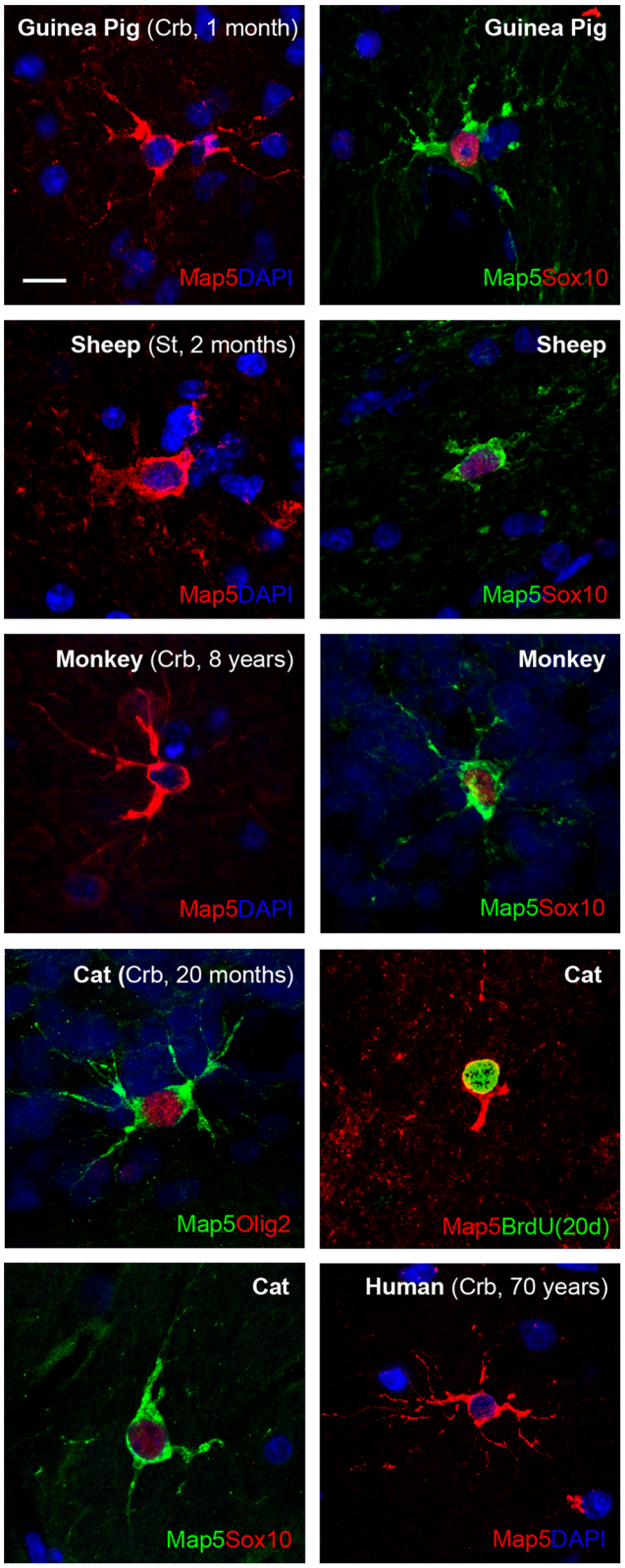

Figure 8. Occurrence of mMap5 in different mammals. Multipolar, Map5+cells showing the same morphology and set of markers found in mouse and rabbit are detectable in various CNS regions of different mammals, including humans. Scale bars: 10 μm.
doi:10.1371/journal.pone.0063258.g008

in neurons, which spans from progenitors of the ventricular zone to fully mature neurons (our data in the adult, but see also [21] in the embryonic ventricular zone), in the glial lineage Map5 is expressed in newly generated cells, but only when they are postmitotic. In other words, although mMap5 cells are not actively proliferating, new mMap5 cells are generated from cycling cells, thus inserting them in a stage-specific state of oligodendroglial-like precursors (deriving from parenchymal progenitors, as confirmed by co-expression of GPR17; see below).

Beside morphological and molecular aspects strongly reminiscent of sinantocytes/polydendrocytes [1,2,5], the mMap5 cells also show different features: their processes are quite smooth, without swelling and varicosities typical of Ng2+cell processes [1]; such difference is qualitative and linked to the cytoskeletal nature of Map5 with respect to the membrane-bound localization of Ng2. Due to the high number of Ng2+cells with complex and overlapping processes, an in vivo quantification of ramifications could be unreliable. In addition, the cytoskeletal protein Map5 could not fill the whole extent of cell process ramifications. For these reasons we chose to quantify the cell somata diameter of both mMap5 and Ng2+cells. Soma diameters of Ng2+cells were prevalently elongated and rather constant in all regions, whereas those belonging to mMap5 cells are more heterogeneous, being prevalently round-shaped in grey matter. As expected, the average soma diameter was not significantly different in the three regions and its mean value (8,9±0,1 μm) is very similar to that recently reported for a subset of premyelinating cells (9,7±0,9 μm; detected in Lucifer yellow-filled cells in PLP-GFP mice [50]). Taken together, these features suggest that mMap5 cells could be elements which derive from parenchymal progenitor cell division, yet being in a non-cycling, more stable and specialized/differentiated stage.

Even though Map5 and Ng2 never co-localize, mMap5 cells may represent a small population of more mature, 'intermediate' cells deriving from Ng2 precursors that have already dowregulated this proteoglycan during their differentiation pathway. Support to this view comes from the expression analysis of GPR17, a membrane G-protein coupled receptor recently deorphanized [76], which has been shown to represent a new Ng2 cell marker [52] and to occur in oligodendrocyte precursor cells [58] as a transiently expressed functional modulator [52,53,77]. In detail, GPR17 has been shown to decorate two distinct differentiation stages of Ng2+cells: an early stage of still proliferating cells expressing GPR17 mainly in the cell body along with other precocious markers (e.g., platelet-derived growth factor receptor alpha, PDGFRα), and a more advanced post-mitotic stage, at which cells are losing Ng2 but GPR17 is maximally upregulated in cell processes [52,53]. Our findings show that GPR17 is also detectable in a proportion of mMap5 cells (Figs. 4 and 5B), thus suggesting that Map5 specifically labels this second transition state (see also below) and is most probably expressed in-between two distinct stages of differentiation (Fig. 5C). GPR17 is upregulated in cells which assume premyelinating features, after having lost Ng2 and having expressed the receptor in the cell processes [53]. Accordingly, we found GPR17 cellular localization in somata and processes of mMap5 cells, which is, again, consistent with a more mature stage.

In order to provide a more complete picture of all newly generated cells in the oligodendroglial lineage, we extended the time-course experiments to the relative markers. In mice injected with BrdU and killed at different survival times, we analyzed and quantified the percentages of co-expression between the proliferation marker and different oligodendroglial cell markers (Fig. 4). Map5 does not colocalize with BrdU prior than 10 days post

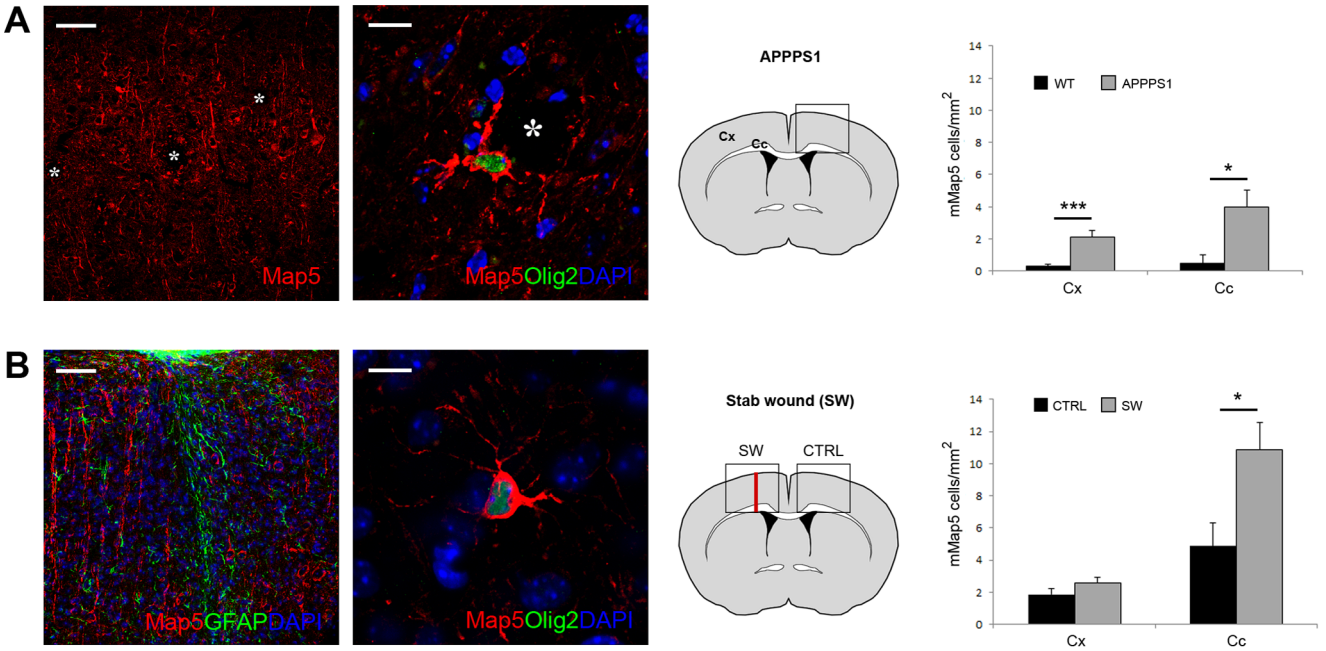

Figure 9. Behavior of mMap5 cells under neurodegenerative and traumatic injury conditions in mice. A, The number of mMap5 is significantly increased in the cerebral cortex (Cx) and corpus callosum (Cc) of APPPS1 trangenic mice. Asterisks, amyloid plaques. B, A slight increase in the amount of mMap5 is detectable after stab wound lesion in the mouse cerebral cortex and corpus callosum. Differences between A and B in the number of mMap5 in WT animals is related to the different ages at which the two lesion models were analysed (12 months for Alzheimer and 3 months for stab wound). Scale bars: Low magnifications, 50 μm; high magnifications, 10 μm.
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injection, thus confirming that at least 1 week is necessary before its expression in newly born mMap5 cells (2 weeks in rabbit, as found in parallel experiments carried out on this species). Starting from the second week, the percentage of Ng2+/BrdU+ double labeled cells decreases in parallel to an increase in the number of BrdU+ mMap5 cells. This trend confirms that new mMap5 cells, although not actively proliferating, are generated from cycling cells (most likely Ng2+progenitors). On the other hand, the increase in percentage of cells co-expressing BrdU and the mature oligodendrocyte marker GST-π detectable at subsequent times, suggests that some mMap5 cells might differentiate into oligodendrocytes. It is important to note that both decrease of Ng2+newly generated cells and increase of mMap5 newly generated cells are far more visible in the white matter (while almost stable in the grey matter; see Fig. 4E). This pattern supports the hypothesis that mMap5 cells are an intermediate stage between Ng2+progenitors and oligodendrocytes in the white matter, yet leaving open possibilities for other roles in the grey matter.

mMap5 cells are reduced in number during adult/old ages but can increase after brain damage

Previous studies indicated that Map5 expression is high during development and at peri-natal stages [11,40], then undergoing an early post-natal reduction to persist at lower levels in the peripuberal and adult CNS (see for example quantitative data on western blots from rat cerebral cortex and spinal cord, in [37]). Here, we confirm that the total amount of Map5 molecule in brain lysates remains substantially unchanged during adulthood, a trend which is clearly due to the prevalent expression of the protein in mature neurons throughout the CNS, as also observed qualitatively in immunocytochemical specimens.

In parallel to the total Map5 expression, as shown by our cell counts, the mMap5 glial cells do represent a very small cell population, even smaller that the NG2+cells (about 1:30; see Figs. 2 and 5). Quantification of the mMap5 cell densities in both mouse and rabbit, revealed that their number decreases dramatically during the peripuberal period (Figs. 6, 7 and 10), at later stages with respect to that observed for neurons, being likely related to the last phases of myelination [78]. Indeed, the drop in mMap5 cells is particularly evident within large white matter tracts, and by comparing the two mammalian species, it starts from absolute values which are higher in mice, then following a similar trend although more diluted in rabbits (Fig. 10B).

Our data indicate that even if the number of mMap5 cells becomes stabilized at low levels at adult/old ages, in some injury/pathology situations their number can increase again (Fig. 10C). This increase seems to be more evident in chronic neurodegenerative states with respect to traumatic events, and, surprisingly, in grey matter with respect to white matter (Fig. 9). In this context, it is interesting to note that while the dramatic decrease observed during postnatal development does occur mainly in white matter (likely linked to the last phases of myelination), the increase detected in APPPS1 mice is higher in the cerebral cortex grey matter. This fact, even taking into account that neurodegenerative lesions are less evident in the white matter, opens the possibility that mMap5 cells could subserve other functions within the lesioned parenchyma. However, the more prominent increase observed in chronic neurodegeneration with respect to acute trauma could be linked to temptative myelinization.

In conclusion, we show that mMap5 cells represent a subset of newly generated cells in the CNS parenchyma. These cells occur in the nervous tissue of most mammalian species, including humans, wherein they can be easily identified with a stage-specific marker, thus allowing further studies on their behavior in different physiological and pathological conditions.

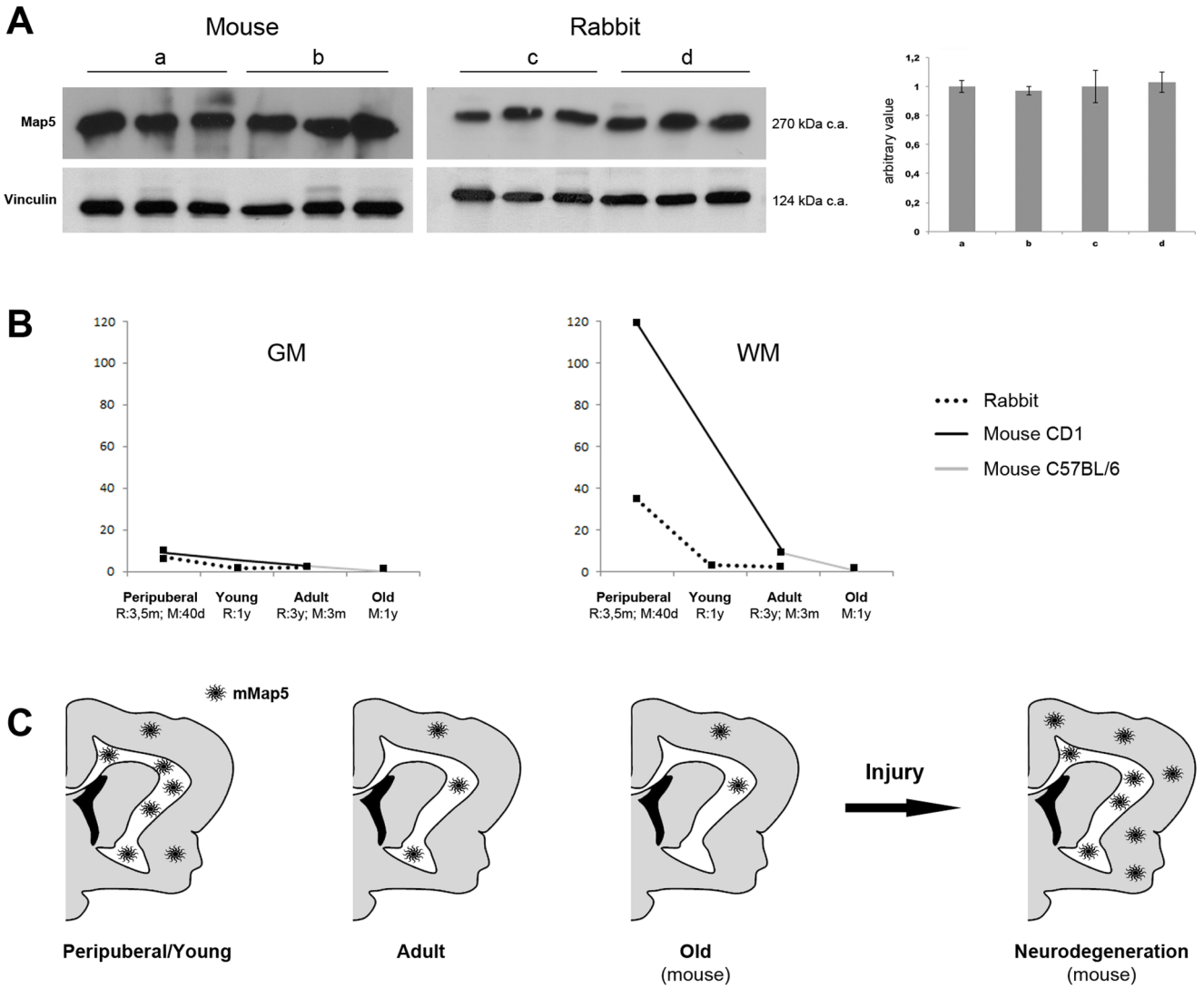

Figure 10. Amount of Map5 molecule and mMap5 cells in the rabbit and mouse CNS at different ages. A, Immunoblot analysis of Map5 (Map1b) expression in different animals at different ages. Autoradiography of the western blot of protein lysate from total brain of mouse 40 days old (a) and 3 months old (b), rabbit 3,5 months old (c) and 1 year old (d), probed with anti-Map5 and anti-vinculin antibodies (3 animals for each condition). On the right, quantitation of the image after normalization with vinculin. The values expressed are a media of the value of the 3 animals for each condition. Values of older animals were expressed relatively to those of young animals of the same species. Level of Map5 in the brain of the older animals does not change significantly with respect to level in the young animals of the same species. B, Trend in the amount of mMap5 cells in the rabbit and mouse CNS at different ages. C, Schematic representation of the data reported in B, and reactivity of mMap5 after injury.
doi:10.1371/journal.pone.0063258.g010

Methods

Animals and tissue preparation

All experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86-609 EU) and the Italian law for the care and use of experimental animals (DL.vo 116/92). Experiments carried out in this study were approved by the Italian Ministry of Health (8 october 2009) and the Bioethical Committee of the University of Turin. Mice and rabbits were raised in the NICO animal facility (authorization n. 182/2010-A). All experiments were designed to minimize the numbers of animals used and their discomfort. Six peripuberal (3,5 months old), three young (1 year old) and three adult (3 years old) female New Zealand White (*Oryctolagus cuniculus*), 3 peripuberal (40 days old) and 12 adult (3 months old) CD-1 mice (*Mus musculus*), were used. In addition, to extend the comparative analysis, some sample tissues of other mammalian species were provided through collaborations with other Institutions. Fixed brain sections were obtained from: one female albino Dunkin-Hartley guinea pig (*Cavia porcellus*, 30 days old) provided by Dpt. of Animal and Human Biology, University of Turin (authorization of the Italian Ministry of Health number 66/99-A); one adult cat (*Felis silvestris catus*, 18 months old) provided by Dept. de Fisiología y Zoología, Sevilla, Spain (Servicio de Animales de Experimentación, Universidad de Córdoba, Spain; approved by the Committee on Bioethics of the València University (R.D. 120/2005 BOE 252/34367-91, 2005); one young female sheep (*Ovis aries*, 2 months old) provided by Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (post-mortem material collected at a local slaughter house: Old Bear S.a.s. Cuneo, Ronchi, Italy); one adult male macaque monkey (*Macaca fascicularis*, 8 years old) provided by Dpt. of Physiology and Pharmacology of the

University of Rome Sapienza, Italy (authorization of the Italian Ministry of Health 27 september 2010; the animal was housed not in single cage but in a large common, enriched environment with other animals, with free access to food and water, in order to minimize any discomfort and suffering; it was sacrificed for the study of cerebral connectivity). An autoptic tissue sample of the human cerebellum belonging to a woman, 70 years old, was kindly provided by Department of Clinical and Biological Sciences, University of Turin, Italy (sample anonymized for analysis, from a tissue bank; procedure approved by the local Ethical Committee - San Luigi Hospital, Orbassano, Italy, n. 191/INT). Three APPPS1 mice (12 months old, raised in the animal house of NICO research center; authorization Italian Ministry of Health, 17 october 2011) were used as a model of chronic damage (chronic amyloid deposition; Radde et al., 2006); we examined three age matched C57BL/6 as wild-type littermates (authorization as for the APPPS1 mice).

Animals were deeply anesthetized (ketamine 100 mg/kg - Ketavet, Bayern, Leverkusen, Germany - and xylazine 33 mg/kg body weight (5 mg/kg in mice) - Rompun; Bayer, Milan, Italy - for rabbit and guinea pig; sodium pentobarbital 50 mg/kg, i.p., for cat; ketamine 5–10 mg/kg, i.m. and metomidine 30 mg/kg, i.m., for monkey) and perfused intracardially (apart from sheep, perfused in the head through the carotid artery after slaughtery) with a heparinized saline solution followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were then postfixed (2 hs, mice; 6 hs, rabbits and guinea pig; overnight, monkey; 48 hs, sheep), cryoprotected with increasing concentration of sucrose/0.1 M PB, till 30%, frozen at −80°C, and cryostat sectioned (40 μm thick). The human cerebellar tissue was fixed in formalin for 1 hr then in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight.

BrdU injections and surgical procedures

Nine mice received one daily injection of BrdU (Sigma-Aldrich; 40 mg/Kg) for 5 consecutive days and then were killed 5 days (n = 3), 10 days (n = 3), 30 days (n = 3), after the last injection. Six rabbits received one daily injection of BrdU for 5 consecutive days and then were killed 5 days (n = 2, peripuberal), 10 days (n = 4; 2 peripuberal, 2 young), after the last injection. The cat received one daily injection of BrdU for 2 consecutive days and was killed 19 days after the last injection.

Stab wound was performed on three 3 months old CD1 mice. Surgical procedures were carried out under deep anesthesia (ketamine, 100 mg/Kg; Ketavet, Bayern, Leverkusen, Germany; xylazine, 5 mg/Kg; Rompun; Bayer, Milan, Italy). A stab-wound in the right cerebral cortex (Bregma from −0.4 mm to −2 mm, laterolateral 1.5 to 2.5 mm) encompassing both gray and white matter was performed, then animals were killed 15 days after lesion. The controlateral part of the cortex was used as control.

Immunohistochemistry

Immunohistochemical reactions were performed on free-floating sections incubated in blocking buffer (5% normal serum, 0.3% Triton X-100 in 0.01 M PBS, pH 7.4) for 1 h at RT, and then incubated for 24–48 h at 4°C in a solution of 0.01 M PBS, pH 7.4, containing 0.1–1% Triton X-100, 2% normal serum and the primary antibodies (Table 2). For BrdU staining, DNA was denatured in 2N HCl, 0.5% Triton X-100 for 30 min at 37°C. Sections were then rinsed in 0.1 M borate buffer, pH 8.5 for 20 minutes. Following primary antisera incubation, sections were incubated with appropriate solutions of secondary AMCA-conjugated (1:200; Jackson ImmunoResearch, West Grove, PA), cyanine 3 (Cy3)-conjugated (1:800; Jackson ImmunoResearch, West Grove, PA), Alexa488-conjugated (1:400; Molecular Probes, Eugene, OR) and dylight 649-conjugated (1:400, Jackson ImmunoResearch, West Grove, PA) antibodies, for 2 hours RT. GD/17 was detected with the high sensitivity Tyramide signal amplification system (Perkin Elmer Life Sciences). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, KPL, Gaithersburg, Maryland USA), mounted with MOWIOL 4-88 (Calbiochem, Lajolla, CA) and examined using an E-800 Nikon microscope (Nikon, Melville, NY) connected to a colour CCD Camera, and a Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany) confocal microscope.

Image processing and data analysis

All images were collected with the confocal microscope. Images were processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA). Only general adjustments to color, contrast, and brightness were made.

Quantitative analysis (cell densities, marker coexpression, soma diameters and cell process lenght) were performed by means of the Neurolucida software (Micro-Brightfield, Colchester,VT), Imaris 7.4.2 software (Bitplane AG, Zurich, Switzerland) or by confocal analysis. Countings has been conducted on 3 to 5 representative coronal (brain) or sagittal (cerebellum) cryostat sections, from each animal. Three animals were analyzed for each time point or experimental condition. For cell densities: the areas of the regions of interest were determined per section using a suitably calibrated Neurolucida-microscope setup. Data are expressed as densities (cell/mm^2) and presented as means ± SEM. For multiple/double staining, the number of inspected cells ranged from 50 to 300 in total / 3 animals (see also S2). For Ki67 and BrdU staining, at least 50 cells in total / 3 animals were analyzed (see also S2). For morphometric analysis (soma diameters, total process lenght), at least 30 cells were randomly chosen in each area analyzed in three animals. We analyzed three different CNS areas: cerebral cortex, corpus callosum and cerebellar cortex. The soma diameter was calculated for each cell by measuring its minimum (min) and maximum extent (max) in two orthogonal directions (Fig. S2B). Average soma diameter was obtained averaging the two values. The total length of mMap5 cell processes was calculated on confocal images processed with Imaris 7.4.2 software, using the "Autopath" filament function, to trace labeled processes. All morphometric data were presented as means± SEM.

Statistical analysis was carried out by the Statistical Package for the Social Science 14.0 (SPSS, Chicago, IL) and included two way ANOVA test followed by Bonferroni's post hoc analysis (to compare mean values) and Student's t test (to compare two groups). $P<0.05$ was considered statistically significant.

Western blots

Animals were sacrificed at 40 days and 3 months (mouse, n = 6), 3,5 months and 1 year (rabbit, n = 6); brains were collected and then immediately homogenized in a glass-Teflon Potter homogenizer in an ice-cold lysis buffer containing 20 mM Hepes, pH 7.5/10 mM KCl/1.5 mM MgCl2/1 mM ethylenediaminetetraacetic acid (EDTA)/1 mM ethylene glycol tetraacetic acid (EGTA)/1 mM DTT/0.5% CHAPS/complete protease inhibitors; Roche Cat. No. 11 697 498 001). The homogenates were centrifuged at 12000 rpm for 15 min at 4°C. Protein concentration was determined using a Bradford assay #23236. Proteins extracts (50 μg) were separated on SDS-PAGE (6% polyacrilamide) and transferred to polyvinylidene difluoride membranes. Then the membranes were blocked in 5% nonfat milk in tris buffered saline (TBS)-T (200 mM Tris and 1.5 M NaCl with 0.1% Tween 20) and were incubated with primary antibody diluted in