Esta es la versión de autor del artículo publicado en:
This is an author produced version of a paper published in:

Journal of Neuroscience 38.22 (2018): 5096-5110

DOI:  http://doi.org/10.1523/JNEUROSCI.3364-17.2018

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R-Ras1 and R-Ras2 are essential for oligodendrocyte differentiation and survival for correct myelination in the central nervous system

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DOI: 10.1523/JNEUROSCI.3364-17.2018
Received: 28 November 2017
Revised: 14 March 2018
Accepted: 10 April 2018
Published: 2 May 2018


Conflict of Interest: The authors declare no competing financial interests.

This work was supported by the Spanish Ministry of Economy and Competitiveness (BFU2015-64829-S and SAF2012-31279) to B.C. and (SAF2015-70368-R) to F.W. Authors wish to thank Drs. M. Izquierdo, P. Bovolenta, and B. Alarcón for helpful scientific discussions. We also want to thank Dr. A. Morales for providing us with the Nkx2.2 antibody, J.R. Perea, S. Gutierrez-Erlandsson, M. Guerra and T. Martin for their technical assistance, M. Sefton for critical reading of the manuscript and Life Science Editors for their editing assistance. The qPCR experimental development and data analysis was provided by the Genomics and NGS Core Facility at the Centro de Biología Molecular “Severo Ochoa”, Madrid, Spain.

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Cite as: J. Neurosci ; 10.1523/JNEUROSCI.3364-17.2018

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Abbreviated title: Essential role of R-Ras1 and 2 in CNS myelination

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Abstract

Rapid and effective neural transmission of information requires correct axonal myelination. Modifications in myelination alter axonal capacity to transmit electric impulses and enable pathological conditions. In the central nervous system (CNS), oligodendrocytes (OLs) myelinate axons, a complex process involving various cellular interactions. However, we know little about the mechanisms that orchestrate correct myelination. Here, we demonstrate that OLs express R-Ras1 and R-Ras2. Using female and male mutant mice to delete these proteins, we find that activation of the PI3K/Akt and Erk1/2-MAPK pathways is weaker in mice lacking one or both of these GTPases, suggesting that both proteins coordinate the activity these two pathways. Loss of R-Ras1 and/or R-Ras2 diminishes the number of OLs in major myelinated CNS tracts and increases the proportion of immature OLs. In R-Ras1/− and R-Ras2/− null mice, OLs show aberrant morphologies and fail to differentiate correctly into myelin-forming phenotypes. The smaller OL population and abnormal OL maturation induce severe hypomyelination, with shorter nodes of Ranvier in R-Ras1/− and/or R-Ras2/− mice. These defects explain the slower conduction velocity of myelinated axons we observed in the absence of R-Ras1 and R-Ras2. Together, these results suggest that R-Ras1 and R-Ras2 are upstream elements that regulate the survival and differentiation of progenitors into OLs through the PI3K/Akt and Erk1/2-MAPK pathways for proper myelination.
Significance Statement

In this study, we show that R-Ras1 and R-Ras2 play essential roles in regulating myelination in vivo and control fundamental aspects of oligodendrocyte survival and differentiation through synergistic activation of PI3K/Akt and Erk1/2-MAPK signaling. Mice lacking R-Ras1 and/or R-Ras2 show a diminished oligodendrocyte population with a higher proportion of immature oligodendrocytes, explaining the observed hypomyelination in main CNS tracts. In vivo electrophysiology recordings demonstrate a slower conduction velocity of nerve impulses in the absence of R-Ras1 and R-Ras2. Thus, R-Ras1 and R-Ras2 are essential for proper axonal myelination and accurate neural transmission.
Introduction

Mammalian plasma membranes from specialized glial cells generate myelin as oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. Axonal myelination is essential to facilitate fast, saltatory conduction of action potentials. Changes in myelin structure, such as sheath thickness and internode length, can alter axonal conduction velocities, which may induce pathologies like leukodystrophies and peripheral neuropathies (Suter and Scherer, 2003; Boespflug-Tanguy et al., 2008).

During development, oligodendrocyte progenitor cells (OPCs), which differentiate into OLs, arise along the caudorostral axis of the ventricular neuro-epithelium of the neural tube at multiple restricted foci distributed along the dorsoventral axis to colonize the entire CNS (de Castro F., 2013; Ono et al., 2017). OPCs then differentiate into mature OLs forming myelin (Nave and Werner, 2014). Various intrinsic and extrinsic cues, like specific growth factors, protein kinases and extracellular matrix proteins regulate the spatial and temporal patterns of OL differentiation by modifying gene expression and cell morphology (Bauer et al., 2009; Emery, 2010).

Among these factors, PI3K/Akt and Erk1/2-MAPK signaling pathways may control OL survival, migration, differentiation and myelination (Ness et al., 2002; Romanelli et al., 2009; Ishii et al., 2014; Murcia-Belmonte et al., 2014; Gaesser and Fyffe-Maricich, 2016; Murcia-Belmonte et al., 2016). Indeed, mutant animals that have constitutively-active PI3K-Akt-mTOR pathways in their OPCs and OLs show dramatically enhanced myelin formation but not changes in OL generation (Flores et al., 2008; Goebels et al., 2010; Harrington et al., 2010). In contrast, inhibition of effectors of the PI3K/Akt pathway such as mTOR causes defective OL differentiation both in vitro and in vivo (Tyler et al., 2009; Wahl et al., 2014). Erk1/2 signaling controls thickness and
maintenance of CNS myelin (Wahl et al., 2014 Ishii 2014), and its inactivation produces a delay in the differentiation and maturation of OLs (Fyffe-Maricich et al., 2011). Hyperactivation of the Erk1/2-MAPK pathway during development drives a transient OPC hyperproliferation without affecting their differentiation or the final number of mature OLs (Ishii et al., 2013). In addition, Erk1/2 activation in OLs drives sheath expansion (Jeffries et al., 2016) (Jeffries et al., 2016). It is known that there is cross-talk between PI3K/Akt and Erk1/2-MAPK (Gaesser and Fyffe-Maricich, 2016; Furusho et al., 2017), though little is known about the mechanisms that mediate the coordinated activity of signaling in these two molecular pathways.

One candidate is the Ras superfamily of GTP-binding proteins. These membrane-anchored intracellular signal transducers that act through both PI3K/Akt and Erk1/2-MAPK pathways (Arimura and Kaibuchi, 2007) to influence various cell functions, including proliferation, differentiation and cell survival (Karnoub and Weinberg, 2008; Pylayeva-Gupta et al., 2011). Members of the classic Ras subfamily (Hras, Kras and Nras) are frequently mutated and constitutively active in human cancers (Chan et al., 1994). GTPases of the Ras-related (R-Ras) subfamily, composed of R-RAS1 (RRas), R-RAS2 (TC21) and R-RAS3 (also called MRas), are less well understood (Ohba et al., 2000; Colicelli, 2004; Gutierrez-Erlandsson et al., 2013). R-Ras proteins share strong homology (55-60% amino acid identity) with classic Ras proteins (Drivas et al., 1990; Colicelli, 2004), and with many effector proteins, including components in PI3K/Akt and Erk1/2-MAPK pathways (Chan et al., 1994; Graham et al., 1994; Graham et al., 1999; Movilla et al., 1999; Rosario et al., 1999, 2001; Delgado et al., 2009). R-Ras1 and R-Ras2 are almost ubiquitously expressed (Komatsu and Ruoslahti, 2005; Larive et al., 2012), whereas R-Ras3 expression is more restricted (Kimmelman et al., 2002; Sun et al., 2006). In the CNS, OLs can express R-Ras1 (Olsen and Ffrench-Constant, 2005),
however its specific function and putative role with R-Ras2 in myelination remains unknown.

Here we investigated the role of R-Ras1 and R-Ras2 in OL differentiation for myelin formation, using three knockout mice: (single R-Ras1 or R-Ras2 null mutants, and a double null mutant). Different levels of hypomyelination in R-Ras1<sup>−/−</sup> and R-Ras2<sup>−/−</sup> mice indicate that both GTPases are essential for proper myelination. Single and double null mutant mice showed diminished OL viability from weaker activation of PI3K/Akt and Erk1/2-MAPK signaling. OL maturation was altered, changing the internode length (myelin segments between nodes of Ranvier), which strongly decreased axonal conduction velocity. Overall, our data strongly suggest that R-Ras1 and R-Ras2 are critical for OL differentiation and CNS myelination through mechanisms using Akt and Erk pathways.
Material and Methods

Animals. Mice were housed in specific pathogen–free conditions, in a humidity and temperature-controlled room on a 12-h light/dark cycle, receiving water and food *ad libitum*. All animal procedures were approved by the corresponding institutional ethical committee (CBMSO) and were performed in accordance with Spanish and European directives. All efforts were made to minimize animal suffering.

*R-Ras1*−/− mice were generated at GenoWay Company (France) using the targeting construction BAL1-HR with a neomycin resistance cassette flanked by FRT sequences inserted in intron 1 and LoxP sites flanking exons 2 and 6. The construction was electroporated into embryonic stem cells derived from mouse 129Sv/Pas and selected by the antibiotic G418. Southern blot was used to verify the correct homologous recombination.

Heterozygous mice were crossed, and offspring littermates were genotyped by PCR (*R-Ras1*−/− FW: 5′-GGAGCAAGAGGAGGGAAGGAATGGG-3′, *R-Ras1*−/− RV: 5′-CCTTCCAGAGGACTCAGTTCAATCC-3′, *R-Ras1*+/+ FW: 5′-CGCTCTAGCTGAGCCTCTGT-3′, *R-Ras1*+/+ RV: 5′-TACAGGTTCTTGTGGGAAA-3′).

*R-Ras2*−/− mice were generated at Lexicon Pharmaceuticals (Texas, USA) and were derived from embryonic stem cell clone OST361011 with insertion of retroviral VICTR37 in the middle of intron 1 of *R-Ras2*. Heterozygous mice were crossed and offspring littermates were genotyped by PCR (primer 1, 5′-TGAAACAGGATCATGTTGTGGAG-3′; primer 2, 5′-CAGGAGGAGTCCAAGAAGAC-3′; primer 3, 5′-ATAAACCCTCTTGCAGTTGCATC-3′) (Delgado et al., 2009). *R-Ras2*+/+ and *R-Ras2*−/− transgenic mice were obtained by crossing of heterozygous mice.
R-Ras1−/− and R-Ras2−/− mice were kindly provided by Prof. Alarcón (CBMSO, Spain). Double-knockout R-Ras1−/−;R-Ras2−/− mice were generated by backcrossing individual lines of R-Ras1−/− and R-Ras2−/−. Animals were maintained in a C57BL6J background.

We have used either, male and female mice to perform the experiments. The morning the vaginal plug appeared was defined as embryonic day (E) 0.5, and newborn was defined as postnatal (P) 0.

Western blotting. Tissue samples (optic nerve, spinal cord and corpus callosum of animals from P30 to P90) were dissected, sonicated in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 2 mM EDTA, 0.1% SDS, 0.5% desoxycholate, Protease Inhibition Cocktail; Roche 11697498001, Basel, Switzerland) and phenylmethane sulfonyl fluoride (PMSF). For detection of phosphorylated proteins, a different buffer was used (40 mM Tris pH 8.0, 300 mM NaCl, 20% Glycerol, 4 mM EDTA, 0.3% Brij 96, 1 mM PMSF, 2 mM Na3VO4, 10 mM NaF, 1 mM apoprotein, 1 mM benzamidine, 1 mM iodoacetamide, and protease inhibitor cocktail. Lysates were denatured by boiling for 5 min in protein loading buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol (BME), 12.5 mM EDTA and 0.02 % bromophenol blue) and resolved in 10-12% SDSP-gels in the presence of BME. Gels were run at constant current starting at 90-100V. After electrophoresis, samples were transferred onto PVDF membranes using a semi-dry electroblotting system (Transblot-turbo, BioRad, California, USA) at 1.2 mA/cm² for 35-40 min. Nonspecific protein binding was blocked by incubating the membrane with 5% non-fat milk or 5% BSA (for detection of phosphorylated proteins) in TBS-Tween-20 for 2 h at room temperature. Membranes were then incubated overnight at 4 °C with the pertinent primary antibodies diluted in blocking buffer: rabbit anti-R-Ras 1:200 (ab154962), rabbit anti-Caspr 1:250 (Abcam Cat# ab34151, RRID: AB_869934, Cambridge, UK), mouse anti-GAPDH (G9) 1:1000.

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(Santa Cruz Biotechnology Cat# sc-365062, RRID:AB_10847862, Dallas, Texas, USA), goat anti-MAG (C-19) 1:200 (Santa Cruz Biotechnology Cat# sc-9544, RRID:AB_670102), rabbit anti-Akt 1:1000 (Cell Signaling Technology Cat# 9272, RRID:AB_329827, Massachusetts, USA), rabbit anti-P-Akt (S473) 1:1000 (Cell Signaling Technology Cat# 4060, RRID:AB_2315049), rabbit anti-P-Akt (T308) 1:1000 (Cell Signaling Technology Cat# 9275), rabbit anti-phospho S6 (S240/244) 1:1000 (Cell Signaling Technology Cat# 5364, RRID:AB_10694233), rabbit anti-p44/42 MAPK (Erk1/2) 1:1000 (Cell Signaling Technology Cat# 9102, RRID:AB_330744), rabbit anti-P-p44/42 MAPK (P-Erk1/2) (T202/T204) 1:1000 (Cell Signaling Technology Cat# 9101, RRID:AB_331646), mouse anti-MBP (aa67-74) 1:200 (Bio-Rad / AbD Serotec Cat# MCA685S, RRID:AB_325009, Raleigh, North Carolina, USA), mouse anti-TCF-4 clone 6H5-3 1:1000 (Millipore Cat#05-511, RRID:AB_309772, Missouri, USA) or rabbit anti-Olig2 1:2000 (Millipore Cat# AB9610, RRID:AB_570666). Specific rabbit antiserum to R-Ras2 (kindly provided by Prof B. Alarcón) was generated by immunization of New Zealand rabbits with a purified GST-TC21 fusion protein (Delgado et al., 2009), and used at a 1:200 dilution. After washing, blots were then incubated for 1 h with appropriate peroxidase-conjugated secondary antibodies (ThermoFisher, Waltham, Massachusetts, USA). Labeled proteins were detected with the chemiluminescence reagent ECL (Amersham Biosciences, USA).

**Immunohistochemistry.** Animals were anesthetized (ketamine/xylazine) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in PBS. Perfused tissues were removed and postfixed in 4% paraformaldehyde at 4 °C overnight, then cryoprotected in 30% sucrose in PBS and embedded and frozen in a 7.5% gelatin in 15% sucrose solution. Then they were
sectioned on a cryostat to produce 15 μM-thick cryosections on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were blocked for 1 h at room temperature with 10% fetal bovine serum in PBS containing 0.5% Triton-X 100 (blocking solution) and then incubated overnight at 4 °C with the primary antibodies (rabbit anti-R-Ras 1:200, rabbit anti-R-Ras2 1:500, mouse anti-Nkx2.2 1:250 (DSHB Cat# 74.5A5, RRID:AB_531794, Iowa, USA), kindly provided by Aixa Morales (Cajal Institute, CSIC, Madrid, Spain), rabbit anti-olig2 1:500, rabbit anti-cleaved caspase-3 (Asp175) 1:500 (Cell Signaling Technology Cat# 9661, RRID:AB_2341188), rabbit anti-MBP 1:300 (Abcam Cat# ab40390, RRID:AB_1141521), mouse anti-NF200 clone NE14 1:300 (Sigma-Aldrich Cat# N5389, RRID:AB_260781, St. Louis, Missouri, USA), rabbit anti-Caspr 1:250, mouse anti-TCF-4 clone 6H5-3 1:200 and mouse anti-O4 1:200 (Millipore Cat# MAB345, RRID:AB_94872) diluted in blocking solution. After 3 washes, fluorescent-tagged secondary antibodies were applied for 1 h at room temperature, and sections were counterstained with DAPI (32670, Sigma-Aldrich, St. Louis, MO, USA) and mounted in Aqua-polymount mounting medium (Cat#18606; Polyscience; Warrington, PA, USA).

BrdU injections were administered intraperitoneally to pregnant mice (100 μg/g) at different stages of embryo development. One hour after BrdU administration, the mother was euthanized and the brains of embryos were fixed overnight in 4% PFA. Staining for BrdU was performed as described (Cubelos et al., 2008). Briefly, slides were incubated overnight at 4 °C with a mouse monoclonal anti-BrdU antibody 1:200 (Becton Dickinson Cat# 347580, RRID: AB_609568, New Jersey, USA). Secondary Alexa 598-conjugated anti-rat antibodies 1:500 (Molecular Probes, Eugene, Oregon, USA) were applied for 2 h at room temperature. Anatomically-matched sections were
selected from each mouse at each stage after BrdU injection (n=3, control mice and n=3, R-Ras1−/−;R-Ras2−/− mice). Identical results were obtained from independent analysis by two investigators. Quantification of positive cells was performed always in the same part of the tissues with ImageJ software (RRID:SCR_003070, Wisconsin-MA, USA).

**Toluidine blue staining.** Mice were anesthetized as indicated above, intracardially perfused with 4% PFA and 2.5% glutaraldehyde in 0.1 M PBS, and brains were dissected and treated in the same fixative overnight. Sections were made with a Leica VT1200 S vibrating blade microtome (100 μm), washed 10 min in PBS and stained for 1 min with 200 μl of 0.1% toluidine blue, and finally washed with distilled water and ethanol for 30 sec. Pictures were taken with a Leica MZ6 magnifying glass for the proper location of implanted recording electrodes.

**Electron microscopy.** Mutant and littermate control mice were anesthetized as indicated above, intracardially perfused with 4% PFA and 2.5% glutaraldehyde in 0.1 M PBS, and treated in the same fixative overnight. Then, optic nerves were removed after several washes in PBS, the sections were postfixed with 1% osmium tetroxide in double-distilled water and 1% potassium ferrocyanide 1 h at 4 °C. After three washes with double-distilled water they were treated with 0.15% tannic acid in PBS (0.1 M pH 7.4) and block-stained with 2% uranyl acetate in distilled water for 1 h at room temperature in darkness. Sections were then washed 3 times with double-distilled water and dehydrated in an ascending series of ethanol dilutions up to 100% at 4 °C. The following infiltration was made with propylene oxide: EtOH (1:1; v:v) for 5 min, propylene oxide 3 times for 15 min each, propylene oxide:Epon (1:1) (epoxy resin TAAB 812, TAAB laboratories, Berkshire, England) 45 min, 100% Epon 1 h, 100% Epon overnight. Encapsulation was made in flat molds where optic nerves were
correctly oriented and then polymerized 48 h at 60 °C. Semi-thin (1 μm) sections were stained with toluidine blue. The optic nerve was cut in 70-80 nm sections on an ultramicrotome (Leica Ultracut UCT, Wetzlar, Germany) with a diamond blade (DIATOME) and collected on Cu-Pd boutonniere grids covered by Formvar. Staining of ultrathin sections was performed by drops of 2% aqueous uranyl acetate for 7 min followed by Reynolds’s lead citrate for 2 min. Ultrastructural analyses were performed with a JEM-1010 electron microscope (Jeol, Japan). For g-ratio analysis, twelve pictures were taken along the section covering the whole diameter of the optic nerve using a CMOS 4K x 4K, F416 of TVIPS camera (Gauting, Germany). From these pictures, six in perfect condition that matched the mutant and control, were used to measure the thickness of the myelin sheath of optic nerve axons by g-ratio analysis (the diameter of axon / the diameter of axon plus myelin sheath). All axons present in the entire image were analyzed (around 800-1000 axons per genotype). To quantify the number of fibers crossing the corpus callosum, pictures were taken in the medial corpus callosum covering its whole width, and the total number of axons were quantified. Three animals per genotype were analyzed, and identical results were obtained from independent analysis by 3 investigators.

Confocal microscopy and imaging. Fluorescence images were obtained using a confocal multispectral Leica TCS SP8 system (Leica Microsystems) equipped with a white-light laser for selection of appropriate fluorochrome excitation lines (488 nm and 550 nm), a 405 nm diode for DAPI excitation and HyD detectors for signal emission capture. Image acquisition was performed sequentially using 20x/0.75 NA dry and 63x/1.4NA oil immersion objectives with LAS X v. 2.0.1 software (Leica Microsystems). All images were processed and quantified using Adobe PhotoShop (Adobe Systems, RRID:SCR_014199, San Jose, CA, USA) and ImageJ.
Primary cell cultures and staining. OPCs were obtained from cortex and optic nerve of P1 control and R-Ras1-/-;R-Ras2-/- littermate mice, following an adapted protocol for OPC isolation by shaking (McCarthy and de Vellis, 1980; Molina-Holgado et al., 2002; Medina-Rodriguez et al., 2013; Murcia-Belmonte et al., 2014). Purified OPCs were placed on 12 mm cover glass pre-treated with 10 μg/ml poly-lysine (#P2636; Sigma-Aldrich; St. Louis, MO, USA) in 0.1 M borate buffer (pH 8.5) and laminin (L2020; Sigma-Aldrich) the same day of the culture. 20000 cells per well were cultured in differentiation buffer containing BME (41010; Gibco; Grand Island, New York, USA), F12 (21765; Gibco), transferrin (T0665; Sigma-Aldrich), putrescin (P5780; Sigma-Aldrich), progesterone (P6149; Sigma-Aldrich), sodium selenite (Sigma-Aldrich), insulin (I1882; Sigma-Aldrich), T4 (T1775; Sigma-Aldrich), glutamine (25030; Gibco), anti-mycotic solution and 0.6% glucose. After 14 days in vitro (DIV) of differentiation, OLs were fixed with 4% PFA for 20 min and washed. OLs were placed in PBS containing 0.5% Triton-X 100 for 20 min and then blocking solution (10% FBS in PBS containing 0.5% Triton-X 100) for 30 min. After that, they were incubated for 2 h at room temperature with primary antibodies (1:500 dilution of mouse anti-CNPase, Biolegend, #SMI-91R, RRID: AB_10122287, St. Diego, California, USA or a 1:250 dilution of rabbit anti-Olig2). After washing, cells were incubated 35 min with fluorescent-tagged secondary antibodies and DAPI (32670; Sigma-Aldrich). OLs from three different experiments were classified according to their morphologies in two different groups (Kremer et al., 2009), one where the number and complexity of processes was very low (simple processes) and another where the processes had a high degree of arborization (complex processes).

RT-qPCR. RNA was extracted from optic nerves from control, R-Ras1-/-, R-Ras2-/- and R-Ras1-/-;R-Ras2-/- mice using an RNeasy kit according to the manufacturer’s
instructions (Qiagen Cat# 74134; Venlo, Netherlands). RNA was reverse-transcribed using random hexamers and SuperscriptIII reverse transcriptase (Superscript III First Strand Synthesis Supermix for qRT-PCR, no amperase UNG, Cat. No. 11752-250, Thermo Fisher; Waltham, MA, USA) according to the manufacturer’s instructions. cDNA was amplified using the BioRadC FX 384 thermocycler and Eva Green Sso Fast (BioRad, California, USA), Power Sybr Green or Taqman Master Mix. The following primers were used: For \textit{R-Ras1} (R-Ras-FW sense 5´-AAAGCAGATCTGGAGAACCA-3´, R-Ras-RV antisense 5´-TGCCTCATCGACATTCAGAC-3´), for \textit{R-Ras2} (R-Ras2-FW sense 5´-CGTGATGAGTTTCCCATGATT-3´, R-Ras2-RV antisense 5´-CAGTGCTGCTCTCTCCCT-3´). All primers were designed to span at least one intron. Expression levels were normalized to GAPDH, ActB, HPRT1, 18S, TBP, ARBP and GUSB expression, and fold-changes were calculated by dividing normalized expression in control (value 1) by that of \textit{R-Ras1}\textsuperscript{−/−} or \textit{R-Ras2}\textsuperscript{−/−}. qPCR data was analyzed with GenEx Professional 5.3.7 software (MultiD, Sweden).

\textit{Electrophysiology}. Control (n=9), \textit{R-Ras1}\textsuperscript{−/−} (n=13), \textit{R-Ras2}\textsuperscript{−/−} (n=13) and \textit{R-Ras1}\textsuperscript{−/−};\textit{R-Ras2}\textsuperscript{−/−} (n=14) mice were prepared for chronic recording of field potentials evoked at the lateral geniculate nucleus by stimulus (flashes of light). For this, animals were anesthetized with 4% chloral hydrate and stereotactically implanted with two recording electrodes in the dorsal part of the lateral geniculate nucleus (2.2-2.5 mm posterior to the bregma, 2.0 mm lateral to the midline and –2.5 mm depth from brain surface (Paxinos and Franklin, 2013). Electrodes were made from 50 μm teflon-coated tungsten wire (Advent Research Materials, Eynsham, UK). Two bare silver wires were affixed to the skull as ground. Electrodes were connected to a 4-pin socket (RS-Amidata, Madrid, Spain) that was latterly fixed with dental cement to the cranial bone. After surgery,
animals were kept for 5 days in independent cages with free access to food and water for a proper recovery. Light stimulation was provided by a xenon arc lamp located 30 cm in front of the animals’ eyes and lasted ~1 ms (Photic stimulator, Cibertec, Madrid, Spain). For recordings, each alert behaving mouse was placed in a transparent box (5 × 5 cm), dark-adapted for >30 min and presented with a total of 20 stimuli at a rate of 3 per min. This box was in the center of a larger (30 × 30 × 30 cm) covered by polished aluminum walls. Photic stimulations were triggered from a programmable CS-20 stimulator (Cibertec). Each animal received two stimulation sessions.

**Statistical Analysis.** Quantitative data are presented as the mean ± standard deviation (SD). The experimental groups were compared using a two-tailed Student’s t-test. Statistical numeric data are provided in the legends. (*) means p<0.05; (**) means p<0.01; (***) means p<0.001.
**Results**

R-Ras1 and R-Ras2 are expressed by oligodendrocytes in myelinated CNS tracts

While OLs may express R-Ras1 (Olsen and Ffrench-Constant, 2005), the distribution of R-Ras2 remains undetermined. So, we precisely defined the expression of R-Ras1 and R-Ras2 in different myelinated areas of the CNS in adult mice (P90). Using immunoblotting, we probed samples from the main myelinated tracts of the CNS for R-Ras1 and R-Ras2, including: the optic nerve (which consists almost entirely of myelinated axons) (Fig. 1A), corpus callosum (Fig. 1B) and spinal cord (Fig. 1C). Given the strong homology between R-Ras1 and R-Ras2, antibodies against these GTPases recognize both proteins. We can easily detect two bands of different molecular weights in Western blots: a 25 kDa band corresponding to R-Ras1 (Iwasawa et al., 2012) and a 21 kDa band corresponding to R-Ras2 (Delgado et al., 2009). In double null mutant mice lacking both R-Ras1 and R-Ras2, no immunoreactivity was observed, confirming the antibodies used are specific for R-Ras1 and R-Ras2 (Fig. 1A-C). We could unequivocally confirm the absence of the appropriate proteins in the single null mutants (Fig. 1A). R-Ras3 expression was not modified in R-Ras1/−/− and/or R-Ras2/−/− mice, showing a band of 24 kDa (Fig. 1A-C).

To generate R-Ras1 deletion mutants, we used a germ-line knockout mouse with exons 2-6 deleted, the region that encodes most of the protein (Fig. 1D). We genotyped mice by PCR using primers 1 and 2 to detect the alleles corresponding to R-Ras1+/+ mice and a mixture of primers 3 and 4 to identify the R-Ras1/−/− mutants (Fig. 1E). We also used a R-Ras2 knockout mouse line produced from an embryonic stem cell clone with a retroviral insert in the first intron of R-Ras2 (Delgado et al., 2009) (Fig. 1F). Finally, we generated R-Ras1/R-Ras2 double null mutant mice by backcrossing the individual R-Ras1/−/− and R-Ras2/−/− lines. These mice were backcrossed for ten generations onto the
C57BL/6J strain to exclude any distortions induced by background variations. Quantitative PCR and immunoblot analysis of the CNS confirmed the absence of expression of these genes in R-Ras1\textsuperscript{-/-}, R-Ras2\textsuperscript{-/-} and double-mutant mice (Fig. 1A,B,C,G).

We then determined whether OLs express R-Ras1 and R-Ras2 using double immunohistochemical staining with antibodies against R-Ras1/R-Ras2 and Nkx2.2, a homeobox transcription factor expressed by oligodendroglial cells (Qi et al., 2001). In longitudinal sections of the optic nerve from adult mice (P90), both R-Ras1 and R-Ras2 co-localized with Nkx2.2 (Fig. 1H), which was also observed in the corpus callosum and spinal cord (data not shown). So, we conclude that R-Ras1 and R-Ras2 are expressed in OLs of the main myelinated CNS tracts.

Oligodendrocyte population is diminished in R-Ras1\textsuperscript{-/-}, R-Ras2\textsuperscript{-/-} and R-Ras1\textsuperscript{-/-};R-Ras2\textsuperscript{-/-} mice

To study whether the absence of R-Ras1 and/or R-Ras2 altered oligodendroglial cells, we utilized immunohistochemical staining of optic nerves with the oligodendroglial-specific Olig2 antibody in longitudinal sections from R-Ras1\textsuperscript{-/-}, R-Ras2\textsuperscript{-/-} and double-mutant adult (P90) mice (Fig. 2A,B). Histological examination revealed a decrease in oligodendroglial cell density in R-Ras1\textsuperscript{-/-} (19.1±7.5% reduction; p=0.02), R-Ras2\textsuperscript{-/-} (33.5±2.1% reduction; p<0.0001) and R-Ras1\textsuperscript{-/-};R-Ras2\textsuperscript{-/-} (42.8±11.8% reduction; p=0.003) mice compared to controls (Fig. 2A,C). The reduction in oligodendroglial cells was more pronounced in double mutants (p=0.02) followed by R-Ras2\textsuperscript{-/-} (p=0.003) compared with R-Ras1\textsuperscript{-/-} (Fig. 2C). We examined whether R-Ras1 and R-Ras2 were essential to maintain the OL population in other myelinated CNS tracts and confirmed the reduction in oligodendroglial population in the double-mutant mice compared to controls (38±15.6% reduction in the corpus callosum, p<0.0001; and 27±6.1% reduction...
These observations demonstrate that R-Ras1 and R-Ras2 jointly maintain the OL populations in the main myelinated CNS tracts.

**Absence of R-Ras1 and R-Ras2 reduces activation of PI3K/Akt and Erk1/2-MAPK signaling pathways**

Given that R-Ras1 and R-Ras2 can activate PI3K/Akt signaling (McFall et al., 2001; Rosario et al., 2001; Murphy et al., 2002; Rong et al., 2002; Delgado et al., 2009) and Erk1/2-MAPK (Graham et al., 1994; Movilla et al., 1999; Rosario et al., 1999), we examined these pathways in the optic nerve of P90 mice by Western blot using antibodies against phosphorylated Akt and Erk (active versions) (Fig. 3A). We found a reduction of both Akt phosphoepitopes T308 and S473 in single and double mutants (Fig. 3A), indicating a decrease in PI3K/Akt activity in R-Ras1- and R-Ras2-deficient adult (P90) mice. We then analyzed S6K activity, a kinase that regulates mTOR, by measuring S6 phosphorylation levels. We confirmed a significant decrease in the activity of the PI3K/Akt pathway in the absence of R-Ras1 and/or R-Ras2, with no change in the total levels of these proteins (Fig. 3A). We also examined activity of the Erk1/2-MAPK pathway in our mutant mice, observing a reduction in Erk1/2 phosphorylation in the single- and double-mutant mice relative to the controls. We found that activation of the PI3K/Akt and Erk1/2-MAPK pathways was weaker in mice lacking one or both of these GTPases, which suggests that both proteins coordinate the activity of these two pathways (Fig. 3B).

Using immunohistochemical staining for Olig2 at different stages of OPC differentiation (E12.5, E14.5, P0, P15), we did not detect differences in oligodendrogenesis between double-mutant and control mice. The Olig2 immunostaining suggested that normal oligodendrocyte migration occurs in double-
mutant mice, as OPCs born at E12.5 and E15.5 normally colonized the optic nerve and corpus callosum tracts (data not shown), as previously reported (Spassky et al., 2002; de Castro F., 2013). We also evaluated OL proliferation using BrdU labelling at different developmental stages (E14.5, E18.5, P0 and P15), finding no differences between double-mutant and control mice (data not shown). These experiments show that the absence of R-Ras1 and/or R-Ras2 does not affect OPC genesis, migration or proliferation, and rather that R-Ras1 and R-Ras2 contribute to postmitotic oligodendroglia differentiation to form mature OLs.

Next, we analyzed whether decreased activation of the PI3K/Akt and Erk1/2-MAPK pathways affected OL survival. We assessed the number of apoptotic cells in the optic nerve and corpus callosum with an antibody against active/cleaved caspase-3 in double-mutant and control mice. Quantification of caspase-3-positive cells in the optic nerve at P30 showed increased apoptosis in double-mutant relative to control mice (45.28±17.39 cells/mm² in double-mutants and 11.13±8 cells/mm² in control mice: p=0.012. Fig. 3C,D). So, dampened Erk1/2-MAPK and/or PI3K/Akt signaling may diminish cell survival, producing fewer OLs in R-Ras1- and R-Ras2-deficient mice.

Expression of typical myelin proteins is also reduced in R-Ras1^−/−, R-Ras2^−/− and R-Ras1^−/−; R-Ras2^−/− double-mutant mice

Given that OLs are the only cell type responsible for myelin production in the CNS, we wanted to determine how the reduced OL populations affected the amount of myelin sheathing the axons of mutant mice. We conducted a histological analysis using coronal sections of the adult brain (P90) and discovered a decrease in the corpus callosum in double-mutant mice compared to controls (Fig. 4A). Quantification revealed a decrease in average corpus callosum thickness in R-Ras1^−/−;R-Ras2^−/− (168±11.5 μm) compared to controls (360±44.5, p<0.0001; Fig. 4B). Since we found no significant reduction in the
number of axons forming this tract (3705±943 axons in control mice and 4377±1137
axons in R-Ras1<sup>-/-;R-Ras2<sup>-/-), its reduced size could reflect a deficit in the amount of
myelin in these double-mutant mice. We then examined the corpus callosum and optic
nerve from adult (P90) mice using immunohistological staining with specific antibodies
against the myelin marker protein MBP (myelin basic protein). We saw a dramatic
reduction in the amount of myelin enveloping axons in double-mutant mice compared
to controls (Fig. 4 C,D).

We confirmed the decrease in myelin from adult (P90) optic nerve homogenates by
Western blots using antibodies against MBP and myelin-associated glycoprotein
(MAG) from adult mice lacking R-Ras1 and/or R-Ras2 and controls (Fig. 4E).
Specifically, levels of MAG and MBP protein were lower in single and double mutants
compared to control mice (MAG: R-Ras1<sup>-/-=27.12±13.28% reduction, p=0.008; R-Ras2
<sup>-/-=36.35±9.45% reduction, p=0.00263; and the double mutant=47.18±13.19% reduction,
p=0.00345. MBP: R-Ras1<sup>-/-=30.76±18.40% reduction, p=0.0057; R-Ras2<sup>-/-
=43.64±20.53% reduction, p=0.00144; and R-Ras1<sup>-/-;R-Ras2<sup>-/-=56.53±19.03% reduction,
p=0.00016 (Fig. 4G). In addition, expression of MAG and MBP in the corpus
callosum (Fig. 4E) and spinal cord (data not shown) confirmed that the levels of myelin
were reduced dramatically in the major myelinated CNS tracts in the absence of R-Ras1
and R-Ras2 (MAG R-Ras1<sup>-/-;R-Ras2<sup>-/- 27.52±15.91% reduction, p=0.004; MBP R-Ras1
<sup>-/-;R-Ras2<sup>-/- 55.48±20.53% reduction, p=0.0001, Fig. 4F).

Myelin sheath thickness is reduced in R-Ras1<sup>-/-, R-Ras2<sup>-/- and R-Ras1<sup>-/-;R-Ras2<sup>-/-
mice

To further examine the ultrastructure of myelin tracts, we analyzed the optic nerves
from R-Ras1<sup>-/-, R-Ras2<sup>-/-, double-mutant and control adult mice (P90) with electron
microscopy (EM). We found that the myelin sheath was thinner in single and double
mutants compared to controls (Fig. 5A). Morphometric quantification of the g-ratios for individual fibers compared to the respective axon diameter in the optic nerve (presented as scatter plots) confirmed the reduction in myelin sheath thickness in R-Ras1−/− and R-Ras2−/− mice that was more pronounced in double-mutant compared to control mice (Fig. 5C). Specifically, we quantified significant increases in the mean value of the g-ratio of single (R-Ras1−/−=0.87±0.04, p<0.0001, and R-Ras2−/−=0.8±0.08, p<0.0001) and double-mutant mice (R-Ras1−/−;R-Ras2−/−=0.89±0.06, p<0.0001) compared to controls (0.75±0.09).

To check whether there was a change in the axonal diameter of double mutants compared with the controls, we classified the axons according to their diameter (Fig. 5F). The double mutants showed an increase in the number of axons of lower caliber associated with a decrease in axons of greater diameter (R-Ras1−/−;R-Ras2−/− = 42% increase in axons <400 nm, p<0.0001 and 29% reduction in axons >1000 nm, p=0.0002).

Regardless of axon caliber, we found a clear thinning of the myelin sheath in single (R-Ras1−/− =105±53 nm, p<0.0001; R-Ras2−/− =159±75 nm, p<0.0001) and double mutants (R-Ras1−/−;R-Ras2−/− =73±40 nm, p=0.0001) relative to controls (251±60 nm) (Fig. 5E).

We quantified the number of axons that were myelinated, finding that in the absence of R-Ras1 there was a decrease in the amount of myelin (Fig. 4E,G, 5A,B,C,E) without changes in the number of myelinated axons (Fig. 5D) which indicates that these axons were covered by a thinner myelin sheath. However, the absence of R-Ras2 produced a 30% reduction in the number of myelinated axons concurrent with a loss of myelin. The most drastic phenotype was presented by the double mutant, in which there was a 75% decrease in number of myelinated axons. In addition, these mice R-Ras1−/−;R-Ras2−/− show a great decrease in the amount of myelin, this indicates that these axons are coated
by a very thin myelin sheath, raising the value of the g-ratio. These results demonstrate the hypomyelination of the optic nerve in mice lacking R-Ras1 and/or R-Ras2. The phenotype of the double mutant corresponds to the sum of the defects observed in the single mutants.

We then analyzed the structure, degree and periodicity of compaction of the myelin sheaths at a higher magnification and found no differences among mutant and control mice (Fig. 5A,B). However, there were clearly fewer turns in the myelin sheaths from single mutants and even fewer in double mutants (Fig. 5B). Thus, we conclude that the reduced myelin thickness arises from fewer myelin layers. The phenotype of the double mutant indicates that R-Ras1 and R-Ras2 play additive roles in myelination.

**R-Ras1 and R-Ras2 are necessary for proper oligodendrocyte differentiation**

To evaluate whether the observed myelin loss arose from altered OPC differentiation into mature OLs, we stained brain sections from mice at the most active stages of myelination *in vivo* (P15). The antibody used was against O4 (a sulfatide and pro-oligodendroblast antigen), which is a marker for cell bodies and processes from late precursors and immature pre-myelinating OLs (Sommer and Schachner, 1981; Dawson et al., 2000; Woodruff et al., 2001; Ramos et al., 2011). We found increased intensity in O4-labeling in the corpus callosum, which suggests an alteration in the degree of OL differentiation in double mutants compared to controls (Fig. 6A,B). We then performed dual immunohistochemistry with anti-Tcf4, a specific marker of pre-myelinating immature OLs (Emery, 2010), and anti-Olig2, which recognizes the complete oligodendrocytic lineage. We found a significant increase in the population of immature OLs (Tcf4^+^) in double-mutant compared to P15 control mice (Fig. 6C). The proportion of immature OLs (Tcf4^+^) relative to the total OL population (Olig2^+^) almost doubled in *R-Ras1^-/-;R-Ras2^-/-* mutants relative to control mice (100±7% for controls and 191±39%
for R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup>, p=0.016; Fig. 6C,D). We observed that the increase in Tcf4 was maintained at P30 (100±20% for controls and 255±14% for R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup>, p=0.002; Fig. 6D) and at P120 (100±20% for controls and 170±53% for R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup>, p=0.017; Fig. 6D). We found a significant increase in the population of immature OLs, not only in early postnatal stages (P15 and P30) but also in adult mice (P120). Besides, double immunohistochemical staining in adult mice (P120) with anti-Olig2 and anti-CC1, a marker of mature OLs, showed, as expected, a significant reduction in the proportion of mature OLs in the double mutant (35.8±8.6% CC1/Olig2 positive cells) compared with the control (83.8±0.9% CC1/Olig2 positive cells, p=0.0006, Fig. 6E,F). Therefore R-Ras1 and R-Ras2 seem to be necessary for the adequate maturation of OLs.

To test whether the lack of R-Ras1 and R-Ras2 dampened OL maturation, we prepared enriched OL cultures from the cortex and optic nerve of newborn R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> mice and their control littermates. We found that by 14 DIV, OLs from the double-mutant mice expressed CNPase and formed a more simplified network of processes than those from control mice, suggesting that OL maturation is affected by loss of R Ras1 and R-Ras2 (Fig. 6G). After labeling with anti-CNPase and anti-Olig2 antibodies, we classified OL morphology as having either simple (primary and secondary) or complex (tertiary or myelin network) processes to reflect the changes associated with OL differentiation. This quantification of cell morphology (introduced by Kremer et al., 2009) revealed distinct differences in OL maturation between R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> and control mice. OLs from R-Ras1- and R-Ras2-deficient mice had more simple processes than control mice at the expense of complex processes (45.7±12.69% increase for R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> relative to the control, p=0.0106; Fig. 6H). So, we conclude that OLs in double-mutant mice acquire a simpler morphology with less complex networks of
processes, indicating that R-Ras1 and R-Ras2 are essential for proper morphological
differentiation \textit{in vitro}.

\textbf{R-Ras1 and R-Ras2 modify the size of the nodes of Ranvier and axon conduction velocity in the optic nerve}

Myelin sheathes axons in segments separated by nodes of Ranvier, which are short and periodic interruptions in the myelin sheath (Fig. 7B). Given the observed hypomyelination in the optic nerve and corpus callosum of the double-mutant mice, we analyzed the gross structure of the nodal region by immunostaining against a protein located in the paranodal region, Caspr (Kamasawa et al., 2005; Murcia-Belmonte et al., 2016). Confocal microscopy of longitudinal sections of the optic nerves from adult mice (P90) immunostained with an antibody against Caspr revealed more paranodal regions in the double-mutant mice than controls (80.85±19.71 paranodes/67x67μm for the double mutant and 54.09±14.22 paranodes/67x67μm for the control, p=0.000124; Fig. 7A,E). Indeed, we detected more Caspr protein in the adult (P90) double mutants relative to control mice after probing immunoblots of optic nerve homogenates for Caspr (p=0.016; Fig. 7C). The paranodal increase in the double mutant indicates a shorter internodal length, generating shorter myelin sheaths compared with controls (Savvaki et al., 2008) (Fig. 7D). Altered myelination may also correlate with aberrant nodal and paranodal structures (Savvaki et al., 2008; Tanaka et al., 2009; Lee et al., 2011; Murcia-Belmonte et al., 2016). After classification of the different nodes and paranodes according to their length, we did not detect differences in paranode length (Fig. 7F). In contrast, we revealed a dramatic increase in the proportion of shorter nodes (less than 0.5 microns) in \textit{R-Ras1}$^{-/-}$;\textit{R-Ras2}$^{-/-}$ mice relative to controls (30±4.5% for double mutant and 7.4±5.1% for controls, p=0.004; Fig. 7A boxed insert, G). Accordingly, these double-mutant mice had fewer long nodes (between 1 and 1.5
microns: 6.95±4.05% for R-Ras1−/−;R-Ras2−/− and 25.65±6.05% for control mice, p=0.011; Fig. 7A boxed insert, G). As the number of nodes of Ranvier and internodes increased, they became closer together (a decrease of approx. 15% in length) in the absence of R-Ras1 and R-Ras2.

The decreased myelin sheath thickness and the presence of shorter nodes could provoke abnormal conduction of nerve impulses in the major CNS myelinated tracts (Richardson et al., 2000; Bakiri et al., 2011). Thus, we measured the conduction velocity of nerve impulses along the optic nerve in vivo. We implanted recording electrodes into the dorsal part of the lateral geniculate nucleus of our four groups of mice (Fig. 8A-C) and stimulated the animals bilaterally with light flashes. As previously described (Wiggins et al., 1982; Meeren et al., 1998), flash stimulation evokes an early positive-negative-positive field potential followed by some late oscillatory components. The early positive component (P1, Fig. 8C) presented latencies similar for the four groups of adult mice (P120) (9.58±1.09 ms for controls; 9.73±1.67 ms for R-Ras1−/−, p=0.542; 10.32±2.38 ms for R-Ras2−/−, p= 0.945; and 9.77±1.35 ms for R-Ras1−/−;R-Ras2−/−, p=0.715; Fig. 8D), which confirmed the presence of unaffected axons in the optic nerve (see above). These latencies were in the range of unitary activation of ganglion cells by photic stimulation in mice (Lintas et al., 2013). The negative component of the evoked field potential presented successive downward sags and a total long duration in mutant mice compared to controls (Fig. 8C). These successive negative waves suggest a delayed depolarization of geniculate neurons from arriving ganglion axon terminals. Indeed, a quantitative analysis of the latency of visually evoked potentials showed a significant increase in their latency in single and double mutant mice relative to control mice (19.19±2.90 ms for controls; 23.08±1.51 ms for R-Ras1−/−, p=0.00081; 24.51±2.71 ms for R-Ras2−/−, p= 0.000385; and 26.36±2.22 ms for R-Ras1−/−;R-Ras2−/−, p=0.0000022; Fig. 8E). Although
we found alterations in conduction velocity in the two single mutant mice, we found the most dramatic differences in the double mutant. These results strongly suggesting that R-Ras1 and R-Ras2 have cooperative and non-redundant functions. Consequently, our observed structural and electrophysiological alterations reveal that R-Ras1 and R-Ras2 are essential to achieve the correct velocity for nerve impulse conduction in myelinated CNS tracts.
Discussion

Here we provide in vivo evidence that R-Ras1 and R-Ras2 play an essential role in regulation of myelination by controlling fundamental parameters of OL survival and differentiation. Our data demonstrate that mice lacking R-Ras1 and/or R-Ras2 showed hypomyelination in major CNS tracts, reducing the conduction velocity of nerve impulse through them.

Loss of either R-Ras1 or R-Ras2 reduced the number of OLs, but a more dramatic reduction occurred with the loss of both. Hence, R-Ras1 and R-Ras2 may cooperatively maintain the OL population. As the double mutant induced the most severe phenotype, R-Ras1 and R-Ras2 do not seem functionally redundant and cannot compensate for each other. We acknowledge that the classical Ras members were not modified and did not compensate for the loss of R-Ras (data not shown).

We also detected clear differences in OL differentiation and maturation in these mutant mice. Indeed, our double-mutant mice more strongly expressed markers of premyelinating OLs, associated with lower expression of markers of maturity. OPCs undergo stereotyped morphological changes as they differentiate (Kremer et al., 2009). Specifically, oligodendroglial cells in dissociated cultures extend cell processes until they acquire a truly branched morphology (Ishii et al., 2012; Zuchero et al., 2015). We found that R-Ras1- and R-Ras2-deficient OLs formed a simpler network of processes than control OLs, suggesting that loss of R-Ras1 and R-Ras2 alters oligodendrocyte maturation in vitro and in vivo.

Defects in OL differentiation and maturation hinder myelination (Wolswijk, 1998; Kuhlmann et al., 2008; Kremer et al., 2011). When we quantified essential myelin proteins in the optic nerve and corpus callosum, we discovered distinct hypomyelination in the absence of R-Ras1 and R-Ras2. Specifically, MAG and MBP expression
gradually decreased in these mutants, while a more significant decrease was evident in the double mutants. Electron microscopy analysis of transverse optic nerve sections from adult mice (P90) illustrated how axons possessed thinner myelin sheaths, regardless of axon diameter. R-Ras1/− and R-Ras2/− showed different g-ratio profiles, which could suggest a specific role for each of these GTPases in specific subpopulations of OLs. While we found a gradual decrease in myelin sheath thickness in single mutants, this decrease was more dramatic in the absence of both R-Ras1 and R-Ras2. Despite the dramatic decrease in axonal myelin thickness in our mutants, we observed no alterations in the degree of sheath compaction. These results suggest that the mechanism responsible for assembling myelin sheaths around axons is independent of R-Ras1 and R-Ras2.

The decrease in myelin production in the absence of R-Ras1 and R-Ras2 was much more drastic than the loss of OLs. This result indicates that the surviving OLs are immature and incapable of myelinating axons correctly. We discovered distinct myelin deficiency in axons of all calibers, although not all fibers had hypomyelination. We propose that CNS axons can differ in their proclivity to acquire a myelin sheath, as suggested by (Nave and Werner, 2014). Although axon diameter was comparable between mutant and control mice, we noted a tendency towards a reduced axon caliber in mutants lacking R-Ras1 and/or R-Ras2, consistent with findings that OLs condition axon caliber (Hildebrand and Hahn, 1978; de Waegh et al., 1992; Hildebrand et al., 1993; Sanchez et al., 1996; Goebbels et al., 2010). Myelination may require communication between axons, astrocytes and OLs to determine myelin sheath thickness (Camargo et al., 2017). However, the contributions of each cell type to sheath thickness remains unknown. The absence of R-Ras1 and R-Ras2 in neuron-free cultures suggests an essential role for these GTPases in OPC differentiation. This hypothesis
accounts for our observed alterations in myelination. We note that the influence of R-Ras1 and R-Ras2 on neurons and/or astrocytes was not explored in detail in our current study.

Thus, downregulation of the PI3K/Akt and Erk1/2-MAPK pathways could induce hypomyelination, smaller OL population, diminished cell survival and increased proportion of immature OLs in the absence of R-Ras1 and R-Ras2. Increased activity of the PI3K/Akt pathway, either by inhibiting PTEN or constitutively activating Akt in OLs, does produce hypermyelination without altering OPC proliferation or the final number of mature OLs (Flores et al., 2008; Goebbels et al., 2010). Moreover, altered PI3K/Akt signaling affects OL differentiation. Specifically, inhibition of mTOR, an effector of this pathway, blocks OL differentiation and preserves them in a premyelinating state associated with no myelin production (Tyler et al., 2009; Gaesser, 2016). Likewise, overactivation of the Erk1/2-MAPK pathway in OLs provokes hypermyelination in major CNS tracts (Ishii et al., 2013). Conversely, loss of Erk1/2 in mature OLs decreases the amount of myelin with a concomitant reduction in the size of OL population due to poor OL viability (Ishii et al., 2014). The phenotype we described in R-Ras1- and R-Ras2-deficient mice could result from a combined effect of deactivation of both the PI3K/Akt and Erk1/2-MAPK pathways. The relationship between R-Ras1 and R-Ras2 to PI3K/Akt and Erk1/2-MAPK signaling activation must be addressed in future studies. However, we do propose that R-Ras1 and R-Ras2 are upstream effectors of both pathways and adequately orchestrate their signaling to maintain not only survival but also correct OL differentiation.

Efficacy of neural transmission occurs through specific variables, such as myelin thickness or node and internode length (Waxman, 1980, 1997; Zimmermann and Dours-Zimmermann, 2008; Bekku et al., 2010). Hypomyelination correlates with slower
conduction velocities, as described in other mouse models that overexpress or lack distinct myelin proteins (Robaglia-Schlupp et al., 2002; Michailov et al., 2004; Lee et al., 2011). Some studies suggest weaker myelination yields slower nerve conduction velocities and abnormal node or paranode structures (Tanaka et al., 2009; Lee et al., 2011). A decrease in newly generated OLs may change myelin structures and induce a progressive decrease in axonal conduction velocity in the corpus callosum (Schneider et al., 2016). Our data demonstrate that in the absence of R-Ras1 and R-Ras2, shortening of the nodes of Ranvier and internodes accompanies hypomyelination, which may cause the reduced conduction velocity we observed in the optic tract. However, we cannot exclude that the absence of R-Ras1 and R-Ras2 affects other factors like ion channel distribution or other structural proteins in the nodal or paranodal regions. This remains an area for future studies.

To-date, R-Ras1 and R-Ras2 activity in leukodystrophias has not been explored. The accepted animal model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), is attenuated in R-Ras1−/− because of an increase in tolerogenic dendritic cells and natural regulatory T cells (Ray et al., 2014). After our current results, we strongly suggest that the roles of R-Ras1 and R-Ras2 in demyelinating diseases should be carefully studied, especially the neuropathological aspects of disease.

In summary, we identified R-Ras1 and R-Ras2 as key elements for OL survival and differentiation, acting through the synergistic activation of the PI3K/Akt and Erk1/2 signaling pathways. These results are separable from the classical Ras proteins, as they were neither modified nor compensated for the lack of R-Ras responsivity. Therefore, we conclude that R-Ras1 and R-Ras2 GTPases are necessary for proper OL-mediated myelination and regulation of correct nerve impulse transmission.
**Figure legends**

**Figure 1.** OLs express R-Ras1 and R-Ras2 in the main myelinated tracts of the CNS. 

_A_, Immunoblot of adult optic nerve lysates show R-Ras1, R-Ras2 and R-Ras3 expression. Two different antibodies against R-Ras1 or R-Ras2 recognized both proteins, a band of 25 kDa corresponding to R-Ras1 and another of 21 kDa corresponding to R-Ras2. No bands were observed in optic nerve lysates from _R-Ras1^-/-;R-Ras2^-/-_ mice. R-Ras3 expression was not modified in _R-Ras1^-/-_ and/or _R-Ras2^-/-_ showing a band of 24 kDa. 

_(n=3)._ **B-C,** Western blot of corpus callosum (_B_) and spinal cord (_C_) extracts from control mice confirmed expression of R-Ras1, R-Ras2 and R-Ras3 in primary CNS myelinated tracts. (_n=3)._ **D,** Schematic representation of the targeting strategy to generate the _R-Ras1_ null mutant. A Neo cassette flanked by FRT sites was inserted into intron 1 together with two loxP sites flanking exons 2 and 6. The position of the primers designed for genotyping are shown by the arrows. **E,** PCR genotyping. A mixture of primer 1 and primer 2 detected the _R-Ras1^+/+_ (~100 bp) allele by PCR and a mixture of primer 3 and primer 4 detected the _R-Ras1^-/-_ target allele (~1000 bp). **F,** Generation of _R-Ras2^-/-_ mice. Illustration of the retroviral insertion site of the _R-Ras2_ gene in intron 1 and the positions of the three primers used for genotyping. **G,** Analysis of the relative expression of _R-Ras1_ and _R-Ras2_ by RT-qPCR in control, _R-Ras1^+/+_, _R-Ras2^+/+_ and _R-Ras1^-/-_;_R-Ras2^-/-_ adult mice normalizing all data to control mice. Values expressed as the mean fold-change of 3 different animals, p<0.0001. **H,** Longitudinal optic nerve sections from control, _R-Ras1^-/-_, _R-Ras2^-/-_ and _R-Ras1^-/-_;_R-Ras2^-/-_ adult mice showing co-localization between R-Ras1 (top) and R-Ras2 (bottom, green) with Nkx2.2, an oligodendroglial cell marker (red). No staining for R-Ras1 or R-Ras2 was observed in the double-mutant mice. (_n=3)._ All samples were obtained from adult mice (P90). Scale bar: 25μm.
Figure 2. OL population is significantly diminished in R-Ras1−/−, R-Ras2−/− and R-Ras1−/−;R-Ras2−/− mice. A, Immunolabeling with anti-Olig2 (green) of longitudinal optic nerve sections from control, R-Ras1−/−, R-Ras2−/− and R-Ras1−/−;R-Ras2−/− adult mice (P90), show a reduction in the OL population in the mutant mice compared with the controls. B, Simplified scheme of the visual tract shows the region of analysis. C, Quantification of the density of Olig2-positive cells per mm² shows a 19.1±7.5% decrease in R-Ras1−/− (p=0.02), a 33.5±2.1% reduction in R-Ras2−/− (p<0.0001) and a 42.8±11.8% reduction (p=0.003) in R-Ras1−/−;R-Ras2−/− mice relative to controls. Reduction of OLs in the R-Ras1−/− mutant mice was significantly less than that observed in the R-Ras2−/− (p=0.003) and R-Ras1−/−;R-Ras2−/− (p=0.02) mutant mice. The bar graph represents the mean and SD for the number of Olig2-positive cells per mm² from six different experiments (n=6). Scale Bars: 75 μm.

Figure 3. R-Ras1 and R-Ras2 are necessary for OL survival driven by PI3K/Akt and Erk1/2-MAPK signaling. A, Western blot analysis of phospho-Akt (T308), phospho-Akt (S473) and phospho-S6 in optic nerve lysates of adult mice (P90) shows the PI3K/Akt pathway was altered in the absence of R-Ras1 and/or R-Ras2. Phosphorylated proteins were significantly reduced in mutant mice compared to controls. (Bottom) Western blot analysis shows decreased phosphorylated Erk1/2 (T202/Y204) in adult optic nerve lysates from R-Ras1−/−, R-Ras2−/− and R-Ras1−/−;R-Ras2−/− mice compared to controls. Graphs show densitometric analysis of phosphorylated Akt, S6 and Erk1/2 normalized to total protein levels compared to controls (100%, n=4): P-Akt (T308) R-Ras1−/− 78.5±6.51% reduction (p<0.0001), R-Ras2−/− 35.3±12.80% reduction (p=0.0035), R-Ras1−/−;R-Ras2−/− 34.5±15.23% reduction (p=0.007); P-Akt (S473) R-Ras1−/− 49.2±1.20% reduction (p<0.0001), R-Ras2−/− 53.2±15.92% reduction (p=0.0044), R-Ras1−/−;R-Ras2−/− 62.9±4.92% reduction (p=0.0001); p-S6 R-Ras1−/− 62.6±6.8% reduction.
(p<0.0001),  
R-Ras2<sup>−/−</sup> 50.9±15.2% reduction (p=0.0002),  
R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> 65.2±23.5% reduction (p=0.0005); p-Erk1/2  
R-Ras1<sup>−/−</sup> 45.4±25.8% reduction (p=0.004),  
R-Ras2<sup>−/−</sup> 55.6±15.3% reduction (p<0.0001),  
R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> 68.5±20.3% reduction (p<0.0001).

B, Schematic representation of the pathways downstream of R-Ras1 and R-Ras2. Discontinuous lines imply indirect activation or inhibition. C, Bar graph shows the mean number of caspase-3-positive cells per mm<sup>2</sup> observed in P30 optic nerves from 
R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> (45.28±17.39) compared with controls (11.14±8.01, p=0.0124; n=3).  
D, Immunostaining of longitudinal sections of optic nerves at P30 with anti-cleaved-caspase-3 reveals increased cell apoptosis in the R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> mice compared to controls (n=3). Scale Bar=25 μm.

Figure 4. Myelin decrease in the absence of R-Ras1 and/or R-Ras2. A, Coronal sections from adult (P90) mouse brains stained with DAPI show a remarkable reduction in the thickness of the corpus callosum in the double mutant compared with the control. Lower-magnification images of the corpus callosum shown in the boxed insert (interaural 4.40-4.76 mm and bregma 0.61-0.97 mm; (Paxinos and Franklin, 2013). B, Quantification of corpus callosum thickness in these coronal sections shows a decrease in R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> compared with control. Corpus callosum thickness was analyzed in several positions: position 0 in x-axis means a measurement in middle line (R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> 169.7±16.5 μm, control 428.6±43.1 μm, p<0.0001); position 0.5 is a measurement in cingulate cortex (R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> 158.7±7.8 μm, control 321.3±40.7 μm, p=0.0002 in right, R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> 155.1±15.8 μm, control 342.4±30.2 μm, p<0.0001 in left); position 1 is a measurement in secondary motor cortex (R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> 184.3±18.2 μm, control 365.9±59.5 μm, p=0.0011 in right, R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> 181.8±29.9 μm, control 408.2±41.6 μm, p<0.0001 in left); position 1.5 is a measurement in primary motor cortex (R-Ras1<sup>−/−</sup>;R-Ras2<sup>−/−</sup> 158.9±12.3 μm, control
Table 1. Comparison of myelin thickness in control and mutant mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Myelin Thickness (μm)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>305.9±66.8</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>R-Ras1−/−</td>
<td>352.5±30.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>R-Ras2−/−</td>
<td>166.7±31.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>R-Ras1−/−;R-Ras2−/−</td>
<td>352.5±30.3</td>
<td>&lt;0.0001</td>
</tr>
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Figure 5. Thinner myelin sheath in R-Ras1−/−, R-Ras2−/− and R-Ras1−/−;R-Ras2−/− adult (P90) mutant mice. A, Transverse sections of the optic nerve from adult (P90) control, R-Ras1−/−, R-Ras2−/− and R-Ras1−/−;R-Ras2−/− mice analyzed by electron microscopy show a significant decrease of myelin in single and double mutants compared with controls. B, Higher magnification images show no differences in myelin structure, compaction and periodicity in mutant mice compared to controls. However, R-Ras1−/−, R-Ras2−/− and more dramatically R-Ras1−/−;R-Ras2−/−, show less wrapping of myelinated axons.
compared to controls. C, Scatter plots show the morphometric quantification of g-ratios (y-axis) of individual fibers relative to the respective axon diameters (x-axis) from optic nerves. Significant increase in g-ratios were found in $R$-Ras1$^{-/-}$ (0.87±0.04, p=0.000012, n=924 axons analyzed), $R$-Ras2$^{-/-}$ (0.8±0.08, p=0.000011, n=1014 axons analyzed) and $R$-Ras1$^{-/-};R$-Ras2$^{-/-}$ (0.89±0.06, p=0.000072, n=1037 axons analyzed) compared to controls (0.75±0.09, n=764 axons analyzed). D, Graph shows the percentage of myelinated axons present in the optic nerve of $R$-Ras1$^{-/-}$ = 102±2.7%, $R$-Ras2$^{-/-}$ = 72.1±5.2% and $R$-Ras1$^{-/-};R$-Ras2$^{-/-}$ = 23.9±2.5% compared to the control = 100±2.8%. Significant differences were observed in the $R$-Ras2$^{-/-}$ (p<0.0001) and the double-mutant mice (p<0.0001). E, Scatter plots show the variation of myelin thickness with respect to the diameter of axons in single and double mutants compared to the control (control = 251±60 nm, n=764 axons analyzed; $R$-Ras1$^{-/-}$ = 105±53 nm, p<0.0001, n=924 axons analyzed; $R$-Ras2$^{-/-}$ = 159±75 nm, p<0.0001, n=1014 axons analyzed; $R$-Ras1$^{-/-};R$-Ras2$^{-/-}$ = 73±40 nm, p<0.0001, n=1037 axons analyzed). F, Graph shows the percentage of axons classified according to their diameters in control and double-mutant mice. Compared to the control, $R$-Ras1$^{-/-};R$-Ras2$^{-/-}$ mice showed a significant increase in the smallest axons, measuring less than 600 nm, (control = 30%, $R$-Ras1$^{-/-};R$-Ras2$^{-/-}$ = 42.6% of axons, p<0.0001) and a significant decrease in axons measuring more than 1000 nm (control = 30.3%, $R$-Ras1$^{-/-};R$-Ras2$^{-/-}$ = 21.5% of axons, p=0.0002). All samples were obtained from adult mice (P90). n=3. Scale Bars= 500 nm in A; 250 nm in B.

**Figure 6.** R-Ras1 and R-Ras2 play a crucial role in OL differentiation. A, Mirror images of coronal brain sections from P15 control and $R$-Ras1$^{-/-};R$-Ras2$^{-/-}$ mice immunolabeled with an anti-O4 antibody (red). Pictures correspond to the corpus callosum (interaural 2.36-2.48 mm and bregma -1.43 mm (Paxinos and Franklin, 2013).
More intense staining occurred in R-Ras1<sup>-/-</sup>;R-Ras2<sup>-/-</sup> mice, indicating an increase in the proportion of pre-myelinating OLs in double mutants relative to controls. **B**, Schematic representation of different O4 immunostaining observed in double mutants compared to controls. Right hemisphere represents R-Ras1<sup>-/-</sup>;R-Ras2<sup>-/-</sup> mice where the corpus callosum shows more premyelinating OLs (red) than controls (left hemisphere). **C**, Dual immunostaining with anti-Tcf4 (red) and anti-Olig2 (green) in the corpus callosum of P15 control (top) and R-Ras1<sup>-/-</sup>;R-Ras2<sup>-/-</sup> (bottom) mice. Double-mutant mice showed more premyelinating OLs than controls (Tcf4/Olig2 positive cells) in P15 mice. **D**, Percentage of premyelinating OLs (Tcf4-positive cells) relative to the amount of OLs (Olig2-positive cells). Double-mutant mice show a significant increase in the number of immature OLs compared to controls. Bar graph represents a significant increase in the percentage of Tcf4-positive cells in double mutants compared with controls. (P15: R-Ras1<sup>-/-</sup>;R-Ras2<sup>-/-</sup>=91% increase p=0.016; P30: R-Ras1<sup>-/-</sup>;R-Ras2<sup>-/-</sup>=155% increase, p=0.002 and P120: R-Ras1<sup>-/-</sup>;R-Ras2<sup>-/-</sup>=70% increase, p=0.017) (n=3). **E**, Double immunostaining with anti-CC1 (red) and anti-Olig2 (green) in the corpus callosum of P120 control (top) and R-Ras1<sup>-/-</sup>;R-Ras2<sup>-/-</sup> (bottom) mice. Double-mutant mice showed a drastic reduction in the proportion of mature OLs compared with the controls (CC1/Olig2 positive cells). **F**, Quantification of the percentage of mature OLs (CC1 positive cells) relative to the total amount of OLs (Olig2 positive cells). A significant decrease in the percentage of CC1 positive cells was found in double mutants compared with controls (R-Ras1<sup>-/-</sup>;R-Ras2<sup>-/-</sup>=57.3% decrease p=0.0006 (n=3). **G**, Representative confocal images of morphologies found in primary cultures stained for CNPase (green) and Olig2 (red). After 14 DIV, OLs from double mutants show simpler morphology than controls. **H**, Schematic representation of enriched primary OL cultures from the cortex of control and R-Ras1<sup>-/-</sup>;R-Ras2<sup>-/-</sup> mice shows a simpler
morphology in double mutants. Proportion of OLs (CNPase and Olig2 positive cells) with simple and complex processes are shown for each genotype in the bar graph. Most double-mutant OLs had simple processes (72.85±12.7%, n=101 OLs quantified) that differed significantly from controls (27.15±12.7%, p=0.0106, n=121 OLs quantified). A few double-mutant OLs had complex processes (24.9±13.3%) that differed significantly from controls (75.1±13.3%, p=0.0106). Scale Bars: 250 μm in A; 25 μm in C and E.

Figure 7. More nodes of Ranvier with shorter length generate more internodes in the absence of R-Ras1 and R-Ras2 in adult mice (P90). A, Double-staining of longitudinal sections of adult (P90) optic nerves with anti-Caspr (green) and anti-NF200 (red) reveal an increase in the number of paranodes (green) in the R-Ras1−/−;R-Ras2−/− mice compared to controls. The distance between paranodes (nodal length) in higher magnification images (boxed insert) was shorter in double mutants than in controls. B, Simplified scheme representing the node of Ranvier between two internodes. C, Western blot of P90 optic nerve lysates shows a significant increase in Caspr protein expression in double mutants compared to controls. Bar graph shows the mean ± SD change in densitometric measurements normalized to GAPDH and relative to controls (p=0.016; n=5). D, Schematic representation of the shorter nodal length and more internodes observed in the R-Ras1−/−;R-Ras2−/− mice compared to control. E, Bar graph shows a significant increase in paranodes found in double mutants (80.85±19.71 paranodes/67×67μm) compared to controls (54.09±14.22 paranodes/67×67μm) (p=0.000124; n=4). F, Paranodal length classification does not reveal any differences in R-Ras1−/−;R-Ras2−/− compared with controls. G, Node classification by their length reveals more short nodes in R-Ras1−/−;R-Ras2−/− mice (30.5±4.5% of nodes <0.5 μm long) than in controls (7.5±5.1% of nodes <0.5 μm long, p=0.004) at the expense of longer nodes.
Figure 8. Axonal conduction velocity was affected by the absence of R-Ras1 and/or R-Ras2. 

A, Experimental design. Alert-behaving mice received a photic stimulator (flash) through a stroboscope located 30 cm in front of them. 

B, Recording electrodes implanted in the left dorsal lateral geniculate nucleus. Photomicrograph illustrates recording electrode location. 

C, Examples of field potentials evoked by light flash stimulation at the dorsal lateral geniculate nucleus. Recordings averaged 20 times and collected from representative control, R-Ras1⁻/⁻, R-Ras2⁻/⁻, and R-Ras1⁻/⁻;R-Ras2⁻/⁻ mice. 

Light flash presentation indicated by an arrow. Latency to P1 and to the latest N component of the evoked field potential indicated. 

D, E, Although no significant differences were observed for latency values from flash presentation to P1, latency values to the last N component of the evoked field potential were significantly larger for the three experimental groups (R-Ras1⁻/⁻, R-Ras2⁻/⁻, and R-Ras1⁻/⁻;R-Ras2⁻/⁻) compared with controls. ***, p<0.001. All mice analyzed were adults (P120).
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Figure 7