The Expression of Tubb2b Undergoes a Developmental Transition in Murine Cortical Neurons

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ABSTRACT
The development of the mammalian brain requires the generation, migration, and differentiation of neurons, cellular processes that are dependent on a dynamic microtubule cytoskeleton. Mutations in tubulin genes, which encode for the structural subunits of microtubules, cause detrimental neurological disorders known as the tubulinopathies. The disease spectra associated with different tubulin genes are overlapping but distinct, an observation believed to reflect functional specialization of this multigene family. Perturbation of the β-tubulin TUBB2B is known to cause polymicrogyria, pachygyria, microcephaly, and axon guidance defects. Here we provide a detailed analysis of the expression pattern of its murine homolog Tubb2b. The generation and characterization of BAC-transgenic eGFP reporter mouse lines has revealed that it is highly expressed in progenitors and postmitotic neurons during cortical development. This contrasts with the 8-week-old cortex, in which Tubb2b expression is restricted to macroglia, and expression is almost completely absent in mature neurons. This developmental transition in neurons is mirrored in the adult hippocampus and the cerebellum but is not a universal feature of Tubb2b; its expression persists in a population of postmitotic neurons in the 8-week-old retina. We propose that the dynamic spatial and temporal expression of Tubb2b reflects specific functional requirements of the microtubule cytoskeleton. J. Comp. Neurol. 523:2161–2186, 2015.

INDEXING TERMS: microtubules; tubulinopathies; GFP-reporter line; developing cortex; neuronal migration; glia;
AB_2068336; AB_2088494; AB_2314535; AB_303395; AB_442102; AB_10048713; AB_570666; AB_310177; AB_2170714; AB_2286684; AB_778267; AB_10615604

The development of the mammalian brain requires a dynamic microtubule cytoskeleton (Ayala et al., 2007). Microtubules establish the mitotic spindle, separate the sister chromatids during mitosis, promote movement of progenitors and postmitotic neurons, and are critical for the outgrowth of neurites (Dent and Gertler, 2003; Kuijpers and Hoogenraad, 2011; Tsai and Gleeson, 2005). It has been demonstrated that microtubule dysfunction can cause detrimental neurological disease in humans (Gleeson and Walsh, 2000; Keays, 2007). The structural subunits of microtubules themselves, the tubulins, have been implicated in a class of neurodevelopmental disorders now categorized as the tubulinopathies (Bahi-Buisson et al., 2014; Breuss and Keays, 2014; Jaglin et al., 2012; Tischfield et al., 2011). The tubulins are a large multigene family, consisting of eight α- and nine β-isoforms in humans that show high

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sequence conservation, partially because of a stereotypic folding pathway that is highly sensitive to amino acid changes (Cleveland et al., 1978, 1980; Cowan et al., 1981; Lewis et al., 1997; Tian et al., 1997). The ongoing debate on whether these isoforms have distinct functions in vivo was fomented by the findings that mutations in the different tubulins result in overlapping, but separate, clinical symptoms (Bahi-Buisson et al., 2014; Breuss and Keays, 2014; Cleveland, 1987; Cushion et al., 2013; Fulton and Simpson, 1976; McKeain et al., 2001).

The idea that different isoforms are functionally distinct is known as the multitubulin hypothesis (Fulton and Simpson, 1976). It is supported by a number of genetic replacement studies in invertebrates, in vitro studies that have revealed that different isoforms affect the processing of microtubule-associated proteins and by rescue experiments following in utero depletion of Tubb3 (Fukushige et al., 1999; Hoyle and Raff, 1990; Hutchens et al., 1997; Saillour et al., 2014; Savage et al., 1989, 1994; Sirajuddin et al., 2014). The origin of some of these functional differences appears to lie within the acidic C-terminal region, which is highly variable between isoforms. In addition, the various isoforms show distinct spatiotemporal expression patterns (Breuss et al., 2012; Burgoyne et al., 1988; Lewis et al., 1985; Villasante et al., 1986; Wang et al., 1986). These patterns appear to correlate with the disease phenotypes observed in patients with tubulin gene mutations. For instance, the postmitotically expressed neuronal isoform TUBB3 is associated with polymicrogyria and axon guidance disorders but not with microcephaly (Bahi-Buisson et al., 2014; Poirier et al., 2010; Tischfield et al., 2010). This contrasts with mutations in TUBB5, an isoform that is expressed in progenitor cells throughout neurogenesis, which is associated with microcephaly and mild cortical malformations (Breuss et al., 2012; Ngo et al., 2014). Along similar lines, mutations in TUBB1, which is expressed specifically in the hematopoietic system, is responsible for causing a rare blood disorder known as congenital macrothrombocytopenia in the absence of neurological phenotypes (Kunishima et al., 2009).

The study of tubulin expression patterns initially relied on northern blot analysis (Lewis et al., 1985; Villasante et al., 1986; Wang et al., 1986) and more recently quantitative RT-PCR (Braun et al., 2010; Breuss et al., 2012). Although these studies have provided valuable insight into the global expression levels of the tubulin genes in any given tissue, they do not provide information about the specific cell types that express a particular isoform. Immunohistochemical methods would provide such insight; however, the high degree of sequence homology between the different isotypes (e.g., TUBB2A, TUBB2B, and TUBB2C) has frustrated attempts to raise isoform-specific antibodies. One way to circumvent this limitation is to employ a transgenic approach in which a reporter is driven by an endogenous tubulin promoter. The Miller group used this to great effect, driving a LacZ transgene under control of the 1.1-kb promoter upstream of Tuba1a. This revealed that Tuba1a expression is coincident with the neuronal lineage and is absent from glia (Bamji and Miller, 1996; Gloster et al., 1994, 1999). Likewise, fluorescent reporter lines that have been generated for Tubb3 and Tubb5 have advanced our understanding of the pathogenic mechanisms underlying tubulin-related disease states and have served as a useful tool for advanced imaging applications (Attardo et al., 2008; Breuss et al., 2012; Liu et al., 2007).

Here we report the generation and characterization of a novel reporter mouse expressing eGFP under control of the Tubb2b regulatory machinery. We focused on this gene because mutations in the human homolog have been shown to cause a wide spectrum of neurological diseases, including lissencephaly/pachygyria, polymicrogyria, axon guidance disorders, and copy number variations of its locus that are associated with autism (Breuss and Keays, 2014; Cederquist et al., 2012; Cushion et al., 2013; Jaglin et al., 2009; Pinto et al., 2010; Romaniello et al., 2012). Our experiments reveal that Tubb2b is expressed in Sox2- and Tbr2-positive neuronal progenitors and, at higher levels, in Dcx-positive postmitotic neurons in the developing mouse neocortex. This expression, unlike that of Tubb3 and Tubb5, is almost completely lost in mature NeuN-positive neurons in the cortex and cerebellum.

**MATERIALS AND METHODS**

**Animals**

Animals were maintained under specific-pathogen-free (SPF) conditions within the animal research laboratories of the Institute of Molecular Pathology on a 14:10-hour light:dark cycle. Food and water were provided ad libitum. The Tg(Tubb2b-eGFP)GlbDAK and Tg(Tubb2b-eGFP)GlcDAK mouse lines were outcrossed to C57BL6/J wild-type animals. The date of vaginal plug detection was considered E0.5. All procedures were carried out according to the existing legal requirements and licenses (M58/006093/2011/13).

**Generation of the Tg(Tubb2b-eGFP)GlbDAK and Tg(Tubb2b-eGFP)GlcDAK mouse lines**

Generation of the mouse lines was performed as described previously (Breuss et al., 2012). In short, the Red/ET system (K001; Gene Bridges) was used to replace the endogenous Tubb2b locus on a bacterial
artificial chromosome (RP23-237E7) with an eGFP-FRT-NeoR-FRT cassette. After excision of the neomycin resistance cassette, with a 707-FLP(2) plasmid (A104; Gene Bridges), pronuclear injection was performed and PCR screening for genomic incorporation was carried out (forward primer CCGGTGGTGACAGATGAATCC, reverse primer CACAGTGGCACGCCAGTGC).

**Real-time quantitative PCR**

Fetal brain (E16.5) and 8-week-old organs were harvested from C57/BL6 animals, dissected, and snap frozen in liquid nitrogen. RNA was isolated with the RNeasy lipid tissue kit (Qiagen, Valencia, CA; catalog No: 74804) and the RNeasy fibrous tissue kit (Qiagen; catalog No: 74704) according to the manufacturer’s handbook. Reverse transcription employed the First Strand Synthesis III kit (Invitrogen, Carlsbad, CA; catalog No: 18080-400). For quantitative RT-PCR analysis of the Tubb2b expression, intron-spanning primers were designed with the Primer3 program and specificity was confirmed by BLAST searches, analysis of melting curves, and gel electrophoresis (Tubb2b forward primer TCATCAGCCACTGACACAG, reverse primer TTCCCAAGATTACCTGTC). After efficiency testing, qPCR was performed with SYBR green on a Bio-Rad cycler, with 0.9% NaCl followed by 4% PFA. Brains of 8-week-old brains and embryos were dissected after perfusion with 0.9% NaCl followed by 4% PFA. Brains were postfixed for 6 hours in 4% PFA, dehydrated in 30% sucrose overnight, and sectioned (40 μm). P21 and 8-week-old stainings were performed on floating sections without antigen retrieval (Table 1). For stainings on embryonic and postnatal tissues (until P10), antigen retrieval with antigen unmasking solution (Vector Laboratories, Burlington, CA; H-3301) was performed by heating slides to 90°C, followed by cooling at room temperature for 30 minutes, before washing with 1x phosphate-buffered saline (PBS) and incubation with the primary antibody at 4°C overnight (14-16 hours; Table 1). On the next day, the sections were washed and incubated with a species-specific secondary antibody for 1 hour at 4°C. Sections were subsequently stained for 5 minutes with Hoescht 33342 fluorescent stain in 1x PBS (1:2,000) before mounting in Dako Fluorescent Mounting Medium (Dako; Art. No. S302380). Microscopy was performed on a Zeiss LSM 710, and low-magnification images of the tissues were captured with a slide scanner (Zeiss).

**Quantifications**

Confocal images were captured with the Zeiss LSM 710. Depending on the abundance of the marker (e.g., Sox2, Dcx, Ki67), one to four images per animal were analyzed (n = 3 animals for all quantifications). For the embryonic cortex, spinal cord, and retina, an area with a width of 100 μm from the apical to the basal surface was drawn prior to quantification, and only cells within this area were counted. For the P0 and P7 cerebellum, an area 250 μm × 250 μm was employed. The number of marker-positive cells and the number colocalizing with eGFP was determined for each animal and the percentage of colocalization averaged. To measure eGFP intensity in the granule cell layer of the dentate gyrus, representative sections of three animals were chosen. Six equally sized boxed regions (1–6) extending from the subgranular zone to the outer granule cell layer were drawn and measured in Imagej (average intensity). For each dorsal blade of the dentate gyrus, two segmented regions were analyzed and averaged before normalization to the intensity of area 1. To quantify the intensity of eGFP in the embryonic cortex, slides were scanned with a Pannoramic Flash 250 II slide scanner with the same settings for all animals at E12.5, E14.5, and E16.5 (exposure time of 5 msec). Six equally sized boxed regions (1–6) with a width of 100 μm extending from the ventricular zone to the molecular cell layer were drawn and measured in Imagej (average intensity). Two regions were analyzed per animal, and the average intensity for each of the six segmented regions was calculated. The data were plotted in GraphPad Prism 6.
Cochlear whole mount

After perfusion with 4% PFA, the petrous portion of the temporal bone was isolated and the cochlea dissected. The organ of Corti from the medial part of the cochlear spiral was stained for 5 minutes in Hoechst 33342 Fluorescent Stain in 1/3 PBS (1:2,000) and mounted on a slide with glycerol. A z-stack of the whole mount was captured with a Zeiss LSM 710 microscope. From this, a z-projection (maximum intensity) was generated in ImageJ and exported to Adobe Illustrator, with adjustments for contrast and brightness.

Permanent retina peroxidase immunostaining and imaging

For the permanent staining, eyes were harvested, postfixed, dehydrated, and embedded in paraffin. Tissue was then sectioned on a microtome (5 μm). Sections were dried at 55°C overnight and stored at RT. Antigen retrieval was performed with Dako Target Retrieval Solution (Dako; S1699) by heating the sections to 90°C with 30 minutes of incubation at RT. Sections were incubated with the primary GFP antibody (1:500) in 0.3% Triton X-100 and 4% milk in 1× PBS at RT. On the next day the sections were washed and incubated for 1 hour with anti-rabbit Ig ImmPress reagent peroxidase (catalog No. MP-7401) at RT. Subsequently, the sections were washed and incubated with the peroxidase substrate solution (ImmPACT DAB; catalog No. SK-4105) until the desired stain intensity developed. Sections were washed and counterstained with nuclear fast red. Images were captured with an Axioplan 2 (Zeiss) and exported, with adjustments for contrast and brightness in ImageJ or Panoramic Viewer.

Clearing and whole mount of Tg(Tubb2b-eGFP)GlbDAK and Tg(Tubb2b-eGFP)GlcDAK embryos

Tg(Tubb2b-eGFP)GlbDAK and Tg(Tubb2b-eGFP)GlcDAK embryos were harvested at E14.5, postfixed in 4% PFA overnight, and washed twice in 1× PBS for 20 minutes on a shaker. For nuclear staining, embryos were placed in 1:5,000 Hoechst in 1× PBS for 20 minutes on a shaker. Subsequently, the embryos were washed in 1× PBS and transferred to Scale A2 solution for clearing (4 M urea, 10% glycerol, 0.1% Triton X-100, pH 7.7; Hama et al., 2011). Embryos were left for 2 weeks at 4°C on a shaker in Scale A2

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**TABLE 1. Primary Antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Supplier and species</th>
<th>Dilution</th>
<th>Triton/PBS (%)</th>
<th>Vector AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin</td>
<td>Recombinant mouse calbindin</td>
<td>Millipore (AB1778), rabbit, polyclonal, RRID: AB_2068336</td>
<td>1:500</td>
<td>0.2</td>
<td>Yes/no</td>
</tr>
<tr>
<td>Dcx</td>
<td>Peptide mapping at the C-terminus of doublecortin of human origin</td>
<td>Santa Cruz Biotechnology (sc-8066), goat, polyclonal, RRID: AB_2088494</td>
<td>1:200</td>
<td>0.3</td>
<td>Yes/no</td>
</tr>
<tr>
<td>GFAP</td>
<td>GFAP isolated from cow spinal cord</td>
<td>Dako (Z0334), rabbit, polyclonal, RRID: AB_2314535</td>
<td>1:500</td>
<td>0.3</td>
<td>Yes/no</td>
</tr>
<tr>
<td>GFP</td>
<td>Highly purified recombinant full length protein made in E. coli; the antibody is directed against the entire GFP molecule</td>
<td>Abcam (ab290-50), rabbit, polyclonal, RRID: AB_303395</td>
<td>1:500</td>
<td>0.3</td>
<td>Yes/no</td>
</tr>
<tr>
<td>Ki67</td>
<td>Prokaryotic recombinant fusion protein corresponding to a 1,086-bp Ki67 motif</td>
<td>Leica Biosystems (NCL-Ki67p), rabbit, polyclonal, RRID: AB_442102</td>
<td>1:200</td>
<td>0.3</td>
<td>No</td>
</tr>
<tr>
<td>NeuN</td>
<td>Purified cell nuclei from mouse brain</td>
<td>Millipore (MAB377), mouse, monoclonal, RRID: AB_10048713</td>
<td>1:400</td>
<td>0.3</td>
<td>Yes/no</td>
</tr>
<tr>
<td>Olig2</td>
<td>Recombinant mouse Olig2</td>
<td>Millipore (AB9610), mouse, polyclonal, RRID: AB_570666</td>
<td>1:1,000</td>
<td>0.3</td>
<td>Yes/no</td>
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<tr>
<td>pH3</td>
<td>Linear peptide corresponding to human histone H3, phosphorylated at Ser10</td>
<td>Millipore (06-570), rabbit, polyclonal, RRID: AB_310177</td>
<td>1:500</td>
<td>0.3</td>
<td>Yes</td>
</tr>
<tr>
<td>Prox1</td>
<td>Recombinant human Prox1 protein</td>
<td>Millipore (MAB5654), mouse, monoclonal, RRID: AB_2170714</td>
<td>1:400</td>
<td>0.3</td>
<td>No</td>
</tr>
<tr>
<td>Sox2</td>
<td>Peptide mapping near the C-terminus of Sox-2 of human origin</td>
<td>Santa Cruz Biotechnology (sc-17320), goat, polyclonal, RRID: AB_2286684</td>
<td>1:200</td>
<td>0.3</td>
<td>Yes/no</td>
</tr>
<tr>
<td>Tbr2(rb)</td>
<td>Synthetic peptide conjugated to KLH derived from within residues 650 to the C-terminus of mouse Tbr2/Eomes</td>
<td>Abcam (ab23345), rabbit, polyclonal, RRID: AB_778267</td>
<td>1:100</td>
<td>0.3</td>
<td>Yes</td>
</tr>
<tr>
<td>Tbr2(ch)</td>
<td>KLH-conjugated linear peptide corresponding to mouse Tbr2</td>
<td>Millipore (AB15894), chicken, polyclonal, RRID: AB_10615604</td>
<td>1:300</td>
<td>0.3</td>
<td>Yes</td>
</tr>
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solution for clearing, and images were captured with a fluorescence stereomicroscope (Zeiss Lumar) and a Zeiss LSM 780 confocal microscope. 3D rendering employed Imaris software.

**Light sheet microscopy**

Samples were imaged with a custom-made light sheet fluorescence microscope. In the setup employed, the imaging objective (Olympus, ×25, N.A. 0.95, W.D. 8 mm, ScaleView immersion) was mounted on an inverted microscope (Olympus iX73), and the excitation objective (Mitutoyo Plan Apo, ×20, N.A. 0.42, W.D. 20 mm, air) was mounted horizontally on a custom-built platform on one side of the inverted microscope. Images were recorded with a scientific CMOS camera (Hamamatsu Orca-Flash4.0) at full frame (2,048 × 2,048 pixels) with a standard eGFP filter set (488 nm ET Laser Filter set; AHF). The measured effective pixel size on the sample was 253 nm, which resulted in a field of view of 518 × 518 μm. The excitation light sheet was generated with a galvanometer scanner to translate the focused attenuated laser beam rapidly (Cobolt Calypso TM 200 491 nm DPSS laser) in the imaging plane at 1 kHz (Keller et al., 2008). Once aligned, both objectives were kept static throughout imaging, and image stacks were acquired by translating the sample vertically through the imaging plane with a long-travel-range piezo stage. All images were deconvolved with a theoretical point spread function (PSF) of the microscope. The PSF was generated with a Born and Wolf 3D optical model with the ImageJ plugin PSF Generator (Kirshner et al., 2013). The deconvolution was done with a Richardson-Lucy algorithm with the plugin DeconvolutionLab (Vonesch and Unser, 2008).
<table>
<thead>
<tr>
<th>E14.5</th>
<th>Tg(Tubb2b-eGFP)GlbDAK</th>
<th>GlicDAK</th>
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<tr>
<td><strong>Control</strong></td>
<td></td>
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<tr>
<td>A</td>
<td>B</td>
<td>B1</td>
</tr>
<tr>
<td>Hoechst</td>
<td>Hoechst</td>
<td>GFP</td>
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<td>GFP</td>
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| Tg(Tubb2b-eGFP)GlbDAK | |
|---|---|---|---|---|
| D | D1 | F | G |
| Hoechst | GFP | GFP | GFP |
| GFP | | | |

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<td>Hoechst</td>
<td>GFP</td>
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<td>GFP</td>
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**Figure 3.**
Every image ran through 10 iterations, and the background was subsequently subtracted. Data sets were stitched together when the region of interest was larger than the field of view of a single image. Stitching of the deconvolved images was done with the program XuvTools (Emmenlauer et al., 2009).

**Antibody characterization**

A list of all antibodies and the conditions used for immunostainings in this study can be found in Table 1. Calbindin (D28K) is a polyclonal antibody raised in rabbit against recombinant mouse calbindin (Millipore, Bedford, MA; AB1778, RRID: AB_2068336). This antibody was previously characterized as labeling a variety of inhibitory neurons, including Purkinje cells in the cerebellum (Nobrega et al., 2013; Swanson and Goldowitz, 2011). When we applied this antibody to P0 brains, we observed the expected pattern of positive cells in the inner granular layer and the labeling of Purkinje cells in 8-week-old animals. Dcx (C-18) is a polyclonal antibody raised in goat that binds to a peptide mapping at the C-terminus of human doublecortin (Santa Cruz Biotechnology, Santa Cruz, CA; sc-8066, RRID: AB_2088494). This antibody that was previously characterized as binding to migrating, postmitotic neurons has been used recently in several studies (Boekhoorn et al., 2008; Vernerey et al., 2013). When we applied this antibody, it yielded the expected cytoplasmic pattern reminiscent of migrating neurons, being widely expressed at later stages and only sparsely in earlier stages of development (data not shown). GFAP is a polyclonal antibody raised in rabbit that binds specifically against glial fibrillary acidic protein isolated from bovine spinal cord (Dako; Z0334, RRID: AB_2314535). Staining was shown to be specific in primary mouse astroglial cell cultures as well as in brain sections of adult rats (Castellano et al., 1991). With our immunostainings, we were able to detect the characteristic staining pattern for GFAP. The signal marked processes near the ventricle, in the hippocampus, throughout the cortex, and near the pial membrane. GFP is a polyclonal antibody raised in rabbit against the entire GFP molecule (Abcam, Cambridge, MA; ab290-50, RRID: AB_303395). It was shown that it binds specifically against eGFP in mouse dorsal root ganglion neurons that were transfected with an adeno-associated viral vector harboring eGFP among other genes of interest (Fagoe et al., 2014). With our immunostaining, we were able to detect signal in those cells that were eGFP positive in cryosections of the same tissue. Ki67 is a polyclonal antibody raised in rabbit against a prokaryotic recombinant fusion protein corresponding to a 1,086-bp Ki67 motif (Leica Biosystems; NCL-Ki67p, RRID: AB_442102). This antibody was previously shown to bind to the Ki67 antigen in the nucleus of proliferating cells in the adult hippocampus (Yutsudo et al., 2013), and we observed a similar staining pattern in the same region. NeuN is a monoclonal antibody raised in mouse against purified cell nuclei from mouse brain (Millipore; MAB377, RRID: AB_10048713). It was characterized previously to recognize specifically the DNA-binding, neuron-specific protein Fox-3 and was observed in most neuronal cell types throughout the nervous system of adult mice (Kim et al., 2009; Mullen et al., 1992). We observed the expected nuclear staining pattern for NeuN on brain sections of adult mouse brains, throughout all the layers of the cerebral cortex (layers i–vi) and in the hippocampus in the neuron-rich pyramidal cell layers. Olig2 is a polyclonal antibody raised in rabbit against recombinant mouse Olig2 (Millipore; AB9610, RRID: AB_570666). Olig2 staining can be detected as relatively sparse signal in the cortex, localizing to the nuclei of cells (Heimer-McGinn and Young, 2011). We observed sparse staining of Olig-2 throughout the cortex, which did not colocalize with neurons. Olig2 signal was strong in white matter tracts, for example, in the corpus callosum and anterior commissure, as would be expected for oligodendrocytes in the mammalian brain. pH3 is a polyclonal antibody raised in rabbit against a linear peptide of human histone H3, phosphorylated at the position Ser10 (Millipore; 06-570, RRID: AB_310177). This antibody was shown to bind to proliferating cells in late G2 and M phases, where the phosphorylation of the serine 10 residue takes place (Hendzel et al., 1997; Yamada et al.,

Figure 3. Tubb2b is expressed in the central and peripheral nervous systems in E14.5 mouse embryos. A–C: Representative images of Scale-cleared whole-mount embryos of the Tg[Tubb2b-eGFP]GlcDAK mouse line (B), the Tg[Tubb2b-eGFP]GlcDAK mouse line (GlcDAK, C), and a control (A) at E14.5. GFP signal is clearly visible throughout the central nervous system, brain, and spinal cord. The individual GFP channel for B is shown in B1. D,E: High-magnification images of the rostral head area (D) and the hind limb (E). The individual GFP channels for D and E are shown in D1 and E1, respectively. GFP signal is present in nerve fiber-like tracts associated with the olfactory epithelium, developing vibrissa, and digits of the hind limb. F,G: 3D reconstructions of eGFP-positive structures in the area of the developing vibrissa (F) and the hind limb (G). H: 3D reconstruction of a SPIM image stack from the surface of the brain. Note that individual cells are visible. I: High-resolution 3D reconstruction of a SPIM image stack of the rostral area of the head containing the developing vibrissa. Hoechst nuclear counterstaining is shown in gray and GFP signal in green in A–E; n = 3 animals for B–E. Scale bars = 500 μm in A (applies to A–C); 250 μm in E1 (applies to D,E); 200 μm in G (applies to F,G); 20 μm in H; 100 μm in I.
Figure 4.
2014). We observed a similar pattern of pH3-positive cells in E14.5 sections in the proliferating ventricular and subventricular zones of the brain. We could further correlate pH3 staining with the occurrence of mitosis based on chromosome shapes judged by Hoechst staining (data not shown). Prox1 is a mouse monoclonal antibody against recombinant human prospero homeobox 1 (Prox1) protein (Millipore; MAB5654; RRID: AB_2170714). On western blot analysis, the antibody recognizes an ~83-kDa protein in fetal mouse brain lysate. It was shown previously to label mature granule cells in the hippocampus of adult mice (Keays et al., 2010). We observed a similar Prox1 labeling in the granular cell layer of the dentate gyrus on our coronal brain sections of adult mice. Sox2 is a goat polyclonal antibody against a peptide mapping near the C-terminus of Sox-2 of human origin (Santa Cruz Biotechnology; sc-17320, RRID: AB_2286684). It was shown previously to recognize cell nuclei in the germinal zone of the adult mouse brain (Mathews et al., 2010) and was used for staining in the adult hippocampus (Navarro-Quiroga et al., 2006). Additionally, the antibody was shown to recognize specifically cells that express a Sox2-GFP reporter following lentiviral infection (Suh et al., 2007). We observed strong Sox2 staining in the ventricular zone at E14.5, where radial glial cells can be found. We previously correlated this staining also with Pax6, another radial glial marker (data not shown). Tbr2(rb) is a rabbit polyclonal antibody against a synthetic peptide conjugated to KLH derived from within residues 650 to the C-terminus of mouse Tbr2/Eomes (Abcam; ab23345, RRID: AB_778267). This Tbr2 antibody was used previously to detect basal progenitors, or intermediate progenitors, that are found in the subventricular zone of the developing brain (Pulvers and Huttner, 2009). In our staining Tbr2 localized reliably to the subventricular zone, where it marks this type of progenitors. Tbr2(ch) is a chicken polyclonal antibody against a KLH-conjugated linear peptide corresponding to mouse Tbr2 (Millipore; AB15894, RRID: AB_10615604). This Tbr2 antibody was used previously to confirm the validity of a Tbr2-Cre driver line (Vasistha et al., 2014). In our staining, Tbr2 localized reliably to the subventricular zone, where it marks intermediate progenitors. We used this antibody in our E16.5 immunostainings.

RESULTS

Tubb2b expression analysis by quantitative RT-PCR

We began by assessing the expression of Tubb2b (RefSeq accession NM_023716) by quantitative RT-PCR. Consistent with previous studies, its expression was high in the developing brain at E16.5 and markedly reduced in the 8-week-old brain (Fig. 1A; Breuss et al., 2012; Jaglin et al., 2009). We extended this analysis to all major organs in the mouse (Fig. 1B). This revealed moderate levels of Tubb2b in the cochlea, eye, lung, and thymus. The remaining tissues analyzed (e.g., heart, liver, testes, pancreas) showed either lower levels of expression or absence of any expression.

Generation of a fluorescent reporter line for Tubb2b

To investigate the expression pattern of Tubb2b further, and specifically to explore in which cell types it is expressed, we generated a transgenic GFP reporter mouse line (Fig. 2). We chose to recombiner a bacterial artificial chromosome (BAC) that contained the Tubb2b gene and approximately 150 kb of upstream and 40 kb of downstream sequence, replacing the coding sequence with enhanced GFP (eGFP) and a neomycin resistance cassette. After excision of the resistance cassette by Flp-recombinase, engineered BACs were injected into pronuclei of mice. Animals positive for the transgene were then screened for GFP expression and the presence of the transgene. This approach resulted in the recovery of two transgenic mouse lines, \( Tg(Tubb2b-eGFP)GlbDAK \) and \( Tg(Tubb2b-eGFP)GlcDAK \).
**Developmental expression of Tubb2b**

To obtain a first insight into the expression of Tubb2b, we performed tissue clearing on Tg(Tubb2b-eGFP)GlbDAK and Tg(Tubb2b-eGFP)GlcDAK embryos at E14.5 and assessed the overall expression of eGFP in whole mounts (Fig. 3A–C; Hama et al., 2011). Expression of the reporter was strongest in the developing central nervous system, brain, and spinal cord. In addition, we observed GFP expression in peripheral nervous structures such as the developing vibrissa and in the limbs (Fig. 3D–G). Selective plane illumination microscopy (SPIM), a technique that allows the acquisition of large three-dimensional images, permitted the visualization of individual GFP-positive cells in the superficial dorsolateral cortex (Fig. 3H) and resolved individual nerve fibers associated with the vibrissa (Fig. 3I). We focused our attention on the developing cortex and analyzed Tg(Tubb2b-eGFP)GlbDAK and Tg(Tubb2b-eGFP)GlcDAK mice at E12.5, E14.5, and E16.5 (Fig. 4). At E12.5, widespread GFP signal could be observed, particularly in the dorsal tip of the epithalamus (Fig. 4B–D). Expression was also detected in the subpallial...
TABLE 2.
Embryonic Quantifications

<table>
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<tr>
<th>Age</th>
<th>Marker</th>
<th>Sox2</th>
<th>Tbr2</th>
<th>Dcx</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(E12.5)</td>
<td>(E14.5)</td>
<td>(E16.5)</td>
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<tr>
<td></td>
<td>n = 3</td>
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<td>525 cells</td>
<td>722 cells</td>
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<tr>
<td>E12.5</td>
<td></td>
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<td>100% ± 0.00%</td>
<td>99.89% ± 0.11%</td>
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<tr>
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<td>469 cells</td>
<td>266 cells</td>
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<tr>
<td></td>
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<td>99.13% ± 0.48%</td>
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<td>99.58% ± 0.42%</td>
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<tr>
<td></td>
<td></td>
<td>98.99% ± 0.69%</td>
<td>98.48% ± 1.52%</td>
<td>100% ± 0.00%</td>
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<td>n = 3</td>
<td>71 cells</td>
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Figure 6. The Tubb2b eGFP reporter colocalizes with cycling progenitors and postmitotic neurons at E12.5 and E16.5. A–T: Representative coronal sections of the cortex of the Tg(Tubb2b-eGFP)GlbDAK mouse line (A–H,K–R) and littermate controls (I,J,S,T) at E12.5 (A–J) and E16.5 (K–T). Immunostainings were performed for Sox2 (A,B,K,L), Tbr2 (C,D,M,N), Dcx (E,F,O,P), and pH3 (G,H,Q,R). High-magnification images of the boxed regions in A, C, E, G, K, M, O, and Q are shown in B, D, F, H, L, N, P, and R, respectively. Gray-scale images of the individual channels are shown in B1, B2, D1, D2, F1, F2, H1, H2, L1, L2, N1, N2, P1, P2, R1, and R2. eGFP-positive cells were found to colocalize with all markers; n = 3 animals for A–H,K–R. Scale bars = 20 μm in A (applies to A,C,E,G,J); 10 μm in B (applies to B,D,F,H,J); 20 μm in K (applies to K,M,O,Q,S); 10 μm in L (applies to L,N,P,R,T).
regions, including the ganglionic eminences, consistent with the basal ganglia phenotypes described in an ENU-induced Tubb2b mouse mutant (Fig. 4P,Q; Stottmann et al., 2013). At E14.5 and E16.5, the GFP signal was detected in the ventricular and subventricular zones as well as the intermediate zone and the cortical plate (Fig. 4G–J,L–O). The expression was notably higher in postmitotic regions dominated by migrating and

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Tg(Tubb2b-eGFP)GlbDAK</th>
<th></th>
<th>Control</th>
<th>Tg(Tubb2b-eGFP)GlbDAK</th>
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<tbody>
<tr>
<td>P0</td>
<td></td>
<td></td>
<td>P7</td>
<td></td>
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Figure 7. The Tubb2b eGFP reporter colocalizes with mitotically active progenitors and postmitotic cells in the developing cerebellum. A–N: Representative coronal sections of the cerebellum of the Tg(Tubb2b-eGFP)GlbDAK mouse line (B–G,I–N) and a littermate control (A,H) at P0 (A–G) and P7 (H–N). A,B,H,I: Hoechst-stained scans of the cerebellum for the two mouse lines. C,J: Magnification of the external and internal granular layers (EGL and IGL, respectively) for P0 and the external and internal granular layers, the molecular layer (ML), and the Purkinje cell layer (PCL) for P7. Note the increased expression of the fluorescent reporter in the IGL compared with the EGL at P0 and P7. Gray-scale images of the GFP channels are shown in C1 and J1. D–G,K–N: Immunostainings were performed for pH3 (D,K), NeuN (E,L), calbindin (F,M), and Sox2 (G,N). Gray-scale images of the individual channels are shown in D1, D2, E1, E2, F1, F2, G1, G2, K1, K2, L1, L2, M1, M2, N1, and N2. eGFP-positive cell were found to colocalize with all markers, although expression levels were markedly reduced in pH3-positive cycling progenitors in the EGL; n = 3 for B–G,I–N. Scale bars = 100 μm in A (applies to A,B); 20 μm in C, 20 μm in D (applies to D–G); 100 μm in H (applies to H,I); 20 μm in J; 20 μm in K (applies to K–N).
Figure 8. The Tubb2b eGFP reporter is highly expressed in the developing spinal cord and dorsal root ganglia. A–I: Representative coronal sections of the spinal cord of the Tg(Tubb2b-eGFP)GlbDAK mouse line. A–C: Hoechst-stained scans of the spinal cord at E12.5 (A), E14.5 (B), and E16.5 (C). Gray-scale images of the GFP channels are shown in A1, B1, and C1. D–I: Representative images stained for the postmitotic marker Dcx and the progenitor marker Sox2 at E14.5. E, F, H, and I show magnifications of the boxed regions in D and G. E and H show magnifications of the spinal cord, F and I of a dorsal root ganglion. Gray-scale images of the individual channels are shown in D1, D2, E1, E2, F1, F2, H1, H2, I1, and I2. eGFP colocalizes with 99.5% of Dcx-positive cells in the spinal cord (n = 761 cells) and 95.2% of Dcx-positive cells in the dorsal root ganglion (n = 300 cells). Although 98.9% of Sox2-positive cells in the spinal cord also express eGFP (n = 253 cells), only 4.6% of Sox2-positive progenitors in the dorsal root ganglion are eGFP positive (n = 189 cells). Solid arrowheads indicate cells positive only for eGFP, open arrowheads those positive only for Sox2; n = 3 animals for A–I. Scale bars = 200 μm in A–C; 50 μm in D (applies to D,G); 20 μm in E (applies to E,F,H,I).
**Figure 9.** *Tubb2b* eGFP reporter expression in the 8-week-old mouse brain. **A:** Representative coronal section of a control (transgene negative) at 8 weeks. **B:** Representative coronal section of the *Tg(Tubb2b-eGFP)GlbDAK* mouse line. **C:** Representative coronal section of the *Tg(Tubb2b-eGFP)GlcDAK* mouse line. **D–I:** Magnifications for regions of the 8-week-old *Tg(Tubb2b-eGFP)GlbDAK* mouse brain. **D:** Scattered eGFP expression was observed in the granular layer (gl) of the olfactory bulb (OB), and signal was also present in the olfactory nerve layer (onl). **E,F:** Scattered expression was observed in the white matter tracks of the anterior commissure (AC; E) and the corpus callosum (CC; F). **G:** In the cortex (Ctx), this was seen throughout layers i, ii–iv, v, and vi and the white matter (wm). **H:** In the hippocampus (Hipp) the gl and molecular layer (ml) of the dentate gyrus and the stratum lucidum (sl) showed strong eGFP expression. **I:** In the cerebellum (Crbl), the ml exhibited broad eGFP expression, whereas the gl, the Purkinje cell layer and the arbor vitae (av) showed scattered signal. Hoechst nuclear staining (shown in blue) can be seen in A–I; n = 3 for B–I. Scale bars = 1,000 µm in A (applies to A–C); 500 µm in D,E,H,I; 200 µm in F,G.
### Figure 10.
eGFP-positive cells colocalize with GFAP- and Olig2-positive macroglia in the 8-week-old cortex.

**A–H:** Representative coronal sections of the cortex of the Tg(Tubb2b-eGFP)GlbDAK mouse line (A–F) and a littermate control (G,H) at 8 weeks. Immunostainings were performed against NeuN (A,B), GFAP (C,D), and Olig2 (E,F). B, D, F, and H show high-magnification images of the cortex. Gray-scale images of the individual channels are shown in A1, A2, B1, B2, C1, C2, D1, D2, E1, E2, F1, and F2. A,B: Only 0.1% of NeuN-positive cells colocalize with GFP (n = 5,079 cells, n = 3 animals). eGFP-positive cells are shown with a solid arrow, and NeuN-positive cells with an open arrow. C,D: eGFP-positive cells colocalized with 71.2% of GFAP-positive astroglia (dashed, open arrowheads; n = 961 cells, n = 3 animals). E,F: eGFP-positive cells colocalized with 70.3% of Olig2-positive oligodendrocytes (dashed, open arrowhead; n = 381 cells, n = 3 animals). Scale bar = 50 μm in A (applies to A,C,E,G); 20 μm in B (applies to B,D,F,H).

<table>
<thead>
<tr>
<th>Tg(Tubb2b-eGFP)GlbDAK</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>A1</td>
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<td>A2</td>
<td>B2</td>
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<tr>
<td>C</td>
<td>D</td>
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<tr>
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<td>D1</td>
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<td>E1</td>
<td>F1</td>
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<tr>
<td>E2</td>
<td>F2</td>
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**Expression Analysis of Murine Tubb2b**

The Journal of Comparative Neurology | Research in Systems Neuroscience
differentiating neurons. The Tubb2b reporter signal in the germinai zones was stronger at E14.5 and E16.5 in comparison with E12.5, suggesting an upregulation of Tubb2b coincident with the commitment to neuron-producing lineages (Fietz and Huttner, 2011; Florio and Huttner, 2014; Gotz and Huttner, 2005; Wang et al., 2011; Fig. 4R). At E14.5, strong expression could also be detected in the lateral telencephalon and the ventral subpallium and, at E16.5, in the developing hippocampus. Analysis of our second line (Tg(Tubb2b-eGFP)GlbDAK) at these time points yielded similar results, although the expression was lower compared with that of the Tg(Tubb2b-eGFP)GlcDAK mouse line, although contrasted with the dorsal root ganglia, where GFP was observed in only 4.6% of Sox2-positive progenitors (n = 189 cells, n = 3 animals) but was found in 95.2% of Dcx-positive neurons (n = 300 cells, n = 3 animals; Fig. 8D,F,G,I). These data show that Tubb2b is expressed in progenitors and postmitotic neurons in the developing cortex, cerebellum, and spinal cord. Consistent with the findings of others, this expression is at higher levels in regions dominated by migrating and differentiating neurons (Jaglin et al., 2009).

### Expression of Tubb2b in the postnatal brain

Next we examined GFP expression in the brain of 8-week-old Tg(Tubb2b-eGFP)GlbDAK mice. We observed numerous GFP-positive cells sparsely distributed throughout the brain (Fig. 9B). This was confirmed with the Tg(Tubb2b-eGFP)GlcDAK mouse line, although expression was reduced (Fig. 9C). Positive cells were enriched in the corpus callosum, anterior commissure; in regions surrounding the lateral ventricles; and within the internal capsule (Fig. 9B,E–G). GFP signal could also be observed along the larger blood vessels and close to the pial surface, areas where astrocytes contribute to the formation of the blood–brain barrier (Abbott et al., 2006). In the hippocampus, there was notable signal in the mossy fiber track, and the molecular and granule cell layers of the dentate gyrus (Fig. 9H). In the olfactory bulbs, GFP-positive cells were confined mainly to the granule layer, although not all of the cells expressed the reporter (Fig. 9D). Tubb2b expression was also detected in the molecular layer of the cerebellum, the Purkinje cell layer, the granule cell layer, and the arbor vitae (Fig. 9I). To ascertain whether these GFP-positive cells are neurons, we performed immunohistochemistry with sera against NeuN. Surprisingly, only 0.15% of NeuN-positive cells within the cortex were also GFP positive (n = 5,079 cells, n = 3 animals; Fig. 10A,B, Table 3). In contrast, staining with glial markers revealed widespread colocalization with the GFP signal (Fig. 10C–F). We observed that, within

### TABLE 3.

Postnatal Quantifications

<table>
<thead>
<tr>
<th>Age</th>
<th>NeuN</th>
<th>GFAP</th>
<th>Olig2</th>
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<tr>
<td></td>
<td>Marker</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P0</td>
<td>P10</td>
<td>P21</td>
</tr>
<tr>
<td></td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
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<td>n = 3</td>
<td>n = 3</td>
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<tr>
<td>1,612 cells</td>
<td>515 cells</td>
<td>182 cells</td>
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<tr>
<td>210 cells</td>
<td>100 cells</td>
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<tr>
<td>360 cells</td>
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<td>230 cells</td>
<td>3 animals</td>
</tr>
<tr>
<td>3 animals</td>
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| 96.3% (P0) and 95.2% (P7) of NeuN-positive postmitotic neurons (P0: n = 322 cells, P7: n = 100 cells, n = 3 animals; Fig. 7D–G,K–N). Tubb2b was highly expressed in the spinal cord throughout development (Fig. 8A–C) and at E14.5 was observed in 98.9% of Sox2-positive stem cells lining the central canal (n = 253 cells, n = 3 animals) and in 99.5% of Dcx-positive postmitotic neurons (n = 761 cells, n = 3 animals; Fig. 8D,E,G,H). This contrasted with the dorsal root ganglia, where GFP was observed in only 4.6% of Sox2-positive progenitors (n = 189 cells, n = 3 animals) but was found in 95.2% of Dcx-positive neurons (n = 300 cells, n = 3 animals; Fig. 8D,F,G,I). These data show that Tubb2b is expressed in progenitors and postmitotic neurons in the developing cortex, cerebellum, and spinal cord. Consistent with the findings of others, this expression is at higher levels in regions dominated by migrating and differentiating neurons (Jaglin et al., 2009).
Figure 11. Tubb2b eGFP reporter expression is gradually lost from neurons postnatally. A–O: Representative coronal sections of the cerebral cortex of the Tg(Tubb2b-eGFP)GlbDAK mouse line (B–E,G–J,L–O) and littermate controls (A,F,K) at P0 (A–E), P10 (F–J), and P21 (K–O). A,B,F,G,K,L: Hoechst-stained scans of the cerebral cortex at the indicated time points. C–E,H–J,M–O: Immunostainings were performed for NeuN (C,H,M), GFAP (D,I,N), and Olig2 (E,J,O). Dashed, open arrows indicate colocalization of a given marker with eGFP, solid, open arrows indicate cells that are solely NeuN positive. Gray-scale images of the individual channels are shown in C1, C2, D1, D2, E1, E2, H1, H2, I1, I2, J1, J2, M1, M2, N1, N2, O1, and O2; n = 3 for B–E,G–J,L–O. P: Quantification of the percentage of NeuN-, GFAP-, or Olig2-positive cells also positive for eGFP for the time points P0, P10, P21, and 8 weeks. Graph shows mean ± SEM (n = 3 animals). Note the dramatic reduction of eGFP/NeuN double-positive cells from P10 to P21. Scale bars = 100 μm in A (applies to A,B); 5 μm in C (applies to C–E); 100 μm in F (applies to F,G); 5 μm in H (applies to H–J); 100 μm in K (applies to K,L) 5 μm in M (applies to M–O).
Figure 12. Expression of the Tubb2b reporter in the 8-week-old cerebellum. A: Representative Hoechst-stained image of the Tg(Tubb2b-eGFP)GlbDAK cerebellum. GFP signal is clearly visible throughout the molecular layer (ML), in a subset of cells in the Purkinje cell layer (PCL), sparsely in the granular cell layer (GCL), and in the arbor vitae (AV). Gray-scale image of the GFP channel is shown in A1. B: Schematic showing the cerebellum, its layers, and the major cell types. Indicated are the Purkinje cells (PC), Bergmann glia (BG), oligodendrocytes (ODC), astrocytes (AC), and granular cells (GC). Gray indicates no expression, whereas green cells show expression of the Tubb2b reporter. C–F: Representative images stained for calbindin (C), NeuN (D), Sox2 (E), and Olig2 (F). Gray-scale images of the individual channels are shown in C1, C2, D1, D2, E1, E2, F1, and F2. Quantitation revealed that 0.9% of calbindin-positive Purkinje cells (n = 121 cells) and 0.2% of NeuN-positive granule cells colocalize with eGFP, whereas 73.2% of Olig2-positive cells (n = 97 cells) and 90.7% of Bergmann glia are positive for eGFP (n = 226 cells). Solid arrowheads indicate cells that are positive for calbindin or NeuN but negative for eGFP. Open, dashed arrowheads indicate cells that are double positive for Sox2/eGFP or Olig2/eGFP; n = 3 animals for all conditions. Scale bars = 50 μm in A; 50 μm in C (applies to C–F).
the cortex, 71.2% of GFAP-positive cells were GFP positive (n = 961 cells, n = 3 animals) and 70.3% of cells positive for the oligodendrocyte marker Olig2 expressed GFP (n = 381 cells, n = 3 animals; Fig. 10C–F, Table 3). These data strongly suggest that in development the expression of Tubb2b is downregulated in the neuronal lineage. To ascertain when this transition occurs, we analyzed eGFP-positive cells in the neocortex of mice aged P0, P10, and P21 (Fig. 11A–P). At P0, we found that 92.9% of NeuN-positive cells colocalized with eGFP (n = 1,612 cells, n = 3 animals), which decreased to 82.7% at P10 (n = 1,084 cells, n = 3 animals) and 15.6% at P21 (n = 498 cells, n = 3 animals; Fig. 11C,H,M). This contrasts with Olig2-positive cells;
we observed 88.4% colocalization at P0 (n = 182 cells, n = 3 animals), 88.8% colocalization at P10 (n = 230 cells, n = 3 animals), and 68.8% colocalization at P21 (n = 70 cells, n = 3 animals; Fig. 11E,J,O, Table 3).

Taken together these data reveal that Tubb2b persists in the glial lineage, contrasting with postmitotic neurons, which transit from high levels of expression at embryonic time points to an almost complete loss of Tubb2b at 8 weeks of age.

Given the abundance of eGFP signal in the developing cerebellum, we then asked whether Tubb2b is also downregulated in mature cerebellar neurons. As in the cortex, at 8 weeks of age, reporter signal was restricted to a subpopulation of cells (Fig. 12). To ascertain the identity of these cells, we used the Purkinje cell marker calbindin (Fig. 12C), the pan-neuronal marker NeuN (Fig. 12D), the oligodendrocyte marker Olig2 (Fig. 12G), and Sox2, which labels Bergmann glia in the Purkinje cell layer (Fig. 12F). We found that only 0.9% of calbindin-positive Purkinje cells (n = 121 cells, n = 3 animals) and 0.2% of NeuN-positive granule cells colocalize with eGFP, whereas 73.2% of Olig2-positive cells (n = 97 cells, n = 3 animals) and 90.7% of Bergmann glia were positive for eGFP (n = 226 cells, n = 3 animals). We conclude that the developmental transition observed in cortical neurons also occurs in the cerebellum.

**Tubb2b is expressed in young neurons in the dentate gyrus**

We turned our attention to the dentate gyrus of the adult hippocampus, one of the few adult regions where neurogenesis persists (Gage, 2000; Malatesta and Gotz, 2013; von Bohlen Und Halbach, 2007; Yu et al., 2014). Neurons are generated by Sox2-positive radial glial-like progenitors residing in the subgranular zone, before a short migration (Dcx-positive) and differentiation into mature Prox-1- and NeuN-positive granular cells (Fig. 13C). Careful analysis of the GFP expression pattern in the dorsal blade of the dentate gyrus showed
Figure 15. Tubb2b reporter expression in the developing eye is largely restricted to postmitotic cells. A–C: Coronal sections of the eye at the indicated developmental time points E12.5, E14.5, and E16.5 for the Tg(Tubb2b-eGFP)GlbDAK mouse line. A1–C1 show gray-scale images for A–C. D,E: Immunostainings for the progenitor marker Sox2 (D) and the postmitotic marker Dcx (E) on E14.5 coronal sections. F,G: Higher magnification images of the Sox2 staining (F,G; gray-scale images are presented in F1, F2, G1, and G2). The open arrowhead indicates a Sox2-positive cell, the solid arrowhead an eGFP-positive cell (G). H,I: Higher magnification images for Dcx staining. Note the colocalization of eGFP and Dcx, indicated with an open, dashed arrowhead (I). Quantitation revealed that only 4.1% of Sox2-positive cells are GFP positive (n = 521 cells), whereas colocalization was observed for 100% of DCX-positive cells (n = 295 cells). Hoechst nuclear staining (shown in blue) can be seen in A–C; n = 3 animals for all conditions. Scale bars = 200 μm in A–C; 50 μm in D (applies to D,E); 20 μm in F (applies to F,H); 10 μm in G (applies to G,I).
The gradual loss of the reporter signal coincident with this maturation (Fig. 13B). We found that 77.9% of Sox2-expressing progenitors (n = 591 cells, n = 3 animals) and 75.0% of the Ki67-positive population were positive for GFP (n = 77 cells, n = 3 animals; Fig. 13D,E). Similarly, 78.4% of Dcx-positive neurons showed GFP expression (n = 190 cells, n = 3 animals), whereas 63.7% of Prox1-positive and 55.0% of NeuN-positive cells were GFP positive (n = 1,312 and n = 1,478, respectively, n = 3 animals; Fig. 13F–H; von Bohlen Und Halbach, 2007). These data suggest that as neurons mature in the dentate gyrus the expression of Tubb2b is downregulated.

**Tubb2b expression in the cochlea and retina**

Our qPCR results revealed that Tubb2b is expressed at moderate levels in the retina and the cochlea at 8 weeks of age (Fig. 1B). We investigated this further by employing the Tg(Tubb2b-eGFP)GlbDAK mice. For the cochlea we observed GFP signal in both the inner and the outer hair cells as well as a subset of support cells known as Hensen cells (Fig. 14A–C). We observed high autofluorescence in retinal preparations, so we employed permanent staining (peroxidase based) to characterize the expression pattern of eGFP. This revealed the presence of the reporter in the inner nuclear layer (that consists of bipolar and horizontal cells) and also in the ganglion cell layer (Fig. 14D–G). We investigated the origin of this postmitotic neuronal expression further by analyzing the developing retina. Tubb2b reporter expression was low at E12.5 and increased at E14.5 and E16.5, restricted at all time points to a subpopulation of cells close to the basal surface of the optic cup (Fig. 15A–C). GFP signal could also be observed in the optic nerve at later time points (see, for example, E16.5; Fig. 15C). Immunohistochemistry at E14.5 revealed that, in contrast to the case in the developing cortex, only 4.1% of Sox2-positive cells were GFP positive (n = 521 cells, n = 3 animals), whereas colocalization was observed for 100% of DCX-positive cells (n = 295 cells, n = 3 animals; Fig. 15D–I). These data suggest that the expression of Tubb2b in progenitor cells and its subsequent reduction in differentiating neurons are not a universal feature of Tubb2b gene regulation.

**DISCUSSION**

We have characterized the expression pattern of the tubulin isoform Tubb2b, a disease-causing gene in humans. By means of a novel GFP reporter mouse line, we have shown that it is expressed at high levels during cortical development in progenitors (Sox2-positive), intermediate progenitors (Tbr2-positive), and migrating...
neurons (Dcx-positive). Surprisingly, this expression is almost completely lost in mature neurons (NeuN-positive) in the 8-week-old cortex, but it persists in astrocytes and oligodendrocytes (Fig. 16). In the cerebellum, there is abundant expression of Tubb2b at birth in all cell types, but at 8 weeks of age Tubb2b is largely absent in NeuN-positive granule cells and calbindin-positive Purkinje cells. This expression pattern is mirrored in the hippocampus, where we observed GFP signal in progenitor cells (Sox2-positive), newly born neurons (Dcx), and some mature granule cells (Prox1-positive). GFP signal is gradually lost, however, in older granule cells, which are located farthest from the subgranular zone. This developmental transition in neurons is not a universal feature of the Tubb2b gene; analysis of the developing retina has revealed that it is largely absent from Sox2-positive progenitors and that this expression persists in neurons located in the ganglion cell layer and a population of cells within the inner nuclear layer in 8-week-old animals.

One caveat that should be kept in mind when interpreting our data is the underlying assumption that the presence of eGFP reflects the presence of mRNA of Tubb2b. It is possible that eGFP persists in the cell longer than the endogenous Tubb2b transcript. However, it should be noted that our results mirror the in situ hybridization and qPCR data generated by the Chelly group (Jaglin et al., 2009). Employing a radioactive probe targeted to the 3'UTR of Tubb2b, they showed that Tubb2b is highly expressed in the developing brain and spinal cord at E14.5 and E16.5. Analogous to the data presented here, expression of Tubb2b was demonstrated in the ventricular zone and intermediate zone, with higher levels of expression in the cortical plate. Similar to our results, their qPCR data showed high levels of Tubb2b expression at embryonic time points (E12.5, E14.5, E16.5) and at birth, which was dramatically reduced in the adult brain. The Chelly group did report Tubb2b expression in the Purkinje cell layer of the adult cerebellum, which they presumed was in the Purkinje cells. We also observe notable staining in the Purkinje cell layer in our Tg(Tubb2b-eGFP)GlbDAK mouse line, but our work has revealed that this is due to the expression of Tubb2b in adjacent Bergmann glia and not the Purkinje cells themselves.

The temporal and spatial expression of Tubb2b is consistent with the anatomical phenotypes described for mice and patients with mutations in this gene (Bahibuisson et al., 2014; Breuss and Keays, 2014). Stottmann and colleagues have described a recessive ENU-induced mutant mouse harboring an N247S mutation that presents with proliferation defects and cortical thinning (Jaglin et al., 2009; Stottmann et al., 2013), and Chelly and colleagues have shown that in vivo knockdown of Tubb2b in the rat cortex impairs neuronal migration (Jaglin et al., 2009). With respect to human disease phenotypes, the high levels of expression in migrating and differentiating neurons correlate with polymicrogyria, agyria/pachygyria, and axon guidance defects described for a number of patients (Cederquist et al., 2012; Cushion et al., 2013; Jaglin et al., 2009; Romaniello et al., 2012). These severe diseases, which have their origins in development, may mask phenotypes associated with the adult expression of TUBB2B. It is conceivable that mutations in TUBB2B might impair glial function and visual and auditory processing.

One aspect that is apparent from this study is that in the cortex Tubb2b is expressed in radial glial cells, shows increased expression levels in migrating and differentiating neurons, and is subsequently lost in mature cortical neurons. This intriguing expression pattern may be correlated with specific cytoskeletal requirements. The Vale group recently reported isotype-specific interaction with different molecular motors (Sirajuddin et al., 2014), and it has also been shown that the localization of Tubb2b mRNA is important for the correct shape and function of the growth cone (Preitner et al., 2014). It is possible that Tubb2b, in concert with specific microtubule associated proteins, confers different dynamic properties on the cytoskeleton, which are important for cells undergoing morphological change but not required in mature cortical neurons.

To explore whether Tubb2b does indeed confer specific properties on the cytoskeleton, genetic replacement experiments in which Tubb2b is depleted and then systemically rescued with a variety of different isoforms might be informative (Saillour et al., 2014). Alongside biochemical studies that assess isotype-specific microtubule binding partners and their association with tubulin posttranslational modifications (Janke and Bulinski, 2011), it should be possible to define the functional repertoire of Tubb2b. Our work provides the foundation for such studies, highlighting the necessity to consider cellular identity and maturation in such experiments.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Designed the research: MB, DAK. Performed the research: JM, MB, TG, ML, TH, SN, AB, KC. Designed and built the selective plane illumination microscope and sample holders: ERS-G, KG. Performed deconvolution: ERS-G. Supervised the development of the SPIM control software: MS, KGH. Analyzed the data: MB, JM, DAK. Wrote the manuscript: MB, JM, DAK.

LITERATURE CITED


