Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning

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Endocannabinoids (eCBs) have recently been identified as axon guidance cues shaping the connectivity of local GABAergic interneurons in the developing cerebrum. However, eCB functions during pyramidal cell specification and establishment of long-range axonal connections are unknown. Here, we show that eCB signaling is operational in subcortical proliferative zones from embryonic day 12.5 in the mouse telencephalon and controls the proliferation of pyramidal cell progenitors and radial migration of immature pyramidal cells. When layer patterning is accomplished, developing pyramidal cells rely on eCB signaling to initiate the elongation and fasciculation of their long-range axons. Accordingly, CB1 cannabinoid receptor (CB1R) null and pyramidal cell-specific conditional mutant (CB1Rf/f,NEX-Cre) mice develop deficits in neuronal progenitor proliferation and axon fasciculation. Likewise, axonal pathfinding becomes impaired after in utero pharmacological blockade of CB1-Rs. Overall, eCBs are fundamental developmental cues controlling pyramidal cell development during corticogenesis.

Pyramidal cell specification follows a sequential scenario in the developing cerebrum: commitment of progenitor cells to the neuronal lineage occurs in the subcortical proliferative ventricular zone (VZ) and subventricular zone (SVZ). Immature pyramidal cells undergo radial migration to populate the cortical plate (CP) (1), where they acquire layer-specific neurochemical and morphological diversity (2). Pyramidal cell positioning and patterning of their corticofugal and intracortical axons is in part achieved via transcriptional control acting throughout cellular identification (2). However, epigenetic microenvironmental cues, provided by neural progenitors, radial glia, and immature neurons, are also fundamental in attaining cortical cell identity with particularly robust effects on pathfinding and directional growth of long-range axons (3).

Endocannabinoids [eCBs; anandamide (AEA) and 2-arachidonoylglycerol] control various forms of synaptic plasticity at cortical glutamatergic synapses in the postnatal brain (4) through functional CB1 cannabinoid receptors (CB1Rs) (5). During brain development, eCBs control neuronal fate decision (6), interneuron migration (7), and axonal specification (8). Developmental eCB actions are underpinned by a temporally defined assembly of functional eCB signaling networks with coincident expression of sn-1-diacylglycerol lipases (DAGLα/β) (9) and N-arachidonoyl-phosphatidyl ethanolamine (NAPE)-selective phospholipase D involved in eCB synthesis, fatty-acid amide hydrolase (FAAH) (an enzyme preferentially degrading AEA), and CB1Rs (8). The selective axonal targeting of CB1Rs and DAGLs in immature neurons suggests that eCBs may function in either cell-autonomous (6, 9) or target-derived (8) manner to control axonal elongation and postsynaptic target selection, respectively.

Although recent findings in both mammals (8) and nonmammalian vertebrates (10) suggest that eCB signaling is required for axonal elongation and fasciculation, eCB functions instructing distinct stages of pyramidal cell development are unknown. Here, we show that eCB signaling is operational in the subcortical VZ/SVZ and drives neural progenitor proliferation and migration, thus contributing to defining the final positions and densities of immature pyramidal cells. Subsequently, eCB signaling in immature pyramidal cells is required for axonal polarization and the formation of long-range glutamatergic axons. Accordingly, genetic and pharmacological disruption of CB1R functions reveals fasciculation deficits and axonal mistargeting. In sum, our data demonstrate that eCB signaling is dispensable for the genesis, proliferation, migration, and axonal behaviors of neocortical pyramidal cells and support the concept that eCBs act as a novel class of morphogens during corticogenesis.

Results

CB1R Expression in Developing Cerebrum. We used in situ hybridization (11, 12) and immunofluorescence (8) histochemistry combined with high-resolution confocal microscopy [see supporting information (SI) Methods and Figs. S1 and S2] to define the identity of cells expressing CB1R and DAGLα/β expression in the developing mouse and human neocortex. CB1R mRNA expression was restricted to telencephalic differentiation zones at embryonic day (E)12.5 in mouse (Fig. 1A and B). By E14.5, significant CB1R mRNA expression was evident in immature pyramidal cells populating the CP and hippocampal primordium (Figs. 1C and Fig. S2 A–D), reached peak expression levels by
approximately E16.5 (Fig. 1D), and gradually declined during pyramidal cell morphogenesis in the late gestational embryo (Fig. S2 G–J). Considerable CB1R mRNA expression in neural progenitors exiting the cortical SVZ was sustained throughout corticogenesis, whereas the VZ was largely devoid of CB1R hybridization signal (Fig. 1E and G). These findings are not restricted to mouse development, because a similar CB1R mRNA expression pattern was seen in the second trimester human fetal telencephalon, comparable to E18/postnatal day (P)0 rodent brain (13). In particular, robust CB1R mRNA expression was detected in the human SVZ, CP neurons, and hippocampal pyramidal cell layers (Fig. 1H and I).

eCB Signaling During Corticogenesis. DAGLβ-like immunoreactivity (i.r.) was present in the cortical VZ/SVZ from E12.5 until birth (Fig. 1J and K and Fig. S3A) (10), suggesting the coincidence of local eCB synthesis with neuronal progenitor proliferation. CB1R i.r. was evident in intermediate progenitor cells (Tbr2/−/H11001/−), known to differentiate into pyramidal cells (2), that had engaged in radial migration toward the CP (Fig. 1L and M and Fig. S3). CB1R mRNA expression by immature pyramidal cells concurred with the targeting of CB1Rs to developing long-range axons between E13.5 and P0 (Fig. 1N–R). Coexistence of DAGLβ and CB1Rs in developing glutamatergic axons (Fig. S2 E and F) reinforces the hypothesis (9) that eCB signaling is required for pyramidal cell development and functional specification.

eCBs Control SVZ Progenitor Proliferation. We tested whether eCBs control neural progenitor proliferation (6) in subcortical VZ/SVZ by analyzing CB1R−/− and wild-type littermates pulsed with BrdU on E14.5. Lack of CB1Rs significantly decreased neural progenitor proliferation (Fig. 2A). Conversely, FAAH−/− (14) increased proliferation of VZ/SVZ progenitors (Fig. 2B). Here, genetic manipulation of FAAH activity was used to elevate eCB levels; however, the particular eCB mediating these phenomena was not identified.

Fig. 1. CB1R localization in the developing brain. (A–G) In situ hybridization demonstrating the spatial and temporal distribution of CB1R mRNA in the mouse brain. Arrows in C and E and G denote CB1R mRNA hybridization signal in pyramidal cells and SVZ progenitors, respectively. (H and I) Distribution of CB1R mRNA in human fetal brain. Nissl/AChE histochemistry reveals territorial boundaries. (J–M) DAGLβ and CB1R expression in mouse VZ/SVZ. Cortical Tbr2+ projection neurons migrating toward the CP express CB1Rs. (N–R) CB1Rs are selectively enriched in axons of cortical projection neurons. Arrows indicate corticothalamic axons, and arrowheads identify axons committed to the thalamus. (Scale bars: E–G and K–M, 30 μm; N–R, 85 μm; A–D, H, and I, 100 μm.) See SI Text for abbreviations.

Fig. 2. eCBs regulate neural progenitor proliferation in cortical VZ/SVZ. (A) CB1R deletion significantly reduces the rate of neural progenitor proliferation, as defined by the density of BrdU+ cells in the VZ/SVZ. (B) Conversely, elevated eCB levels in FAAH−/− mice (14) significantly increase neural progenitor proliferation. (C) Conditional CB1R deletion in subcortical SVZ progenitors (arrows) (16) decreases the rate of Ki67+ progenitor proliferation (n = 3 per genotype). **, P < 0.01, compared with wild-type littermates. (Scale bar, 100 μm.)
eCB functions specifically underpinning pyramidal cell progenitor proliferation were elucidated in mice with conditional deletion of CB1Rs through Cre recombinase expressed under the control of regulatory sequences of NEX, a neuronal basic helix-loop-helix protein (15). Prominent Cre activity is observed by approximately E11.5 in cortical progenitors in NEX-Cre mice (15), ensuring the lack of CB1Rs in pyramidal cells at all developmental stages studied here. In these mutants (16), VZ/SVZ progenitor proliferation was significantly impaired, as indicated by reduced Ki67 and GOLGA5 cell density in proliferative zones (Fig. 2C and Fig. S4A). Because NEX is expressed by pyramidal progenitors in SVZ but not in VZ (16), a coincident proliferation deficit in CB1R<sup>f/f</sup>NEX-Cre and CB1R<sup>R<sup>−/−</sup></sup> mice suggests that eCBs exert differential control on subcortical progenitor pools. Therefore, we analyzed BrdU<sup>+</sup> progenitor proliferation rates separately in SVZ and VZ of CB1R<sup>−/−</sup> (SVZ, 226%; VZ, 150% of control) as well as AM251 significantly inhibited neurochemical differentiation in SVZ/SVZ progenitor pools. Therefore, we analyzed BrdU<sup>+</sup> progenitor proliferation rates separately in SVZ and VZ of CB1R<sup>−/−</sup> (SVZ, 226%; VZ, 150% of control). Thus, progenitor proliferation in SVZ may rely more on autocrine eCB regulation, whereas non-cell-autonomous signaling predominates in VZ.

Our genetic data were further validated by HU-210 (synthetic CB1R agonist) and URB597 (FAAH inhibitor) treatment of E14.5 organotypic slices revealing increased VZ/SVZ progenitor proliferation upon sustained cannabinoid receptor stimulation (Fig. S4B).

**Pyramidal Cell Specification Relies on eCB Signaling.** Exogenous CB1R agonists have been identified (7, 8) as morphogens and chemotropic guidance cues for cortical interneurons. However, it is unknown whether eCBs affect pyramidal cell development. Therefore, we exposed pyramidal cells isolated from E14.5 neocortex to NFG (100 ng/ml), a differentiation promoting neurotrophin (17), AEA (200 nM) (8), or AM251 (1 μM), a CB1R inverse agonist. NFG increased axonal arbor activity of VGLUT1<sup>+</sup> pyramidal cells (Fig. 4A and B). In contrast, AEA induced the elongation of a leading axon while inhibiting axon branching. AM251 effects were reminiscent of those of NFG: an expansion of axonal arbor and reversal of AEA effects (Fig. 4A and B). Disrupting CB1R signaling by coapplication of AEA and AM251 significantly inhibited neurochemical differentiation by decreasing the density of VGLUT1<sup>+</sup> neurons (Fig. 4B).

Our data also support that pyramidal cells require an intrinsic “eCB tone” to initiate axonal polarization and neurochemical differentiation. Both DAGLαs were expressed by pyramidal cells (Fig. 4C and Fig. S5A) and targeted to the axon (Fig. 4D and E) and growing navigation cones (Fig. 4F). Notably, DAGLα was detected in elongating axon shafts and growth cones (including filopodia) and exhibited proximal localization to CB1R (Fig. S5A), whereas DAGLβ-like i.r. was limited to axonal shafts. DAGLα levels inversely correlated with axon development; initially a random DAGLα distribution was seen in quiescent axons, followed by DAGLα concentrating in axon varicosities (18) (Fig. S5C). These data reveal that axonal DAGLα levels remain high in developing axons and undergo redistribution during axon maturation and synapse specification (Fig. S5C). DAGL inhibition by O-3841 (19) significantly reduced VGLUT1 expression in pyramidal cells, thus supporting the involvement of eCB signaling during the acquisition of a glutamatergic phenotype (Fig. 4G). Moreover, O-3841-induced increased synaptogenesis in vitro (Fig. S5D) suggests that impaired temporal and spatial integrity of eCB signaling in pyramidal cells may disrupt postsynaptic targeting of glutamatergic axons.

**CB1R Deletion Reveals Fasculation Deficits.** We assessed the in vivo significance of our findings in CB1R<sup>f/f</sup>NEX-Cre (15) in new-born CB1R<sup>f/f</sup>NEX-Cre (5A and B’) but not CB1R<sup>f/f</sup>NEX<sup>Cre/+</sup> mice (Fig. S6), L1 neuronal cell adhesion molecule (L1-NCAM)<sup>+</sup> pyramidal axons formed bundles with aberrant trajectories in the corpus callosum, failed to invade the dorsal striatum, and exhibited a change in their striatal paths. In CB1R<sup>R−/−</sup> (null) mice, axonal fasciculation deficits were pronounced at E14–16 (Fig. 5C) with a significant degree of compensation by birth. Nevertheless, corticofugal axons invariably failed to invade the dorsal striatum in CB1R<sup>R−/−</sup> neonates (Fig. 5D).

**In Utero CB1R Blockade Disrupts Neurodevelopment.** CB1R function in long-range axon fasciculation was tested also by i.c.v. injection.
of SR141716 (10 mM in 1 μl) in utero at E13.5, as a proof-of-concept for CB1R antagonist actions. Analysis of corticofugal connectivity at P8 revealed enlarged lateral ventricles (Fig. 5E) in conjunction with impaired progenitor proliferation in the subcortical VZ/SVZ (DMSO, 39 ± 3%; SR141716, 27 ± 3% BrdU cells; P < 0.05; n = 4), resulting in cortical delamination. Expansion of the corpus callosum was due to a deficit in axonal pathfinding, as indicated by a reduced commitment of pyramidal cell axons to the developing callosal trajectory (Fig. 5F and G). Overall, data obtained in genetic models and after in utero SR141716 treatment confirm that proliferation, migration, and axonal pathfinding decisions of pyramidal cells are reliant on eCB signaling through CB1Rs.

**Discussion**

The molecular mechanisms of eCB actions in the adult brain are well appreciated. However, a dearth of knowledge exists on the cellular specificity of eCB actions in the developing brain (20). In this article, we show the onset and spatial restriction of eCB signaling during embryogenesis and demonstrate the cellular roles of eCB signaling on neurochemically identified cortical pyramidal cells and their progenitors. These data, together with prior results on eCB control of neuronal progenitor cell fate and GABA interneuron development, define the complexity of eCB signaling in the developing cerebrum and identify eCBs as developmental signals indispensable for cortical neuron specification and connectivity patterning (Fig. S7).

eCBs Exert Differential Control on Cortical Neurons.** The role of eCBs in cortical neuron specification depends on the molecular identity of neurons. Pyramidal cells express CB1Rs as postmitotic progenitors in the subcortical V2/SVZ. However, GABAergic progenitors in ganglionic eminences lack this receptor, and immature GABAergic interneurons express CB1Rs only during and after intracortical (radial) migration (8). Postmitotic, pyramidal cell lineage-committed neurons harbor the capacity of eCB synthesis throughout their morphological and functional specification. In contrast, GABAergic interneurons seem to lack, except for sporadic NAPE-selective phospholipase D expression, eCB synthetic enzymes. Functional implications of these findings include the following. First, eCB signaling is a key determinant of the number of pyramidal cells destined to particular cortical laminae. Second, eCB control of axonal elongation in pyramidal cells is a primarily cell-autonomous mechanism, with DAGLs and CB1Rs being in close proximity predominantly along the axon stem allowing direct ligand-receptor coupling. Conversely, CB1Rs concentrate in axonal growth cones of GABAergic interneurons (8), suggesting that
CB₄R Knock-Outs Develop Axonal Deficits. Significant axon fasciculation deficits were observed in neonatal CB₂R<sup>f/f</sup>,NEX-Cre mice. Notably, CB₂R<sup>−/−</sup> mice exhibited disrupted axon development at approximately E15 that was reminiscent of the phenotype in conditional mice; however, in CB₂R<sup>−/−</sup> mice the deficit normalized by birth. These differences may demonstrate pleiotropism of the eCB system at the receptor level and suggest that the expression of non-CB₂Rs on neurons or redundancy of signaling pathways (e.g., overt expression of other chemotropic/repressive guidance cues) may compensate for the complete loss of CB₂R-mediated signaling in CB₂R<sup>−/−</sup> mice. The finding that pyramidal cell CB₂Rs are only abundantly expressed during the restricted period of their morphological and functional specification (E14.5–E18.5) argues that targeted disruption of CB₂R signaling during this period will selectively disrupt axon elongation and targeting and impose permanent deficits to a restricted set of neocortical pyramidal cells in CB₂R<sup>f/f</sup>,NEX-Cre conditional mutants.

Overall, our present and previous (6–8) data define discrete spatial and temporal niches for eCB action and identify the cellular basis of their dichotomy on glutamatergic and GABAergic cortical neurons. Although the identity of eCB(s) mediating particular cellular actions has not been elucidated, developmental effects of manipulating both AEA and 2-arachidonoylglycerol levels suggest that both ligands may control pyramidal cell specification through their promiscuity at the CB₂Rs. Considering the high degree of phylogenetic conservatism of eCB signaling, both at enzyme and receptor levels, across vertebrate species (23), data described herein on mammalian neurons (8) together with those on zebrafish and chick neurons (10) formulate the unifying concept that eCBs are key developmental cues establishing neuronal diversity and synaptic connectivity in the developing brain.

Methods

Animals. The generation and genotyping of glutamate decarboxylase 67-GFP (GAD67gfp<sup>/−</sup>) (8), CB₂R<sup>−/−</sup> (11), FAAH<sup>−/−</sup> (14), CB₂R<sup>f/f</sup>,NEX-Cre (11), and respective littermate controls has been reported elsewhere. Mouse and Sprague-Dawley rat embryos and tissues were obtained from timed matings.

Neuroanatomy. In situ hybridization in mouse and human fetal brains was performed as described (8, 11, 12). Multiple immunofluorescence labeling, acetylcholinesterase histochemistry, Hoechst 33,258 (Sigma) and Nissl stains were performed with quality-controlled immunoreagents (6, 8) (Fig. S1) with n = 2–5 brains per group analyzed by laser-scanning microscopy.

Cell Proliferation, Migration, and ex Vivo Electroporation. Cell proliferation and migration was determined after i.p. BrdU injection (100 µg/kg) of pregnant females at E14.5. For proliferation assays, embryos were harvested 2 h after labeling. For migration experiments, pups were killed at P2 [n ~ 6/6 (wild type/CB₂R<sup>−/−</sup>)]; n ~ 7/7 (wild type/FAAH<sup>−/−</sup>)], from three litters. Cortical layers were identified by their discrete cell densities as visualized by Hoechst 33,258 (Sigma) and βIII-tubulin (TuJ1; Promega) counterstaining. Ex vivo electroporation was performed by using a pCiG2-GFP vector (24) and slice cultures were maintained in semidry conditions in wells containing neurobasal medium (1% B27/1% N2/1% penicillin/streptomycin/1% fungizone/5 µg/ml ciprofloxacin). pCiG2-GFP vector was also used to overexpress FAAH in an iRES-EGFP cassette under the control of a CMV enhancer and chicken β-actin pro-
motoneurons. For proliferation studies, brain slices in 6 per condition) were cultured overnight, stimulated, and pulsed with BrdU (10 μM; 2 h). Cyclohexylcarbamic acid 3-carboxymethyl-biphenyl-3-yl ester (URB597) was obtained from Cayman Chemical. A minimum of six slices per treatment were analyzed (6).

**Pyramidal Cell Cultures.** Rat cortices were isolated at E14.5, cells were dissociated by trypsin digestion (0.1%; 5 min) and plated at a density of 25,000 or 200,000 cells per well for morphometry or biochemical analysis, respectively. Cultured neurons were maintained in DMEM/F12, supplemented with 1% B27/1% glutamine/1% penicillin/streptomycin for 3–6 days (7). Ligands were first added 12 h after cell seeding and replenished every other day (7), except for O-3841 (1 μM) (19), which was used in the hippocampus and cerebellum. Density of glutamatergic neurons was defined as a ratio of VGLUT1/H11001, Western blotting of SNAP25 and DAGL/H11350, and the lateral ventricle of the embryo. A single hemisphere was injected, whereas Mulder et al. (10) randomly selected view fields per coverslip. Morphometric analysis was performed as described (7, 8), with also defining the longest axon segment. Western blotting of SNAP25 and DAGL/H11032 was used to verify morphometric assessment (7).

**In Utero SR141716 Treatment.** The uteruses of anesthetized pregnant mice (E13.5) were externalized, and a glass micropipette filled with 1 μM SR141716 (10 mM) or DMSO (vehicle) was advanced through the uterine wall and into the lateral ventricle of the embryo. A single hemisphere was injected, whereas the other served as an internal control. After drug infusion, the uterus was gently repositioned and the abdominal wall was sutured. Mice were transcardially perfused on P8 and processed (Fig. S1).

**Statistics.** Data were expressed as means ± SEM. Statistical analysis was performed by either ANOVA with Student–Neuman–Keuls post hoc tests or two-tailed unpaired Student’s t test on independent samples. P < 0.05 was considered statistically significant.

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