CB₁ Cannabinoid Receptor-Dependent Activation of mTORC1/Pax6 Signaling Drives Tbr2 Expression and Basal Progenitor Expansion in the Developing Mouse Cortex

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The CB₁ cannabinoid receptor regulates cortical progenitor proliferation during embryonic development, but the molecular mechanism of this action remains unknown. Here, we report that CB₁-deficient mouse embryos show premature cell cycle exit, decreased Pax6- and Tbr2-positive cell number, and reduced mammalian target of rapamycin complex 1 (mTORC1) activation in the ventricular and subventricular cortical zones. Pharmacological stimulation of the CB₁ receptor in cortical slices and progenitor cell cultures activated the mTORC1 pathway and increased the number of Pax6- and Tbr2-expressing cells. Likewise, acute CB₁ knockdown in utero reduced mTORC1 activation and cannabinoid-induced Tbr2-positive cell generation. Luciferase reporter and chromatin immunoprecipitation assays revealed that the CB₁ receptor drives Tbr2 expression downstream of Pax6 induction in an mTORC1-dependent manner. Altogether, our results demonstrate that the CB₁ receptor tunes dorsal telencephalic progenitor proliferation by sustaining the transcriptional activity of the Pax6–Tbr2 axis via the mTORC1 pathway, and suggest that alterations of CB₁ receptor signaling, by producing the misexpression of progenitor identity determinants may contribute to neurodevelopmental alterations.

Keywords: basal progenitors, cannabinoid, corticogenesis, Eomes, mTORC1

Introduction

In the mammalian developing cortex, neural progenitor cells are differentially distributed in the ventricular and subventricular zones (VZ and SVZ). Apical and basal progenitor cells are identified by their selective expression of the transcription factors Pax6 and Tbr2/Eomes, respectively (Guillemot et al. 2006; Osumi et al. 2008). On one hand, the heterogeneity of apical VZ progenitor subpopulations contributes to the acquisition of the projection neuron diversity of the mature six-layered neocortex (Gal et al. 2006). In addition, intermediate/basal progenitors in the mouse SVZ can undergo additional rounds of division and contribute to the number and diversity of pyramidal neurons in the mature mammalian neocortex (Noctor et al. 2004; Hansen et al. 2010). Pax6, a paired domain-containing transcription factor, master regulates the network of neural stem cell decision-making genes (Sansom et al. 2009; Asami et al. 2011), being essential for the maintenance of the VZ radial glial cell pool and the transition from apical to basal progenitors by driving Tbr2 expression (Warren et al. 1999; Englund et al. 2005). Likewise, Tbr2 is essential for neuronal amplification and tightly controls the balance between neural stem cell renewal and neurogenesis (Guillemot et al. 2006). Thus, the Pax6–Tbr2 transcription factor axis exerts a pivotal role in the maintenance of cortical progenitor cell populations and in the appropriate timing of cortical neurogenesis (Englund et al. 2005; Sessa et al. 2008).

Whereas these and other endogenous fate determinants have been extensively studied (Guillemot et al. 2006), how extrinsic signals from neurogenic niches tune the appropriate coordination of neuronal generation, maturation and circuit establishment remains largely unknown. Synaptic and nonsynaptic neuronal activities constitute physiological inputs that tune neurogenesis during development and in the adult brain (Ge et al. 2007; Ben-Ari 2008). Thus, different neurotransmitters and neuromodulators have been shown to influence neural progenitor proliferation and neurogenesis. In particular, the CB₁ cannabinoid receptor, the most important mediator of endocannabinoid actions on key neurobiological processes (Castillo et al. 2012), exerts a regulatory role in neuronal differentiation and long-range connectivity (Berghuis et al. 2007; Mulder et al. 2008; Díaz-Alonso et al. 2012). In addition, CB₁ receptors are known to control neural progenitor cell proliferation in vitro and in vivo, both in the developing brain and in adult neurogenic areas (Jin et al. 2004; Aguado et al. 2005, 2007; Trazzi et al. 2010). However, the molecular mechanism of CB₁ receptor action on cortical progenitor expansion during embryonic development remains unknown.

Here, we show that CB₁ receptor signaling exerts a crucial role in mouse cortical progenitor expansion by promoting the generation of basal progenitors from radial glial cells. This CB₁ receptor-mediated effect occurs through the induction of Pax6 transcriptional activity via the mammalian target of rapamycin complex 1 (mTORC1) pathway. Thus, gain or loss of CB₁ receptor function enhanced or reduced, respectively, the activation of the mTORC1 pathway and Pax6⁺ radial glial cell progression to Tbr2-expressing basal progenitors. These findings contribute to unveiling the molecular basis of cortical progenitor cell generation and may provide mechanistic clues to understand the origin of neurodevelopmental alterations origi-

ated by unbalanced progenitor expansion.

Materials and Methods

Materials

The following materials were kindly donated: Tbr2-promoter luciferase construct (M. Götz and L. Pinto, Ludwig Maximilians University Munich, Germany), Pax6 transcriptional activity reporter constructs (A. Stoykova and T. Tuoc, Max Plank Institute for Biophysical Chemistry, Göttingen, Germany), Pax6 expression vector (M. Nieto, National Center Biotechnology, Madrid, Spain), CB₁fl/fl and CB₁f/f, Nest-Cre+ colony-founding mice and CB₁ in situ hybridization (ISH) probes (B. Lutz and C. Hoffmann, Mainz, Germany), and HU-210 (R. Mechoulam, Hebrew University, Jerusalem, Israel).
Animals
Experimental designs and procedures were approved by the Complutense University Animal Research Committee in accordance with the European Commission regulations. All efforts were made to minimize the number of animals and their suffering throughout the experiments. Mice were maintained in standard conditions, keeping littersmates grouped in breeding cages, at a constant temperature (20 ± 2 °C) on a 12-h light/dark cycle with food and water ad libitum. The generation and genotyping of CB1−/− and CB1f/f, Nex-Cre/+ (Nex-CB1−/−), and their respective wild-type (WT) littermate controls, has been reported elsewhere and was performed accordingly (Monory et al. 2006). Mouse embryonic tissues were obtained upon timed mating as assessed by vaginal plug observation (E0.5). For CB1 expression analyses, we compared WT, CB1−/−, CB1f/f, and Nex-CB1−/− embryos. After carefully ascertaining that WT and CB1f/f expression levels were indistinguishable, only the comparison among CB1f/f, Nex-CB1−/−, and CB1−/− embryos is shown in Figure 1.

Immunoﬂuorescence and Confocal Microscopy
Cell proliferation was determined after intraperitoneal iodo- and bromo-deoxyuridine (IdU, BrdU) injection (100 μg/g body weight) of pregnant females at E12.5 and E13.5 as indicated. Coronal brain slices (14-μm thick) were processed as previously described (Diaz-Alonso et al. 2012). Cortical layers were identified by their discrete cell densities as visualized by DAPI counterstaining.

Figure 1. The CB1 receptor is expressed, albeit at low levels, in proliferative areas of the developing mouse cortex. (A) CB1 mRNA was quantified by qPCR in E13.5 cortical extracts of CB1f/f, Nex-CB1−/−, and CB1−/− embryos (n = 4 for each group). **P < 0.01 versus CB1f/f extracts; ###P < 0.01 versus Nex-CB1−/− extracts. (B and C) Representative raw and binaryISH images in the developing cortex of the same 3 genotypes at E14.5. Semiquantitative analysis of CB1-transcript levels was performed to estimate the relative presence of CB1 transcripts in each compartment (n = 4 for each group). *P < 0.05, **P < 0.01 versus the respective VZ+SVZ; #P < 0.05 versus Nex-CB1−/−VZ+SVZ; $P < 0.05 versus CB1f/f VZ+SVZ. (D) Representative images of CB1 receptor immunoreactivity in E14.5 CB1f/f, Nex-CB1−/−, and CB1−/− cortical sections. Cell nuclei were counterstained with DAPI. Insets of the indicated areas are shown. CP, cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular/subventricular zone. Scale bars: B, 25; D, 25 and 10 μm (insets).
Immunofluorescence was performed, after blockade with 5% goat serum, by overnight incubation at 4 °C with the indicated primary antibodies (Table 1), followed by incubation for 1 h at room temperature with secondary antibodies. The appropriate anti-mouse, rat, guinea pig, and rabbit highly cross-adsorbed AlexaFluor 488, AlexaFluor 546, Alexa Fluor 594, and AlexaFluor 647 secondary antibodies (Invitrogen, Carlsbad, CA, USA) were used. Confocal fluorescence images were acquired by using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with 2 passes by Kalman filter and a 1024 × 1024 collection box. Immunofluorescence data were obtained in a double blind manner by an independent observer, and all quantitative data were obtained from a minimum of 6 sections from 1-in-10 series per mouse. Immunofluorescence of cortical sections was performed along the rostral to caudal axis and the quantifications were carried out in equivalent sections from the mediolateral area of the rostro-medial cortex. Quantifications of cortical progenitor cell populations in the VZ/SVZ were performed in a 275-μm-wide 180-μm-high cortical box. Positive cells for the corresponding markers were quantified. In CB1 receptor knockdown experiments by in utero and ex vivo electroporation, cells positive for the indicated markers were quantified within the GFP+ cell population.

**In Situ Hybridization**

Coronal sections (14 μm) of E14.5 or E16.5 mouse embryonic brains were obtained and processed for ISH as described (Diaz-Alonso et al. 2012). Representative ISH images were processed with Image J software, and binary images were obtained after application of the same background subtraction. Binary images were employed for semiquantitative determination of the ISH signal present in the different cortical compartments. In some cases, CB1 ISH was followed by immunofluorescence detection of the GFP protein (as described above) to identify the electroporated area.

**In Utero and Ex Vivo siRNA Electroporation**

siCB1 and siControl (Thermo Scientific) were electroporated together with pCAG-GFP and Fast Green in the lateral ventricle of E13.5 embryos, either ex vivo, followed by coronal slicing and organotypic culture for 2 days in vitro (DIV), or in utero, as described (Diaz-Alonso et al. 2012). In utero electroporated embryos were analyzed 3 days later, at E16.5.

**Primary Cortical Progenitor and Organotypic Cultures**

Cortical progenitors were obtained from dissected cortices isolated from E13.5 CB1+ embryos and grown as neurospheres as previously described (Aguado et al. 2005). For pharmacological regulation experiments, neurospheres were dissociated with accutase (Sigma) and mechanically plated on polylysine and laminin-coated coverslips at a density of 50,000 cells/cm², and grown in a chemically defined medium composed of DMEM/Hams F-12 medium supplemented with N2 (Millipore), glutamine and basic FGF. For acute CB1 receptor genetic ablation studies, CB1+ neurospheres were subjected to nucleofection (Amaxa nucleofector, Lonza, Spain) with either pCAG-Cre-GFP or pCAG-GFP prior to plating. Proliferation assays were performed after 1 day of preincubation with BrdU (10 μg/mL). The progenitor identity was assessed after 2 DIV by quantification of highly immunoreactive Tbr2+ cells from ≥10 randomly selected view fields/coverslips after DAPI counterstaining. Slice cultures were maintained in semidry conditions on Millicell membranes (Millipore) containing neurobasal medium, B27 (Invitrogen, 1%), N2 (1%), glutamine (1%), and penicillin/streptomycin (1%). Pharmacological manipulation was performed at 1 DIV with the indicated drugs at the indicated concentrations. CB1 receptor expression rescue in CB1−/− neural progenitors was performed by nucleofection with pCAG-CB1−GFP or pCAG-GFP as a control.

**Gene Promoter Activity Assays**

Primary cortical progenitors were transiently transfected with the Tbr2-promoter luciferase reporter constructs (Pinto et al. 2009) by nucleofection, and P19 mouse embryonic carcinoma cells were transiently transfected (Lipofectamine 2000, Invitrogen) with a Pax6 expression vector and a Pax6-binding sequences (pCON/P5) reporter construct (Tuoc and Stoykova 2008). Firefly and renilla (as internal transfection control) luciferase activities were measured using Dual-Luciferase Reporter assay system (Promega) in a Lumat LB9507 luminometer (Bertech Technologies, Bad Wildbad, Germany).

**Real-Time Quantitative PCR and Chromatin Immunoprecipitation**

RNA was isolated using RNeasy Plus kit (Qiagen). cDNA was obtained with Transcriptor (Roche). Real-time quantitative PCR (qPCR) assays were performed using the FastStart Master Mix with Rox (Roche) and probes were obtained from the Universal Probe Library Set (Roche). Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Applied Biosystems). Each value was adjusted by using 18S RNA and β-actin levels as reference. For chromatin immunoprecipitation (ChiP) analysis, WT and CB1−/− cortices were dissected from E14.5 mouse embryos and diced in ice-cold Hanks’ buffered saline solution. Samples were processed as recommended by the manufacturer (EZ-Chip, Millipore) and immunoprecipitation was performed with an anti-Pax6 antibody (Millipore) or a nonspecific rabbit IgG as control. Pax6-bound DNA was determined by qPCR analysis. Primers were designed from published data for the amplification of Pax6-binding DNA sequences (Sansom et al. 2009) in the promoter region of several genes (Tbr2, Nf1, neurogenin3, Hex7, and Rap1b). Reactions were performed in triplicate on 3 independent ChiP samples per genotype (n = 12 cortices for each genotype). The enrichment for each gene was calculated by normalizing the Pax6/input ratio with the Pax6/input ratio of a DNA region that does not bind Pax6 (Gad1 gene promoter).

**Table 1**

Antibodies employed in this study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td>Rat monoclonal</td>
<td>Abcam</td>
<td>1:250</td>
</tr>
<tr>
<td>BrdU/IdU</td>
<td>Mouse monoclonal</td>
<td>Becton/Dickinson</td>
<td>1:200</td>
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<td>CB1</td>
<td>Rabbit polyclonal</td>
<td>Frontier Institute</td>
<td>1:500</td>
</tr>
<tr>
<td>CB1</td>
<td>Guinea pig polyclonal</td>
<td>Frontier Institute</td>
<td>1:500</td>
</tr>
<tr>
<td>GFP</td>
<td>Rabbit polyclonal</td>
<td>Chemicon</td>
<td>1:250</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Rabbit polyclonal</td>
<td>Neomarkers</td>
<td>1:200</td>
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<tr>
<td>Nestin</td>
<td>Mouse monoclonal</td>
<td>Chéromics</td>
<td>1:200</td>
</tr>
<tr>
<td>Pax6</td>
<td>Mouse monoclonal</td>
<td>Developmental Studies</td>
<td>1:50</td>
</tr>
<tr>
<td>Pax6</td>
<td>Rabbit polyclonal</td>
<td>Millipore</td>
<td>(10 μg ChIP reaction)</td>
</tr>
<tr>
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<td>Mouse monoclonal</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>RC2</td>
<td>Mouse monoclonal</td>
<td>Developmental Studies</td>
<td>1:50</td>
</tr>
<tr>
<td>pS6</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling</td>
<td>1:250 (IgF)</td>
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<tr>
<td>pS6-AlexaFluor488 conjugate</td>
<td>Rabbit polyclonal</td>
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<td>1:1000 (WB)</td>
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<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>1:500</td>
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<tr>
<td>α-Tubulin</td>
<td>Mouse monoclonal</td>
<td>Sigma</td>
<td>1:4000 (WB)</td>
</tr>
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**Data Analyses and Statistics**

Results shown represent the means ± SEM, and the number of experiments is indicated in every case. Statistical analysis was performed by one- or two-way ANOVA, as appropriate. A post hoc analysis was made by the Student–Neuman–Keuls test.

**Results**

**The CB1 Cannabinoid Receptor is Expressed, Albeit at Low Levels, in Proliferative Areas of the Developing Mouse Cortex**

To investigate the role of CB1 receptor signaling in cortical progenitor cell populations, it was crucial to determine first the
expression levels of this receptor in the VZ/SVZ. Previous studies had indicated the presence of CB1 receptors in proliferative cells of the mouse developing cortex in vivo and in vitro (Aguado et al. 2005; Mulder et al. 2008). However, the expression levels of the CB1 receptor in the cortical VZ/SVZ in vivo may have been overestimated owing to the recently described lack of specificity of commercially available anti-CB1 antibodies (Morozov et al. 2013). We quantified CB1 transcripts by qPCR in cortical extracts derived from CB1<sup>fl/fl</sup>, conditional Nex-CB1<sup>−/−</sup>, and complete CB1<sup>−/−</sup> embryos at E13.5. CB1 transcripts were undetectable in CB1<sup>−/−</sup> samples, while low but significant CB1 transcript levels were consistently observed in Nex-CB1<sup>−/−</sup> cortical extracts (Fig. 1A). This remainder (~25%) of CB1 transcripts present in the latter samples conceivably corresponds to progenitor cells owing to the postmitotic neuron selectivity of Nex-driven recombinase expression (Goebbels et al. 2006; Díaz-Alonso et al. 2012).

The presence of CB1 transcripts in the developing mouse cortex was also analyzed by ISH (Fig. 1B). CB1 receptor expression in CB1<sup>−/−</sup> animals showed a typical inside-out expression gradient, with higher levels in postmitotic cells. In addition, CB1 transcripts were present, albeit at low levels, in the VZ/SVZ. The CB1<sup>−/−</sup>-transcript signal was essentially absent in complete CB1<sup>−/−</sup> mice, thus confirming the specificity of the CB1 ISH. Semiquantitative analysis of the ISH signal was subsequently performed to estimate the relative presence of CB1 transcripts in the progenitors’ compartments (VZ/SVZ) and the postmitotic areas (IZ/CP) (Fig. 1C). Using stringent image acquisition and processing settings, the CB1 ISH signal in CB1<sup>−/−</sup> within the VZ/SVZ accounted for a ~17% of total (VZ/SVZ + IZ/CP) CB1 signal, a value that is not far from that of CB1 mRNA levels remaining in the Nex-CB1<sup>−/−</sup> cortices as determined by qPCR (Fig. 1A). Of note, in Nex-CB1<sup>−/−</sup> mice, the VZ/SVZ compartment preserved its CB1 mRNA expression (Fig. 1C, VZ+SVZ white columns) and only postmitotic neurons (Fig. 1C, IZ+CP black columns) lost receptor expression.

Similar to the aforementioned ISH analyses, the characterization of CB1 receptor protein expression by confocal microscopy proved the efficient ablation of CB1 in the postmitotic area of Nex-CB1<sup>−/−</sup> and CB1<sup>−/−</sup> mouse cortices (Fig. 1D, upper panels). In line with the ISH data, in the VZ/SVZ of CB1<sup>−/−</sup> and Nex-CB1<sup>−/−</sup> animals, the presence of CB1 receptors was low but clearly distinguishable from the faint unspecific reaction observed with the anti-CB1 antibody in CB1<sup>−/−</sup> mice (Fig. 1D, lower panels) (Morozov et al. 2013).

**The CB1 Cannabinoid Receptor Regulates Pax6–Tbr2 Cortical Progenitor Expansion**

To investigate the role of CB1 receptor signaling in cortical progenitors, we performed pharmacological manipulation studies in organotypic cultures of E13.5-WT embryonic brain cortices. The synthetic cannabinoid agonist HU-210 increased apical (Pax6<sup>+</sup>) and basal (Tbr2<sup>+</sup>) progenitor cell populations in a CB1 receptor-dependent manner, as evidenced by the ability of the CB1 antagonist rimonabant (SR141716) to prevent cannabinoid-induced increase in progenitor cell number and mRNA levels (Fig. 3A,D). We confirmed the involvement of CB1 receptor signaling in the regulation of the Pax6–Tbr2 transcription factor axis by siRNA interference assays. Thus, CB1 receptor knockdown by siCB1 ex vivo electroporation (Supplementary Fig. 2) was accompanied by reduced Pax6 and Tbr2 mRNA levels (Fig. 3E). The transcript levels of Sox2, a transcription factor essential for neural stem cell proliferation and self-renewal (Guillemot et al. 2006), were also reduced in siCB1-electroporated cortices. Moreover, HU-210-induced increase in Tbr2<sup>+</sup> cell number in cortical slices was prevented by siCB1 electroporation (Fig. 3F,G). To further support the role of CB1-mediated signaling in cortical progenitor expansion in vivo, acute elimination of CB1 receptors was conducted by in utero electroporation of siCB1 or siControl at E13.5, and embryos were analyzed at E16.5. Importantly, ablation of CB1 receptor signaling reduced the Tbr2<sup>+</sup> population within the GFP<sup>+</sup> cell population when compared with siControl-electroporated embryos (Fig. 3H,I).

**The CB1 Cannabinoid Receptor Regulates mTORC1 Signaling in Progenitors of the Developing Mouse Cortex In Vivo**

To study the mechanism of CB1-mediated regulation of cortical progenitors in vivo, among the various signaling pathways activated by CB1 receptors (Harkany et al. 2007), we analyzed...
the mTORC1 pathway, as the CB1 receptor is known to activate mTORC1 in neurons (Puighermanal et al. 2009), and this pathway exerts key pleiotropic actions in the control of neural cell fate during brain development (Crino 2011). The mTORC1 pathway was found to be active in the VZ/SVZ of the developing cortex, as evidenced by the phosphorylation of the ribosomal protein S6 at Ser 235/236, a canonical readout of mTORC1 activation, and genetic inactivation of CB1 receptor signaling reduced the number of phospho-S6+ cells in the VZ and SVZ of E14.5 cortices (Fig. 4A, B). Proliferating cells in the VZ/SVZ co-localized to a large extent with S6 phosphorylation, and loss of CB1 receptor function decreased phospho-S6 immunoreactive Ki67+ cycling progenitors of the VZ/SVZ (Fig. 4C, D). Double immunofluorescence with an anti-Pax6 or anti-Thrb2 antibody,

Figure 2. The CB1 receptor controls neural progenitor populations and cell cycle during cortical development. (A–C) Pregnant CB1+/− female mice crossed with CB1+/− male mice were injected with IdU and BrdU at E12.5 and E13.5 gestational days (GD), respectively, and embryonic cortices analyzed at E14.5. Quantification of IdU+ BrdU+/IdU+ and BrdU+ Ki67+/BrdU+ cells was performed, allowing to assess cell cycle maintenance ratio between E12.5 and E13.5 and between E13.5 and E14.5, respectively (n = 4 for each group). Representative images of BrdU+ (green) and Ki67+ (red) cell distribution at E14.5 are shown. Empty arrowheads indicate double-positive cells, white arrows point BrdU-only cells. (D–G) Quantification of highly immunoreactive Pax6- and Thrb2-expressing cells in a 275-μm-wide 180-μm-thick box placed adjacent to the ventricular wall (covering the VZ and SVZ) of CB1−/− and WT littermates at E14.5 and E16.5 (n = 7 and 8; 6 and 5, respectively, for each group). Representative images are shown. (H–J) Quantification of nestin- and RC2-immunoreactive (IR) area in the VZ/SVZ of CB1−/− and WT littermates at E14.5 (n = 3 for each genotype). Representative images are shown. Scale bars: C, 50 and 20 μm (insets); D and F, 50 μm; H, 25 μm. *P < 0.05; **P < 0.01 versus WT mice.
Figure 3. CB1 receptor signaling drives Pax6\(^{+}\) and Tbr2\(^{+}\) cortical progenitor expansion in organotypic cultures. (A–D) Cortical organotypic cultures from E14.5-WT cortices were exposed for 48 h to vehicle (Veh) or HU-210 (1 \(\mu\)M), alone or combined with SR141716 (SR, 25 \(\mu\)M), and highly immunoreactive Pax6- and Tbr2-positive cells (green and red, respectively) quantified in the ventricular and subventricular zones (VZ and SVZ). Representative immunofluorescence images are shown. Pax6 and Tbr2 mRNA levels were also determined by qPCR after pharmacological manipulation of cortical slices (B and D, right panels). (E) Organotypic cultures prepared from CB1 siRNA (siCB1) or control siRNA (siC) and pCAG-GFP-electroporated E14.5-WT cortices and the transfected (GFP\(^{+}\)) areas were dissected and analyzed after 48 h by qPCR for the indicated transcripts. (F–G) WT cortices were electroporated ex vivo at E14.5 with siCB1 or siControl and pCAG-GFP after 1 day in vitro (DIV), treated with HU-210 (1 \(\mu\)M) or vehicle. Tbr2\(^{+}\) cells (red) were quantified in the transfected GFP\(^{+}\) cell population after 2 DIV. Empty arrowheads indicate double-positive cells, white arrows point GFP-only cells. Results are represented as the Tbr2\(^{+}\) GFP\(^{+}\) cells/total GFP\(^{+}\) cells ratio in HU-210-treated slices normalized to the corresponding vehicle-treated slices. Results correspond to 4 independent experiments. (H–I) In utero electroporation of siControl or siCB1 together with pCAG-GFP was performed at E13.5 and Tbr2\(^{+}\) cells (red) in the ventricular and subventricular zone (VZ/SVZ) were quantified at E16.5 and referred to the GFP\(^{+}\) electroporated cell population. Empty arrowheads indicate double-positive cells, white arrows point GFP-only cells. Embryos (n = 3) from at least 2 different litters per condition. *P < 0.05; **P < 0.01 versus vehicle-treated or control siRNA-electroporated slices. a.u., arbitrary units. Scale bars: 25 \(\mu\)m.
Figure 4. The CB1 receptor controls mTORC1 signaling in cortical progenitors in vivo. (A and B) Phospho-S6-positive (pS6, red) cells were quantified after immunofluorescence in the developing dorsal telencephalon of CB1−/− and WT littermates at E14.5 and referred to total cell number in the analyzed cortical column. Empty arrowheads point to representative pS6+ cells. (C and D) mTORC1 activation status in proliferative progenitor cells was assessed by double immunofluorescence analysis of pS6 and Ki67 in the VZ and SVZ of E14.5 CB1−/− and WT littermates (n = 3 embryos for each genotype). (E and F) mTORC1 signaling in Pax6+ radial glial cells was assessed by the quantification of phospho-S6+ cells highly immunoreactive for Pax6 (green) and referred to Pax6 highly immunoreactive total cell number in the VZ. Empty arrowheads point to Pax6+pS6+ cells, whereas white arrows indicate Pax6+pS6− cells (n = 10 embryos for each group). (G–I) mTORC1 activation status in intermediate progenitor cells labeled with an anti-Tbr2 antibody (green) was assessed with pS6 immunoreactivity in the VZ and SVZ (H and I, respectively) of CB1−/− and WT littermates (n = 6 for each group). Empty arrowheads point to Tbr2+pS6+ cells, whereas white arrows indicate Tbr2+pS6− cells. *P < 0.05; **P < 0.01 versus WT mice. Scale bars: A 100 and 25 μm (insets); E and G, 25 μm.
together with an anti-phospho-S6 antibody, showed that a large fraction of Pax6+* cells have an active mTORC1 pathway (47 ± 3% Pax6+*pS6+/Pax6+; n = 10), while mTORC1 activity in Tbr2+ cells of the SVZ cells was notably lower (13 ± 4% Tbr2+*pS6+/Tbr2+; n = 6). Interestingly, ablation of the CB1 receptor decreased the activation of the mTORC1 pathway in VZ Pax6+ cells (Fig. 4E,F) as well as in the recently generated Tbr2+ cells located at the VZ (Fig. 4G,H), but not in the SVZ (Fig. 4I). These results indicate that CB1 receptor-driven mTORC1 activity contributes to the apical to basal progenitor transition.

**The CB1 Cannabinoid Receptor Activates mTORC1 Signaling in Proliferating Cortical Progenitors**

To investigate the mechanism of CB1 receptor-induced expansion of neural progenitor cells, we performed pharmacological manipulation experiments in E13.5 progenitor cultures. First, we confirmed that CB1 receptors regulate cortical progenitor identity in vitro in a similar manner than in vivo. Treatment with HU-210 increased the generation of Tbr2+ cells, and SR141716 prevented this effect (Fig. 5A,B). As revealed by immunofluorescence (Fig. 5C,D), HU-210 increased S6 phosphorylation in proliferating cells that express the endogenous marker PCNA. HU-210-induced S6 phosphorylation in cortical progenitor cultures was prevented by co-administration of SR141716 and rapamycin (a widely employed mTORC1 inhibitor). Western blot analysis confirmed the activation of the mTORC1 pathway by CB1 signaling in cortical progenitor cells. Thus, the HU-210-induced increase of phosphorylated S6 was prevented by SR141716 and rapamycin (Fig. 5E,F). In addition, the phosphorytalininositol 3-kinase (PI3K) inhibitor LY-294,002 and the MEK inhibitor UO126 prevented HU-210-induced mTORC1 activation. To unequivocally address the role of CB1 receptor signaling in the control of the mTORC1 pathway in neural progenitor cells, we performed an acute genetic ablation strategy by means of pCAG-Cre-GFP or pCAG-GFP nucleofection in CB1-f/f-derived neurospheres. Loss of CB1 receptor function reduced cell proliferation as quantified by BrdU+ cell immunofluorescence (Fig. 5G,H), while in agreement with pharmacological gain of function experiments, CB1 ablation by Cre-expressing vector nucleofection restored phospho-S6+ cell number (Fig. 5I,J).

We furthermore confirmed the involvement of CB1 receptors in regulating mTORC1 activity in proliferating cells by ex vivo siRNA electroporation. CB1 receptor knockdown prevented the HU-210-mediated increase in pS6+ PCNA+ cell number in cortical slices (Fig. 6A,B). As expected, inhibition of mTORC1 by rapamycin abrogated basal and HU-210-induced phospho-S6 cells (Supplementary Fig. 3). In addition, acute CB1 knockdown in utero decreased pS6-positive cells within the transfected cell pool in the VZ, confirming the involvement of CB1 receptors in the regulation of mTORC1 activity in cortical progenitor cells in vivo (Fig. 6C,D). Finally, mTORC1 inhibition by rapamycin blunted the HU-210-induced increase in Tbr2+ cell number (Fig. 6E,F), confirming the importance of this signaling pathway in CB1-induced basal progenitor expansion. In summary, these results demonstrate that CB1 receptor signaling drives the expansion of cortical Tbr2+ progenitor cells through the activation of the mTORC1 pathway, at least in part, in a cell-autonomous manner.

**The CB1 Cannabinoid Receptor Drives Tbr2 Expression Through the Stimulation of Pax6 Transcriptional Activity**

To examine in detail the molecular mechanism of CB1 receptor action in cortical progenitors, we sought to investigate its impact on the intrinsic determinants of neural progenitor identity. For this purpose, primary progenitor cell cultures were nucleofected with a luciferase construct under the control of the Tbr2 promoter (Pinto et al. 2009). CB1 receptor activation by HU-210 increased Tbr2-promoter activity in WT, but not in CB1-deficient cells (Fig. 7A). Importantly, rescue of CB1 receptor expression in CB1−/− progenitors after pCAG-CB1 nucleofection restored basal and cannabionid-induced Tbr2-promoter-driven luciferase activity (Fig. 7B). HU-210-induced Tbr2-promoter luciferase activity was prevented by SR141716 and rapamycin (Fig. 7C), pointing to the involvement of the mTORC1 pathway in CB1 receptor-induced Tbr2 expression.

Considering the prominent role of the transcription factor Pax6 in cortical neurogenesis, its pivotal action in the transition from apical to basal progenitor cell populations and its role in driving Tbr2 expression (Englund et al. 2005; Tuoc and Stoykova 2008; Sansom et al. 2009; Georgala et al. 2011), we sought to investigate whether Pax6 is involved in CB1 receptor-mediated upregulation of Tbr2 expression. Transfection of a construct with the Pax6 consensus DNA-binding sites of its paired-box and paired-like homeodomains (Pcon and P3 sites, respectively) controlling luciferase expression was performed in P19 mouse embryonic carcinoma cells. HU-210 promoted Pax6 transcriptional activity in a CB1 receptor-dependent manner, as shown by the prevention of HU-210-induced pCON/P3 activity by SR141716 co-incubation (Fig. 7D). In addition, rapamycin blunted HU-210-induced Pax6 activity, thus indicating that the CB1 receptor drives Pax6 transcriptional activity in an mTORC1-dependent manner.

Next, we confirmed that Pax6 is an effective regulator of Tbr2 in our model. Pax6 overexpression in neural progenitors increased Tbr2-promoter luciferase activity (Fig. 7E). We subsequently analyzed Pax6 binding to the Tbr2 promoter by performing chromatin immunoprecipitation (ChIP) analysis in WT and CB1-deficient embryonic cortical extracts. Pax6-bound DNA was obtained and qPCR analysis revealed a significant enrichment of the Tbr2 promoter in WT extracts (Fig. 7F) while, in CB1-deficient extracts, Pax6 binding to the Tbr2 promoter was significantly reduced. Overall, these results support that CB1 receptor signaling drives Tbr2 expression by increasing Pax6 transcriptional activity in an mTORC1-dependent manner.

**Discussion**

Here, we show that CB1 receptor signaling in vivo and in vitro drives the expansion of cortical progenitor cells by inducing the activity of the Pax6–Tbr2 transcription factor cassette via mTORC1 signaling. Pharmacological and acute genetic CB1 receptor manipulation evidenced that CB1-mediated Tbr2 expression occurs downstream of Pax6 induction in an mTORC1-dependent manner. Thus, in the absence of CB1 receptor signaling, the Tbr2 basal progenitor cell population is reduced as a consequence of decreased Pax6 activity. These findings delineate the signaling mechanism involved in CB1 receptor-mediated regulation of cortical progenitor expansion in the developing telencephalon.
Figure 5. CB₁ receptor signaling regulates mTORC1 activity in proliferating cortical progenitors. (A and B) Cortical progenitor cultures from E14.5-WT embryos were treated for 24 h with vehicle (Veh) or HU-210 (100 nM), alone or combined with SR141716 (SR, 1 μM), and highly immunoreactive Tbr2+ cells were quantified. Total cell numbers were assessed by DAPI counterstaining. Positive cells are indicated by empty arrowheads and Tbr2-negative cells by white arrows. (C and D) Cortical progenitors were treated as above with HU-210, alone or in the presence of SR or rapamycin (Rapa, 100 nM). Immunofluorescence was performed with antibodies against PCNA (green) and S6 phosphorylated at Ser235/236 (red) and pS6+ cells were quantified in the PCNA+ cell population. Representative double-positive cells are pointed by empty arrowheads, while PCNA-only positive cells are indicated by white arrows. (E and F) Western blot analysis was performed with anti-phospho-S6 antibody in cortical progenitor extracts after 30 min of CB₁ stimulation with HU-210 preceded, where indicated, by 1 h preincubation with CB₁ antagonist (SR), or PI3K (LY294-002), MEK (UO126), or mTORC1 (Rapa) inhibitors. Loading control was performed with anti-α-tubulin antibody. Quantification of the relative phosphorylated protein and α-tubulin optical density is given in arbitrary units (a.u.). (G and H) E14.5 cortical progenitors were obtained from CB₁ f/f embryos, grown as neurospheres and nucleofected with pCAG-Cre-GFP or pCAG-GFP and cultured for 2 days in vitro (DIV). Immunofluorescence was performed with an anti-BrdU antibody (red) and BrdU+ cells quantified in the electroporated GFP+ cell population. Representative double-positive cells are pointed by empty arrowheads, while GFP-only positive cells are indicated by white arrows. (I and J) E14.5 cortical progenitors were obtained from CB₁ f/f embryos, grown as neurospheres and nucleofected with pCAG-Cre-GFP or pCAG-GFP and cultured for 2 DIV. Immunofluorescence with anti-pS6 (red) and anti-CB₁ (blue) antibodies and pS6+ cells were quantified. Representative triple-positive cells are pointed by empty arrowheads, while GFP-only positive cells are indicated by white arrows. *P < 0.05; **P < 0.01 versus vehicle-treated cells and slices or control-nucleofected cortical progenitors. Scale bars: A, C, G, and I, 25 μm. Results correspond to 4 independent experiments.
The Pax6–Tbr2 transcription factor axis is an essential regulator of the balance between self-renewal and neurogenesis, controlling the transition of radial glial to basal progenitors, and from proliferative progenitors to postmitotic neural cells (Hevner et al. 2001; Englund et al. 2005; Arnold et al. 2008; Sessa et al. 2008). Pax6 levels regulate radial glial cell cycle in a highly dose-dependent manner (Sansom et al. 2009). Pax6-
mediated regulation of radial glial cell proliferation involves the control of symmetrical versus asymmetrical mode of cell division (Estivill-Torrus et al. 2002; Asami et al. 2011), and both gain and loss of function result in accelerated cell cycle exit and premature progenitor pool exhaustion (Quinn et al. 2007; Sansom et al. 2009). The complexity of the Pax6 role in regulating progenitor cell fate is highlighted by the diversity of cellular outcomes observed upon its manipulation, which can result in alterations of cell proliferation, cell cycle, and apoptosis (Osumi et al. 2008; Georgala et al. 2011). The puzzling pleiotropic actions of this transcription factor have been ascribed to a number of factors including, but not limited to, selectivity provided by different Pax6 subdomains, direct and indirect Pax6 targets, and context-dependent differences (Berger et al. 2007; Mi et al. 2013; Walcher et al. 2013). Likewise, Tbr2 deficiency results in defective SVZ cell proliferation and reduced neurogenesis, contributing to developmental alterations (Arnold et al. 2008; Sessa et al. 2008). Overall, altered activity of these transcription factors may not only compromise the appropriate number of neurons in the mature cortex, but also affect and restrict the diversity of neuronal identities acquired by their progeny (Franco et al. 2012). Consequently, alterations of different progenitor subpopulations contribute to cortical developmental disorders, including autism, Down syndrome and intellectual disability (Elsen et al. 2013; Tyler and Haydar 2013).

Results shown here demonstrate that CB1 receptors are expressed, albeit at low levels, in the germinal zone of the mouse developing cortex. CB1 receptors have been shown to be functional in cultures of neural progenitors from different origins, thus suggesting cell-autonomous actions in progenitor cells (Galve-Roperh et al. 2013). Nonetheless, the low expression levels of CB1 receptors in the VZ/SVZ suggest that CB1 receptors present in differentiated neurons may also contribute to the regulation of progenitor cell fate. Thus, the CB1 receptor emerges as a novel signaling platform that coordinates progenitor cell expansion and neurogenesis by transducing information from the endocannabinoid tone present in neurogenic niches to endogenous neural identity determinants.

**Pathophysiological Implications of CB1 Cannabinoid Receptor-Mediated Regulation of mTORC1 Signaling**

The endocannabinoid tone, via CB1 receptors, regulates neural progenitor proliferation (Jin et al. 2004; Agudo et al. 2005; Mulder et al. 2008). However, the molecular mechanism of CB1 receptor-mediated signaling in cortical progenitor expansion has remained elusive. Our new findings show that CB1 receptor activation drives basal progenitor expansion and Tbr2 identity downstream of Pax6 induction by engaging the PI3K/Akt/mTORC1 signaling pathway. In agreement, CB1 receptor-induced neurite outgrowth relies on the regulation of a transcription factor network that includes Pax6 activation via the PI3K/Akt/mTORC1 signaling pathway (Bromberg et al. 2008) and in neurons, CB1 receptor activates mTORC1 via PI3K/Akt (Puiuhermanal et al. 2009). In cerebellar progenitors, CB1 receptor-induced cell proliferation is mediated by the PI3K/Akt pathway that, as a consequence of GSK3β inhibition, increases β-catenin nuclear translocation and cyclin D1 expression (Trazzi et al. 2010). Likewise, the CB2 receptor, normally absent from mature neuronal cells but expressed in undifferentiated neural progenitors, promotes cell proliferation via PI3K/Akt/mTORC1 signaling (Palaizuels et al. 2012). In addition to the involvement of PI3K/Akt signaling in CB1-mediated mTORC1 activation, a potential contribution of other pathways, such as the ERK cascade, is plausible.

mTORC1 signaling regulates neural cell fate, and a fine tuning of this signaling pathway is essential for appropriate cortical development (Crino 2011; Han and Sahin 2011). In the developing cortex, ablation of Raptor, one of the mTORC1 components, results in reduced cortical cell number and size, as well as microcephaly, a phenotype that, at least in part, is due to aberrant progenitor cell proliferation, cell cycle...
alterations in the VZ/SVZ, and interference with apical Sox2+ and basal Trb2+ progenitor cell populations (Cloetta et al. 2013). Our findings indicate that, in the absence of CB1 signaling, mTORC1-driven Pax6 activity is impaired, and this may shift the mechanism of cell division toward neurogenesis (at the expense of the generation of intermediate progenitor cells), and leading to premature progenitor cell exhaustion. Noteworthy, mTORC1 activation in intermediate progenitor cells in adult neurogenic brain areas reverts progenitor quiescence in the aged brain (Paliouras et al. 2012). Thus, mTORC1 activation by CB1 and likely also CB2 cannabinoid receptors may contribute to injury-induced neural progenitor priming (Aguado et al. 2007; Palazuelos et al. 2012) and alleviate aging-associated decline of neurogenesis (Goncalves et al. 2008; Marchalant et al. 2009).

Alterations of intrinsic fate determinants that control neural progenitor identity, by either gain or loss of function, interfere with proper cortical development and ultimately have important consequences in neuronal excitability, cognition, and mood-anxiety disorders (Ramocki and Zoghbi 2008). Interference with Pax6 and Tbr2 expression exerts severe consequences on neurogenesis that, in turn, produce behavioral alterations (Sisodiya et al. 2001; Baala et al. 2007; Tuoc et al. 2009; Saito et al. 2011). Conditional cortical Pax6 knockout mice display strong behavioral deficits, including cognitive function and sensorimotor integration (Tuoc et al. 2009). Likewise, Tbr2-deficient mice show enhanced aggressiveness and hyperactivity (Baala et al. 2007; Arnold et al. 2008). Thus, deregulated activity of cell fate determinants can contribute to different neurodevelopmental disorders.

The instructive role of CB1 receptor signaling in regulating the P13K/Akt/mTORC1 pathway, and its impact on the Pax6–Tbr2 transcription factor code depicted here, point to a potential contribution of the endocannabinoid system to some of these neurodevelopmental disorders. Mutations of different upstream signaling elements of the mTORC1 pathway (e.g., tuberous sclerosis complex proteins Tsc1/Tsc2, Rheb1 and others) lead to the mTORC1 overactivation characteristic of tuberous sclerosis complex and focal cortical dysplasia patients (Crino 2011). While focal mTORC1 hyperactivation in the neocortex frequently results in intractable epilepsy, in the cerebellum, it may contribute to certain autism characteristics (Tsai et al. 2012).

CB1 and CB2 cannabinoid receptors have been shown to be expressed in developmental focal cortical alterations. In particular, increased CB1 receptor expression was observed in type II focal cortical dysplasia lesions (Zurolo et al. 2010). CB1 signaling may thus contribute to mTORC1 pathway overactivation and may interfere with the appropriate expression of progenitor identity determinants that is essential for progenitor expansion and coordination of cell cycle exit with radial migration (Kim et al. 2010; Orlova et al. 2010; Magri et al. 2011). Future studies are required to elucidate the possible contribution of CB1 receptor signaling to the appearance of developmental pathologies characterized by an overactive mTORC1 signaling. In any case, understanding the role of the CB1 receptor in cortical neurogenesis can contribute to increase our understanding of the behavioral consequences evoked by cannabinoid exposure of the developing brain on the predisposition to epilepsy and psychiatric disorders (Jutras-Aswad et al. 2009).

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