INTRODUCTION

In addition to filamentous actin (F-Actin) and intermediate filaments, microtubules (MTs) are core components of the cytoskeleton in all eukaryotic cell types, providing structure and shape to the cytoplasm (Pollard & Goldman, 2018). They are highly dynamic elements that mediate a variety of cellular functions, including cell division, cell migration, and transport (Fletcher & Mullins, 2010). MTs form the spindle apparatus during mitosis, which is essential to segregate the chromosomes (Petry, 2016). They constitute the internal structure of cilia and flagella (Loreng & Smith, 2017), underlying cell mobility.
and migration and further represent the tracks for intracellular motor proteins, such as kinesins or dynein (Vale, 2003). In neurons, MT-based transport mediates the delivery of mRNAs, proteins, and organelles over long distances in axons and dendrites (Hirokawa, Niwa, & Tanaka, 2010; Kapitein & Hoogenraad, 2015; Knneussel & Loebrich, 2007).

MTs consist of heterodimers of multiple alpha- and beta-tubulins in a 1:1 stoichiometry (Montecinos-Franjola, Chaturvedi, Schuck, & Sackett, 2019). They polymerize in a GTP-dependent manner to form protofilaments, which further assemble into the functional microtuble, a hollow tube consisting of usually 13 protofilaments in mammals (Chaaban & Brouhard, 2017; Mitchison & Kirschner, 1984). It is incompletely understood, whether similar alpha- and beta-tubulin dimers can adapt to the diversity of MT-based cellular functions (Roll-Mecak, 2019). So far, eight individual genes encoding alpha-tubulin and eight genes encoding beta-tubulin have been described in the mouse. They share up to 99% identity among each other and with human tubulin genes (Khodiyar et al., 2007). Based on the high degree of identity between individual tubulin isotypes, redundant functions have been discussed. Moreover, the differences among isotypes have been highly conserved in evolution, suggesting that they mediate a functional significance.

Polymorphisms in human tubulin genes participate to neuronal disease (Chakraborti, Natarajan, Curiel, Janke, & Liu, 2016). For instance, different mutations in the neuron-specific gene encoding beta3-tubulin (TUBB3) result in malformation of cortical development and affect neuronal migration (Tischfield et al., 2010; Whitman et al., 2016). Likewise, mutations in alpha4a-tubulin (TUBA4A), associated with a familial form of amyotrophic lateral sclerosis (ALS), cause neurodegenerative defects (Smith et al., 2014). MT dysfunctions in neurons, therefore, point to important and distinct roles of tubulin isotypes at different stages of development, differentiation, and function.

Tubulin is subject to different forms of posttranslational modifications (PTMs), including tyrosination, de-tyrosination, acetylation, polyglutamylation, polyglycylation, polyamination, and phosphorylation, most of them being reversible (Song & Brady, 2015). Interestingly, a number of the PTMs modify the C-terminal tail of tubulin, which is exposed to the outer surface of the microtuble (Nogales, Wolf, & Downing, 1998; Redeker, 2010; Redeker, Rusconi, Mary, Prome, & Rossier, 1996; Wall et al., 2016). Based on the combinatorial diversity of tubulin isotypes and PTMs, the so-called tubulin code hypothesis has evolved (Janke & Knneussel, 2010; Roll-Mecak, 2019). It suggests that cells form highly specialized MTs or MT-subdomains, either by combining different tubulins, and/or by addition of specific patterns of tubulin PTMs. Individual MTs may, therefore, have a unique identity with respect to their binding to microtuble-associated proteins (MAPs), molecular motors or MT-stabilizing/-destabilizing proteins (Bodakuntla, Jijumon, Villablanca, González-Billault, & Janke, 2019).

The MT cytoskeleton plays a fundamental role during the development of axons, dendrites, and neuronal synapses and is a critical regulator of neuronal plasticity at mature stages (Dent et al., 2007; Hu et al., 2011; Jaworski et al., 2009; Yau et al., 2014). Presently, the spatio-temporal expression pattern of tubulin isotypes in neurons and brain tissue is barely understood. This limitation is not at least due to the lack of suitable antibodies and many cross-reactivities of individual sera.

With the aim to provide a structured overview of neuronal tubulin isotypes and the available tubulin antibodies, we (i) compared the amino acid sequences of mouse alpha- and beta-tubulins, (ii) reviewed tubulin mRNA data from GEO and ENCODE databases and (iii) validated a number of tubulin-specific antibodies. Finally, (iv) we analyzed the protein expression levels of individual tubulin isotypes at different developmental stages using cultured neurons and ex vivo brain lysates. Together, we provide an overview of tubulin isotype-specific expression patterns during brain development in mice, which may serve as a resource for future studies.

2 MATERIALS AND METHODS

2.1 Protein sequence alignment and analysis

Protein sequence alignment was performed using the software application MegAlign (DNASTAR, version 15.3.0). Alignment was performed with the MUSCLE algorithm option. Source and accession numbers of the analyzed sequences are summarized in Figures 1b and 2b. For multiple alignments, a percent identity value was displayed for each pair of sequences. Percent identity for two amino acid sequences was calculated as follows: (Matches × 100)/length of aligned region including gaps. Identity scoring only counts perfect matches, and does not consider the degree of similarity of amino acids to one another. Note, that only internal gaps were included. If MegAlign deduced the pairwise score from adjacent alignments, a pairwise alignment was not calculated. In these cases, the percent identity is based on the two sequences, as they appeared in the multiple alignment. Blocks of shared gaps that may occur in the multiple alignment were not included in the length of the aligned region.

2.2 Analysis of mRNA transcript levels

Gene expression levels of mouse tubulin isotypes were quantified based on two published data sets: (i) for mRNA expression levels in the hippocampus (C57BL/6 wild-type mice at postnatal days (P) 10, P 15, P 18, P 20, P 28, and P 32),
the GEO DataSet_604 provided by NCBI (series GSE1482), was reanalyzed with respect to tubulin isotypes (Tuba1a, Tuba1c, Tuba3b, Tuba4a, Tuba8). Values are given as arbitrary units (a.u.) based on the Affymetrix murine genome U74A version 2 array platform. (ii) For mRNA expression levels from mouse
brain (embryonic day E 14.5 and at 8 weeks), the BioProject PRJNA66167 data set, a component of the mouse ENCODE (ENCyclopedia Of DNA Elements) project (Yue et al., 2014) provided by NCBI, was reanalyzed with respect to tubulin isotypes (Tuba1a, Tuba1b, Tuba1c, Tuba3a, Tuba3b, Tuba4a, Tuba8, Tubb1, Tubb2a, Tubb2b, Tubb3, Tubb4a, Tubb4b,
The analysis was restricted to data of the whole brain at E 14.5 and to averaged values of the “cerebellum adult”, “cortex adult”, and “frontal lobe adult” datasets, summarized as “whole brain”. For both data sets, individual tubulin expression values were normalized against the corresponding GAPDH value prior to analysis. The results are presented as arbitrary units (a.u.). The ENCODE data set is published under the following creative common license deed: https://creativecommons.org/licenses/by-nc-sa/3.0/.

2.3 | Antibodies

All tubulin antibodies used in this study are summarized in Table 1. In addition, the following primary and secondary antibodies were used: mouse anti-beta-actin (Sigma; AC-15, #A5441, WB 1/5,000); mouse anti-γ-adaptin (BD Biosciences; #610386; WB 1/5,000); donkey anti-mouse HRP-conjugated (Dianova; #715-036-151; WB 1/10,000); donkey anti-rabbit HRP-conjugated (Dianova; #711-036-152; WB 1/10,000); donkey anti-rat HRP-conjugated (Dianova; #712-036-153; WB 1/10,000).

2.4 | DNA constructs

Used primers and cDNAs are summarized in Table 2. RNA primers was performed from tissue, as indicated, using TRIzol™ Reagent (Invitrogen; #15596026), according to the manufacturer's instructions. Afterwards, Reverse Transcription PCR (RevertAid H Minus First Strand cDNA Synthesis Kit Thermo fisher; #K1632) was performed using Random Hexamer Primers. Resulting cDNA was used for PCR amplification. Constructs were cloned via HindIII/ SalI into pAcGFP-C3. Tubb4a was cloned EcoRI/KpnI into pAcGFP1-C2. Tuba1b-de-Tyr was cloned via BglII/BamHI into pAcGFP-C1. The cDNA of de-tyrosinated Tuba1b (mCherry-Tuba1b-Delta1-pcDNA3.1+) was previously published (Aillaud et al., 2016).

2.5 | Culture of HEK293 cells and transfection

Human embryonic kidney 293 cells (HEK293, ATCC; #CRL-1573) were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO2 in DMEM, high glucose, GlutaMAX™ (Invitrogen; #61965-026), supplemented with 50 U penicillin/ml, 50 μg/ml streptomycin, and 10% (v/v) bovine serum (all Invitrogen). Cells were grown in 6-well plates (coated with poly-L-lysine) to 80% confluence. Cells were transfected by a calcium phosphate co-precipitation protocol, using GFP-tubulin fusion constructs, as indicated. Two days after transfection, cells were harvested as described below (Preparation of Primary Hippocampal Cell Lysates).

2.6 | Primary culture of hippocampal neurons

One day prior to the preparation, 12 mm sterile glass coverslips (Carl Roth) were placed into 24-well plates and coated with poly-L-lysine (Sigma-Aldrich) (50 μg/ml) over night at 37°C. Coverslips were rinsed twice with 500 μl sterile H2O. About 1 ml of preheated complete neurobasal medium (Thermo Fisher; #A3582901) supplemented with L-glutamine (#25030024) and B-27 (#A3582801) was added to each well. Embryos at stage E 15 were dissected in HBSS (Gibco, Thermo Fisher Scientific) at 4°C. Hippocampi of C57BL/6 mice were dissected, transferred to 1 ml 0.05% trypsin/EDTA solution (Invitrogen), and incubated for 5 min at 37°C. After removal of trypsin, the hippocampi were washed with DMEM-F12-containing serum (Gibco, Thermo Fischer Scientific) and kept in HBSS medium. Hippocampi from both sexes were gently dissociated into cells, using fire polished Pasteur pipettes. Cell density was calculated using a Neubauer Counting Chamber (Marienfeld, Lauda-Königshofen). 60,000 cells were plated per well in 24-well plates and kept at 37°C in a humidified atmosphere of 95% air and 5% CO2 in a cell incubator.

2.7 | Preparation of primary hippocampal cell lysates

To quantify the protein expression levels of tubulin in primary hippocampal neurons, cultures were harvested after 1, 3, 7, 14, or 20 days in vitro (DIV), respectively. The adherent cells were washed once in ice-cold PBS and harvested in PBS lysis buffer, containing 1% (v/v) Triton X-100 (Sigma), PhosSTOP phosphatase inhibitor (Roche), Complete protease inhibitor (Roche), and 1 mM PMSF (Applichem). After incubation for 30 min on ice, lysates were centrifuged at 1,000 x g for 10 min at 4°C. Resulting supernatants were boiled in SDS sample buffer after adjustment of protein concentrations using a BCA assay (Pierce Biotechnology). About 3 µg of total protein per sample per time point (days in vitro) were subjected to SDS-PAGE. Subsequently, samples were analyzed by western blotting.

2.8 | Preparation of hippocampal tissue lysates

To quantify the protein expression levels of tubulin, hippocampi of mice at embryonic day (E) 15, postnatal days...
TABLE 1   Antibodies against alpha- and beta-tubulin isotypes. Summary of antibodies against (A) alpha-tubulin and (B) beta-tubulin isotypes, validated and used in this study. The immunogenic isotype, the source or company, the host species (mouse (ms), rabbit (rb) or rat), the catalogue number, the clonality (monoclonal (mono) or polyclonal (poly)) and the dilution used for western blotting, are listed. If available, clone names, RRID numbers and the immunogen (including accession numbers), according to manufacturer’s information, are given. Specificity statements are based on the data shown in Figure 4 and Supplemental Figure S2. Antibodies used to analyze the protein expression levels are marked (x). Isotype-specificity is indicated in bold. Amino acid (aa).

| Isoform   | Company     | Species | Catalog #     | Clonality | Clone     | RRID          | Immunogen                              | Specificity        | Dilution (WB) | Used for analysis |
|-----------|-------------|---------|---------------|-----------|-----------|---------------|----------------------------------------|-------------------|---------------|------------------|
| Tuba1a    | Santa Cruz  | ms      | sc-134237     | Mono      | 7-RY28    | AB_2212295    | aa 352–451 of human TUBA1a             | No signal         | 1/1,000       |                  |
| Tuba1b    | Abcam       | rb      | ab200216      | Poly      | –         | –             | aa 423–451 of human TUBA1a             | Not specific       | 1/1,000       |                  |
| Tuba1b    | Abcam       | rb      | ab108629      | Mono      | EPR1333   | AB_10866252   | aa 400–500 of human TUBA1b             | Not specific       | 1/10,000      |                  |
| Tuba1b    | Origene     | rat     | SM568P        | Mono      | YOL1/34   | AB_342597     | Yeast tubulin                          | Not specific       | 1/1,000       |                  |
| Tuba1b    | Origene     | ms      | TA506582      | Mono      | OTI3G3    | AB_2623807    | Full length of human TUBA1b            | No signal         | 1/1,000       |                  |
| Tuba3     | Santa Cruz  | ms      | sc-134240     | Mono      | 10J1      | AB_2210044    | Full length of human TUBA3c            | Not specific       | 1/1,000       |                  |
| Tuba3c    | Sigma       | ms      | SAB1402410    | Mono      | 2D2       | AB_10645777   | aa 352–451 of human TUBA1a             | Not specific       | 1/500         |                  |
| Tuba3c    | Abcam       | rb      | ab116219      | Poly      | –         | AB_10900045   | aa 412–441 of human TUBA3c/d           | Recognizes Tuba3b, Tuba1a/b | 1/500 | x  |
| Tuba4a    | Abcam       | rb      | ab177479      | Mono      | EPR13477(B) | –             | aa 50–150 of human TUBA4a             | **Specific for Tuba4a** | 1/5,000 | x  |
| Tuba8     | Novus       | ms      | NBP2-37535    | Mono      | 2D6       | –             | Fragment of human TUBA8                | Recognizes Tuba8 and Tuba3b | 1/1,000 | x  |
| Tuba8     | Bioserv     | ms      | Custom (Diggle et al.) | Mono | – | – | aa 35–45 (TFGTQASKIND) of murine Tuba8 | **Specific for Tuba8** | 1/100 | x  |
| Tuba-pan  | Sigma       | ms      | T9026         | Mono      | DM1A      | AB_477593     | Microtubules derived from chicken embryo brain; epitope within aa 426–450 of alpha tubulin | Recognizes all Tuba isotypes | 1/5,000 |                  |

B

| Tubb1     | Novus       | ms      | NBP2-46245    | Mono      | OTI4A3    | –             | Full length of human TUBB1             | **Specific for Tubb1** | 1/1,000 | x  |
| Tubb2     | Abcam       | rb      | ab151318      | Poly      | –         | –             | aa 243–445 of human TUBB4b             | Not specific       | 1/1,000 |                  |
| Tubb2     | Abcam       | rb      | ab179512      | Mono      | EPR16773  | –             | aa 400 to C-term of human TUBB2B       | **Specific for Tubb2a/b** | 1/1,000 | x  |

(Continues)
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The tissue was immediately suspended in lysis buffer (320 mM sucrose, 1 mM NaHCO₃, 1 mM MgCl₂, 500 µM CaCl₂, and 1 µM PMSF) in the presence of protease inhibitors (Complete; Roche) and lysed with 12 strokes at 800 rpm using a 2 ml Teflon potter. Subsequently, lysates were centrifugated at 1,400 ×g for 10 min at 4°C. After adjustment of protein concentrations using a BCA assay (Pierce Biotechnology), resulting supernatants were boiled in SDS sample buffer. About 1 µg of total protein per sample per time point (age) were subjected to SDS-PAGE. Subsequently, samples were analyzed by western blotting.

2.9 | Statistical analysis

2.9.1 | Gene expression levels

We analyzed the potential changes in RNA expression levels of tubulin isotypes over time, using a Univariate Analysis of Variance (Uni-ANOVA). The RNA expression levels are the “dependent variable”, postnatal days are the first “between-subject-factor” and tubulin isotypes are the second “between-subject-factor”. Based on a strong interaction between “postnatal days” and “tubulin isotypes”, a restricted analysis for each isotype was performed to compare expression levels of individual tubulins, over time. Data were analyzed using SPSS (PASW) Statistics 19.0 (Version 19; SPSS). Comparisons were considered as statistically significant if p-values were below *p < .05, **p < .01, and ***p < .001. n-Values are given in the figure legends. Averaged data are presented as mean ± SD, as indicated in figure legends.

2.9.2 | Western blot analysis

For detection of relative immunoblot signal intensities, images were acquired using a Chemo-Cam Imager ECL HR 16-3200 (Intas). Signal intensities were analyzed using the ImageJ software (version 1.38, National Institutes of Health, NIH). Data were analyzed using SPSS (PASW) Statistics 19.0 (Version 19; SPSS). Statistical significance between groups (days in vitro or age) was determined by repeated measurement analysis of variance (repeated measurement ANOVA), as indicated in figure legends. Comparisons were considered as statistically significant if p-values were below *p < .05, **p < .01, and ***p < .001. n-values are given in the figure legends. Averaged data are presented as mean ± SEM as indicated in figure legends.
### TABLE 2  GFP-tubulin fusion proteins. Summary and cloning details of GFP-tubulin isotype expression vectors, used in this study. Specific tubulin cDNAs were amplified (i) based on tagged open reading frame clones, purchased from ORIGENE (Rockville, USA), (ii) based on published and provided expression vectors or (iii) after RNA extraction and reverse transcription PCR, using tissue lysate, as indicated. Primers and the corresponding nucleotide sequences are listed. Names of constructs, corresponding isotypes and target expression vectors are listed.

| Construct                  | Isotype    | Vector      | Tissue or cDNA clone          | Primer name       | Primer sequence 5′–3′                                                                 |
|----------------------------|------------|-------------|------------------------------|------------------|---------------------------------------------------------------------------------------|
| GFP-Tuba1a                 | Tuba1a     | pAcGFP1-C3  | Cerebellum Adult mouse       | PB-Tuba1a_s      | TTAAGTCGACTTATGATATCCCTCTCTCTTCTCCTCCC                                                  |
|                            |            |             |                              | PB-Tuba1a_as     | AATAAAGGCCTATGAGGAGATGCAATTCACC                                                      |
| GFP-Tuba1b                 | Tuba1b     | pAcGFP1-C3  | cDNA clone MR207205 origene  | PB-Tuba1b_s      | ATAAAGCCTATGAGGAGATGCAATTCACC                                                  |
|                            |            |             |                              | PB-Tuba1b_as     | TTTTGTCACTTATGACTCCTCTCTCTCTCTCTCT                                               |
| GFP-Tuba1b-de-Tyr          | Tuba1b-de-Tyr | pAcGFP1-C1 | cDNA provided by Marie-Jo Moutin |                 |                                                                                       |
|                            |            |             |                              | –                |                                                                                       |
|                            |            |             |                              | –                |                                                                                       |
| GFP-Tuba1c                 | Tuba1c     | pAcGFP1-C3  | cDNA clone MR207167 origene  | PB-Tuba1c_s      | TTAAGTCGACTTATGAGGAGATGCAATTCACC                                                  |
|                            |            |             |                              | PB-Tuba1c_as     | AATAAAGGCCTATGAGGAGATGCAATTCACC                                                   |
| GFP-Tuba3b                 | Tuba3b     | pAcGFP1-C3  | Testis Adult mouse           | PB-Tuba3b_s      | ATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
|                            |            |             |                              | PB-Tuba3b_as     | TTTTGTCACTTATGACTCCTCTCTCTCTCTCT                                               |
| GFP-Tuba4a                 | Tuba4a     | pAcGFP1-C3  | Hippocampus Adult mouse      | PB-Tuba4a_s      | GCAAAGCTATGCGAGTGCACATTGACC                                                       |
|                            |            |             |                              | PB-Tuba4a_as     | CTAGTCGACCTACTCTCTCCTCCTCTCTCTCT                                                   |
| GFP-Tuba8                  | Tuba8      | pAcGFP1-C3  | Testis Adult mouse           | PB-Tuba8_s       | AATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
|                            |            |             |                              | PB-Tuba8_as      | AATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
| GFP-Tubb1                  | Tubb1      | pAcGFP1-C3  | Spleen Adult mouse           | PB_msTubb1_s     | AAATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
|                            |            |             |                              | PB_msTubb1_as    | AAATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
| GFP-Tubb2a                 | Tubb2a     | pAcGFP1-C3  | Whole brain E17 mouse        | PB_msTubb2a_s    | AAAAAGCCTATGAGGAGATGCAATTCACC                                                   |
|                            |            |             |                              | PB_msTubb2a_as   | AAAAAGCCTATGAGGAGATGCAATTCACC                                                   |
| GFP-Tubb2b                 | Tubb2b     | pAcGFP1-C3  | Whole brain E17 mouse        | PB_msTubb2b_s    | AAAAAGCCTATGAGGAGATGCAATTCACC                                                   |
|                            |            |             |                              | PB_msTubb2b_as   | AAAAAGCCTATGAGGAGATGCAATTCACC                                                   |
| GFP-Tubb3                  | Tubb3      | pAcGFP1-C3  | Hippocampus Adult mouse      | Tubb3-s          | GCAAAGCTATGAGGAGATGCAATTCACC                                                       |
|                            |            |             |                              | Tubb3-as         | AATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
| GFP-Tubb4a                 | Tubb4a     | pAcGFP1-C2  | Hippocampus Adult mouse      | Tubb4-s          | GCAAAGCTATGAGGAGATGCAATTCACC                                                       |
|                            |            |             |                              | Tubb4-as         | AATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
| GFP-Tubb4b                 | Tubb4b     | pAcGFP1-C3  | cDNA clone MR207096 origene  | PB-Tubb4b_s      | TTAAGTCGACTTATGAGGAGATGCAATTCACC                                                   |
|                            |            |             |                              | PB-Tubb4b_as     | TTAAGTCGACTTATGAGGAGATGCAATTCACC                                                   |
| GFP-Tubb5                  | Tubb5      | pAcGFP1-C3  | Whole brain E17 mouse        | PB_msTubb5_s     | AATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
|                            |            |             |                              | PB_msTubb5_as    | AATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
| GFP-Tubb6                  | Tubb6      | pAcGFP1-C3  | Bladder Adult mouse          | PB_msTubb6_s     | AATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
|                            |            |             |                              | PB_msTubb6_as    | AATAAAGCCTATGAGGAGATGCAATTCACC                                                   |
3 | RESULTS

3.1 | Mouse tubulin isotypes: identity and difference

To compare the protein sequence of individual mouse tubulins, we plotted all alpha-tubulin isotypes described, including Tuba1a, Tuba1b, Tuba1c, Tuba3a, Tuba3b, Tuba3l, Tuba4a, and Tuba8 (Figure 1a,b). By sequence comparison, we identified amino acid (AA) identities of the selected isotypes in the range of 76% to 100% (Figure 1c). Three tubulin domains are typically characterized: (i) the N-terminal domain (AA 1 to 221), (ii) the intermediate domain (AA 222 to 400) and (iii) the C-terminal domain (AA 401 to >450) (Nogales et al., 1998). While Tuba3l and Tuba8 contain insertions in their N-terminal domain, compared to the other alpha-tubulins, only very few AAs differ in the intermediate tubulin domain, which is involved in alpha-/beta-dimerization and mediates lateral contacts between the protofilaments (Nogales, Wolf, Khan, Luduena, & Downing, 1995). Notably, most of the differences between alpha-isotypes are located at the extreme C-terminus that has been identified as the outside surface of the microtubule (Nogales et al., 1998). Interestingly, TubaL3 is a unique isotype, which lacks most of these C-terminal AAs (Figure 1a).

We further compared the protein sequence of all mouse beta-tubulins described, namely Tubb1, Tubb2a, Tubb2b, Tubb3, Tubb4a, Tubb4b, Tubb5, and Tubb6 (Figure 2). Within their N-terminal domains, beta-tubulins show an explicitly higher variability, as compared to alpha-tubulins, whereas their intermediate domain is equally conserved. Similar as identified for alpha-tubulins, the extreme beta-tubulin C-terminus (>position 430) is highly unique (Figure 2a,b). This region, as it forms the microtubule surface, is suggested to interact with MAPs and motor proteins (Larcher, Boucher, Lazereg, Gros, & Denoulet, 1996; Nogales et al., 1998). It is also subject to different posttranslational modifications that further modulate MT stability, transport, and severing (Janke & Bulinski, 2011; Janke & Kneussel, 2010).

Based on their N-terminal and C-terminal variability across isotypes, the degree of identity between the selected beta-tubulins ranges from 79% to 100% (Figure 2c).

3.2 | Tubulin mRNA levels in brain

To compare the tubulin gene expression in the mouse central nervous system, we initially screened the NCBI Gene Expression Omnibus (GEO) and the UCSC ENCODE Database, which previously published quantitative mRNA profiles from hippocampus and whole brain, respectively. GeneChip arrays had been used to analyse the gene expression in defined tissues. To this end, extracted mRNA was transcribed into fluorescent cDNA probes, which after fragmentation, were hybridized with a commercial array of short 25-mer DNA fragments, representing target regions of the genes of interest (Bumgarner, 2013). Probe-target hybridization was quantified by detection of the fluorophore to determine the relative abundance of nucleic acid sequences in the target. For both data sets, we normalized the tubulin expression values against the corresponding GAPDH value.

Among the alpha-tubulins analyzed, Tuba1a and Tubaalc reveal highest transcript levels in the postnatal hippocampus, whereas Tuba3b, Tuba4a, and Tuba8 are just marginally detectable (Figure 3a). However, over the time course from postnatal day 10 (P 10) to P 20, Tuba4a revealed a significant increase in mRNA expression levels (Figure 3a and S1a). Moreover, Tuba1a and Tuba1c transcript levels significantly decrease until P 20, but still remain at a relatively high level (Figures 3a). mRNA quantification from whole brain confirm prominent levels of Tuba1a, Tuba1b, and Tuba1c at embryonic stages, which are explicitly reduced at postnatal week 8 (Figure 3b). Although Tuba4a and Tuba8 mRNAs are detected in postnatal brain, their expression values are an order of magnitude lower than those of the Tuba1 isotypes (Figure 3b, inset).

In the hippocampus, Tubb2a, Tubb3, Tubb4a, Tubb5, and Tubb6 mRNAs were detected with Tubb2a representing highest transcript levels. Notably, the transcripts of all beta-tubulin isotypes, except of Tubb4a, are significantly reduced between P 10 and P 32, however remain to be present at later stages (Figures 3c and S1b). Data from whole brain confirm high Tubb2a mRNA levels and show that Tubb2b transcripts are equally available. In addition, Tubb3 and Tubb5 are highly abundant, whereas Tubb4a and Tubb4b copy numbers are much lower (Figure 3d). A detailed analysis of Tubb6 mRNA shows that transcript levels are small and even undergo a reduction until postnatal week 8. In contrast, Tubb1 mRNAs are hardly detectable in brain (Figure 3d, inset). Together, both databases (GEO and ENCODE) reveal differential tubulin transcript levels during the CNS development. It should be noted that both datasets do not distinguish between neurons and non-neuronal cells, such as glia. Moreover, mRNA half-lives of tubulin isotypes may significantly differ and transcript levels may therefore not be proportional to protein expression levels.

3.3 | Tubulin-specific antibodies

A technical requirement for the analysis of protein expression levels is the availability and quality of suitable antibodies. To test the specificity and cross-reactivity of available mouse tubulin antibodies (Table 1), we individually expressed GFP-fusion proteins (Table 2) of tubulin isotypes (Tuba1a, Tuba1b, Tuba1c, Tuba3b, Tuba4a, Tuba8 and Tubb1, Tubb2a, Tubb2b, Tubb3, Tubb4a, Tubb4b, Tubb5,
and Tubb6) in HEK293 cells. In this assay, the presence of the GFP-tag increases the molecular mass of tubulin and allows to distinguish it from endogenous tubulin in western blots.

Out of 21 tubulin antibodies tested, only seven turned out to be suitable to identify the correct isotype (Figure 4). Others were cross-reactive by detecting one or more alternative tubulin isotypes or did not detect the expressed tubulin fusion protein at all (Figures 4, S2 and Table 1).

A commercial Tuba3c antibody (ab116219) detects a closely related GFP-Tuba3b fusion protein (Figure 4a, star) and endogenously expressed tubulin (Figure 4a, rhomb). However, the antibody is cross-reactive and also detects Tuba1a and Tuba1b.

The Tuba4a antibody (ab177479) specifically detects a GFP-Tuba4a fusion protein (Figure 4b, star), while Tuba4a is not endogenously expressed in HEK293 cells (Figure 4b, rhomb). Since Tuba4a does not contain a tyrosin residue at the very C-terminus (compare with Figure 1a), it differs from other alpha-tubulin isotypes. To exclude a potential antibody cross-reactivity of the Tuba4a-specific antibody with de-tyrosinated tubulins, we also tested this antibody against de-tyrosinated Tuba1b (Tuba1b-de-Tyr). However, no cross-reactivity could be observed (Figure S3).
A Tuba8 antibody (NBP2-37535) detects the GFP-Tuba8 fusion protein, but in addition binds to GFP-Tuba3b (Figure 4c, star). Tuba3/Tuba8 are endogenously expressed in HEK293 cells (Figure 4c, rhomb).

In contrast, another Tuba8 antibody, kindly provided by Diggle and colleagues (Diggle et al., 2017), turned out to be highly specific and did not detect any other tubulin isotype (Figure 4d). As we could not detect the Tuba8 expression in HEK cells (rhomb) using this antibody, we conclude that the HEK cell signal of the former antibody (compare with rhomb in Figure 4c) exclusively represents the Tuba3b protein expression.

Analysis of a Tubb1 antibody (NBP2-46245) revealed a specific detection of GFP-Tubb1 (Figure 4e, star), while Tubb1 is not endogenously expressed in HEK293 cells (Figure 4e, rhomb).

A Tubb2 antibody (ab179512) recognizes both a Tubb2a and Tubb2b epitope, as detected by expression of the respective GFP-fusion proteins (Figure 4f, star). It also detects endogenous Tubb2 in HEK293 cells (Figure 4f, rhomb).
The widely used Tubb3 antibody (801202), which is often used as a neuronal tubulin marker (also known as TUJ1), is a highly specific antibody, exclusively detecting GFP-Tubb3 (Figure 4g, star). Consistent with Tubb3 representing a neuron-specific tubulin isotype, it is not expressed in HEK293 cells (Figure 4g, rhomb).

Tubb4 (NB-120-11315) detects the endogenous tubulin signals (Figure 4h, rhomb), but also multiple unspecific epitopes. Upon expression of GFP-Tubb4a and GFP-Tubb4b, we observed a specific band that runs slightly lower than the unspecific signal (Figure 4h, star), suggesting that this antibody is suitable to detect Tubb4 proteins.

Finally, the Tubb5 antibody turned out to be highly specific for GFP-Tubb5 (Figure 4i, star) and also detects endogenous Tubb5 expressed by HEK293 cells (Figure 4i, rhomb).

For GFP-Tubb1, we observed a lower molecular weight, as compared to other GFP-tubulin fusion proteins (Figure 4e–i). Since the sequence of our fusion construct was confirmed to be correct, this difference may be due to a different posttranslational modification.

In summary, only some of the available tubulin antibodies can be used to specifically study tubulin isotypes, while others should be considered as pan-tubulin antibodies. Table 1 presents an overview of the tubulin antibodies tested in this study. It highlights the ones used for further analysis and indicates isotype-specificity.

### 3.4 Tubulin protein expression levels in cultured neurons

To assesse the expression levels of individual tubulin isotypes in neurons, equal amounts of total protein from cultured hippocampal neurons at DIV 1–20 (days in vitro after plating) were subjected to SDS–PAGE and analyzed by western blotting (Figure 5). In all experiments, γ-adaptin served as a loading control (Figures 5 and S4a).

Cultured hippocampal neurons contain just minor numbers of astrocytes and mainly consist of neuronal cells. The neurons develop an axon from DIV 1.5, start to grow dendrites from DIV 4 and are considered as mature cells from DIV 7 onwards (Banker, 2018). Synaptogenesis, the formation of either spine or shaft synapses, is initiated around DIV 7, whereas mature synapses are detectable from DIV 10 onwards. Synapses undergo plastic changes while their numbers gradually increase between DIV 14 and DIV 20 (Graber, Vaida, Bockmann, & Boeckers, 2009).

Tuba1/Tuba3 protein expression (compare with Figure 4a) showed a peak around DIV 3–7 when neurons develop multiple neurites (Figure 5a–j). In contrast, the Tuba4a expression was almost absent at very early stages, but gradually increased from DIV 7 to DIV 20 (Figure 5b–j), a time period when synapses are developed and maintained. Tuba3/Tuba8 detection (Figure 5c–j) revealed a similar expression pattern, as compared to Tuba1/Tuba3, suggesting that it is mainly reflected by Tuba3.

In fact, this was confirmed using the highly specific Tuba8 antibody (Diggle et al., 2017) (compare with Figure 4d). Since we could not detect any Tuba8 protein expression in cultured hippocampal neurons, compared to the loading control (Figure 5d), the former tubulin signals in Figure 5c represents the Tuba3 expression.

Out of the beta-tubulin isotypes analyzed, the Tubb1 expression peaked at DIV 7 and was significantly downregulated thereafter (Figure 5e,k). As suggested by the mRNA data (Figure 3c,d), Tubb2 protein was prominently expressed at all stages analyzed and its expression level did not significantly change between DIV 1 and DIV 20 (Figure 5f,k). Interestingly, the neuronal marker Tubb3 peaked around DIV 7. It was downregulated at later stages, however remained to be detectable to a lesser extent at DIV 20 (Figure 5g,k). Tubb4 revealed a similar expression pattern as Tubb1 (peak at DIV 7) (Figure 5h,l). Finally, Tubb5 turned out to be highly expressed from the very beginning, even before neurons develop axons and dendrites, however was downregulated from DIV 7 onwards (Figure 5i,l).

In summary, we conclude that hippocampal cultures express several alpha- and beta-tubulins between DIV 1 and DIV 20, with the exception of Tuba8 (Figures 3a and 5d). It should be noted that some tubulins are highly expressed before and during neurite outgrowth (e.g., Tuba5), whereas others show prominent levels at mature stages (e.g., Tuba4a).

### 3.5 Tubulin protein expression levels in the hippocampus

To check for tubulin expression in the developing hippocampus, we also analyzed protein levels of individual isotypes from hippocampal lysates up to 3 months of age (Figure 6). In all experiments, actin served as a loading control (Figures 6 and S4b). As suggested by the neuronal culture data (Figure 5), all tendencies with respect to the up- or downregulation of tubulin isotypes could be confirmed (compare Figure 5j,k,l with Figure 6j,k,l). Due to the wide developmental range of the ex vivo samples analyzed, the expression patterns of individual tubulin isotypes appeared to be more distinct, as compared to the culture-derived samples. At the prenatal stage E 15, we revealed Tubb5 as a prominent tubulin isotype (Figure 6i,l). About 1 week later, at postnatal stage P 5, Tuba1/Tuba3 (Figure 6a–j), Tuba3/Tuba8 (Figure 6c–j), Tubb2 (Figure 6f–k), Tubb3 (Figure 6g–k), and Tubb5 (Figure 6i–l) were highly abundant. Out of these isotypes, Tubb2 remained to be highly expressed (Figure 6f,k), whereas Tubb5 was significantly downregulated at mature stages (Figure 6i,l). Furthermore,
at P 20, Tubb1 was a prominent tubulin expressed in the hippocampus (Figure 6e,k), while Tuba4a (Figure 6b,j) and Tubb4 (Figure 6h,l) show increasing expression levels in young adult mice at 3 months. Similar as observed in cultured hippocampal neurons (Figure 5), Tuba8 was not detected at any of the developmental stages analyzed in this study (Figure 6d,j). Out of all tubulins investigated, Tubb2 showed the most constant expression across the different time points, suggesting a more general function for this tubulin.

Together, our data reveal distinct hippocampal protein expression patterns of individual tubulin isotypes over different developmental stages, suggesting for distinct cellular functions.
Our study summarizes quantitative tubulin mRNA and protein data from mouse brain, hippocampus and cultured hippocampal neurons. It further provides information about tubulin antibody suitability and cross-reactivity. A number of previous reports have investigated tubulin isotypes in neurons and other cell types at different developmental stages in health and disease (Arai, Maruo, Ara, Uehara, & Matsuda, 2001; Guo, Qiang, & Luduena, 2011; Hallworth & Luduena, 2000; Jiang & Oblinger, 1992; Lewis, Lee, & Cowan, 1985; Moody, Miller, Spanos, & Frankfurter, 1996; Wang, Villasante, Lewis, & Cowan, 1986). While most studies focused on either single tubulins or compared beta-tubulin isotypes in a specific functional context, it turned out to be difficult to get a broad overview about the different alpha- and beta-tubulins and their respective antibodies.

For the neuron-specific Tub3 isotype, a differential expression has been reported (Guo et al., 2011; Jiang & Oblinger, 1992). This isotype is mainly expressed during the
early stages of neurite outgrowth and dropped to lower levels at mature stages. The same was observed for Tubb5, however its expression almost disappeared in neurons of adult mice (Guo et al., 2011). Our data reflect these findings, indicating consistency between the different studies.

The available transcript data often correlate with the protein expression results observed in our study. For instance, Tubb2a is a major mRNA at later embryonic stages and around birth, while it is downregulated thereafter. Likewise, Tubb5 is downregulated postnatally, whereas Tubb4 is slightly upregulated at young adult stages. Protein expression data from hippocampal lysate confirm these tendencies, suggesting that mRNA and protein levels are largely proportional to each other. Our data further show that protein expression levels of individual tubulins correlate to each other, independent of the fact that they are derived from tissue lysate (containing neurons and glia) or cultured neurons (mainly containing neurons). The expression levels reported here, therefore for the most part represent neuronal tubulins, although tubulins expressed by glial cells contribute to the results of Figure 6. Since the data from dissociated neurons resemble those from neuronal tissue, neuron cultures are a suitable model to study neuronal tubulin functions at the single cell level.

The development of cultured hippocampal neurons from DIV 1–20 is well-defined (Banker, 2018; Grabrucker et al., 2009), ranging from the formation of minor processes to axons, dendrites, and synapses. Our protein expression data from neuronal cultures reveal that some tubulin isotypes peak in a narrow time window that corresponds to a specific stage of development. For instance, Tubb5 expression reaches a maximum at DIV 3, whereas Tubb3 is highly expressed at DIV 7. In contrast, Tubb2 is equally expressed throughout all stages analyzed. Although these observations are correlative, they suggest that individual tubulin isotypes may participate in specific cytoskeletal processes, while others may mediate general roles.

For example, during neuronal saltatory migration, a specific role of Tuba1a has been reported (Belvindrah et al., 2017). In this study, Tuba1a-mutant neurons revealed decelerated cell migration, in correlation with increased straightness of newly polymerized MTs. Interestingly, the same missense mutation leads to cortical malformations (Keays et al., 2007). However, Tuba8, another tubulin isotype previously associated with impaired brain development does not compensate for the observed cellular phenotypes (Abdollahi et al., 2009). In line with this, Diggle and colleagues (Diggle et al., 2017) recently indicated a possible role of Tuba8 in spermatogenesis, but not brain development. This is supported by a very low transcript copy number of Tuba8 in whole brain (Figure 3) and the inability to detect Tuba8 protein expression in hippocampal neurons (Figures 5 and 6). Furthermore, mutations and polymorphisms in tubulin genes contribute to specific neuronal diseases, indicating distinct functions of specific isotypes (Chakraborti et al., 2016). For instance, several mutations in TUBB3 cause defects in axon targeting to the oculomotor muscles, resulting in congenital fibrosis of the extraocular muscles (CFEOM) (Tischfield et al., 2010). This developmental function of Tubb3 fits to its high expression levels during early postnatal stages, similar as observed in the present study. In another example, defined mutations in TUBB4 participate to dystonia, an adult onset neurodegenerative disorder (Hersheson et al., 2013). Accordingly, our data also revealed a very restricted expression of this tubulin isotype at the adult stage (Figure 6h). Together, these examples indicate that although some functional aspects of MTs may be compensated by related tubulins, others are unique and cannot be counterbalanced.

The combinatorial use of individual alpha- and beta-tubulins in MTs regulates functional MT parameters. For instance, MTs composed of recombinant Tuba1b/Tubb1 + Tubb4b heterodimers show a faster in vitro growth rate, as compared to a combination of Tubal/Tubb3 (Vemu, Atherton, Spector, Moores, & Roll-Mecak, 2017; Vemu et al., 2016). Furthermore, Tubb3 seems to determine the protofilament number of MTs, leading to less stable MTs assembled by Tuba1b/Tubb3, in comparison to Tuba1b/Tubb2b assemblies (Ti, Alushin, & Kapoor, 2018). These studies help to understand, how tubulin isotype composition tunes microtubule dynamics. To which extent other tubulin isotypes affect MT binding affinities and MT polymerization rates and their dynamics, requires further investigation.

Notably, tubulin PTMs have the potential to change MT binding affinities and to control motor-protein transport parameters. Consequently the delivery of cargoes to specific subcellular destinations, such as neuronal synapses, can be affected (Maas et al., 2009; Magiera et al., 2018; Sirajuddin, Rice, & Vale, 2014). Tubulin PTMs may also contribute to structural plasticity, regulating dendrite branching and/or the development of dendritic spine protrusions (Dent, 2017; Hu et al., 2011; Jaworski et al., 2009; Yau et al., 2014). In general, the combinatorial use of tubulin isotypes and specific PTMs offers a great potential in fine-tuning of MT and/or MT-dependent functions.

In summary, our study provides an overview of tubulin spatio-temporal expression patterns in brain and may help to address open questions in the microtubule field. A clear limitation is that many available antibodies to study tubulin isotypes are cross-reactive. Although many open questions remain in the microtubule field, future studies should also produce better tools to label and distinguish individual tubulin isotypes in vitro and in vivo.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
T. J. H. and M. K. designed the study. T. J. H., J. R., F. L. L. and P. B. performed experiments. T. J. H., J. R., F. L. L. and P. B. analyzed data. T. J. H. and M. K. wrote the manuscript.

DATA AVAILABILITY STATEMENT
Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author.

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