Supporting Information

Appendix S1a: Abbreviations

ACN, acetonitrile; AD, Alzheimer's disease; AP, alkaline phosphatase; CaMKII, Ca2+/calmodulin-dependent protein kinase II; 6CDFDA, 2,7-dichlorodihydrofluorescein diacetate; Cdc2, cell division control protein 2 homolog; Cdc42, cell division control protein 42 homolog; CDK2, cyclin dependent kinase 2; CDK5, cyclin dependent kinase 5; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CNP, -2',3'-cyclic nucleotide 3'-phosphodiesterase; CNT, control; CRMP-2, collapsin response mediator protein-2; DAPI, 4', 6' diamidine-2'phenylindole dihydrochloride; Dcc, deleted in colorectal cancer; 2D-DIGE, bidimensional difference gel electrophoresis; DMEM, Dulbeco's Modified Eagle's Medium; Edg8, endothelial differentiation gene 8; EGFP, enhanced green fluorescent protein; ERK, extracellular-signal-regulated kinase; FCS, fetal calf serum; Fyn, tyrosine protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GSK3β, glycogen synthase kinase 3β; IPG, immobilized pH gradient; LPA, lysophosphatidic acid; LRRK2, Leucine Rich Repeat Protein Kinase-2; MAPK, Mitogen-activated protein kinase; MBP, myelin basic protein; MNK1, MAP kinase-interacting serine/threonine-protein kinase 1; MS/MS, tandem mass spectrometry; NAC, N-acetyl-L-cysteine; 3-NP, 3-nitropropionic acid; N-WASP, neuronal Wiskott–Aldrich Syndrome protein; OLGs, oligodendrocytes; OLN-93, oligodendroglial cell line; OPCs, oligodendrocyte precursor cells; PBS, phosphate buffered saline; PKA, protein kinase A; PRK2, protein kinase C-related kinase; Rac1, ras-related C3 botulinum toxin substrate 1; RhoA, ras homolog gene family, member A; ROCK, rho kinase; rpSA, ribosomal protein SA; ROS, reactive oxygen species; ROSC, roscovitine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sema3A, semaphorin3A; S1P, sphingosine-1-phosphate; TFA, trifluoroacetic acid; VAMP2, vesicle-associated membrane protein 2; Veh, vehicle; WT, wild type

Appendix S1b: Supplementary Materials and Methods

Reagents

Neutral Red; 4', 6' diamidine-2'phenylindole dihydrochloride (DAPI); poly-L-lysine; transferrin; insulin; sodium selenite; biotin; hydrocortisone; T3; N-acetyl-L-cysteine (NAC), 3-nitropropionic acid (3-NP); Trolox (hidrosoluble vitamine E analog), Y27632 (ROCK inhibitor), roscovitine (cdk5 inhibitor), NaF; sodium orthovanadate; protease inhibitor cocktail; acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Sigma. Penicillin was from UPS and streptomycin was from Gibco. 6CDFDA (2, 7-dichlorodihydrofluorescein diacetate) was obtain from Cayman. CHAPS detergent, immobilized pH gradient (IPG) strips and Cy2, Cy3 and Cy5 N-hidroxysuccinimidyl ester dyes were from GE Healthcare. C-18 ZipTip was from Millipore. Calf intestinal alkaline phosphatase was from New England BioLabs and restriction enzyme DpnI was from Invitrogen.

Cell cultures and treatments

Primary rat OLGs were isolated from newborn Wistar rats. Protocols for the care and use of animals were approved by the Institutional Committee. Briefly, the meninges, midbrain and olfactory bulbs were removed and dissociated cortices were grown at 37°C with 5% CO_2 for 15 days in DMEM-F12 containing 10% FCS. OPCs were derived from mixed glial cultures containing OPCs and microglia grown on an astrocyte monolayer by first shaking for 30 min at 240 rpm and then, after replacing cell culture medium, for 18 h at 190 rpm. Cells were filtered through a 16 μm mesh and then let to adhere for 10 min at 25°C. For immunocytochemistry, 2.5 x 10^3 cells per well were seeded on poly-L-lysine-coated coverslips. For Western blots and 2-D-DIGE, cells were plated at 2 x 10^6 cells on 100 mm dishes. Cells were differentiated in GDM medium (1% FCS, 1% Penicillin-Streptomycin, 12.5 μg/μl transferrin, 5 μg/μl insulin, Na selenite 0.008 μg/ml, 0.01 μg/ml biotin, 1 μg/μl hydrocortisone and 0.01 μg/ml T3 in DMEM-F12) for 5 days. OLG purity was estimated by immunofluorescence with anti-MBP, anti-GFAP and anti-βIII tubulin used as OLG, astrocytic and neuronal markers, respectively. In addition, nuclear staining with 4', 6' diamidine-2'phenylindole dihydrochloride (DAPI) and phase contrast examination did not reveal the presence of fibroblasts. OLN-93 rat oligodendroglial cell line (Richter-Landsberg & Heinrich 1996) was cultured in Dulbeco's Modified Eagle's Medium (DMEM) containing; 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in 10% CO_2. For measurement of process length, OLN-93 cells were plated at a density of 10^4 cells/well in 8-well Nunc Chamber.

For Western blot, cells were plated at a density of 3 x 10^5 cells per well in 35 mm dishes. OLGs and OLN-93 were exposed to the indicated concentrations of 3-NP, an irreversible inhibitor of mitochondrial complex II known to induce the accumulation of superoxide (Bacsi *et al.* 2006). To assess the effect of antioxidants and kinase inhibitors, these were added 1 h before 3-NP at the following concentrations: 10 mM N-acetyl-L-cysteine (NAC); 1 μM Trolox; 10 μM Y27632 and 50 μM roscovitine (Sigma).

Viability assay

OLGs were plated at 1.5 x 10^5 cells per well in poly-L-lysine-coated p24 multi-well plates. At the end of 3-NP treatment, cells were washed once with PBS and 500 μl of neutral red in DMEM-F12 were added. After incubation for 2 h at 37ºC, the culture was washed once with PBS and then 200 μl containing 1% acetic acid / 49% ethanol (in water) were added for 10 min under shaking in the dark. One hundred and fifty μl were loaded onto a 96-multi-well plate and OD measured at 570 nm in a microtiter plate reader 550 (Bio-Rad).

Assessment of ROS accumulation

OLGs were incubated with PBS containing 1 mM 3-NP or PBS alone for 30 min after which 100 μM of 6CDFDA was added to the culture medium and incubated at 37ºC under 5% CO_2 in the dark for additional 30 min. OLGs were washed three times with PBS, fixed with 4% paraformaldehyde and immunocytochemistry for MBP was performed using Alexa-Fluor-568-conjugated antibodies (GE Healthcare).To analyze accumulation of ROS in mature OLGs cells were examined under epifluorescence (Axiovert 200M, Zeiss, Germany).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.1% Triton X-100, and blocked in 5% normal goat serum (NGS) in PBS at room temperature. For surface Dcc receptor labeling, cells were subjected to immunofluorescence without detergent permeabilization, following the protocol described in Bouchard et al. 2004. Cells were incubated with primary antibodies overnight at 4ºC, and proteins were visualized by fluorescence of Alexa Fluor 488- or Alexa-Fluor-568-conjugated antibodies (GE Healthcare). Nuclei were stained with

DAPI. Signals were analyzed on a fluorescence microscope (Axiovert 200M, Zeiss, Germany) or a confocal Pascal LSM5 microscope (Zeiss) using a 3-frame filter and Zeiss LSM5 image examiner.

Morphometric analysis

A grid of concentric circles with increasing diameters (10 μm each) was divided orthogonally in 16 sections and superimposed on each viable MBP-positive OLG with a fully identifiable circular arborization. The outer circle reached by the longest process in each of the 16 sections per cell was used to calculate the mean diameter of the OLG expressed in microns. Branching was assessed by counting the number of intersections of α-tubulin and CRMP-2-positive processes with the concentric circles as a function of distance (in μm) from the center of the cell (Appendix S2 Fig. S1). OLN-93 process length was measured starting from the cell body with ImageJ software and expressed in microns. At least twenty five cells per condition were analyzed in at least 3 independent experiments.

Analysis of Dcc receptor immunoreactive puncta on cell surface

The number of surface Dcc receptor positive puncta was measured using ImageJ software. Images were taken with the same setting parameters and converted to 8 bit. Threshold was adjusted to obtain discreet points consistent across all images. The area and the number of particles were counted automatically on individual cells. The number of particles was divided by the cell area to control for variance in cell size. At least 25 cells per condition were analyzed in at least 3 independent experiments.

2-D difference gel electrophoresis (DIGE)

Mature OLGs with at least 95% purity were treated with 1 mM 3-NP or PBS for 1 h, harvested and cell pellets containing approximately 6 x 10^6 cells for each condition were kept at -80°C. This procedure was repeated 8 times. Pellets were pooled and solubilized into a buffer containing 7 M urea; 2 M thiourea and 4% CHAPS detergent. The samples, each containing 100 μg of protein (determined by amino acid analysis), were differentially labeled with Cy2, Cy3 and Cy5 N-hydroxysuccinimidyl ester dyes as described previously (Wu 2006). For the first dimension gel electrophoresis the labeled samples were pooled and mixed with rehydration

buffer containing 7 M urea; 2 M thiourea; 4% CHAPS; 1% DTT; 2% Pharmalytes pH 3−10 and loaded onto 24 cm pH 3-10 linear immobilized pH gradient (IPG) strips. Isoelectric focusing was performed on an Ettan IPGphor 3 (GE Healthcare) for approximately 60 kVh at 20 °C. For the second dimension gel, IPG strips were applied to 22 × 24 cm SDS-PAGE gels (12% T; 2.6% C). For image acquisition, gels were scanned using a Typhoon 9410 Imager (GE Healthcare) and image analysis performed using DeCyder v6.5, (GE Healthcare) software using the Differential In-gel Analysis module.

Mass spectrometry analysis of digests

Spots of interest were subjected to robotic tryptic (Promega) digestion on a GE Healthcare Ettan TA Digester and desalted using a C-18 ZipTip. Peptides/proteins were eluted with 3 µl of 60% ACN, 0.1% TFA containing 3.5 mg/ml ά-cyano-4-hydroxy cinnamic acid matrix and 0.8 µl were loaded onto the MALDI target plate. MALDI-Tof/Tof protein identification was performed on an Applied Biosystems (AB) Model 4800 MALDI-Tof-Tof mass spectrometer. MS/MS spectra were used to search for protein candidates with the NCBI-nr database using the MASCOT search engine (http://www.matrixscience.com). Peptide tolerance was set at +/- 20 ppm and MS/MS tolerance at +/- 0.2 Da.

SDS-PAGE and Western blots

Homogenates were sonicated for 15 sec, incubated 30 min on ice and centrifuged for 15 min at 10,000 x g at 4ºC. Protein concentration in supernatants was determined using bicinchoninic acid (BCA) protein assay kit (Pierce). Lysates were resolved on 7.5-10% polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked in 10 mm Tris-HCl, pH 7.4, 150 mm NaCl (Tris-buffered saline) with 0.1% Tween 20 and 5% nonfat milk or bovine serum albumine (BSA) and then probed with the different antibodies. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Dako), and proteins were visualized with enhanced chemiluminescence using a Storm-840 imager. Protein bands were quantified by the ImageQuant software (GE Healthcare).

Appendix S2: Supplementary Figure Legends and Supplementary Figures

Figure S1. Representation of the methods used for measuring OLG diameter and branching. **A)** Immunofluoresence with anti-MBP. White dots depict the intersections with concentric circles of the longest process in each section. Diameters of those circles were averaged to estimate OLG diameter. **B**) Immunofluorescence with anti-α- tubulin. White dots depict intersections of each process with concentric circles used to estimate the number of crossovers at increasing distance from the center. Scale bars=10μm.

Figure S2. Immunofluorescence with anti-MBP of OLGs incubated with: **A)** Veh (PBS); **B)** Trolox, prior to the treatment with 3-NP; **C)** NAC, prior to the treatment with 3-NP. Note that the diameter of 3-NP treated cells in the presence of antioxidants is similar to the control. Scale bars=10μm.

Figure S3. Representative Western blot and quantification of immunoreactivity of the bands with anti-CNP normalized by anti-GAPDH on the same membrane of homogenates from: **A)** Mature OLG; **B)** OLN-93 cells. No significant changes in CNP levels respect to GAPDH levels were observed in both cell cultures after 3-NP treatment.

Figure S4. Immunofluorescence with anti-α-tubulin of OLN-93 cells incubated with: **A**) Veh; **B**) 1 mM 3-NP; **C)** preincubated with Y27632 prior to the addition of 3-NP; **D**) preincubated with NAC prior to the addition of 3-NP. Arrows indicate the shortening of the process in the presence of 3-NP. Scale bars=10μm.

Figure S5. A) Immunofluorescence with anti-extracellular Dcc of OLN-93 cells incubated with Veh (upper left panel) or 1 mM 3-NP (upper right panel). Arrows indicate Dcc at the plasma membrane. Bars=10μm. Lower left panel, quantification of the number of particles by area expressed in arbitrary units (A.U.). Bars represent the mean ± SEM of 50 cells for each condition. Three independent experiments were done. Lower right panel, estimation of the process length from phase contrast microphotografies of the cells probed with anti-Dcc. **p<0.001 Student's t test as compared to control. **B)** Left panel, representative Western blot probed with anti-phospho-Tyr416-Fyn (p-Fyn) of homogenates from OLN-93 cells incubated with PBS in the absence (-) or presence (+) of 3-NP. After stripping, anti-total Fyn (t-Fyn) in the

same membrane was tested. On the left, molecular mass in kDa. Right panel, bars represent the mean ± SEM of the immunoreactivity of p-Fyn normalized by t-Fyn expressed in arbitrary units (A.U.).

FIGURE S1

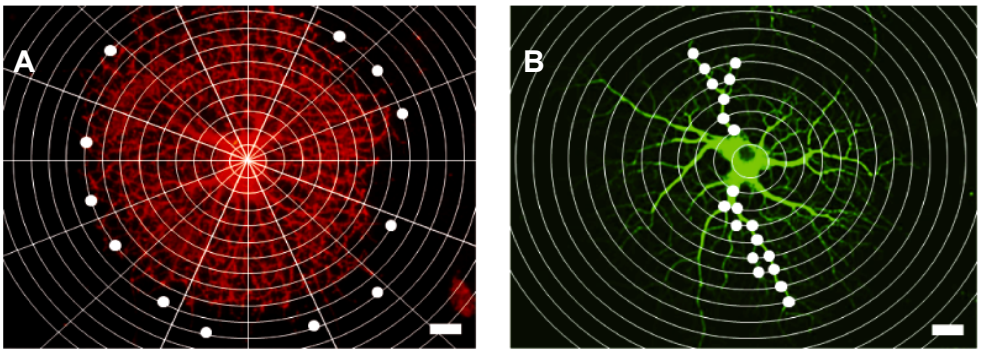


FIGURE S2

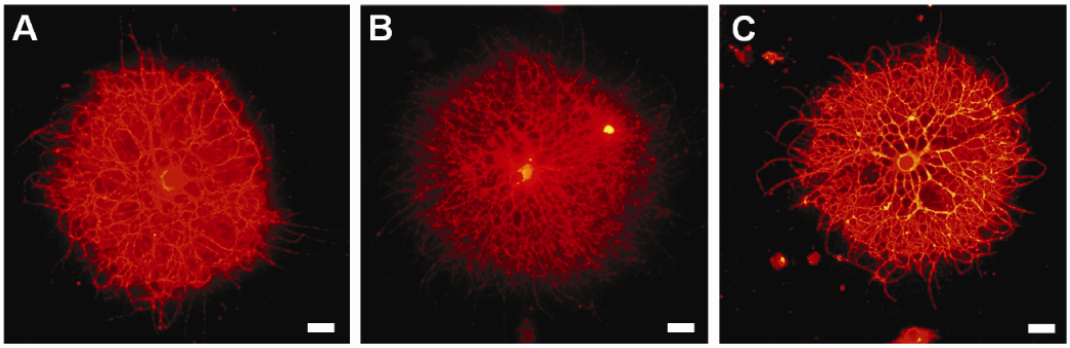


FIGURE S3

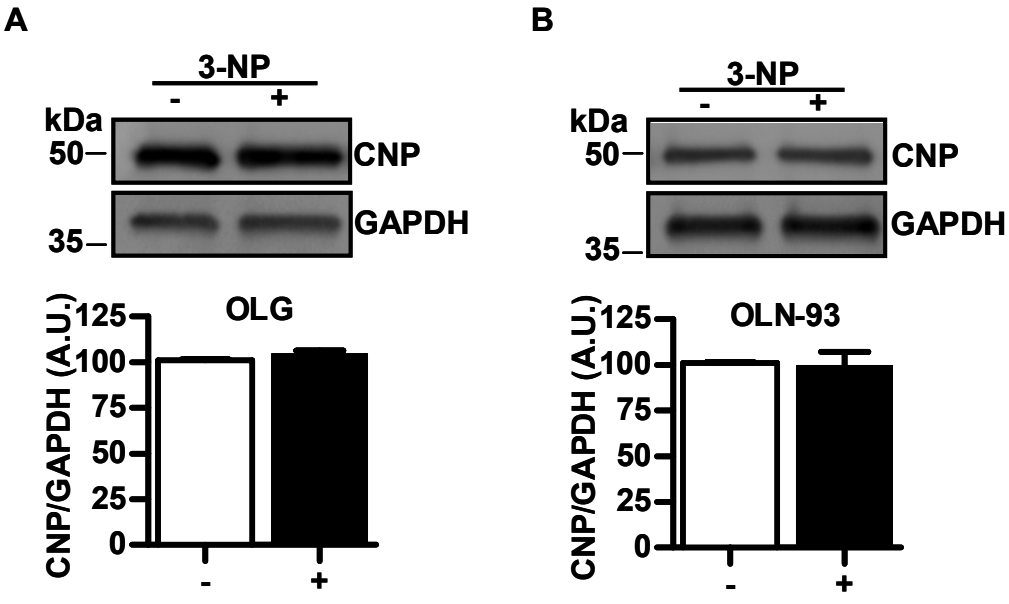


FIGURE S4

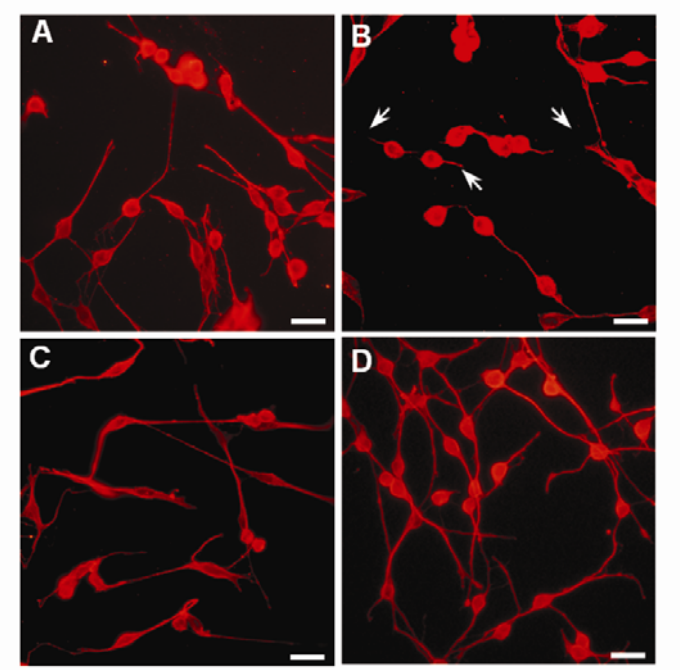
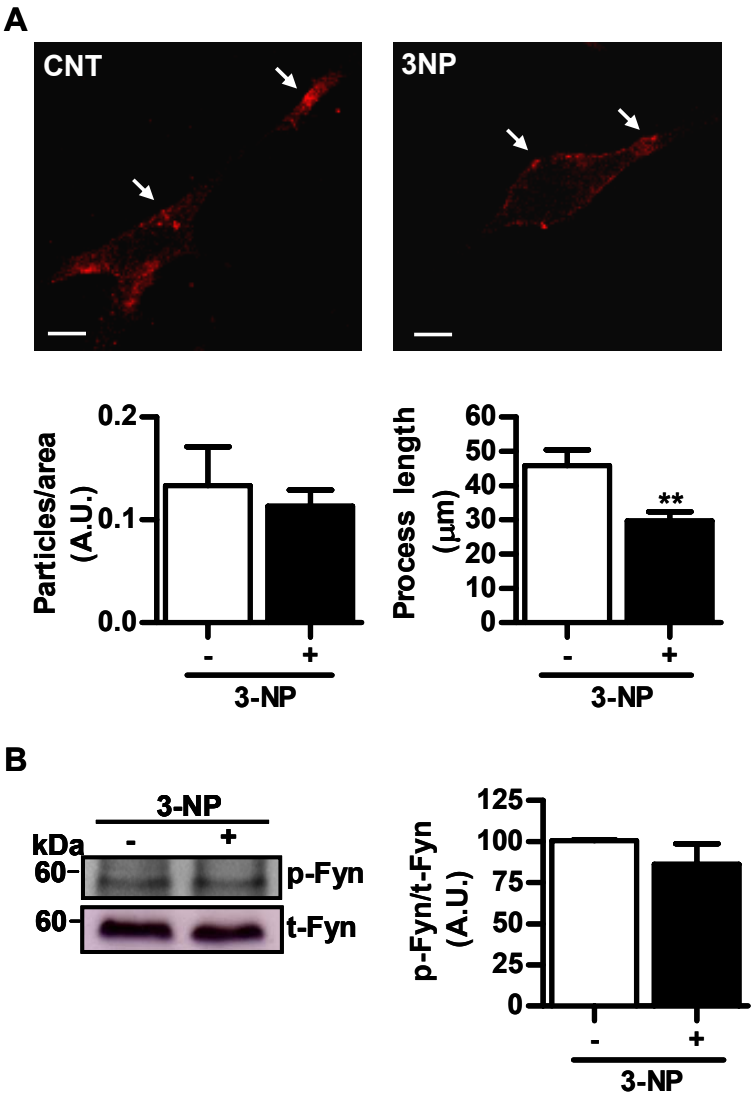


FIGURE S5



Appendix S3: Supplementary Table

Table I. Mass Spectrometry Protein Identification of 2D-DIGE differential spots from lysates of primary OLGs after treatment with 3-NP

Spot	Protein ID (Uniprot)	Protein Name (origen)	DB-SS*	Percentage of Coverage	N° of Peptides identified	Length (a.a)	Cy5/Cy3 ratio
1	P47942	CRMP-2 (rat)	180	35	15	572	1.76
2	P35527	Keratin 9 (human)▲	335	25	14	623	4.5
3	P35527	Keratin 9 (human)▲	343	26	14	623	4.1
4	P31000	Vimentin (rat)	633	81	39	466	2.2
5	P31000	Vimentin (rat)	199	55	23	466	-1.9
6	P31000	Vimentin (rat)	562	87	31	466	-1.88
7	P31000	Vimentin (rat)	264	66	23	466	-1.72
8	P38983	rpSA (rat)	321	72	10	295	-2.0

CRMP-2, Collapsin response mediator protein-2; **rpSA,** ribosomal protein SA; **▲,** Products of contamination during gel processing; **DB-SS*,** Data base search score considered significant when >80; **a.a,** aminoacids.