC metabolism during the adolescent dosing period, with higher levels of 11-OH-THC<sup>82,83</sup>, a metabolite with significant CB<sub>1</sub>R activity<sup>84</sup>. These sex differences are in line with greater susceptibility for divergent psychiatric vulnerability in humans, where females with cannabis use show greater susceptibility for depression, and males

appear more prone to addiction, including greater risk of development of cannabis use disorder<sup>72</sup>.

In addition to modulating the ECS, adolescent THC exposure has profound effects seen in adulthood on the excitatory and inhibitory mechanisms, which have been studied mainly in the PFC. Chronic exposure to escalating THC levels during adolescence significantly downregulates GAD67, the rate-limiting enzyme for GABA synthesis<sup>85</sup>, suggesting reduced GABA tone later in life. Interestingly, activating GABA<sub>A</sub> receptors in the PFC reverses THC-induced anxiety, a phenotype linked to reduced GABA levels<sup>86,87</sup>. In contrast, excitatory tone in the adult PFC is sensitized with adolescent THC exposure, with medial PFC pyramidal neurons demonstrating greater spontaneous firing<sup>86</sup>. The *in vivo* induction of glutamate release, by pharmacological inhibition of NMDA receptor, is also potentiated in medial PFC neurons in THC-exposed animals<sup>87</sup>. Adolescent THC exposure also increases the levels of postsynaptic glutamatergic markers in the PFC, including PSD95 (REF.<sup>88</sup>), NMDA receptor subunits GluN2A and GluN2B, and the AMPA receptor GluA1 subunit<sup>89,90</sup>. Importantly, adolescent THC exposure not only increases the overall levels of these glutamatergic markers in adulthood but also alters the normal developmental trajectory of the expression of glutamate receptor subunits that have functional significance for synaptic plasticity. For example, GluN2A, a subunit whose level progressively increases during development, reaches its highest levels in late adolescence in rats exposed to a low dose of THC. In contrast, GluN2B, a marker of developing neurons, whose level usually declines from midadolescence into adulthood, remains at an elevated level as a consequence of prior THC exposure. These findings indicate that adolescent THC exposure not only leads to premature maturation of key plasticity markers, which matches the premature pruning of dendritic spines<sup>91</sup>, but also simultaneously attenuates the normal reduction in NMDA receptor GluN2B content, delaying another facet of normal development. Altogether, the resulting abrogation of GABAergic markers when paired with elevated levels of glutamatergic effectors suggests significantly increased pyramidal excitatory output. Additionally, high-dose adolescent THC-induced reduction of eCB-mediated LTD from layer 5 pyramidal cells is reversed by increasing AEA bioavailability<sup>75</sup>. Furthermore, dendritic spines and arborizations in pyramidal layer 2/3 neurons (which project to subcortical structures such as the amygdala) are significantly reduced even after low-dose adolescent exposure<sup>74,92</sup>, accompanied by a marked reorganization of genes related to neuronal development and synaptic plasticity in young adulthood<sup>91</sup>. Altogether, these results suggest increased excitatory tone (FIG. 2), but potentially reduced top-down control and diminished intercortical coordination of output signals.

Collectively, adolescent THC-induced alterations in excitatory–inhibitory balance have significant implications for behavioural outcomes, which are particularly relevant to psychosis-related conditions, for which a substantial body of evidence documents improper processing of the cortical signal-to-noise ratio<sup>93,94</sup>. A battery of rodent studies demonstrate that adolescent THC



exposure increases psychosis-like behavioural phenotypes, including greater sensitivity to phencyclidine-induced locomotor activity, prepulse inhibition impairments, social avoidance and working memory deficits. Activation of GABA<sub>A</sub> receptors in the medial PFC of THC-exposed rats reversed these behavioural effects<sup>86</sup>. Dopaminergic activity also plays a key role in the ‘noisy cortex’ hypothesis of psychosis, wherein dopaminergic activity leads to greater sensitivity of glutamatergic neurons and increased salience attribution. Consistently, a history of adolescent THC exposure sensitizes VTA dopaminergic cell firing and excitability<sup>80</sup>. Moreover, oral consumption of THC during adolescence reduces CB<sub>1</sub>R expression in the VTA, specifically on glutamatergic inputs, which would be expected to render excitatory inputs to the VTA less sensitive to eCB inhibition, resulting in enhanced glutamatergic tone and increased dopamine levels<sup>78</sup>. Interestingly, the marked reorganization of developmentally regulated THC gene networks in layer 2/3 pyramidal neurons of the adolescent exposure rat model mimics gene networks dysregulated in the PFC of individuals with diagnosed schizophrenia<sup>91</sup>.

Beyond affective and psychosis-like conditions, a history of adolescent cannabis use is associated with increased susceptibility to drugs of abuse. Preclinical models have recapitulated these results, with adolescent THC exposure associated with increased sensitivity to cocaine<sup>95</sup>, heroin<sup>28,96–98</sup> and cannabinoids<sup>99</sup> later in life. In addition to the changes to the dopaminergic system described above, which has long been implicated in mediating the formation and maintenance of pro-addictive behaviour<sup>100</sup>, adolescent THC exposure also increases expression of the gene encoding endogenous opioid proenkephalin (*Penk*) in the NAc<sup>28</sup>, and increased *Penk* expression is causally associated with increased heroin self-administration<sup>98</sup>. Aspects of genetics and behavioural traits also appear to play a role in adolescent THC-induced opioid sensitivity. For instance, high-dose

adolescent THC exposure increased heroin responding in ‘addiction-prone’ rats (Lewis rats)<sup>96</sup> compared with Wistar rats<sup>97</sup>. Overall, collective results in the field suggest that the long-term impact of adolescent THC/cannabis exposure on future drug sensitivity may depend on multiple factors, including behavioural traits, genetics, amount of THC (including frequency of exposure) and sex.

### Epigenetics, the link across time

The long-term consequences of developmental cannabinoid exposure naturally raise questions about the underlying molecular mechanisms that maintain such protracted effects. Epigenetic factors are considered the most likely candidates to explain enduring phenotypic changes since the epigenome provides the cellular context for environmental influences, including cannabinoid exposure, to functionally alter genes and related behaviours<sup>101–103</sup>. The original definition in the 1950s stated “an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence”<sup>104,105</sup>. Today, the term ‘epigenetics’ is typically used to describe molecular biological mechanisms that modulate gene expression without altering the genetic code. The main epigenetic mechanisms implicated in the effects of cannabinoids are summarized in BOX 2. Epigenetics is not only crucial for normal development but can also create the most well-characterized biological frameworks known to maintain aberrant neuronal processing and plasticity over long periods. Generally, the interaction between genes and regulatory genomic DNA elements, epigenetic modifiers and transcriptional factors determines the expression state of genes. The network of these processes is tightly coordinated during development and within different organ systems, including the brain<sup>106,107</sup>. The ECS, similarly to other biological systems, is highly regulated by epigenetics<sup>108,109</sup>, and these processes are critical in controlling various aspects of both short-term and long-term synaptic communication and plasticity in early life<sup>110</sup>, the adult brain<sup>111</sup> and various neuropsychiatric disorders<sup>91</sup>.

Despite the role of epigenetic factors in neurodevelopmental processes and in maintaining molecular memories throughout life, there remains a surprising dearth of studies regarding epigenetic mechanisms in relation to developmental cannabis/THC exposure (TABLE 2). Of the known epigenetic mechanisms, most information relevant to the neurodevelopmental effects of cannabinoids has been accrued regarding histone modifications. Our group revealed distinct alterations in the histone modification profile of the NAc in adult rats with prenatal THC exposure<sup>26,112</sup>. The disturbances were characterized by decreased levels of the trimethylation of lysine 4 on histone H3 (H3K4me3; a transcriptionally permissive mark), increased levels of dimethylation of lysine 9 on histone H3 (H3K9me2; a repressive mark) and decreased RNA polymerase II association with the promoter and coding regions of the dopamine D<sub>2</sub> receptor gene (*Drd2*) in the NAc. Importantly, THC-related chromatin modifications, which would be predictive of reduced transcription, were linked to disturbances of the *Drd2* mRNA expression in both humans and rodents

#### Box 2 | Main epigenetic mechanisms implicated in the effects of cannabinoids

##### DNA methylation

Methyl groups are added directly to the DNA. The role of DNA methylation is dependent upon genomic location, developmental stage, cell type and disorder. Methylation in promoter regions and transcriptional regulatory sequences has frequently been associated with gene silencing, whereas methylation within the gene body is less understood and may act as either a positive effector or a negative effector<sup>159,160</sup>.

##### Histone modifications

Histone proteins are associated with ~150 bp of DNA to form the nucleosome, the basic unit of chromatin. Histones are subject to well-characterized covalent modifications, including acetylation, methylation, phosphorylation, ubiquitination and sumoylation<sup>161</sup> and a variety of more recently discovered structures in the brain<sup>162</sup>. The modifications can influence both the accessibility of genomic regions and the binding of transcription factors to the DNA.

##### Non-coding RNAs

These functional RNA molecules are transcribed from DNA and regulate gene expression at the transcriptional and post-transcriptional levels<sup>163</sup>. While the exact genomic targets of specific non-coding RNAs remain to be characterized, the multiple non-coding RNAs are mechanistically intriguing given the variety of tissue-specific cellular and developmental processes that can be influenced by them<sup>164</sup>. Some non-coding RNAs even persist during the maturation of germ cells and in early embryo development and thus are interesting candidates for the propagation of cannabinoid effects across generations<sup>165</sup>.

Table 2 | Overview of studies on the epigenetic dysregulation of the brain in association with cannabinoid exposure

Cannabinoid	Exposure period	Brain region and rodent subject studied	Epigenetic alteration	Effect <sup>a</sup>	Ref.
THC	Prenatal	Adult male rat NAc	H3K4me2, H3K9me3 Promoter, gene body	↓ <i>Drd2</i> mRNA levels	26
THC	Adolescent	Adult male rat NAc shell	H3K9me2, H3K9me3 Promoter, gene body	↑ <i>Penk</i> mRNA levels	98
THC	Adolescent	Adult female rat PFC	H3K9me3 Global levels, promoters	↓ mRNA expression of genes related to ECS and synaptic plasticity	75b
WIN-55,212-2 cannabinoid agonist	Adolescent	Adult male mouse hippocampus	DNA methylation Intragenic	↑ DNA methylation and ↓ mRNA expression of <i>Rgs7</i>	147
WIN-55,212-2 cannabinoid agonist	Adolescent, adult	Adult male rat PFC	Chromatin accessibility (ATAC-seq) Promoter, gene body	↑ Accessibility at <i>Npas2</i> and splicing	115
HU-210 cannabinoid agonist	Adolescent	Adolescent male rat entorhinal cortex	microRNAs	Altered expression of various microRNAs	117
THC	Adolescent, adult	Adolescent and adult female rat hippocampus, NAc and amygdala	H3K4me2, H3K9me3, H3K14ac Global levels	Brain region-specific and age-specific alterations of histone modifications at different times after exposure	90b
THC	Adolescent; Preconception (both parents)	Adult male and female rat NAc	CpG DNA methylation at promoters, intergenic regions, especially in gene bodies	Altered methylation at loci implicated in synaptic plasticity, including the <i>Dlg4</i> gene network	120
THC	Preconception (father)	Adult rat NAc with THC exposure (father only)	DNA methylation Promoter	↓ DNA methylation of synaptic <i>Dlgap2</i>	139
WIN-55,212-2 cannabinoid agonist	Preconception (father)	Adult male rat PFC	DNA methylation Global levels	↑ DNA methylation and ↑ DNMT expression	148

ATAC-seq, assay for transposase-accessible chromatin using sequencing; DNMT, DNA methyltransferase; ECS, endocannabinoid system; H3K4me2, dimethylation of lysine 4 on histone H3; H3K9me2, dimethylation of lysine 9 on histone H3; H3K9me3, trimethylation of lysine 9 on histone H3; H3K14ac, acetylation of lysine 14 on histone H3; NAc, nucleus accumbens; PFC, prefrontal cortex; THC, Δ<sup>9</sup>-tetrahydrocannabinol. <sup>a</sup>Arrows indicate the direction of change; in several large-scale studies different directions were observed, depending on the conditions, locus or RNA. <sup>b</sup>The functional significance of histone modifications can be transcriptional repression (H3K4me2 and H3K9me3) and transcriptional activation (H3K14ac). Enhanced chromatin accessibility correlates with increased mRNA expression. The potential roles of DNA methylation differ by locus and can regulate both transcription and splicing. MicroRNAs are involved in post-transcriptional repression. <sup>c</sup>Very high dose THC (in excess of levels normally used by humans) used in the study.

and persisted into adulthood. These findings are intriguing since reduction of the dopamine D<sub>2</sub> receptor function and mRNA levels is characteristic of individuals with substance use disorders<sup>113</sup>, suggesting that early cannabinoid exposure potentially enhances addiction vulnerability through altering the epigenetic processes at the *Drd2* locus. Unfortunately, this remains the only published study to date to investigate direct epigenetic effects of prenatal THC exposure. Thus, the epigenetic relationship to other molecular alterations documented with prenatal THC exposure is currently unknown.

Knowledge is also limited regarding epigenetic factors in relation to adolescent THC exposure, but several reports have described persistent disturbances associated with exposure during this period. For instance, enduring reduction of repressive epigenetic marks, H3K9me2 and H3K9me3, has been detected at the opioid neuropeptide *Penk* locus in the NAc of adult rats following adolescent THC exposure, concomitant with increased *Penk* mRNA levels and enhanced heroin self-administration behaviour in adulthood<sup>98</sup>. One of the strongest pieces of evidence for a critical role of epigenetic disturbances linked to developmental THC exposure comes from leveraging an unbiased strategy of RNA sequencing of pyramidal layer 2/3 neurons<sup>111</sup>. The results revealed

that low-dose THC exposure during adolescence leads to a profound reorganization of the transcriptome (only 5% overlap with the normal developmental gene expression signature) predominantly related to histone modification, chromatin remodelling and synaptic plasticity gene networks, matching morphological alterations of dendritic spines. Importantly, the history of adolescent THC exposure substantially enhanced the coordination between epigenetic and dendritic/synaptic plasticity-related genes, with the prominent epigenetic modifications associated with these networks being H3K4me3 and KMT2A, a histone methyltransferase with specific activity at H3K4 (REF.<sup>111</sup>). These findings are intriguing given that H3K4 methyltransferases are essential for neurogenesis<sup>114</sup>.

A comprehensive set of investigations by Prini et al. has also highlighted epigenetic differences on specific global chromatin modifications within mesocorticolimbic brain regions (PFC, hippocampus, NAc and amygdala) depending on whether THC exposure (escalating moderate to high dose) occurred during adolescent or adulthood<sup>75,90</sup>. In line with the concept of higher vulnerability of the immature brain to external stimuli, there was a more robust and complex response to adolescent THC exposure as compared with adult exposure

on repressive histone methylation (H3K9me2 and H3K9me3) and an acetylation mark of active transcription (H3K14ac). Moreover, alterations of H3K9me3 in the PFC were associated with cognitive impairments<sup>75</sup>, and pharmacological blockade of H3K9me3 during adolescent exposure to THC prevented the development of cognitive deficits, demonstrating the causal contribution of epigenetic mechanisms to the long-term behavioural effects induced by adolescent THC exposure.

An apparent greater sensitivity of the epigenetic machinery to adolescent versus adult cannabinoid exposure was also demonstrated with WIN-55,212-2 (REF.<sup>115</sup>). Adolescent, but not adult, rats exposed to this CBR agonist developed increased sensitization to cocaine correlated with chromatin remodelling, histone hyperacetylation and decreased levels of histone deacetylase (HDAC6) expression. There were also alterations of chromatin accessibility involving genes such as *Npas2*, which encodes a transcription factor that regulates GABAergic neurotransmission. Once again, these pre-clinical data demonstrate that adolescent cannabinoid exposure significantly reprogrammes the epigenetic and behavioural responses to cannabinoids.

In addition to chromatin alterations, direct methylation of the DNA also occurs as a consequence of adolescent WIN-55,212-2 exposure, which led to memory deficits in adulthood accompanied by increased hippocampal AEA levels<sup>116</sup>. DNA hypermethylation was observed at the intragenic region of *Rgs7*, a gene involved in synaptic plasticity via G-protein-coupled receptor signalling cascades, and the deficits observed in hippocampus-dependent learning and memory processes were attributed to reduced expression of *Rgs7*. Abnormal expression of short non-coding microRNAs has also been detected in adult animals with adolescent cannabinoid treatment, which was exacerbated by prior maternal stress<sup>117</sup>, but the functional developmental consequences remain to be elucidated.

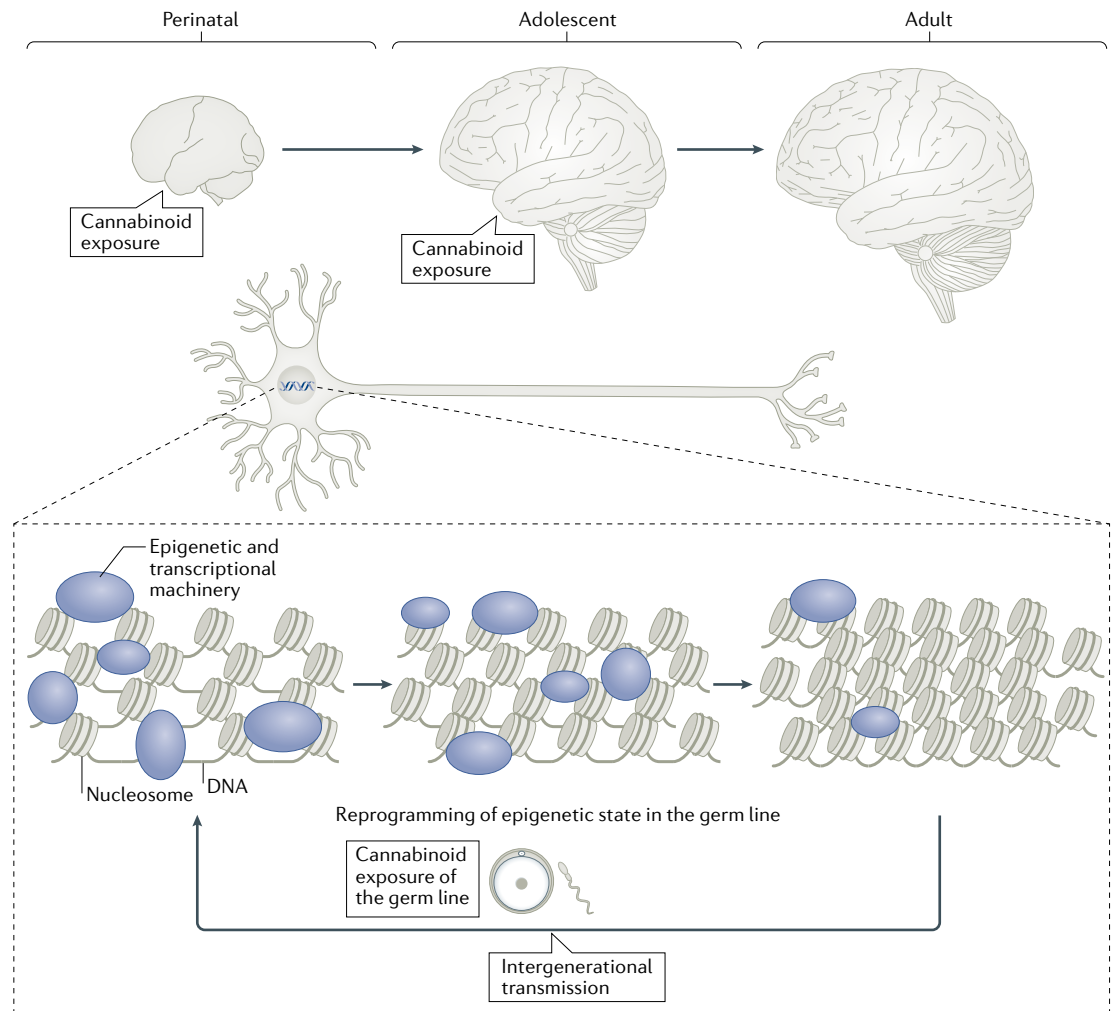
Although not extensive to date, current research suggests that cannabis exposure during multiple stages of development can alter epigenetic mechanisms to change the trajectory of individual vulnerability to neurobiological and phenotypic dysregulation later in life. FIGURE 3 illustrates a simplified model based on various lines of evidence for the role of chromatin accessibility and other epigenetic mechanisms as major factors in the regulation of normal brain development<sup>118</sup>, which can be affected by developmental cannabinoids. The fact that epigenetic modifications are linked to THC-induced neural and behavioural phenotypes has important treatment implications and can account for some inconsistent findings regarding developmental effects of cannabis/THC since epigenetic modifications are reversible and thus sensitive to environmental conditions.

### Intergenerational impact

In addition to the direct effects of cannabis exposure during development on the brain, there is growing evidence that cannabis may also act through the germ line to shape synaptic development and behaviour across generations. The first attempt to address this possibility

was our cross-generational study where male and female rats were intermittently exposed to a low dose of THC during adolescence and mated during adulthood. Surprisingly, their F1 male progeny displayed increased work effort to self-administer heroin and abnormal behaviours during heroin withdrawal<sup>119</sup>. As male offspring (females were not tested) were cross-fostered in these studies, these intergenerational phenotypes were likely inherited through the germ line. Neurobiologically, the adult F1 generation with parental adolescent THC exposure also had abnormal mRNA expression of CBR, dopamine receptor and glutamatergic receptor genes in the striatum and altered LTD measured electrophysiologically<sup>101</sup>. Both male and female progeny exhibit significant glutamatergic disturbances in the dorsal striatum in adulthood, and these effects appear stronger in females<sup>101</sup>. Genome-wide alterations of DNA methylation in the NAc have also been documented in adult offspring of THC-exposed parents, indicating robust intergenerational epigenetic reprogramming<sup>120</sup>. These effects were more prominent for synaptic plasticity-related genes in a functional network centred on *Dlg4* (REF.<sup>120</sup>). *Dlg4* encodes PSD95, a membrane-associated guanylate kinase scaffolding protein located in neural postsynaptic densities and important for regulating dopamine–glutamate interactions<sup>121</sup>. Epigenetic dysregulation of *Dlg4* has been linked to abnormal glutamatergic transmission involved in morphine conditioning<sup>122</sup>, consistent with the earlier observation of increased heroin self-administration in adult offspring with parental THC exposure<sup>119</sup>. Many of the differentially methylated genes in the *Dlg4* network have been speculated to be susceptibility genes for psychiatric conditions such as schizophrenia, depression, autism and obsessive–compulsive disorder<sup>123,124</sup>.

Multiple other studies addressing intergenerational effects of cannabinoids have now been reported but with key differences between experimental paradigms and in outcomes. For instance, a study similarly examining adolescent THC exposure in both parents, but in a different rats strain, reported reduced heroin reinforcement behaviour in male offspring<sup>125</sup>. The effects of THC exposure before conception in either the father or the mother have also been evaluated in independent studies. THC exposure limited to females (when as adolescents) diminished the reward-facilitating effects of THC and amphetamine in their male offspring (females were not tested)<sup>126</sup>. In contrast, adolescent exposure of female rats to WIN-55,212-2 long before conception increased morphine sensitivity in both male and female offspring<sup>127,128</sup>. Comparatively, males exposed to low or moderate doses of THC during adulthood sired offspring with reduced attentional performance, adolescent hyperactivity, and impaired learning and memory in both sexes<sup>129,130</sup>. More research is clearly needed, but these findings suggest there may be unique cross-generational effects of cannabinoid exposure imparted through the male and female germ lines. Additionally, the cross-generational effects of THC documented thus far pertain to intergenerational effects (that is, in the F1 generation) where the offspring are derived from germ cells directly exposed



**Fig. 3 | Overview of proposed epigenetic effects induced by exogenous cannabinoids on the developing brain and epigenetic reprogramming of the germ line.** Epigenetic mechanisms, which occur on DNA in the nucleus of cells to regulate gene expression, are sensitive to cannabinoid exposure. During perinatal and adolescent stages of development, the chromatin structure of gene loci functionally related to synaptic plasticity (depicted by the synapse) is in a more open state, regulated by epigenetic and transcriptional machinery (represented by various coloured shapes) that can be altered by external stimuli such as cannabinoid exposure. As development progresses, this higher sensitivity of epigenetic responsiveness declines, rendering the adult brain less vulnerable to external influences. Marked epigenetic remodelling occurs during germ cell production, and reprogrammed germ cells are sensitive to the effects of cannabis. Such epigenetic interference by cannabis may escape the normal reprogramming of the germ line and thereby lead to intergenerational transmission, affecting the offspring from the earliest stages of development. The exact epigenetic mechanisms contributing to intergenerational transmission remain to be elucidated.

to THC. It will be important to determine whether the effects of THC can be transferred transgenerationally (that is, in the F2 generation for males and the F3 generation for females) independently of this direct germ line exposure.

The observations of cross-generational THC effects have spurred intense interest in the underlying epigenetic mechanisms allowing germ line transmission. Until recently, male gametes were believed to deliver little to no epigenetic memory to the fertilized oocyte. However, with the advancement of next-generation sequencing technology allowing enhanced resolution in methylation, chromatin modification and non-coding RNA analyses, studies are beginning to characterize a unique, environmentally responsive and functional

epigenetic landscape in sperm<sup>39</sup>. Comparatively, environmentally responsive epigenetic mechanisms in the female germ line have been understudied, in part due to technical challenges<sup>131</sup> that should diminish as single-cell/low-cell-number technologies become more widely adopted.

The ECS plays critical roles not only in the development of a variety of somatic cells and physiological systems but also in reproduction. An extensive literature has detailed the importance of the ECS to germ line development and function<sup>132,133</sup>. It is known that both male and female reproductive tissues express CBRs and eCBs. Cannabis-using women are known to produce lower-quality oocytes, which is associated with lower pregnancy rates<sup>134</sup>. In males, THC can disrupt

the normal development of sperm cells<sup>135,136</sup>, with both cannabis use in humans and THC exposure in rodents associated with reduced sperm counts and altered sperm motility<sup>137</sup>. Recent evidence also extends these documented effects to epigenetic mechanisms; cannabis use is associated with altered DNA methylation in human sperm<sup>138</sup>. Furthermore, THC exposure directly affects methylation at several gene loci in the rat model, including some genes differentially methylated in the sperm of cannabis users<sup>138</sup>. Most notably, *Dlgap2*, which has neuronal functions, including synapse organization and signalling, was hypomethylated in human cannabis user sperm, a finding validated in THC-exposed rat sperm<sup>139</sup>. Intriguingly, this locus was similarly hypomethylated in THC-exposed rodent NAc<sup>139</sup>. Moreover, several of the THC-affected loci in rat sperm were associated with genes differentially methylated in the offspring brain of adolescent THC-exposed rats identified by Watson et al.<sup>120</sup>, suggesting a relationship between THC-responsive epigenetic patterning in the germ line and THC responsive epigenetic patterning in the adult brain<sup>39</sup>.

Of other germ line epigenetic mechanisms, sperm microRNAs and other types of non-coding RNA have gained recognition as causal mechanisms driving intergenerational effects of paternal preconception exposures such as chronic stress and high-fat diet in rodents<sup>140–145</sup>. Additionally, recent studies have revealed that the sperm features a complex chromatin organization at genomic regulatory regions, such as enhancers that associate with chromatin structure in somatic cells during postnatal development<sup>146</sup>. Thus, future studies exploring the constellation of these implicated mechanisms will elucidate how the THC-responsive germ line epigenome may reshape early neuronal development to promote sustained alterations in synaptic function and behaviour across the lifetime.

## Conclusion

While debates mount regarding the developmental effects of cannabis, accumulating evidence from various lines of research demonstrates that there is potential for exposure during prenatal and adolescent periods to change the trajectory of various neurobiological systems. The evidence also clearly identifies perturbation of synaptic plasticity as a common characteristic of the adult brain despite THC exposure limited to early developmental periods. Importantly, the causal disturbances of neural processes in adulthood by early cannabinoid exposure are linked to behavioural phenotypes predictive of psychiatric and addiction risk. Despite the growing literature, there nevertheless remain large gaps in knowledge regarding individual differences related to early cannabinoid exposure, including genetics, sex (including aspects of puberty that have not been addressed), other environmental insults during development (for example, trauma/stress, toxins and other drugs) and the amount/frequency of exposure (including the combination of various cannabinoids with THC in different strains being consumed today). Systematic studies of these factors are also critical to identify individuals at potential risk so as to optimize early interventions that could prevent the full onset of adult psychopathology. Nevertheless, it is important to emphasize that any such psychopathology need not be deterministic. The marked epigenetic reprogramming induced by THC, and that could account for the parental environment to be transmitted across generations, emphasizes that the observed neurobiological disturbances and behaviours can be reversed. The dynamic nature of epigenetic mechanisms in contrast to static genetic inheritance could thus provide opportunities to counter negative consequences of developmental cannabis exposure.

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