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The genetic signature of perineuronal oligodendrocytes reveals their unique phenotype

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Abstract

Oligodendrocytes – best known for assembling central nervous system myelin – can be categorized as precursors, myelin-forming cells and non-myelinating perineuronal cells. Perineuronal oligodendrocytes have been well characterized morphologically and ultrastructurally, but knowledge about their function remains scanty. It has been proposed that perineuronal oligodendrocytes support neurons and, following injury, transform into myelin-synthesizing cells. Recent findings implicating perineuronal oligodendrocytes in cytoarchitectural abnormalities in the prefrontal cortex of schizophrenia and other psychiatric disorders shed new light on these cells. We have obtained the genetic signature of perineuronal oligodendrocytes by identifying gene expression differences between oligodendrocyte subpopulations using cell-specific tags, microarray technology, quantitative time-resolved polymerase chain reaction and bioinformatics tools. We show that perineuronal cells are the progeny of oligodendrocyte progenitors and, hence, are members of the oligodendrocyte lineage. Physiologically they exhibit a novel phenotype. Their expression of PDGFR- $\alpha\beta$ and its growth factor ligand PDGF-CC sets them apart from members of

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. List of transcripts that exhibit a two-fold difference between A2B5⁺/OTMP⁺ and A2B5⁺ cells.

Data S2. List of transcripts that exhibit a two-fold difference between A2B5⁺/OTMP⁺ and O4⁺ cells.

Fig. S1. The polyclonal Ab OTMP identifies perineuronal oligodendrocytes, but does not recognize perineuronal astrocytes or perineuronal microglial cell.

Fig. S2. Developmental distribution of A2B5⁺/OTMP⁺ cells in different brain compartments.

Fig. S3. Participation of sonic hedgehog (Shh) in the A2B5⁺/OTMP⁺ phenotype.

Fig. S4. Molecules implicated in the interaction between the A2B5⁺/OTMP⁺ cell and its extracellular matrix (ECM).

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their lineage as this receptor precludes their response to the same growth factors that act on myelinating cells. Their coordinate expression and context-specific usage of transcription factors Olig2, Ascl1 and Pax6, together with the prominent presence of transcription factors Pea3, Lhx2 and Otx2 – not hitherto linked to the oligodendrocyte lineage – suggested a cell with features that blur the boundary between a neuron and a glial cell. But they also maintain a reservoir of untranslated transcripts encoding major myelin proteins presumably for a demyelinating episode. This first molecular characterization of perineuronal oligodendrocytes revealed the striking difference between the myelinating and non-myelinating phenotypes.

Keywords

cell fate specification; glial cells; oligodendrocyte–neuron interaction; rat; remyelination; signaling; transcription factors

Introduction

Oligodendrocytes (OLGs) are a heterogeneous population of central nervous system (CNS) cells best known for their role as facilitators of fast nerve conduction, which they achieve by surrounding axons with myelin sheaths. The diversity of OLGs was captured by del Rio Hortega (1928) who described four subtypes of myelinating, plus a class of non-myelinating OLGs that either appose neuronal somata (perineuronal) or envelop blood vessels (perivascular).

Oligodendrocytes progenitors (OLPs) have diverse developmental origins. This is true for spinal cord and hindbrain (Cai *et al.*, 2005; Vallstedt *et al.*, 2005), for forebrain (Marshall & Goldman, 2002; Kessaris *et al.*, 2006) and for cortical OLPs (Gorski *et al.*, 2002), establishing the notion that the genetic program of OLPs is heterogeneous. Nonetheless, no functional correlate to each of these subpopulations has been identified. Rather, the view is that they are functionally equivalent when tested for their capacity to make myelin (Richardson *et al.*, 2006). New findings are beginning to uncover the genetic underpinning of the various myelinating subtypes. In this context, it was reported that in the spinal cord, ventrally and dorsally derived progenitors differ in their preference for the type of axons they myelinate (Tripathi *et al.*, 2011).

The issue that every OLP can make myelin may merely reflect the plasticity of these cells, i.e. we may conjecture that even non-myelinating OLGs may, under stress, become myelinating OLGs. Indeed, Ludwin (1979) proposed that perineuronal oligodendrocytes (pN-OLGs) have the potential to remyelinate axons denuded of their myelin. The implication is that pN-OLGs might possess a latent myelinating machinery that can be activated after a demyelinating episode, but is blocked during development as is the case for satellite Schwann cells.

pN-OLGs are well defined morphologically and ultrastructurally; their physiological function, however, remains unknown. Recent findings implicating pN-OLGs in cytoarchitectural abnormalities in the prefrontal cortex of schizophrenia and other psychiatric disorders (Vostrikov *et al.*, 2007; Kim & Webster, 2010, 2011) shed new light

on these cells. The consistent findings that such abnormalities are accompanied by a reduced number of pN-OLGs (Vostrikov *et al.*, 2007) implicate them as players – either on their own or through their support of neurons – during development and homeostasis of the prefrontal cortex.

To provide a genetic framework for a functional investigation of pN-OLGs, and to test the concept that non-myelinating OLGs have the capacity to transform into myelinating cells, we obtained their gene expression profile. We chose pN-OLGs as representatives of non-myelinating cells because we had a polyclonal antibody (Ab), OTMP, that recognizes them. This Ab was generated against a synthetic peptide modeled after the predicted amino acid sequence of a cDNA isolated from an ovine OLG cDNA library (Szuchet *et al.*, 2001). Here we present the gene expression profile of pN-OLGs – defined as $A_2B_5^+$ /OTMP⁺ – relative to an $A_2B_5^+$ OLP and to an O_4^+ OLG. We show that pN-OLGs and O_4^+ cells are progeny of $A_2B_5^+$ precursors. Despite this close lineal relationship, pN-OLGs have orchestrated a genetic program that sets them apart from both $A_2B_5^+$ precursors and O_4^+ OLGs.

This work has unraveled a novel OLG lineage phenotype. This is the first molecular characterization of pN-OLGs. This research should open up new avenues for examining the function of these cells and aid in clarifying their mode of action in psychiatric and demyelinating disorders.

Materials and methods

Immune-purified polyclonal antibody, OTMP

A 20-amino-acid peptide of sheep OTMP (Szuchet *et al.*, 2001) was synthesized, coupled to a carrier and injected into two rabbits (Biosource Inc., Camarillo, CA, USA). Following testing by enzyme-linked immunoassay and immunohistochemistry, the most active batch of serum was purified on a peptide-affinity column.

Immunocytochemistry of cultured live rat oligodendrocytes

$A_2B_5^+$ OLPs purified via fluorescence-activated cell sorting were maintained as such by supplementing the medium with growth factors by way of a conditioned medium from a neuroblastoma (B104) cell line. The cells switch to an OLG phenotype (differentiate) following removal of the conditioned medium. The two phenotypes have characteristic morphologies and express different surface markers. The progenitor is mostly bipolar and is marked by the mAb A_2B_5 (Eisenbarth *et al.*, 1979), whereas the committed OLG is mainly multipolar and is stained by the mAb O_4 (Sommer & Schachner, 1981; Gard & Pfeiffer, 1990). We double stained live cells with either A_2B_5 / OTMP or O_4 /OTMP. For this we used the regular medium containing either mAb A_2B_5 or mAb O_4 (1 : 500) and polyclonal anti-OTMP (1 : 100) and cells were placed back in the incubator for 30 min. Medium was then removed and cultures were washed twice with phosphate-buffered saline (PBS) (Ca^{2+} + Mg^{2+}), fixed with 4% paraformaldehyde in PBS (Ca^{2+} + Mg^{2+}) for 10 min, and washed with PBS 3 × 10 min each. The secondary Abs, goat α -mouse IgM (A_2B_5 or O_4) Alexa-Fluor 488 (green) / goat α -rabbit IgG (OTMP) Alexa-Fluor 594 (red) at 1 : 700 in PBS containing 3% normal goat serum (NGS; Gibco, Long Island, NY, USA) were applied for 2 h before slides

were washed once for 15 min and 4×5 min, then mounted with Vectashield containing diamidino 2-phenylindol-HCl (DAPI) (Vector Labs, Burlingame, CA, USA).

Immunohistochemistry of rodent and human brain

Rat brain—Animals were handled according to NIH guidelines. C57BL / 6 mice or Sprague Dawley rats (Taconic Farms, Germantown, NY, USA) were anesthetized by intraperitoneal injection of xylazine plus ketamine and perfused through the heart with PBS followed by 4% paraformaldehyde in PBS. Brains were removed and sectioned with a vibratome to 15- to 20- μ m sections. Free-floating sections were blocked with 3% NGS in PBS containing 0.1% Triton X-100 for 1.5 h at room temperature. Primary Abs were applied overnight at 4 °C. We used affinity-purified polyclonal rabbit anti-OTMP peptide (1 : 200) in combination with Abs for the identification of: (i) neurons (NeuN, 1 : 500; Chemicon, Temecula, CA, USA); (ii) oligodendrocytes [anti-MBP, (1 : 1000, Covance, Princeton, NJ, USA); CC1 / Ab7 / APC (1 : 350, Oncogene Research Products, Boston, MA, USA), anti-CNP (1 : 500, Sternberger Monoclonals, Lutherville, MD, USA)]; (iii) microglial cells, (Iba1; Wako Chemicals, Richmond, VA, USA); (iv) astrocytes (anti-GFAP, 1 : 1000; Sigma, St. Louis, MO, USA). After three washes with PBS, sections were incubated for 1.5 h with secondary Abs anti-rabbit IgG labeled with Alexa Fluor 594 (1 : 800) and anti-mouse IgG labeled with Alexa Fluor 488 (1 : 800) (Molecular Probes, Eugene, OR, USA). The sections were washed with PBS, mounted onto slides and covered using Vectashield with DAPI. Two types of controls were included: (i) primary Ab was replaced by pre-immune serum; and (ii) primary Ab was omitted. Images were captured with an Olympus fluorescence microscope fitted with a Cool Snap digital camera (Photometrics, Tucson, AZ, USA). Confocal images were taken with a Zeiss LSM 410 laser scanner microscope with a $25 \times$ Plan Neofluor Objective and a $2 \times$ or $3 \times$ zoom (Carl Zeiss, Thornwood, NY, USA). The excitation wavelengths used were 413 nm for DAPI, 488 nm for green fluorescence and 543 nm for red fluorescence.

We utilized a different protocol to demonstrate that perineuronal OLGs are $A_2B_5^+$ /OTMP⁺ cells and to confirm that they carry a selected set of proteins. For this we purchased slides with $2 \times 10 \mu$ m cryosections from P7 rat brains that had been perfused and post-fixed with 4% paraformaldehyde (Zyagen, San Diego, CA, USA). Sections were left to thaw at room temperature for 30 min in a closed slide-mailer and for another 10 min exposed to air. After washing with PBS for 2×2 min, sections were permeabilized with 0.1% Triton X-100 for 5 min and processed as follows. Sections were double and triple stained with different combinations of Abs to identify the proteins and aid in their localization to specific cell types. An A_2B_5 mAb (1 : 50 / 100; gift from the late Dr S. Pfeiffer) was used in combination with NeuN (1 : 500) and OTMP (1 : 100 / 300) or with a polyclonal Ab against CXCR4 (1 : 2000; Abcam, Cambridge, MA, USA) was tested together with OTMP. Secondary Abs were Alexa Fluor 488, 546, 594, 633 and 647 at concentrations from 1 : 600 to 1 : 800, combined appropriately. When enhancement of the signal to noise ratio was deemed necessary, we added a tyramide amplification step (Molecular Probes). Sections were examined with a Leica SP2 laser scanning microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) at the University of Chicago Integrated Microscopy Core Facility and processed with IMAGEJ software.

Human brain—Primary motor cortex samples originated from individuals whose autopsies revealed no CNS disorder. The tissue was supplied by the Department of Forensic Medicine, Semmelweis University Medical School, and was handled in Budapest (Hungary), an activity reviewed by the Office of Human Subjects Research, NIH (OHSR no. 2481). The dissected tissue blocks were fixed in 4% paraformaldehyde for 2 weeks. Free-floating brain sections (15 μ m) were treated with 10% NGS for 1 h to reduce non-specific binding followed by incubation for 48 h at 10 °C with primary Abs diluted in PBS containing 1% NGS –OTMP (1 : 300), NeuN (1 : 600) or CC1 (1 : 400). Immunoreactivity was visualized as described above. Slides were examined with an inverted epifluorescence microscope (Olympus BX51). Images were acquired using a cooled digital camera controlled by Analysis Lab Digital imaging software. The contrast and brightness of the digitally acquired images were adjusted with Adobe Photoshop 5.5.

Fluorescence-activated cell sorting (FACS) of rat oligodendrocytes

Whole brains were dissected from P0, P7 and P14 rats, and the cerebellum and meninges were removed. The brains were kept at 4 °C in wash buffer (Hanks buffer, pH 7.4, 20 mM HEPES, 50 μ g / mL gentamicin and 0.1% bovine serum albumin). Dissociation of brains, cell isolation, labeling and subsequent cell sorting were performed as previously described by Nielsen *et al.* (2006). Cell Quest Acquisition and analysis software was utilized to quantify fluorescence signal intensities and cell numbers in each cell population. Labeled cell suspensions were analysed using a FACSTAR⁺ flow cytometer (Becton Dickinson, Mountain View, CA, USA). Three independent experiments were performed on P7 animals and two independent experiments on P0 and P14 animals.

Microarray and microarray data analysis

We used three biological replicates for the microarray experiments. Each replicate was obtained from one litter of ten P7 rat pups. Ten pups were required to isolate enough RNA from the FACS-fractionated cell populations. Every experiment was run in triplicate. Total RNA was extracted using the RNeasy micro kit (Qiagen, Valencia, CA, USA). The quality of total RNA was assessed with Agilent's Bioanalyzer microchip (Palo Alto, CA, USA). One hundred nanograms of total RNA was amplified following Affymetrix's small sample labeling protocol (vII). The protocol contains two rounds of reverse transcription and *in vitro* transcription with the biotin label being incorporated during the second round of *in vitro* transcription. For the microarray experiments and data analysis, we followed the protocols given by Nielsen *et al.* (2006). The data were normalized employing a per-chip normalization (normalized to the 50th percentile) and per-gene normalization (normalized to the median). A two-fold difference in normalized expression value (up or down) was used to identify differentially regulated transcripts. In addition, a Welch *t*-test was performed without an assumption of equal variances. The Benjamini and Hochberg multiple testing correction was used with a false discovery rate of 0.05. The two-fold cut-off and the statistical test were used to obtain the differentially regulated gene lists (Supporting Information Data S1 and S2). The raw data were deposited in the Gene Expression Omnibus (GEO accession number – GSE11277) accessible at <http://www.ncbi.nlm.nih.gov/geo/> (Barrett *et al.*, 2005). Pathway and network analysis were performed with KEGG (Kanehisa *et al.*, 2006) and Ingenuity (Redwood City, CA, USA).

Validation of microarray data by qRT-PCR

Total RNA from the three biological replicates – each replicate originated from a litter of ten P7 rat pups – of A₂B₅⁺/OTMP⁺ and of A₂B₅⁺ FACS cells used for the microarray experiments were also utilized for the quantitative time-resolved polymerase chain reaction (qRT-PCR). The corresponding cDNAs were obtained according to the protocol provided with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). Because the total amount of cDNA was insufficient to perform the desired number of experiments, we pre-amplified all the cDNAs by following the TaqMan® PreAmp Master Mix protocol (Applied Biosystems). We selected the following rat genes: *Pea3* (rat *Etv4*), *Cldn10*, *Cxcr4*, *Cdh10*, *Ret*, *Sema4*, *Cxcl12*, *Pdgfra*, *Pdgfrβ*, *Egfr*, *Bmpr1*, *Npl1*, *Npl2* and *Ascl* because of their potential relevance to the phenotype of the cells and *β-actin* as the endogenous control. Their expression was determined with the TaqMan® Gene Expression Assay (Applied Biosystems). All six replicate cDNAs were pre-amplified with the same pool containing probes for each of the target genes in a PCR reaction that consisted of a 10-min hold at 95 °C and 14 cycles of 15 s at 95 °C and 4 min at 60 °C. The pre-amplified cDNAs were diluted 20-fold for the qRT-PCR. Each A₂B₅⁺/OTMP⁺ pre-amplified cDNA was paired with one of the pre-amplified A₂B₅⁺ cDNA and run together in a 96-well plate following the protocol provided by the supplier. Each experiment included seven target genes plus an endogenous control, all in quadruplicate. We used the Applied Biosystems 7900HT thermal cycler and their standard program, which consists of 2 min at 50 °C and 10 min at 95 °C, respectively, and 40 cycles of 15 s at 95 °C and 4 min at 60 °C. Data were analysed with the 2^{-C_T} (where C_T is the threshold cycle) method (Livak & Schmittgen, 2001). Briefly, amplified target genes from A₂B₅⁺/OTMP⁺ and A₂B₅⁺ cells were each normalized relative to their respective endogenous controls (i.e. *β-actin*). The fold increase / decrease in gene expression of A₂B₅⁺/OTMP⁺ cells was calculated utilizing the corresponding values of A₂B₅⁺ cells as calibrators. For the calibrator $C_T = 0$ and $2^0 = 1$.

Results

The polyclonal antibody OTMP highlights perineuronal oligodendrocytes

In vitro, the immuno-purified polyclonal Ab, OTMP, co-localizes with mAb A₂B₅ on the surface of cultured live rat OLPs, but not with O₄⁺ committed OLGs (Fig. 1). On rat brain sections, two patterns of OTMP staining can be discerned. One reveals A₂B₅⁺/OTMP⁺ cells with elongated nuclei and single-process morphology (Fig. 2A–H). These cells resemble A₂B₅⁺ cells that bear the CXCL12 chemokine receptor CXCR4 (Fig. 2I–L) – a receptor implicated in guiding the migration of granule cells (Hagihara *et al.*, 2009) and neural progenitor cells in the normal (Lazarini *et al.*, 2003; Ni *et al.*, 2004) and diseased brain (Cayre *et al.*, 2009). The two sister cells shown in Fig. 2M indicate that these cells may still be mitotic. These features are reminiscent of migrating OLPs (Dugas *et al.*, 2006; Cayre *et al.*, 2009). The other pattern displays the OTMP Ab highlighting pN-OLGs in their varied morphologies, as illustrated in Fig. 3A, B, D for rat and Fig. 3C for human cortices. This Ab does not recognize neurons (Fig. 3) or differentiated OLGs (Fig. 4G–I). As both astrocytes and microglial cells may abut neuronal soma (arrowhead in Fig. 3C), although less frequently than OLGs (Polak *et al.*, 1982; Raine, 1997), we wanted to confirm that OTMP does not label perineuronal astrocytes and / or perineuronal microglial cells. We therefore

co-stained rat cortex with either OTMP / GFAP to detect astrocytes (Supporting Information Fig. S1A) or OTMP / Iba1 to identify cortical microglial cells (Supporting Information Fig. S1B). These figures show that there is no co-localization of OTMP with these antibodies. Independently, Takasaki *et al.* (2010) reported that pN-OLGs do not synthesize GFAP or Iba1. Jointly, these results establish the OTMP Ab as a selective marker for pN-OLGs.

OTMP⁺ perineuronal cells are of oligodendrocyte lineage but do not synthesize the major myelin / oligodendrocyte proteins

To investigate the lineage of pN-OLGs, we stained the cortex of a 7-day-old CNP / EGFP transgenic mouse with anti-GFP / OTMP. Because EGFP is driven by the CNP promoter (an OLG protein), the co-localization of these Abs (Fig. 4A–C) defines pN-OLGs as originating from OLPs. This was substantiated by demonstrating that OTMP⁺ pN-OLGs are also A₂B₅⁺ – a marker of OLPs (Fig. 4D–F). Moreover, pN-OLGs carry transcripts for all the major myelin proteins (Table 1). We then asked – are these transcripts translated? Myelin basic protein (MBP) – the best characterized of the OLG / myelin proteins – was not detected immunohistochemically in pN-OLGs at P14 (Fig. 4G–I), a time point when myelinating OLGs have accumulated vast amounts of this protein. This was also the case for a number of other OLG / myelin proteins tested, such as proteolipid protein, CC1 and CNP (data not shown). Collectively, these outcomes portray pN-OLGs to be OLG lineage cells of a non-myelinating phenotype but with the potential to mount a myelinating program. This observation may be of significance for demyelinating diseases such as multiple sclerosis.

A₂B₅⁺/OTMP⁺ cells are scattered throughout the developing brain

To monitor the developmental stages of non-myelinating cells, we co-labeled acutely isolated P7 and P14 rat brain cells with Abs OTMP and A₂B₅. The rationale for a dual label strategy stems from the *in situ* (Fig. 2A–H) and *in vitro* (Fig. 1A–C) evidence that a subpopulation of progenitors has both epitopes. FACS of this subpopulation revealed heterogeneity in the intensity of staining within both the single-labeled as well as the double-labeled cells (Fig. 5). Quantitatively, there were no appreciable changes in the dispersion of these groups between P7 and P14. This suggested that differences in the stages of development are not responsible for the observed heterogeneity. However, because the cells were derived from whole brain preparations, variation in regional compartments could account for cell diversity.

To assess this possibility, we dissected the CNS tissue into spinal cord, brain stem, basal ganglia, corpus callosum and cortex at P0, P7 and P14 and repeated the experiments outlined above. Heterogeneity persisted in every compartment examined, but there were temporal and spatial differences in the distribution of the A₂B₅⁺/OTMP⁺ cells (Supporting Information Fig. S2). Temporally, there was a sizable increase in the number of A₂B₅⁺/OTMP⁺ cells from a scattered few at P0 to an average of 12% at P7 with only a slight change by P14. Regional differences were minimal in the A₂B₅⁺/OTMP⁺ group throughout the brain, but their presence in the spinal cord was markedly low (Supporting Information Fig. S2). Together, these data showed the A₂B₅⁺/OTMP⁺ population to be scattered throughout the brain. It remains to be seen whether A₂B₅⁺/OTMP⁺ is a signature underlying

a broad class of OLG lineage cells with the common denominator being that of belonging to the non-myelinating type or whether it represents solely pN-OLGs.

The $A_2B_5^+$ /OTMP $^+$ cell depicts a unique oligodendrocyte lineage phenotype

There is reliable experimental evidence that OLPs originate from various sites and migrate throughout the CNS (Marshall & Goldman, 2002; Cai *et al.*, 2005; Miller, 2005; Vallstedt *et al.*, 2005; Kessaris *et al.*, 2006). However, what is still an open question is whether they constitute distinct subpopulations. Functionally, there are at least three OLG subgroups: precursor, myelinating and non-myelinating. Our discovery of OTMP as a tag for non-myelinating cells allowed us to compare the molecular features of each of these OLG subpopulations. We employed the Affymetrix microarray technology to identify gene expression patterns for cells recognized by the molecular markers $A_2B_5^+$, $A_2B_5^+$ /OTMP $^+$, O_4^+ and OTMP $^+$. A two-fold difference in normalized expression value (up or down) was used to identify differentially regulated transcripts. In addition, a Welch *t*-test was performed without an assumption of equal variances and the Benjamini and Hochberg multiple testing correction was applied with a false discovery rate of 0.05. We chose the P7 stage of development because it still contains progenitors while myelination has also begun. Hence, P7 rat brain dissociates were labeled with each of these four markers and were individually purified by preparative FACS. Total RNA was isolated from each of the purified fractions and processed as described in the Methods.

Principal components analysis on gene expression data disclosed a sharp demarcation among the four populations. Notably, the OTMP Ab identified a subpopulation of $A_2B_5^+$ precursors with a distinct phenotype (Fig. 6A). The transition from an $A_2B_5^+$ precursor to an O_4^+ -committed OLG was analysed by Nielsen *et al.* (2006), who showed that the O_4^+ cell completely reorganizes its metabolism to assemble myelin. To bring to light the genetic signature of the $A_2B_5^+$ /OTMP $^+$ phenotype, we analysed its gene expression profile relative to gene expression patterns of both the $A_2B_5^+$ precursor and the O_4^+ -committed cell, using bioinformatics tools. Two general gene expression profiles describe the $A_2B_5^+$ /OTMP $^+$ phenotype. In one, a cluster of genes exhibit higher levels of expression in the $A_2B_5^+$ /OTMP $^+$ cell compared with either $A_2B_5^+$ or O_4^+ cells (Fig. 6B). The other displays a shallow positive slope between $A_2B_5^+$ and $A_2B_5^+$ /OTMP $^+$, but a steep declining slope from $A_2B_5^+$ /OTMP $^+$ to O_4^+ (Fig. 6C). Perineuronal OLGs are representatives of the $A_2B_5^+$ /OTMP $^+$ population by virtue of carrying the A_2B_5 and OTMP epitopes (Fig. 4D–F) and, as such, can now be defined genetically as cells of a non-myelinating $A_2B_5^+$ /OTMP $^+$ phenotype. Here we identify genes presumed to be crucial to the biology of the $A_2B_5^+$ /OTMP $^+$ cells.

Quantitative time-resolved polymerase chain reaction

We used qRT-PCR to set the microarray data on a sound quantitative scale and to underline genes whose transcription might represent a turning point in the fate of an $A_2B_5^+$ progenitor to acquire an $A_2B_5^+$ /OTMP $^+$ non-myelinating phenotype or an O_4^+ myelinating phenotype. With this goal in mind, we selected 14 genes plus β -actin as the endogenous control that would not only validate the expression patterns illustrated in Fig. 6B and C, but could also foretell function. Figure 7 shows the qRT-PCR data analysed by the 2^{-CT} method where

the $A_2B_5^+$ cell is taken as the calibrator, i.e. $C_T = 0$ and $2^0 = 1$ (Livak & Schmittgen, 2001).

What guided our choice of the genes presented in Fig. 7? The transcription factor *Pea3* was an obvious candidate for at least two reasons. First, it has not hitherto been associated with the OLG lineage. Second, receptors and adhesion molecules such as RET, SEMA3E and CDH8 –functionally linked to PEA3 (Livet *et al.*, 2002; Cowden Dahl *et al.*, 2007) – are also enriched in $A_2B_5^+/OTMP^+$ cells. This is illustrated in Fig. 7. Not only are *Pea3* (*Etv4*) transcripts four-fold higher in $A_2B_5^+/OTMP^+$ cells relative to the $A_2B_5^+$ progenitors, but so are transcripts for *Ret*, *Cad10* and *Sema4B* (family members of *Cad8* and *Sema3E*, respectively). Their presence suggests a pivotal role for PEA3 in the biology of the non-myelinating phenotype. *Cldn10* was singled out because its isoform, *Cldn11*, has been heralded as an OLG-specific protein (Morita *et al.*, 1999; Dugas *et al.*, 2006; Nielsen *et al.*, 2006). Indeed, it appears necessary for normal CNS function (Gow *et al.*, 1999). Significantly, *Cldn11* was not detected in $A_2B_5^+/OTMP^+$ cells, while *Cldn10* is shown to have a solid presence (Fig. 7A). The PDGFR α has long been acknowledged as crucial to the events that guide an OLP towards the myelinating phenotype. This fact, and the microarray detection of *Pdgfra* and *Pdgfrb* transcripts in the $A_2B_5^+/OTMP^+$ cells (Supporting Information Data S1 and S2), pointed to PDGFR α as a potential fate-determining candidate for these cells. Figure 7B affirms the micro-array results by revealing a > 3-fold increase in *Pdgfra* and *Pdgfrb* transcripts as OLPs transition to the $A_2B_5^+/OTMP^+$ non-myelinating phenotype. The microarray data indicated high levels of *Egfr* transcripts in the $A_2B_5^+/OTMP^+$ cells; given that this receptor has been implicated in signaling myelination or remyelination by OLGs (Aguirre *et al.*, 2007), it was important to quantify changes in its expression in these cells (Fig. 7B).

The selection of the monogamous *Cxcr4* and its chemokine ligand, *Cxcl12* (Fig. 7B), stems from their implication in cell migration (Lu *et al.*, 2001; Schmucker & Zipursky, 2001; Dziembowska *et al.*, 2005; Hagihara *et al.*, 2009). The receptors *Npl1* and 2 were added because of their relevance to semaphorin signaling. The microarray data revealed richness in extracellular matrix (ECM) transcripts and their cognate receptors. As bone morphogenetic proteins (BMPs) were prominent among the ECM molecules, we wanted to verify whether its receptor, *Bmpr1*, was also upregulated. Figure 7B attests this to be the case. Finally, *Ascl1* was included as a representative of a number of transcription factors important in the OLG lineage.

The values depicted in Fig. 7 match those predicted by the microarray experiments, thus providing a measure of authenticity to the latter (Supporting Information Data S1 and S2). Furthermore, the two general patterns of gene expression deduced from transcripts obtained by the microarray technology (Fig. 6B and C) are also confirmed in Fig. 7. Most of the transcripts shown in Fig. 7 fit the pattern illustrated in Fig. 6B, i.e. they are upregulated. This is not only of significance because it reveals an abrupt and sharp divide between $A_2B_5^+$ and $A_2B_5^+/OTMP^+$ cells but, perhaps more importantly, it bestows functional relevance on these genes in the implementation of the $A_2B_5^+/OTMP^+$ non-myelinating phenotype. The transcription factor *Ascl1* and the receptor *Npl1* (Fig. 7B) follow the trend outlined in Fig. 6C.

Transcription factors expressed by A₂B₅⁺/OTMP⁺ cells

Transcription factors are the ultimate determinants of cell identity. We compared the A₂B₅⁺/OTMP⁺ transcriptome database with that of the A₂B₅⁺ progenitor or the committed O₄⁺ OLG singling out transcription factors that had significant differences in their level of expression in the three phenotypes. Interestingly, we could separate these transcription factors into two categories. In one set, we assembled transcription factors such as DLX1 / 2, OLIG1, OLIG2, SOX10 and ASCL1, known to play critical roles in the acquisition of the myelinating fate. In the other, we grouped transcription factors PEA3 (aka ETV4), LHX2 and OTX2 – not previously linked to the OLG lineage, but known to be essential for motor neurons and Schwann cells.

The transcripts for *Dlx1* / 2, *Olig1*, *Olig2*, *Sox10* and *Ascl1* – all relevant to the process of driving OLPs toward myelin-forming OLGs – reveal an interesting pattern of expression – they vary little during the switch from the A₂B₅⁺ progenitor to the A₂B₅⁺/OTMP⁺ cells, but undergo drastic changes when acquiring O₄ (Fig. 6D). Petryniak *et al.* (2007) investigated the combinatorial expression and function of DLX1 / 2, OLIG2 and ASCL1 in the embryonic ventral telencephalon and proposed distinct roles for each of them in regulating the transition of OLPs toward myelinating OLGs. When our results (Fig. 6D) were analysed in light of this report, it became apparent that the interplay among these transcription factors in A₂B₅⁺/OTMP⁺ cells had a different context. Thus, while the high level of *Dlx1* transcripts found in A₂B₅⁺/OTMP⁺ cells (Fig. 6D) agrees with its presumed role as an inhibitor of the myelinating phenotype, the amount of *Olig2* stays invariant in the three cell types (data not shown). Similarly, the drop of *Ascl1* transcripts in O₄⁺ cells compared with A₂B₅⁺/OTMP⁺ cells (Fig. 6D) does not fit its presumed role as a promoter of OLG differentiation. Moreover, *Pax6* is 10-fold higher in A₂B₅⁺/OTMP⁺ than in O₄⁺ cells (Supporting Information Data S2). In contrast, *Olig1* and *Sox10*, whose transcripts are increased approximately three-fold and > 30-fold, respectively, in O₄⁺ cells (Fig. 6D) conform to their accepted role in determining the myelinating phenotype.

Considering the other transcription factors, the PEA3 group (ERM, PEA3 and ETV1) is a member of the Ets domain of transcriptional regulators. In specific motor neuron pools, PEA3 controls central position and terminal arborization – a crucial step in the assembly of neuronal circuits (Livet *et al.*, 2002). We observed a 52-fold increase in *Pea3* mRNA expression in A₂B₅⁺/OTMP⁺ compared with O₄⁺ cells (Supporting Information Data S2). This suggested *Pea3* as being a beacon of the non-myelinating phenotype. PEA3 shares this status with another family member, ERM, which is expressed in peripheral satellite glia but not in myelinating Schwann cells (Hagedorn *et al.*, 2000). In motor neurons, the tyrosine receptor kinase, RET, the type II intercellular adhesion molecule cadherin-8 (CDH8) and semaphorin-3E (SEMA3E) – a secreted guidance protein – are closely associated with PEA3 function. Significantly, A₂B₅⁺/OTMP⁺ cells transcribe *Ret*, *Cdh10* (also a type II cadherin) and *Sema4B*, a transmembrane semaphorin (Fig. 7A; Supporting Information Data S2). In which way – if any – do these proteins contribute to the function of PEA3 in A₂B₅⁺/OTMP⁺ cells is an intriguing question for future research to address.

The homeodomain transcription factors, LIM-HD, are a family of genes, which apart from the DNA-binding homeodomain, carry two copies of a specialized zinc-finger motif called the LIM domain that mediates protein–protein interaction. In the case of neurons, it is the combinatorial expression of these genes, referred to as the LIM code, that specifies subclasses of motor neurons (Shirasaki & Pfaff, 2002). $A_2B_5^+/OTMP^+$ cells express a single member of the Apterous group, LHX2, and one of its selective binding factors (Supporting Information Data S2). It was reported that LIM-domain-associated factors confer transcriptional synergism between LIM-HD and OTX2 (Bach *et al.*, 1997). Intriguingly, *Otx2* has as high a presence in $A_2B_5^+/OTMP^+$ cells as *Lhx2* (Supporting Information Data S2). These two transcription factors have important functions during development. Of potential relevance here are the reports that LHX2 is involved in selective cell recognition by controlling cell adhesion (Hobert & Westphal, 2000) and that OTX2 regulates NCAM (Nguyen Ba-Charvet *et al.*, 1999). Whereas *Ncam* was not found in $A_2B_5^+/OTMP^+$ cells, two family members, *L1cam* and *Icam*, are highly represented (Supporting Information Data S2).

Signaling pathways that may be determinants in specifying the $A_2B_5^+/OTMP^+$ cell

To illuminate our understanding of the cellular processes that guide an A_2B_5 precursor towards either a non-myelinating ($A_2B_5^+/OTMP^+$) or a myelinating (O_4) phenotype, we started by comparing, in pairs (i.e. $A_2B_5^+/OTMP^+/A_2B_5^+$ or $A_2B_5^+/OTMP^+/O_4^+$), the expression profiles of molecules known to play crucial roles in patterning the CNS. Sonic hedgehog (Shh) and Wingless (Wnt) fall into this category. There is a large body of experimental evidence demonstrating the vital importance of these signaling molecules in specifying oligodendrogenesis (Nery *et al.*, 2001; Murray *et al.*, 2002; Agius *et al.*, 2004; Kasai *et al.*, 2005; Shimizu *et al.*, 2005), but the full range of their action has yet to be unraveled (Fancy *et al.*, 2009). However, the core molecular players have been identified. We used this knowledge to assess whether these morphogens have any part in specifying the $A_2B_5^+/OTMP^+$ cell. Shh is required for the expression of *Pdgfra* (Nery *et al.*, 2001), considered to be one of the earliest markers for OLPs. Significantly, we found a > 3-fold increase in *Pdgfra* transcripts in the $A_2B_5^+/OTMP^+$ relative to $A_2B_5^+$ OLPs (Fig. 7B) and > 7-fold when compared with O_4^+ cells (Supporting Information Data S2) with concomitant high expression of Shh receptors and downstream effectors (Supporting Information Fig. S3). But of greater biological relevance is our observation that $A_2B_5^+/OTMP^+$ cells use this receptor in a cell-specific fashion. In addition to *Pdgfra* (Fig. 7B), the cells transcribe *Pdgfr β* (Fig. 7B), and the growth factor *Pdgfc* – a high-affinity ligand for PDGFR $\alpha\beta$ and a strong mitogen (Reigstad *et al.*, 2005). We have employed a well-characterized interacting network to compare the Shh signaling pathways between the non-myelinating $A_2B_5^+/OTMP^+$ cells and the myelinating O_4^+ cells. Figure 8A depicts the differences. Implied in Fig. 8A is that the binding of secreted PDGFCC to the α - and β -PDGFRs affects their association into the PDGFR $\alpha\beta$ heterodimer, thus silencing the response of $A_2B_5^+/OTMP^+$ cells to PDGFAA – a ligand for the myelinating phenotype. Tacitly, this may well be one of the $A_2B_5^+/OTMP^+$ fate-specifying events. Use of the growth factor PDGFC creates an autocrine loop that serves not only to control receptor activity but also cell numbers. Although the molecular mechanism underlying these interactions remains to be solved, the

interactions *per se* can be viewed as a step toward establishing the $A_2B_5^+/OTMP^+$ phenotype.

Turning to Wnt, it is perhaps one of the most versatile morphogens, regulating as it does gene expression, cell fate, cell adhesion and cell polarity. All three vertebrate Wnt pathways use the frizzled (Fzd) receptor but the adaptor proteins vary (Schambony *et al.*, 2004; Cadigan & Liu, 2006). The Wnt / β -catenin pathway directs the sequential onset of neurogenesis and gliogenesis (Kasai *et al.*, 2005), and prevents the differentiation of OLPs (Shimizu *et al.*, 2005). To appreciate how critical Wnt signaling must be to the biology of $A_2B_5^+/OTMP^+$ cells, it suffices to note that the *Fzd2* transcript is 14-fold higher in $A_2B_5^+/OTMP^+$ than in O_4^+ cells (Supporting Information Data S2). To derive a cell-specific role for Wnt signaling, we analysed the gene expression profiles of $A_2B_5^+/OTMP^+$ cells vs. O_4^+ cells using interactive networks. Setting aside the well-known gene repertoire controlled by Wnt – all of which are enhanced in $A_2B_5^+/OTMP^+$ cells (Fig. 8B) – one protein, NrCAM, appeared as a likely downstream target of Wnt signaling (Fig. 8B) because of the known LEF1 binding to the *Nrcam* promoter (Conacci-Sorrell *et al.*, 2002). NrCAM – an adhesion molecule – is found in neurons and Schwann cells, but not in myelinating OLGs. Uncovering the function of NrCAM in pN-OLGs is worth pursuing further.

As illustrated in Fig. 3, pN-OLGs either appose neuronal somata or contact them through their processes. Hence, it was relevant to investigate signaling pathways that are transduced by direct cell to cell contact. The Notch and the Eph / ephrin pair act in this manner. In the fly, Notch inhibits differentiation by lateral signaling and controls cell fate by inductive interactions (Louvi & Artavanis-Tsakonas, 2006). In vertebrates, it may additionally diversify progenitor populations (Yoon & Gaiano, 2005). The network shown in Fig. 9A reveals three distinguishing aspects of Notch signaling in $A_2B_5^+/OTMP^+$ cells. First, we note a transcriptional activation of the transcription factor *Ascl1* (Fig. 9A). During neurogenesis, there is a tight cross-regulation between Notch and the two transcription factors, ASCL1 and DLX1 / 2, which results in sequential specification of progenitors (Yun *et al.*, 2002). Whether or how these factors specify the $A_2B_5^+/OTMP^+$ cell remains unknown. Nonetheless, as pointed out before, our results support the idea that in $A_2B_5^+/OTMP^+$ cells these two transcription factors, together with OLIG2, engage in a context-specific interplay that differs from their behavior in OLGs (Fig. 6D). Second, we observe the upregulation of *Hes5* (Fig. 9A), a Notch target and an inhibitor of myelin gene expression. In pro-myelinating OLGs, HES5 acts by controlling two pivotal genes, *Ascl1* and *Sox10*; it does this by different mechanisms (Liu *et al.*, 2006). In the $A_2B_5^+/OTMP^+$ cells, *Sox10* transcripts are expressed at a very low level, *Hes5* is 13-fold higher and, unexpectedly, *Ascl1* is approximately six-fold higher than in O_4^+ cells (Fig. 6D; Supporting Information Data S2). On the premise that HES5 is responsible for keeping myelin transcripts at bay in $A_2B_5^+/OTMP^+$ cells, it follows from these results that its transcriptional code has to be tailored for the non-myelinating phenotype. There is a large body of research demonstrating the importance of the so-called epigenetic regulators in defining cell identity (Liu *et al.*, 2007). In this context, it is interesting that the genes *Smarca2* and *Smarca3* that encode ATP-dependent chromatin remodeling proteins have increased expression in $A_2B_5^+/OTMP^+$ relative to O_4^+ cells and might possibly be involved in HES5 action (Fig. 9A).

Notch1 appears to be implicated in the augmented transcription of lipocalin-prostaglandin D2 synthase (L-PTGDS) in $A_2B_5^+/OTMP^+$ cells (Fig. 9A). This is puzzling as Notch1 has been shown to decrease PTGDS expression (Fujimori *et al.*, 2005), but the $A_2B_5^+/OTMP^+$ cells reveal high levels of both Notch1 and PTGDS transcripts. Indeed, the differentiation of the $A_2B_5^+$ progenitor into $A_2B_5^+/OTMP^+$ cells results in an order of magnitude higher transcription of L-PTGDS (Supporting Information Data S1) as well as synthesis of the protein (Fig. 9A, inset).

The Eph receptors and the ephrins are membrane tyrosine kinases. Their binding and subsequent clustering transduce bidirectional signals that regulate cell migration, adhesion or repulsion (Poliakov *et al.*, 2004; Pasquale, 2005; Goldshmit *et al.*, 2006). Analysis of the $A_2B_5^+/OTMP^+$ gene expression profile utilizing the Eph / ephrin canonical pathway revealed a selected transcription of the *ephA2*, 4 and 5 class of receptors and class B3 *ephrins* in the $A_2B_5^+/OTMP^+$ cells (Fig. 9B). As their interaction is mostly between members of the same class, this allows the $A_2B_5^+/OTMP^+$ cells to be receivers of either forward or reverse signaling. That the latter might be of physiological significance for these cells can be surmised both from the expression of the G protein-coupled *Cxcr4* receptor and its ligand *Cxcl12* (Fig. 7A) and from evidence of their participation, together with ephrin-B, in controlling cell migration (Lu *et al.*, 2001; Schmucker & Zipursky, 2001). One attractive feature of the Eph / ephrin signaling is that it fosters cell to cell attachment and detachment. The action is not strictly reversible as cell separation requires endocytosis or proteolysis. Nevertheless, it does provide flexibility for cells to engage or disengage when circumstances demand it.

The $A_2B_5^+/OTMP^+$ cell amasses a complex ECM

Examination of the $A_2B_5^+/OTMP^+$ transcripts reveals an unusual richness of adhesion molecules, receptors and ECM molecules. Their levels of expression range from > 3 to > 7 compared with either $A_2B_5^+$ or O_4^+ cells. It is reasonable to assume that such a metabolic investment signifies that the corresponding proteins were synthesized. The importance of ECM molecules in modulating signaling pathways in glial cells is well documented (Taveggia *et al.*, 2010). The novel aspect is that – not unlike Schwann cells – $A_2B_5^+/OTMP^+$ accumulate, extracellularly, collagens, including the unusual Col18A1 (an HSPG), nidogen and reelin. This accumulation was measured relative to the $A_2B_5^+$ precursor (Supporting Information Fig. S4). The stage in the transition from one phenotype to the other at which these ECM changes take place remains to be defined. Members of the BMP family, their receptors (Fig. 7B), the inhibitor noggin and the receptor endoglin are also upregulated (Supporting Information Data S1). It is the interplay and fine tuning of these molecules together with other morphogens that, ultimately, demarcate cell fate (Yung *et al.*, 2002; Hall & Miller, 2004).

Discussion

In this work we sought to obtain the genetic signature of pN-OLGs – defined as $A_2B_5^+/OTMP^+$ cells – by tabulating only those genes that differed significantly (> 2 -fold up or down) from either the $A_2B_5^+$ OLP or the O_4^+ OLG. The outcome revealed an OLG lineage

cell with a novel phenotype (Fig. 6). This phenotype highlights a cell with molecular characteristics not previously seen in the OLG lineage. By adopting the PDGFR $\alpha\beta$ and its high-affinity ligand, PDGFCC (Figs 7B and 8A), the A2B5⁺/OTMP⁺ cell has effectively separated itself from the other members of its lineage. But the most distinguishing feature of this phenotype is that it has amalgamated genes pertaining to both neuronal and Schwann cell lineages. Use of the Ets domain transcription regulator, PEA3, mimics a class of motor neurons as well as satellite Schwann cells (Hagedorn *et al.*, 2000; Livet *et al.*, 2002; Tran *et al.*, 2007). Likewise, the secretion of fibrous collagens, nidogen (Supporting Information Fig. S4) and the expression of NrCAM (Fig. 8B) by A2B5⁺/OTMP⁺ cells are atypical for OLG lineages but are part of the Schwann cell repertoire. Notwithstanding these similarities, pN-OLGs and satellite Schwann cells function in a distinctive fashion (Court *et al.*, 2006). We interpret these results as indicating that pN-OLGs have developed a code for communicating with neurons that differs significantly from the one used by myelinating OLGs.

A₂B₅⁺/OTMP⁺ cells have evolved a unique set of transcriptional codes

The timing of differentiation of specific cell lineages is a highly complex event resulting from, as yet, little known interactions of genetic and epigenetic factors (Liu *et al.*, 2007). What has come to light is that stage- and region-specific cell fate genesis is controlled by a dynamic pattern of co-expression of different transcription factors, co-factors, inhibitors and accessory molecules that together constitute a lineage-specific transcriptional code (Gokhan *et al.*, 2005; Battiste *et al.*, 2007; Sugimori *et al.*, 2007). During development, it is the action of these transcriptional codes that dictates and controls the spatiotemporal differentiation of distinct cell populations. For example, it was shown that interaction between the so-called 'patterning factors' OLIG2, MASH1 and PAX6 (Sugimori *et al.*, 2007) can direct the fate of a cell to become a TuJ1⁺ neuron (PAX6⁺/MASH1⁺) or an O₄⁺ OLG (OLIG2⁺/MASH1⁺). Against this backdrop, our identification of an OLG lineage cell where these transcription factors, i.e. PAX6, MASH1 and OLIG 2 are coordinately expressed and combined to transmit a novel code, highlights two important issues. First, it calls attention to our scant understanding of the factors that determine the fate of OLG subtypes. Second, it stresses the need to acknowledge that OLGs are phenotypically heterogeneous.

The other group of transcription factors comprising PEA3, LHX2 and OTX2 – adapted from neuronal cell lineages, and expressed by orders of magnitude higher in the A2B5⁺/OTMP⁺ cell than in the O₄⁺ cell – have not hitherto been associated with the OLG lineage. In motor neurons, PEA3 has been shown to be tightly controlled spatially and temporally (Wang & Scott, 2007). The glial cell-derived neurotrophic factor (GDNF), from target cells, emerged as the signal required for the precise induction of PEA3 in neurons (Haase *et al.*, 2002). *In vitro*, ERM expression by satellite Schwann cells requires a constant input of the neuregulin1 isoform GGF2 (Hagedorn *et al.*, 2000). While the PEA3 trigger, its origin and time of action in A2B5⁺/OTMP⁺ cells remain unknown, it is noteworthy that molecules such as the receptor kinase RET, CDH10 and SEMA4B (Fig. 7A) – seemingly critical to PEA3 function in motor neurons – are highly transcribed by these cells. Whether and how they are used by A2B5⁺/OTMP⁺ cells remains an intriguing question.

Transcripts of the adhesion molecule NrCAM are upregulated in $A_2B_5^+/OTMP^+$ cells (Fig. 9B). Because the *Nrcam* promoter is a target of LEF-1 (Conacci-Sorrell *et al.*, 2002) and because PEA3 can synergize with β -catenin-LEF1 to activate transcription (El-Tanani *et al.*, 2004), it may be envisioned as a participant in *Nrcam* transcription. Interestingly, $A_2B_5^+/OTMP^+$ cells have upregulated transcripts, relative to the other members of its lineage, of plexin A2 and D1 as well as neuropilins (Fig. 7B) – mediators of signals from secreted SEMA3A-G. It is the interaction between SEMA3A-G, neuropilin, plexin and NrCAM or L1CAM that results in the assembly and activation of a holoreceptor complex whose composition dictates how guidance signals are integrated with the promotion of cellular outgrowth, cell attachment and migration (Tran *et al.*, 2007). The presence of all the necessary components to form a holoreceptor argue in favor of a hypothesis that $A_2B_5^+/OTMP^+$ cells are programmed to receive and transduce cues from SEMA3A-G. If this hypothesis were to be confirmed, it would position PEA3 as having a role in cell guidance.

Another potential function of PEA3 in $A_2B_5^+/OTMP^+$ cells can be inferred from the observation that it is a target of the EGFR, which promotes its nuclear localization and binding to the *Mmp14* endogenous promoter, thereby eliciting a migratory and invasive phenotype (Cowden Dahl *et al.*, 2007). Significantly, transcripts for *Egfr* and *Mmp14* are 32- and approximately seven-fold higher, respectively, in $A_2B_5^+/OTMP^+$ cells than in the O_4^+ cells (Supporting Information Data S2), indicating that PEA3 may be involved in the upregulation of *Mmp14*. Jointly, these data support the concept that PEA3 may occupy a commanding role in the events that guide the migration, process extension and interaction of $A_2B_5^+/OTMP^+$ cells with neuronal somata. This is a fertile area for future research.

The prominent presence of these transcription factors, their effectors and accessory molecules confer distinguishing characteristics on the $A_2B_5^+/OTMP^+$ cell. Uncovering their combinatorial codes would illuminate the mechanism whereby the non-myelinating phenotype is created.

Oligodendrocyte lineage progression

The embryonic and early postnatal $A_2B_5^+$ cell is highly heterogeneous and generates a varied progeny (Gregori *et al.*, 2002; Strathmann *et al.*, 2007). $A_2B_5^+$ cells – long-accepted as OLG progenitors – have been shown to be a necessary intermediate in the path towards a myelinating phenotype (Baracska *et al.*, 2007). But here we demonstrate that such cells can also take a different route and become $A_2B_5^+/OTMP^+$ cells, i.e. non-myelinating. Given that the $A_2B_5^+$ cell is the founder of both phenotypes, the challenge remains to discover at what stage of development they emerge. Is lineage progression linear or branched? And, more importantly, what triggers the signal? Is it cell-autonomous or does it depend on an extracellular induction?

Which are the critical players that set in motion the segregation and shaping of oligodendrocyte phenotypes?

A comparative analysis of gene expression profiles of different CNS cell types (Dugas *et al.*, 2006; Cahoy *et al.*, 2008; Nielsen *et al.*, 2006) highlights genes critical to the segregation and shaping of the various phenotypes. In this context, and with emphasis on the divergence

between the myelinating and non-myelinating phenotypes, we noted several mechanisms at play. One takes advantage of protein isomers to achieve functional diversity, as exemplified by the usage of PDGFR $\alpha\beta$ and CLDN10 by the non-myelinating cell and PDGFR $\alpha\alpha$ and CLDN11 by the myelinating one. Another consists of adopting a novel set of genes. Noteworthy examples of the non-myelinating phenotype are the transcription factors PEA3, OTX2 and LHX2, the adhesion molecules NrCAM and CDH10 and the receptor kinase RET. A third way for cells to establish identity is by transcriptional upregulation of specific genes. Representative examples are the *Egfr* and the transcription factor *Hes5* for the non-myelinating cells and the set of myelin-specific genes for the myelinating cells. Lastly, there is the enhancement of specific signaling pathways; prominent among them are Shh (Fig. 8A), Wnt (Fig. 8B) and Notch (Fig. 9A). The challenge is to discover how these and all other genes and pathways come together to generate a cell that preserves many of the characteristics of its progenitor (Fig. 6C), presumably to allow it to revert and assume a myelinating role when it is needed, but is also programmed to exert a physiological function of its own.

The function of perineuronal oligodendrocytes

A long-standing challenge has been to define the function of pN-OLGs. It has been reported that, after injury, pN-OLGs protect themselves and neurons from apoptosis by upregulating L-PTGDS (Taniike *et al.*, 2002). L-PTGDS has a restricted distribution and a dual function (Urade *et al.*, 1993). As an enzyme, it catalyses the conversion of prostaglandin H₂ to D₂, but as a lipocalin, it is a transporter of hydrophobic molecules. In brain cell lines, L-PTGDS is activated by protein kinase C through a mechanism of de-repression of NOTCH-HES signaling (Fujimori *et al.*, 2005). As shown (Fig. 9A, inset), L-PTGDS is synthesized by pN-OLGs but its role in these cells is still unknown. The work by Yamazaki *et al.* (2005) showing a bidirectional interaction between perineuronal astrocytes and neurons in the CA1 region of the rat hippocampus raises the intriguing possibility that pN-OLGs may engage in a similar activity. Indeed, synaptic signaling between GABAergic interneurons and OLPs has been reported by Lin & Bergles (2004). There is a growing number of reports on synaptic contacts between NG2⁺ cells and both myelinated and unmyelinated axons as well as cell to cell contacts in different parts of the brain (Paukert & Bergles, 2006; Trotter *et al.*, 2010). However pN-OLGs differ from NG2⁺ cells in more than one way. First, the NG2 proteoglycan is not detected on their surface (Takasaki *et al.*, 2010), and second they use the PDGFR $\alpha\beta$ (Fig. 7B) and their expression of transcription factor Sox10 is at the same level as that of the A₂B₅⁺ progenitor (Fig. 6D). This is in sharp contrast to NG2⁺ cells, which express PDGFR $\alpha\alpha$ and Sox 10 (Trotter *et al.*, 2010). Moreover, Takasaki *et al.* (2010) show that pN-OLGs are not cast for synaptic transmission but, in lieu, are fitted metabolically to sustain the survival, differentiation and function of neurons.

New findings claim a role for pN-OLGs in the development and homeostasis of the prefrontal cortex as their demise leads to cytoarchitectural abnormalities associated with mental disorders (Vostrikov *et al.*, 2007; Kim & Webster, 2010, 2011). These reports raise a couple of intriguing questions. First, what is the contribution of pN-OLGs to these events? Second, is their action direct or do they function in a supportive role? A glutamine transporter gene – *Slc38A1* – was significantly correlated with the decrease in the number of

pN-OLGs in psychiatric disorders (Kim & Webster, 2011). Interestingly, the level of expression of this gene is an order of magnitude higher in pN-OLGs than in myelinating OLGs (Supporting Information Data S2). The gene expression profile of normal pN-OLGs described herein should be an invaluable source for helping to elucidate their role in health and disease.

Is there a role for perineuronal oligodendrocytes in myelin repair?

The data presented attest to the fact that pN-OLGs have the molecular armamentarium to respond to a demyelinating eventuality. They retain the capacity for migration and proliferation; they have receptors, signaling and ECM molecules they would need but, most importantly, they carry transcripts for all the major myelin proteins.

Indeed, two intriguing features suggest that pN-OLGs are poised for myelin repair. First, they possess an autocrine loop consisting of PDGFR α , PDGFR β (Fig. 7B) and their ligand PDGFC (Fig. 8A) that allows them to control their numbers. However, activation of this loop is regulated. Second, their expression of Cxcr4 and its ligand Cxcl12 (Figs 7A and 9) – another autocrine system – endows them with what might be a tightly controlled migratory capacity. We may speculate on how these two systems could come into action after an insult, e.g. a bout of demyelination. PDGFC has a CUB domain (Li *et al.*, 2000; Gilbertson *et al.*, 2001), which can fulfil two functions: (i) it can keep PDGFC close to the cell surface by fostering its interaction with ECM proteins; and (ii) it maintains PDGFC in an inactive state. Enzymes released during tissue damage would proteolytically remove the CUB segment, allowing PDGFC to bind to and heterodimerize PDGFR α plus PDGFR β , thereby releasing a strong proliferation signal to the cells. Asymmetric cell division would generate one cell to be a pN-OLG and another to become a myelinating OLG. The CXCR4 / CXCL12 pair would then take care of transporting the latter to the axon in need of repair. Whether and how these two systems might interplay with one another are important issues for further research.

The conspicuous presence of the transcription factor HES5 (Fig. 9A) – a global inhibitor of myelin gene expression – earmarks it as a potential candidate for stifling the translation of myelin proteins, thus enabling pN-OLGs to exert their, as yet, unknown physiological function. Unraveling this code of repression would open up new approaches to elicit remyelination as it could aid in the design of strategies for interfering with the molecular cues that stop pN-OLGs from synthesizing myelin proteins; the cells could thus be induced to activate an endogenous remyelination program, an issue of great importance for demyelinating diseases such as multiple sclerosis.

The determination herein of the genetic signature of pN-OLGs and the identification of potentially critical players in fate specification have broadened our way of thinking about the OLG lineage and opened up new vistas to investigate the mechanism underlying the segregation of OLG phenotypes.

Conclusions

The morphological heterogeneity of OLGs as well as their varying modes of interaction with neurons has been described. We document, for the first time, the genetic program underpinning part of this heterogeneity. We highlight genes, such as *Pdgfra*, *Pdgfrβ*, *Pdgfc*, *Cldn10*, *Pea3*, *Dlx1*, *Ascl1*, *Pax6*, *Cxcr4* and *Cxcl12*, and signaling pathways that may be critical for the segregation and shaping of OLG phenotypes. Moreover, we show that the non-myelinating OLG, while maintaining low levels of myelin protein transcripts – possibly as a reserve for a demyelinating eventuality – has evolved into a cell with a physiological platform of its own even though it has some features that are reminiscent of neurons and Schwann cells. Knowledge of the genetic signature of non-myelinating OLGs – cells that may prove fundamental to the normal functioning of the brain – should serve as the foundation upon which the molecular mechanisms underlying their function can be built.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ab	antibody
BMP	bone morphogenetic protein
CNS	central nervous system
ECM	extracellular matrix
FACS	fluorescence-activated cell sorting
GDNF	glial cell-derived neurotrophic factor
MBP	myelin basic protein
NGS	normal goat serum
OLGs	oligodendrocytes
OLPs	oligodendrocyte progenitors
pN-OLGs	perineuronal oligodendrocytes
qRT-PCR	quantitative time-resolved polymerase chain reaction

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Fig. 1.

The antibody OTMP recognizes live rat oligodendrocyte progenitors. Live rat oligodendrocyte lineage cells were co-stained with mAb A₂B₅ (green) or mAb O₄ (green) and polyclonal Ab OTMP (red). (A–C) Progenitors; note the co-localization of A₂B₅ and OTMP (arrow in B). (D–F) Cells at different stages of O₄ expression, i.e. differentiation. Note the converse expression of OTMP and O₄ (arrowheads in D and E). Once the progenitor becomes an O₄⁺-committed OLG (F) there is no co-localization with OTMP. Scale bar = 50 μ m.

Fig. 2.

mAb A₂B₅ colocalizes with OTMP and is coexpressed with the chemokine receptor CXCR4 in cells with a polarized morphology. Confocal overlay XY projections of 10- μ m frozen sections of P7 rat brain double stained with either polyclonal Ab OTMP plus mAb A₂B₅ (A–H), polyclonal Ab CXCR4 plus mAb A₂B₅ (I–L) or polyclonal Ab OTMP (M). Examining panels A–C and E–G reveals overlap between A₂B₅ and OTMP. This co-localization occurs in the cell soma and in the process, as visualized in the Z projection through the white line in C and G, and is clearly visible in panels D and H. CXCR4 and A₂B₅ are coexpressed in the same cell (I–K), but do not appear to be colocalized. The Z projection in L attests to this conclusion. Interestingly, all positive cells (A–C, E–G and I–K) exhibit a similar morphology consisting of an elongated nucleus and bearing a single process. These features are reminiscent of migrating OLPs. Panel M presents a confocal section of two sister cells expressing OTMP, indicating that these cells are still mitotic. Scale bar = 10 μ m.

Fig. 3.

The antibody OTMP marks perineuronal oligodendrocytes in cortical gray matter but does not stain neurons. Rat (A, B, D) and human (C) brain sections were labeled for OTMP (red, A, B; green, C), NeuN (green, A–C; red, D), and DAPI (blue) for confocal (A, B, D) or epifluorescence (C) microscopy. Note that OLGs indent the neuronal soma (arrows in A and C) and their processes envelop the neuron (arrowheads in B). Arrowhead in C points to a nucleus abutting a neuron that is not stained by OTMP (astrocyte or microglial cell). There is no overlap between OTMP and NeuN, i.e. OTMP does not stain neurons. Scale bars = 10 μm .

Fig. 4.

OTMP⁺ perineuronal cells are of oligodendrocyte lineage but do not synthesize myelin proteins. Arrows in A–C point to a perineuronal cell in the cortex of a 7-day-old CNP / EGFP transgenic mouse double stained with anti-GFP (A, green) and OTMP (B, red). Whereas GFP is distributed within the cell and its process (A), OTMP – being a membrane protein – is confined to a rim surrounding the cell; the process is also stained (B). The co-expression of the two in the same cell (C) demonstrates that pN-OLGs are progeny of oligodendrocyte precursors. Note that the labeling with OTMP is restricted to pN-OLGs (A and C). The green oligodendrocyte (*) in C that is not associated with a nucleus is not stained with OTMP (B). Panels D–F demonstrate that OTMP⁺ pN-OLGs are also A₂B₅⁺. These panels present confocal overlay XY projections of 10-μm frozen sections of P7 rat brain stained with Ab OTMP (D), mAb A₂B₅ (E), mAb NeuN (F) and DAPI (F). Panel F depicts the superposition of all the images. F shows that the OTMP⁺ cell and its processes overlap with the A₂B₅⁺ cell and its processes. This A₂B₅⁺/OTMP⁺ cell abuts and envelops NeuN⁺ neurons (arrows in F). Arrows in G–I point to a pN-OLG from a P14 rat cortex double labeled for OTMP (G) and MBP (H). Note the absence of MBP in the perineuronal cell and the strong presence of MBP in the myelinated fibers (arrowheads in G and I). Frame I (merged) confirms that pN-OLGs do not synthesize MBP. Scale bars = 10 μm.

Fig. 5.

A₂B₅⁺/OTMP⁺ cells are scattered throughout the developing brain. Panels A (P7) and B (P14) illustrate a representative FACS experiment, which addresses the distribution of acutely isolated OLG progenitors double labeled with A₂B₅ and OTMP. Each graph is divided into upper left (UL), upper right (UR), lower left (LL) and lower right (LR) quadrants, depicting OTMP⁻/A₂B₅⁺, OTMP⁺/A₂B₅⁺, OTMP⁻/A₂B₅⁻ and OTMP⁺/A₂B₅⁻ cell phenotypes, respectively. The percentage of cells found in the individual quadrants is indicated as the percentage gated. There is heterogeneity in the intensity of staining within both the single and the double labeled cells. Quantitatively there are no appreciable changes in cell dispersion between P7 and P14.

Fig. 6.

The $A_2B_5^+/OTMP^+$ cell depicts a unique oligodendrocyte lineage phenotype. We used Affymetrix microarray technology to identify gene expression patterns for P7 cells recognized by four molecular markers: $A_2B_5^+$, $A_2B_5^+/OTMP^+$, O_4^+ and $OTMP^+$. Principal components analysis (A) revealed a sharp demarcation among the different populations. Note that the OTMP antibody identifies a subpopulation of $A_2B_5^+$ precursors with a different phenotype. B and C illustrate two general gene expression patterns of the $A_2B_5^+/OTMP^+$ phenotype compared with both $A_2B_5^+$ and O_4^+ cells. In D the expression patterns of transcription factors demonstrated to be critical for the development of OLGs are compared for the three subpopulations. $A_2OT = A_2B_5 / OTMP$.

Fig. 7.

qRT-PCR quantification of the transcription of 14 genes plus an endogenous control using total RNA obtained from A₂B₅⁺ and A₂B₅⁺/OTMP⁺ cells. Data were analysed by the 2^{-CT} method where the A₂B₅⁺ cell is taken as the calibrator, i.e. $CT = 0$ and $2^0 = 1$. The values depicted in A and B match – with a few exceptions – those predicted by the microarray experiments as attested by the numbers given here: *Etv4* = 6.1; *Cldn10* = 3.9; *Cdh10* = 3.2; *Ret* = 3.6; *Sema4b* = 2.3; *Cxcl12* = 2.3; *Pdgfrβ* = 2.7; *Egfr* = 3.3; and *Bmpr* = 3.3, thus providing a measure of validity to the latter.

Fig. 8.

Shh and Wnt signaling pathways of non-myelinating oligodendrocytes relative to corresponding ones of their myelinating counterpart. We have used the Ingenuity software interacting networks to analyse $A_2B_5^+/OTMP^+$ gene expression data relative to those of O_4^+ committed OLGs. Red denotes up-regulation and green down-regulation of transcripts in the $A_2B_5^+/OTMP^+$ relative to O_4^+ . Edge types: solid line = direct action; broken line = indirect action; arrow from A to B = act on; line joining A to B = binding only; line ending in vertical dash = inhibits. (A) This network shows the predicted sonic hedgehog pathway in

the non-myelinating phenotype. Note the upregulation of transcripts for *Pdgfra* (arrowhead), *Pdgfrβ* (arrowhead) and the growth factor *Pdgfc* (asterisk). Their interaction should result in the expression of the heterodimer PDGFRαβ on the surface of the A₂B₅⁺/OTMP⁺ cells, silencing their response to PDGF-AA – a ligand for myelinating OLGs, and thereby segregating the two cell types. Note also the downregulation of *Pdgfa* (double arrow). The growth factor receptor (double asterisks) implicated in the regulation of the other components remains to be elucidated. A cross-talk with Notch signaling (> 4-fold increase in *Hey1*; large arrow) is hinted by its apparent involvement in the increased expression (3.3-fold) of phospholipase C-γ (small arrow). (B) This interacting network illustrates A₂B₅⁺/OTMP⁺ transcripts pertaining to Wnt signaling. Asterisks mark the receptors *Fzd* (frizzled), co-receptor *Lrp6* and the protein DVL1 (dishevelled); they are all upregulated. Note that NrCAM (arrowhead) appears as a cell-specific target of Wnt signaling.

Fig. 9.

Notch and Eph / ephrin signaling pathways of non-myelinating oligodendrocytes relative to corresponding ones of their myelinating counterpart. Edge types are the same as in Fig. 8. (A) This network illustrates three aspects of Notch signaling (asterisk): 1, (large arrow) up-regulation of ASCL1 / MASH1; 2, (arrowhead) increased expression (> 13-fold) of HES5; 3, (small arrow) enhanced transcription of lipocalin-prostaglandin D2 synthase (L-PGDS). Inset – an OTMP⁺/L-PGDS⁺ pN-OLG demonstrating that the L-PGDS protein is synthesized by these cells. (B) The canonical pathway for the Eph / ephrin tyrosine kinases was used to highlight A₂B₅⁺/OTMP⁺ transcripts relative to the O₄⁺ transcripts. Small arrows point to the up-regulated EphA receptors. Arrowhead signals the ephrin B receptor. Asterisks denote the G-coupled CXCR4 receptor and its ligand CXCL12 / SDF-1, which together with ephrin B may play a role in cell migration.

Table 1

Transcripts for the major myelin proteins

Gene name	A ₂ B ₅	A ₂ OT (A ₂ B ₅ / OTMP)	O ₄	Fold diff. O ₄ / A ₂ OT
Cyclic nucleotide phosphodiesterase 1	1.1	0.9	24.8	27.6
Myelin and lymphocyte protein	0.8	0.8	96.3	127.7
Myelin basic protein	0.9	1.1	6.0	5.7
Myelin oligodendrocyte glycoprotein	1.0	1.2	45.6	39.0
Myelin-associated glycoprotein	0.8	1.4	25.9	19.2
Myelin-associated oligodendrocytic basic protein	0.9	1.1	11.3	10.6
Oligodendrocyte myelin glycoprotein	0.6	0.7	6.2	8.5
Proteolipid protein	0.9	1.1	4.3	3.9
SRY-box containing gene 10	1.1	1.0	30.1	30.2