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Molecular model of cannabis sensitivity in developing neuronal circuits

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Abstract

Prenatal cannabis exposure can complicate in utero development of the nervous system. Cannabis impacts the formation and functions of neuronal circuitries by targeting cannabinoid receptors. Endocannabinoid signaling emerges as a signaling cassette to orchestrate neuronal differentiation programs through the precisely timed interaction of endocannabinoid ligands with their cognate cannabinoid receptors. By indiscriminately prolonging the 'switched-on' period of cannabinoid receptors, cannabis can hijack endocannabinoid signals to evoke molecular rearrangements, leading to the erroneous wiring of neuronal networks. Here, we formulate a hierarchical network design necessary and sufficient to describe molecular underpinnings of cannabis-induced neural growth defects. We integrate signalosome components deduced from genome- and proteome-wide arrays and candidate analyses to propose a mechanistic hypothesis on how cannabis-induced ectopic cannabinoid receptor activity overrides physiological neurodevelopmental endocannabinoid signals, affecting the timely formation of synapses.

Keywords

drug abuse; metabolome; transcriptome; synapse

Endocannabinoids: gatekeepers of neuronal development

Molecular cloning of the CB_1 cannabinoid receptor (CB_1R) [1], and its functional characterization as the major target of Δ^9-tetrahydrocannabinol (THC) from cannabis [2] led to a sea-change in the understanding of the molecular mechanisms of this psychoactive drug s actions on neuronal structure and function in brain regions controlling memory, cognition, movement and pain perception [3]. These findings, coupled with the discovery that the CB_1R functions as an essential signal transducer in an elaborate molecular network relying on "endogenous cannabinoids" (endocannabinoids) to modulate the plasticity of many

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synapses [4] prompted a remarkably vibrant discipline of contemporary neurobiology. Ensuing developmental biology studies concerned with the formation of endocannabinoid signaling networks [5–9], the role of endocannabinoids [10–19] and, consequently, the molecular blueprint of prenatal cannabis abuse [16,20–23] in the developing nervous system soon followed. Our understanding so far is that endocannabinoids can act as focal instructive signals that affect neural progenitor proliferation [18] and neuron *vs*. glia fate decisions [24], as well as the differentiation programs of forebrain neurons (including but not restricted to cell migration, axonal growth and synapse development) [24].

Here we ask: "Are the molecular mechanisms of endocannabinoid signaling (that is, the dynamic arrangements to the enzymatic control of focal endocannabinoid availability and signaling at the CB_1R) necessary and sufficient to establish can*nabis sensitivity in developing neurons?*" We present a series of arguments to pinpoint the nascent axon as a structural substrate of cannabis action [6,13,15,17], and to suggest a causal link between compartmentalized endocannabinoid signaling and cannabis (or cannabinomimetic)-driven modifications to the wiring of emergent neuronal networks in the fetal brain [6,7,13]. Our molecular model reconciles competing and provocative hypotheses on the mode(s) of action of endocannabinoids, the cellular configuration of their metabolic machinery, and signaling by intra- *vs*. extracellular CB_1Rs.

The 'high-way' of brain development: clinical considerations

THC can enter the fetal circulation with rapid onset [25] *via* efficient transfer through the placenta [26]. THC levels in the amniotic fluid and fetus remain elevated up to 5h, followed by gradual clearance within 48h after exposure [25]. Exceptionally high THC doses (>100 mg/kg) may be teratogenic and induce *in utero* death [25]. However, cannabis use during pregnancy can lead to growth retardation [27] and is associated with adverse neurodevelopmental outcomes [28]. The delay in nervous system development upon *in utero* cannabis exposure in humans can impair cognitive performance [29–31], visual-motor coordination [32,33], and social behaviors [29,34], and increase the incidence of drug seeking [35], attention deficit [36], anxiety and depression [37] among affected neonatal or adolescent offspring.

It is becoming evident that not only THC but any plant-derived or synthetic drug – alone [16,38] or in mixture [39] –, which displays significant potency and efficacy at the CB_1R might evoke significant modifications of neuronal differentiation [12,16] and synapse physiology [40,41] by disrupting normal patterns of endocan signaling.

Endocannabinoids in the nervous system

In addition to the best known ligands, 2-arachidonoyl glycerol (2-AG) [42–44] and *N*-arachidonoylethanolamine (AEA) [45], the list of possible endocannabinoids includes a growing number of structurally related ligands with appreciable pharmacological efficacy at the CB_1R or CB_2 cannabinoid receptor (as well as having other targets; Box 1, Table 1). However, the concentration, regionalized distribution, metabolic and signaling interactions of these endocannabinoid-like substances remain as yet largely elusive.

Box 1

Molecular complexity and diversity of endocannabinoid metabolism

2-Arachidonoyl glycerol (2-AG) selectively activates CB_1R and CB_2R with high efficacy, typically as a full agonist [62]. 2-AG is produced by Ca^{2+}-dependent sn-1-diacylglycerol lipase (DAGL) α and β following the PLC-dependent hydrolysis of membrane phospholipid precursors to PIP_2 anddiacylglycerol (DAG) [54] (Table 1).

Other pathways to generate 2-AG include sequential reactions by phospholipase A_1 and lysophosphatidylinositol-specific phospholipase C (lyso-PLC), and phosphatase-mediated conversion of 2-arachidonoyl lysophosphatidic acid to 2-AG [46]. Monoacylglycerol lipase (MAGL) primarily catalyzes 2-AG hydrolysis, with limited contributions by α/β-hydrolase domain-containing 6 and 12 (ABHD6, ABHD12) hydrolases [92]. N-arachidonoylethanolamine, (anandamide, AEA) be-haves as an agonist at the CB_1R, CB_2R and transient receptor potential cation channel V1 (TRPV1) channels. Several pathways are involved in AEA synthesis [46,93] (Table 1). First, Ca^{2+}-stimulated N-acyltransferase generates N-arachidonoyl phosphatidylethanolamine (NAPE), followed by N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD)-mediated NAPE hydrolysis yielding AEA. Second, ABHD4 deacylates NAPEs [93] with subsequent conversion of glycerophospho-NAEs by the glycerophosphodies-terase GDE1 to generate AEA. Third, PLC-mediated cleavage of NAPE can yield bioactive phospho-AEA intermediates that are dephosphorylated by phosphatases (PTPN22). AEA is primarily degraded by fatty-acid amide hydrolase (FAAH) [46]. FAAH can also hydrolyze the ester bond in 2-AG in vitro but its in vivo contribution to 2-AG hydrolysis seems limited. In addition, N-acylethanolamine-hydrolyzing acid amidase (NAAA) can degrade AEA [94]. Alternatively, cyclooxygenase-2 (COX-2) - and lipoxygenases - can oxidize both 2-AG and AEA to prostaglandin-glycerol esters and ethanolamines, respectively.

Other putative neuroactive lipids recovered from adult mammalian brain and exhibiting some efficacy at the CB_1R [46] are also listed at the bottom of Table 1.

Endocannabinoids function as retrograde messengers to modulate the plasticity of many synapses in the adult brain [3]. Coupled with in-depth neurophysiology analyses at many brain regions [46], the understanding of the molecular principles governing activity-dependent endocannabinoid synthesis and utilization have rapidly expanded. However, the molecular architecture of endocannabinoid signaling networks in developing neurons substantially differs from their adult counterparts.

This review focuses on 2-AG, because most available data emphasize the involvement of 2-AG in both developmental processes [13,18] and the control of synaptic neurotransmission [3]. This is largely due to the relative lack of information on the molecular identity, use-dependent recruitment, and cellular localization of metabolic enzymes controlling AEA bioavailability in the nervous system (Box 1, Table 1). However, recent data functionally implicating AEA in retrograde signaling [47] or AEA-induced activation of Ca^{2+}-permeable TRPV1 channels to regulate 2-AG synthesis [48] may soon change this view. This latter pathway may be controlled by feedback from AEA/2-AG-activated CB_1R to limit Ca^{2+} flux through TRPV1 channels [49] (Figure 1b).

Molecular organization of endocannabinoid signaling in developing neurons

Neurogenic commitment differentially regulates the expression of the CB_1R [7], *sn*-1-diacylglycerol lipase α and β isoforms (DAGLα/β) [11] and monoacylglycerol lipase (MAGL) [50], 2-AG synthesizing and degrading enzymes, respectively. Neuronal differentiation up-regulates CB_1R expression [7], whereas cell-cycle exit of neural stem cells represses DAGLs [11]. During neuronal polarization, this signaling triad is co-transported along the nascent axon (Figures 1a,b) [6,13,15,16]. Although the CB_1R can be expressed on the surface of the axon stem, they are preferentially trafficked to the growth cone, including motile filopodial extensions, positioned to sense 2-AG [15,17]. Activation of

the CB_1R elicits growth cone collapse [15,17] suggesting that endocannabinoids can act as chemotropic guidance cues.

Both DAGLα and DAGLβ are expressed in the developing brain. Scaffolding proteins (e.g., homer-1/-2b) are likely to determine the subcellular sites of 2-AG synthesis by anchoring DAGLα [51]. Because DAGLβ lacks a homer-binding domain and is redistributed upon DAGL knock-out [18], a candidate mechanism to limit the subcellular dispersion of DAGLβ is its association with DAGLα. The axonal growth cone is particularly enriched in DAGLs. Here, the enzymatic activity of Ca^{2+}-dependent DAGLs may be spatially confined ("domain-specific") because Ca^{2+} signaling is lateralized in the growth cone during turning (that is, $[Ca^{2+}]$ is increased in the domain facing the attractive gradient [52]). Thus, DAGLs may function as coincidence detectors by focally controlling 2-AG availability and autocrine signaling at the CB_1R to limit the process of growth cone turning.

MAGL is a cytosolic enzyme that can be recruited to the inner leaflet of the plasmalemma to inactivate 2-AG at the plasma membrane [53]. MAGL undergoes focal and rapid proteasomal degradation in the motile neurite tip [13]. The physiological significance of MAGL microgradients tailing off in the distal axon segment may be to enrich the growth cone in signaling-competent 2-AG.

Synaptogenesis coincides with MAGL entering into the growth cone [13], where it may act as a "stop" signal by eliminating growth-promoting 2-AG. Once synaptogenesis concludes, DAGLs redistribute into the somatodendritic domain of neurons to provide 2-AG for retrograde signaling [54]. In contrast, both the CB_1R and MAGL remain localized to presynaptic terminals [13].

Endocannabinoids: intracellular or extracellular signals?

Endocannabinoids are viewed as lipophilic ligands whose ability to disperse in an aqueous extracellular environment may be limited. However, this view is challenged by the complete lack of retrograde synaptic signaling in $DAGL\alpha^{-/-}$ mice [18,55]. Because retrograde signaling relies on facilitated transsynaptic 2-AG diffusion to activate presynaptic CB_1Rs, data from $DAGL\alpha^{-/-}$ models unequivocally identify 2-AG as an *extracellular* retrograde messenger. 2-AG can also signal intracellularly [46]. Therefore, 2-AG s physicochemical properties could suffice to sustain both cell-autonomous and intercellular signaling mechanisms in the developing nervous system.

Is endocannabinoid signaling indispensable for brain development?

The answer might be ambiguous for several reasons. First, developmentally redundant signaling cassettes rely on promiscuous ligand-receptor interactions to sustain signaling efficacy even if a component of a signaling system is compromised. Therefore, using constitutive and global gene knock-outs to determine the function of a single gene may be limited to decipher the absolute contribution of a candidate mechanism to homeostatic control pathways [18,55,56]. Second, DAGL functions may be more essential for neuronal metabolism than previously thought, because in addition to lower levels of 2-AG, $DAGL\alpha^{-/-}$ and $DAGL\beta^{-/-}$ mice have significantly lower arachidonic acid (AA) and AEA levels (~80% and ~40% for $DAGL\alpha^{-/-}$, respectively) in the brain [18]. AA is often a metabolite of 2-AG [18]. Therefore, the decrease of AA in the absence of 2-AG highlights the close relationship between 2-AG, AA and AEA metabolism, likely incorporating yet unidentified metabolic routes. This emphasizes that only a fraction of 2-AG might be used for inter-cellular signaling. Instead, the bulk of 2-AG could be immobilized in, e.g., the plasmalemma or lipid droplets [57], for intermediary metabolism or other processes. Third, genetic disruption of endocannabinoid signaling networks may modify, yet not fully repress,

a specific developmental response pattern [18]. This may be interpreted such that endocannabinoids are *gatekeepers* of developmental processes, and impairments of their signaling must coincide with secondary insults (e.g., maternal deprivation [58], stress or seizures) to impose enduring modifications to neuronal circuitry.

CB_1R can diversify the ontogenic impact of endocannabinoid signals

The CB_1R can exhibit tremendous signaling complexity. The simplest functional unit of a GPCR, (including the CB_1R), is a homodimer that recruits a heterotrimeric G protein [59]. However, the CB_1R is physiologically "dominant" because it can becoupled to $G_{i/o}$-proteins even in the absence of an agonist ("constitutive activity"), thereby depleting the common intracellular $G_{i/o}$ pool and limiting the biological signals of other $G_{i/o}$-coupled receptors [60]. GPCRs can also heterodimerize with other receptors, leading to a combinatorial recruitment of second messengers [61]. Evidence exists for the assembly of CB_1R-μ-opioid, CB_1-OX_1 orexin, CB_1-β_2 adrenergic, CB_1R-D_2 dopamine, CB_1R-A_2A adenosine and CB_1R-tyrosine kinase B (TrkB) receptor dimers, among others [62].

The C-terminus of the CB_1R contains phosphorylation sites for GPCR kinases (GRKs) leading to β-arrestin-dependent desensitization (AAs 426/430) [63], as well as an internalization (AA460–473) domain [64]. Once internalized, the CB_1R is either recycled to the membrane or tagged by GPCR-associated sorting protein-1 (Figure 2) and targeted for proteasomal degradation [65]. The AA465–473 extremity of the CB_1R can bind adaptor proteins [66] such as CB_1R interacting protein 1a (CRIP1a) and 1b. This latter interaction with the CB_1R may be restricted to the adult brain because CRIP1a is targeted to the somatodendritic domain of neurons, and its protein expression profile is different from that of the CB_1R in fetal brain [13].

Signaling complexity at the CB_1R is further enhanced by endogenous agonists or antagonists, whose synthesis and degradation are distinct from those of the endocannabinoids. These compounds can modulate the availability and signaling competence of the CB_1R. Hemopressin is an endogenous nonapeptide derived from α-hemoglobin (Figure 1b). Although hemopressin itself is recognized as an inverse agonist, its truncation products reportedly function as CB_1R agonists [67]. Hemopressin derivatives structurally and energetically fit the ligand-binding pocket of CB_1R [68], affect CB_1R trafficking, and modulate CB_1R-induced neurite outgrowth [67]. The lack of hemopressin effects in $CB_1R^{-/-}$ mice suggest that hemopressin may efficiently modulate neuronal CB_1Rs *in vivo* [69]. Although full-length hemopressin was isolated from brain homogenates, it remains uncertain whether short peptide fragments cleaved from the parent peptide might instead confer its biological activity [68,69].

Finally, the fetal brain can be perceived as a rapidly changing kaleidoscope of neural activity, as a plethora of molecular control switches are turned "on" and "off" in a precise sequence. Maternal cannabis abuse can be viewed as a pathogenic stimulus to derail the physiological output of the CB_1R by forcing it to signal in (a)synchrony with other receptors [70], thus overriding physiological endocannabi-noid signal cascades.

Signaling pathways linking the CB_1R to cell proliferation/survival and neurite outgrowth

Classical G_i protein-mediated signaling at the CB_1R is well suited to activate effectors that couple endocannabinoid signaling to cell survival, proliferation, and differentiation (Figure 2). Here we discuss novel facets of understanding of cell state- specific signaling events and

emphasize the importance of CB_1R signaling in relation to axonal growth and guidance [15,17,71,72], which have evolved since a recent survey of available data [62].

A major branch of CB_1R signaling is directed towards controlling the population size of neural progenitors, including those of cerebellar granule cells and olfactory neurons [73]. This is achieved upon Akt/PKB-mediated inactivation of glycogen synthase kinase-3 through phosphorylation [74], thus allowing anti-apoptotic signal progression through the nuclear import of stabilized β-catenin to regulate cell survival (Figure 2). CB_1R activation can also couple to the accumulation of ceramide, a lipid mediator critical to control senescence, as well as differentiation [62].

It is becoming clear that neuronal CB_1 activates a hierarchical signaling network to induce neurite outgrowth (Figure 2). The classical signaling pathway that couples nthe CB_1R to neurite outgrowth is through the Gβγ subunit-dependent, sequential recruitment of phospholipase C (PLC), protein kinase C(ε) [75] or Fyn, a Src-family tyrosine kinase, to activate the extracellular signal-regulated kinase pathway [15,76]. Signaling through Gβγ can also activate Akt/PKB kinase, with cAMP response element-binding (CREB) and Pax6 transcription factors acting as activators coupling the CB_1R to axonal growth [20]. Alternatively, Akt/PKB can suppress BRCA1 signaling to disinhibit signal transducer and activator of transcription 3 (STAT3) phosphorylation, regulating transcription [20].

$G\alpha_{i/o}$ activation downstream from the CB_1R can induce STAT3 upon upstream activation of Ras-like protein (Ral) or Rac GTPase/c-Jun N-terminal kinase pathways converging at the level of Src [77] (Figure 2). The CB_1R induces focal cytoskeletal remodeling [17] by coupling to small Rho GTPases [15]. In particular, $G\alpha_i$-dependent induction of G protein-regulated inducer of neurite outgrowth 1 (GRIN1) can signal *via* cdc42 to activate Arp2/3 or cofilin [77]. Otherwise, $G\alpha_i$- dependent guanine nucleotide exchange factor induces cofilin-dependent actin remobilization through RhoA. The physiological outcome of CB_1-stimulated Rho GTPase-dependent signaling events may be the CB_1R-dependent collapse of axonal growth cones [13,15,17].

Receptor interactions sensitizing developing neurons to endocannabinoids

Molecular arrangements exerting upstream control upon endocannabinoid signaling during neurite outgrowth are essential to define the ultimate physiological outcome. An appealing hypothesis is to implicate receptor tyrosine kinases (Trks) (e.g. the fibroblast growth factor receptor) as they induce Ca^{2+} mobilization through PLCγ [78]. Elevated intracellular Ca^{2+} can activate DAGLs to generate 2- AG and trigger CB_1R activation [24]. The robustness of Trk signaling is epitomized by *i*) the ability of brain-derived neurotrophic factor (BDNF) to increase cellular CB_1R mRNA content through TrkB receptors [50], *ii*) the ability of BDNF to sensit- ize neurons to endocannabinoids (that is, in the presence of BDNF subphysiological endocannabinoid concentrations promote Akt phosphorylation) [50], *iii*) the fact that TrkB can assemble into signaling multimers with the CB_1R, can be phospho-rylated in a BDNF-independent manner upon CB_1R activity, and can recruit Src kinases [14], *iv*) the finding that BDNF-stimulated endocannabinoid release at inhibitory cortical synapses [79]. The latter mechanism is well poised to link endocannabinoid-driven growth cone turning responses to inhibitory synapse formation *in vivo* [15].

The coincidence of endocannabinoid and interleukin-6 (IL-6) signaling was recently highlighted as a means to modify the developmental efficacy of CB_1R activation. The simultaneous presence of a CB_1R agonist and IL-6 at subthreshold concentrations synergizes to activate CREB and STAT3 [19]. This signal convergence in turn inhibits SHP2

phosphatase to unmask morphogenic PLC signaling. Thus, integration of multiple protein kinases and transcription factors from GPCRs and cytokine receptors is sufficient to evoke neurite outgrowth. This interaction might be significant during intrauterine infection or autoimmune pathologies by modifying cell-cycle control, neuronal migration and neurite growth in response to IL-6-like cytokines (e.g., IL-11, ciliary neurotrophic factor or leukemia inhibitory factor) [80].

The cell-surface receptor DCC (deleted in colorectal cancer) functions as an integral component of a receptor complex that mediates the axonal chemoattractive response towards netrin-1 [81] in a Fyn kinase-dependent fashion [82]. Within the visual system, netrin-1/DCC interactions are particularly important to guide retinal ganglion cell (RGC) axons to innervate relay neurons in the dorsolateral geniculate nucleus [17]. Ocular dominance and non-overlapping topology of the terminal fields of individual RGCs in the geniculate nucleus are established through progressive pruning of their axons. Recently, the CB_1R was found to modulate RGC growth cone morphology through the cAMP/protein kinase A (PKA) pathway [17]. An endocannabinoid-netrin signaling interplay to control the growth cone morphology of RGC axons was suggested since CB_1R activity affected PKA-dependent targeting of DCC to the plasma membrane (CB_1R antagonists increase DCC s insertion into the plasma membrane) [17]. However, the morphological outcome of this CB_1R/DCC interaction is ultimately growth arrest; this also manifests in the lack of eye-specific segregation of retinal projections in $CB_1R^{-/-}$ mice *in vivo* because the CB_1R drives this interaction by decreasing intracellular cAMP levels *via* $G_{i/o}$ proteins.

Compartmentalized signaling by the CB_1R

Any instructive signal must be spatially restricted to encode positional information. This can be achieved either by limiting the expression, cell-surface presentation and extracellular spread of a ligand or by compartmentalizing ligand-receptor interactions. Neurotrophin receptors, DCC, and probably the CB_1R can be clustered by function as “dependence” (or survival) receptors because they create cellular states of dependence towards their cognate ligands by inducing apoptosis if left unoccupied [81]. A common characteristic of these receptors is their association with cholesterol- and sphingolipid-enriched membrane microdomains, that is, lipid rafts, to initiate second messenger signaling [81–83]. Lipid rafts are particularly abundant in regions of high membrane turnover, (e.g. growth cones [84]). They can sequester many kinases, particularly those belonging to the Src family (e.g., Src, Fyn, focal adhesion kinase (Fak)) and are required for axon guidance [82]. The timing and signaling properties of a receptor may be substantially different in raft and non-raft portions of the plasma membrane [82]. Compartmentalized signaling upon focal enrichment of the CB_1R in lipid rafts may be particularly efficacious to control growth cone steering decisions by promoting the formation of submembrane signal transduction complexes specialized for fast signal coupling.

THC can hijack physiologically silent CB_1Rs

In developing cortical axons, MAGL forms a 2-AG-inactivating barrier in the established axon segment, and can function as a *switch-off system’* to terminate 2-AG signaling (Figure 3a) [13]. We postulate that the role of MAGL in elongating axons is to prevent lipophilic 2-AG from accessing and prematurely engaging intracellular CB_1R in the axon stem as they undergo axonal transport.

The differential recruitment of DAGLs and MAGL along the axon might serve several independent modes of endocannabinoid signaling, whose outcome - irrespective of the underlying molecular sequence of events–will be neurite outgrowth. The putative molecular cascade of cell-autonomous 2-AG signaling during axonal growth and guidance has recently

been reviewed [24]. Here, we discuss whether intercellular communication in the presence or absence of extracellular 2-AG gradients can be equally successful.

The canonical paradigm of axon guidance implies the presentation of chemotropic guidance cues as local gradients. In the developing brain, the expression of DAGLs shows considerable regionalization [13,16,54] suggesting that 2-AG concentrations may substantially vary whilst an axon traverses to innervate its target cell. 2-AG can either be presented by cells lining the path of the axonal trajectories or originate from $DAGL^+$ processes, when multiple neighboring axons extend simultaneously in the process of fasciculation [6,13]. These cellular arrangements can establish a 2-AG gradient "map" to preferentially activate the CB_1R within forward-facing filopodia (Figure 3a,c), thus inducing rapid cytoskeletal reorganization within the growth cone. This model suggests that CB_1Rs transported along the axon and distal to the chemotropic 2-AG gradient might remain silent even if left unprotected by insufficient 2-AG-degrading activity (MAGL/ABHD6).

Compartmental MAGL localization during axonal growth [13] suggests that an alternative scenario may be physiologically favored. When axons navigate over surfaces that present quasi-homogeneous 2-AG micro-patterns, the enzymatic activity of MAGL might be necessary and sufficient to establish a cell-autonomous 2-AG gradient. This is because the 2-AG concentration within the axonal plasmalemma and intracellularly inversely correlates with the focal 2-AG-degrading capacity of MAGL (Figures 3a,c_1). Thus, 2-AG concentrations within the growth cone may be equivalent to that in the immediate microenvironment of the developing axon (Figure 3c_1). Inhibition of axonal growth upon disrupting the intracellular gradient of MAGL emphasizes the physiological significance of the above metabolic arrangements [13]. By making extracellular endocannabinoid gradients dispensable, this model reconciles the discrepancy between the limited propensity of endocannabinoids to diffuse over considerable distances and gradient requirements of axonal growth (Figure 3c_1).

Unlike 2-AG, THC is not degraded by MAGL. Therefore, prenatal THC exposure can have at least two cellular foci of action: *i*) it can displace 2-AG from the CB_1R in motile growth cones (Figure 3b), thus modifying (or occluding) second messenger signaling to alter directional axonal growth [15], and *ii*) THC can bypass the axonal MAGL barrier to hijack CB_1Rs as they are being trafficked in axons, thus disrupting the spatial specificity of endocannabinoid signaling by activating "silenced" CB_1Rs (Figure 3b,c_2). Notably, this molecular scenario can also explain how metabolically stable synthetic CB_1R antagonists (e.g., SR141716A [16]) can induce axonal defasciculation and mistargeting, and delay synapse formation [12,16] by compromising the spatial and temporal precision of morphogenic endocannabinoid signals. We propose that the cellular basis of THC-induced axonal growth and guidance errors is the indiscriminate activation of CB_1Rs otherwise kept muted during the physiological process of neurite outgrowth (Figure 3b).

The molecular fingerprint of prenatal cannabis abuse

If THC affects developmental processes, then its molecular fingerprint must involve developmentally regulated genes. The cumulative complexity of this gene/protein network will be reflective of the relative "power" of THC to affect developmental processes. Global genome and proteome profiling after exposure to THC or CB_1R agonists *in utero* (or during adolescence) increasingly support this notion by identifying a largely invariable cluster of target molecules (Figure 4).

Disrupting the temporal precision of CB_1R activity can affect 2-AG degradation by repressing MAGL expression [85,86], reinforcing the functional backbone of our model of THC sensitivity in developing neurons. Cannabinomimetics can up-regulate the expression

or enhance the stability of G protein subunits (including $Go_{\alpha 1}$) [87] and growth-associated second messengers [87,88], suggesting altered signal coupling of GPCRs and growth responses, respectively. THC increases the expression of neurotrophins (BDNF [50], Fgf1 [21]) whose signal transduction cascades (e.g., TrkB) can significantly alter CB_1R-mediated growth responses [14,50,78]. These changes, together with THC s potency to alter the expression of cell-adhesion molecules (L1-NCAM) [89] and cytoskeletal proteins (β-III-tubulin) [88] essential to maintain neuronal polarity and cell-cell interactions, further emphasize its impact on neuronal morphology. The precise dynamics of Ca^{2+} signaling in neurons are integral to regulate growth cone steering decisions and to maintain synaptic neurotransmission at mature synapses. Disrupted signaling down-stream from the CB_1R changes the expression of Ca^{2+}-binding proteins [22,85,87,89], which act as either intracellular Ca^{2+} buffers or sensors, members of the presynaptic vesicle trafficking machinery – including proteins implicated in both synaptic vesicle docking/exocytosis (SNAP-25, synaptophysin) [88] and endocytosis (dynamin) [90] – and postsynaptic scaffolding proteins (PSD95; [90], suggest significant modifications to the structural establishment and function of synapses. Glutamatergic (excitatory) neurotransmission may be particularly affected at multiple levels (glutamate metabolism, AMPA receptor subunit expression [21,91]) recapitulating findings from earlier neurophysiology studies linking altered CB_1R function to deficits of hippocampal long-term potentiation, memory encoding and glutamate release.

Concluding remarks

Whether THC is an agonist or antagonist at the CB_1R during development, when neurons require a high intrinsic endocannabinoid tone to sustain growth processes, is hotly debated [24]. Nevertheless, the concept that THC exerts its detrimental effects by disrupting the temporal and spatial cohesion of endocannabinoid signaling has recently gained significant momentum [12,14,24]. Based on the evidence from cell and systems biology presented herein, we conclude that the functional redundancy of the many endocannabinoid ligands, receptors, metabolic and signaling pathways has evolved to enable endocannabinoids to drive context- and cell state-dependent specification programs in the developing nervous system. This redundancy may be particularly important to prevent developmental defects when the contribution of one or more molecular endocannabinoid components is compromised. Prenatal cannabis exposure can lead to growth defects during formation of the nervous system. The cellular basis of errant neuronal wiring upon cannabis exposure may be due to the ability of THC and related phytocannabinoids to circumvent the spatially precise metabolic control of 2-AG signaling (Figure 3a,b,c_2), thus altering positional signaling downstream from cannabinoid receptors. Ectopic CB_1R activity, whether on the cell surface or intracellularly (Figure 1), appears to powerfully perturb nervous system patterning and intercellular communication. Understanding the functional significance of molecular changes upon exposure to cannabis *in utero* or during the adolescent critical period of brain development might not only provide new insights in endocannabinoid functions but also prompt future investigations to decipher the molecular basis of cannabis-induced psychiatric illnesses in affected offspring.

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Glossary

Cell cycle exit	This term refers to the event when a cell permanently leaves the cell cycle to adopt a terminal differentiation program. To achieve this, cells become refractory to proliferative signals
Fate decision	The point when a progenitor cell commits towards and initiates an intrinsic specification program to generate a terminally differentiated cell
Lateral geniculate nucleus	This nucleus resides within the thalamus and functions as the primary relay center for visual information received from the retina of the eye
Growth cone	A specialized region at the tip of a growing neurite that is responsible for sensing the local environment and for guiding the axon through the transduction of attractive and repulsive extracellular guidance cues toward a target cell
Intercellular (synaptic) communication	Information exchange between neurons *via* neurotransmitter release at a specialized junction, the synapse
Neurite outgrowth	Sequential process including the specification, elongation, and branching of developing axons and dendrites
Neurogenesis	The process by which neurons are created irrespective of the specific region where these cells are generated or their specific functions within the nervous system
Progenitor cell	An early descendant of a stem cell that can proliferate and differentiate. A progenitor cell is more limited than a stem cell in the lineages of cells it can generate
Retrograde signaling	A phenomenon during which a signal molecule travels from the postsynaptic neuron towards the presynaptic one with its direction opposing that of the relevant synaptic neurotransmitter
Synaptogenesis	The formation of functional synapses

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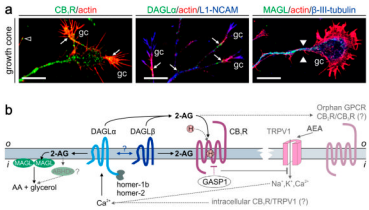

Figure 1. Molecular organization of 2-AG signaling networks in developing neurons

(a)Receptor and metabolic enzyme components of 2-AG signaling coexist in developing neurons and are preferentially targeted towards their axons and growth cones (gc). The CB_1R and DAGLα both accumulate in the central domain and actin-rich filopodia of growth cones (*arrows*) [15]. By contrast, MAGL accumulates in the tubulin-rich axon stem with its levels descending towards the growth cone forming a decreasing gradient of 2-AG hydrolysis activity (arrowheads indicate the start of this gradient) [13]. (**b**) Molecular organization of 2-AG signaling in developing neurons. 2-AG, produced by DAGLα or β, can activate the CB_1R either from the extracellular space or by lateral diffusion in the plasmalemma. Homer scaffolds can anchor DAGLα at preferred signaling positions [51]. The lack of homer binding sites in DAGLβ suggests that DAGLα and DAGLβ may directly associate, anchoring DAGLβ and spatially restricting 2-AG biosynthesis. CB_1R trafficking and/or signaling might be modulated by hemopressin (H) [95], or C-terminal interacting proteins. GPCR-associated sorting protein 1 (GASP1) is one such example [96]. MAGL [56] or ABHD6 [92] can hydrolyze 2-AG into arachidonic acid (AA) and glycerol to terminate signaling. Potential, yet controversial, involvement of other endocannabinoids (e.g., AEA), CB_2Rs, orphan GPCRs and TRPV1 are shown in grey. AEA-activated TRPV1s are particularly intriguing because they could serve on either the plasmalemma or intracellular membranes as Ca^{2+} sensors controlling DAGL activity [48]. *Scale bars* = 10 μm.

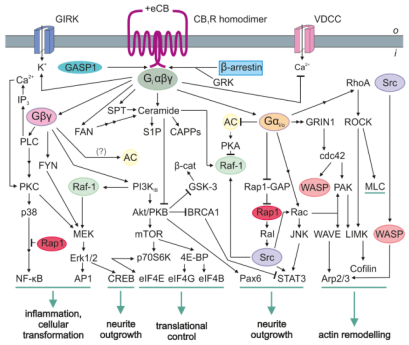

Figure 2. Agonist-induced combinatorial second messenger signaling at the CB_1R

The contemporary view of GPCR signaling identifies receptor homodimers as signaling units [59]. Recruitment of signal effectors is cell state-specific in developing neurons with the active signaling cascade directly determining the physiological outcome (*green*). The CB_1R can inhibit voltage-dependent Ca^{2+}-channels (VDCC) or activate G protein-coupled inward rectifying potassium channels (GIRKs) through Gβγ subunits [62]. β-Arrestins participate in CB_1R internalization and desensitization, while GPCR-associated sorting protein 1 (GASP1) can direct this receptor towards lysosomal degradation [96]. It is being recognized that the CB_1R can recruit mTOR signaling to regulate protein translation through activation of mammalian elongation initiation factors (eIF4E/B/G) [97]. *Abbreviations*: 4E-BP, eukaryotic translation initiation factor 4E-binding protein 1; AC, adenylyl cyclase; Akt/PKB, protein kinase B; AP1, activator protein-1; Arp2/3, actin related protein 2/3; β-cat, β-catenin; BRCA1, breast cancer susceptibility protein 1; CAPPs, ceramide activated protein phosphatases; Cdc42, cell division control protein 42 homolog; CREB, cAMP response element-binding protein; EGF, epidermal growth factor; Erk1/2, extracellular signal-regulated kinase 1/2; FAN, factor associated with neutral sphingomyelinase activation; FYN, member of Src family tyrosine kinases; GRIN1, G protein-regulated inducer of neurite outgrowth 1; GRK, G protein-coupled receptor kinase; GSK-3, glycogen synthase kinase-3; IP_3, inositol 1,4,5 triphosphate; JNK, c-Jun N-terminal kinase; LIMK, LIM motif-containing protein kinase; MEK, Erk kinase; MLC, myosin light chain; mTOR, mammalian target of rapamycin; Nf-κB, nuclear factor κB; p38, mitogen-activated protein kinase (MAPK); p70S6K, serine/threonine kinase; Pak, p21 activated kinase; Pax6, paired box gene 6; PI3K, phosphoinositide-3 kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; Rac, member of Rho family GTPases; Raf-1, MEK kinase; Ral, Ras-like protein; Rap-1, Ras related protein-1; RAP1-GAP, RAP1 GTPase activating protein; RhoA, Ras homolog gene family, member A; ROCK, Rho-associated protein kinase; S1P, sphingosine 1-phosphate; SPT, serine palmitoyltransferase; Src, v-src sarcoma viral oncogene homolog; STAT3, signal transducer and activator of transcription 3; WAVE/WASP, Wiskott-Aldrich syndrome family protein.

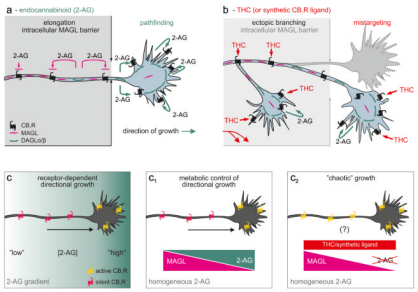

Figure 3. Hypothetical model of phytocannabinoid or cannabinomimetic-induced neuronal wiring defects

(**a**) During cortical axonal development, MAGL forms an intracellular enzymatic barrier to prevent 2-AG-driven activation of the CB_1R transported along the axon [13]. Thus, we recognize MAGL as a metabolic checkpoint to control 2-AG-dependent formation of axon collaterals. A decrementing MAGL gradient towards the motile growth cone will allow sufficient 2-AG accumulation to activate the CB_1R to gain signal competence towards 2-AG, thus impacting growth cone steering decisions. (**b**) Prenatal exposure to phytocannabinoids, particularly Δ^9-tetrahydrocannabinol (THC), or other cannabinomimetics can override this endogenous mechanism since these ligands are resistant to MAGL. Thus, THC can hijack axonal CB_1R *en route* to their signaling positions and induce errant second messenger signaling. (**c–c$_1$**) An extracellular 2-AG gradient may be dispensable for CB_1R-mediated axon guidance. (**c**) Polarized axonal distribution of the CB_1R is sufficient to induce directional growth on an extracellular 2-AG microgradient. (**c$_1$**) Compartmentalized 2-AG degradation by MAGL within the axon stem will be sufficient to focally restrict 2-AG-induced CB_1R activity on a homogeneous 2-AG background. (**c$_2$**) THC might alter the trajectory of growth and synapse formation by ectopic CB_1R activation irrespective of the pattern of 2-AG presentation.

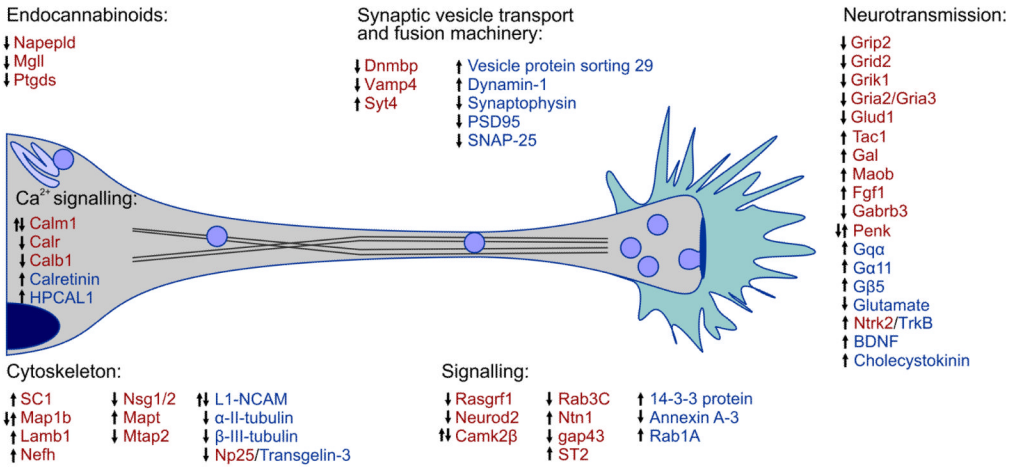

Figure 4. Molecular blueprint of CB_1R activation by THC or synthetic agonists in relation to axonal growth and synapse development, deduced from genome and proteome-wide arrays

Red and blue colors indicate molecular entities identified through genomic [21,22,85–87,89,98–100] and proteomic approaches [14,16,87,88,90,91], respectively. Corresponding gene and protein clusters are indicated. Arrows indicate the direction of regulation upon drug exposure. Double arrows suggest divergent responses due to experimental conditions [21,22], age [89,99], sexual dimorphism [100] or length [89,98] of treatment.

Table 1

Endogenous neuroactive lipids with agonist potency at the CB[1] cannabinoid receptor (CB_1R), and their metabolic pathways.

Precursor	Biosynthesis	Endocannabmoids and some related lipids	Transporter[1]	Degradation	Receptor
DAG 2- Arachidonoy1-lysophospholipid 2-Arachidonoyi-sn-glycero3-phosphate	DAGLα and β PhospholipaseA,/ Lyso-PLC Uncharactertized LPA phosphatase	2-arachidonoyl glycerol (2-AG)	Facilitated diffusion Membrane transporter	MAGL1/2 ABHD6.ABH012 COX-2 FAAH1/2	CB,R CB,R orphan GPCRs
NAPEs	NAPE-PLDor ABHD4/GDE1 or PLC/phosphatase (PTPN22) pathways	N-anchidonoylethanolamine[2] (anandamide, AEA)	Facilitated diffusion Membrane transporter	FAAH1/2 NAAA COX-2	CB,R CB.R TRPV1 GPR55 ion channels
NAPEs acyl-CoA +amino acid		2-Arachidonyl glycerol ether *O*- Arachidonoylethanolamine (Virodhamine) *N*-Palmitoylethanolamide (PEA) *N*-Arachidonoyl-L-serine *N*-Arachidonoyl -L-doparo«no *N*-Arachidonoyl -L-gryane *N*-Acyl-taurines	shared with AEA (?)	shared with AEA (?) NAAA	CB,R CB,R GPR55 ion channels TRPV1 GPR18

[1] Although a membrane transport mechanism facilitating endocannabinoid liberation and uptake has been proposed, the existence and molecular identity of this transporter remain to be elucidated.

[2] In addition to *N*-acyl ethanolamines, such as AEA, *N*-acyl-taurines are also FAAH substrates.